Chapter 17
Mechanisms of Metastasis

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Abstract Metastatic disease is the culmination of cancer and its most common life-threatening manifestation. The highly complex process by which cancer cells disseminate to and successfully colonize organs distant from the primary tumor has been divided into stages, collectively termed the metastatic cascade. Decades of research into metastasis biology has yielded several proposed models, each of which address experimental and clinical observations and contribute mechanistic insight to the metastatic cascade. Despite major advances in dissecting and identifying associated molecular pathways, many details remain to be clarified about the mechanisms that enable tumor cells to form these life-threatening lesions. The lack of a comprehensive understanding of the mechanisms of metastasis has thus delayed advancement of therapeutic strategies for late stage cancer. Here, we review the leading models describing tumor progression and provide an overview of the current state of the scientific community's understanding of metastasis.

Introduction

Despite recent advances in cancer biology and therapeutics, disseminated metastatic disease persists as an insurmountable challenge in the oncology clinic. It is estimated that 577,000 Americans will die of cancer in 2012 accounting for 25 % of all deaths in the USA [1], the vast majority of which will be the result of metastatic disease.

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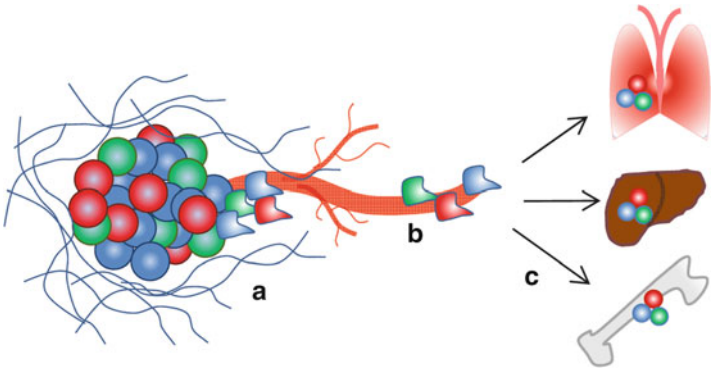

Fig. 17.1 Simplified steps of the invasion–metastasis cascade. (*a*) Invasion of cells into the surrounding tissue. (*b*) Entrance into the bloodstream. (*c*) Extravasation, entrance into distant organ parenchyma, and successful establishment of metastasis

The process by which a tumor that has initiated at a primary site, such as the breast, colon, or prostate gland, spreads to secondary organs is termed the invasion–metastasis cascade. The steps of the invasion–metastasis cascade are poorly understood but evidence suggests that it involves the complex interplay between tumor-intrinsic and host-derived factors. In order for a tumor cell of epithelial origin to form a clinically relevant metastatic lesion, cells from the primary tumor are thought to invade beyond the epithelial basement membrane, gain access to vasculature, survive in the lymphatic or blood stream, and arrest at the target organ, all the while evading innate and adaptive host immune surveillance mechanisms [2, 3].

Upon arrival at this secondary site, the tumor cell must adapt to survive and proliferate in an environment distinct from its tissue of origin, a step commonly termed metastatic colonization (Fig. 17.1). Further adding to the complexity of metastasis are observations that tumors originating in distinct organs have differential organ tropism and that metastases of the same primary tumor employ distinct cellular mechanisms and gene expression programs to colonize different secondary sites [4, 5]. Evidence suggesting that fewer than 0.01 % of cells that reach the bloodstream form macrometastases highlights the extreme inefficiency of the metastasis cascade [6]. Yet despite this inefficiency, metastasis continues to be the primary cause of cancer-related death.

While this admittedly simplistic model of the metastatic process has gained wide acceptance and many of the cellular and molecular determinants of metastasis are continually being identified, the persisting dearth of therapies for metastatic disease underlines the remaining gaps in our understanding of metastasis. Several models elaborating on the invasion–metastasis cascade have been proposed, each of which addresses particular clinical and experimental observations and provides detailed mechanistic insight. Here, we review the previously and recently proposed models of metastatic progression and provide an overview of the current state of the community's understanding of metastasis.

Clonal Selection Model

The most widely accepted model of metastasis is the clonal selection model. Successful dissemination to a secondary site involves many steps and it is thought that tumor cells acquire traits that allow them to initiate invasion, survive within the blood stream, extravasate, and grow in a foreign tissue environment. The clonal selection model postulates that genomic instability in the primary tumor leads to stochastic mutations that result in clonal selection of highly metastatic tumor cells [7–9]. This provides an explanation for why only a subset of cells succeeds in forming lesions at the secondary site. Since genomic instability within the primary tumor results in heterogeneity of neoplastic cells, not all tumor cells will acquire the necessary advantageous mutations and therefore only a small subset of them obtains full metastatic potential (Fig. 17.2a). This model thus attempts to explain the heterogeneity observed in primary tumors as well as the inefficiency of the metastatic process.

Based on the concept of tumor heterogeneity, Fidler and Kripke were the first to suggest this model by showing in animal models that a minute proportion of tumor cells were capable of successfully disseminating to the distant organ [10]. They demonstrated that clones from a parental murine tumor varied in their metastatic potential indicating the existence of metastatic heterogeneity within the primary tumor. In a separate study, using irradiation to induce unique chromosome rearrangements in cells, it was shown that each metastatic lesion was clonal [11]. Thus, these studies concluded that the ability to survive this process was not due to random selection. Since then, many other studies have validated these findings, further supporting that clonal selection occurs, which allows only specific cells to gain full metastatic potential. A recent paper provided support for the clonal selection model in medullablastoma [12]. By using the sleeping beauty transposon method, the authors recapitulated tumorigenesis and subsequent metastasis in mice. Analysis of the primary tumor and the matched metastatic tumor revealed only a small overlap indicating that they are genetically different. However, since common insertion sites existed in the primary tumor and the metastasis, they concluded that the secondary tumor arose from a common progenitor cell which had undergone divergence. Furthermore, the investigators showed analogous findings in human medullablastoma samples. These and other studies indicate that the clonal selection model provides a general mechanism for metastasis [13–15].

Additional evidence for the clonal selection model came with the birth of genomics: microarray analyses of primary tumor samples showed that genetically heterogeneous cellular populations exist within any given tumor [9, 16, 17]. These analyses demonstrated that particular genetic signatures underlie organ-specific metastasis and that not only do tumors exhibit heterogeneity but also that certain genetic mutations account for specific tissue tropism of tumor cells [5]. Further, recent advances in sequencing technology have enabled the analysis of whole genome sequencing of tumors [18]. Using second generation sequencing Ding et al. examined a single patient's peripheral blood, primary basal-like breast tumor, and matched brain metastasis [19]. They found

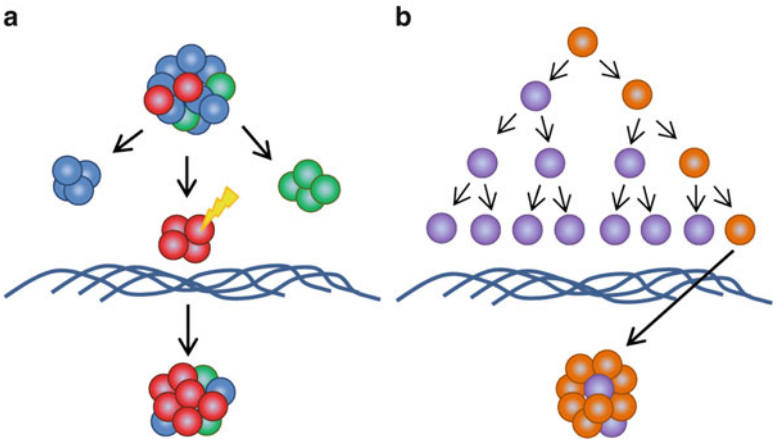

Fig. 17.2 Clonal selection model and cancer stem cell model. (**a**) The clonal selection model proposes that genomic instability within the primary tumor results in tumor cell heterogeneity such that only a subset of tumor cells acquire the mutations (*lightning bolt*) that endow them with metastatic capability. (**b**) The cancer stem cell model suggests that only cancer stem cells (*orange*) have the capability to form metastatic lesions

a wide range of mutations in the primary tumor supporting genetic heterogeneity within the sample. Analysis of the metastasis showed an enrichment of a subset of mutations, suggesting a subset of cells within the primary tumor had metastasized to the brain. The xenograft derived from the patient's primary tumor was shown to contain a mutational profile that overlapped with the metastasis, further supporting the notion that a minority population of cells arose within the primary tumor with an enhanced metastatic capability.

Similarly, in a study by Navin et al. the investigators inferred tumor evolution using single-cell sequencing [20]. Employing flow-assisted cell sorting with subsequent genome amplification and sequencing of 100 single cells from a heterogenic breast cancer sample, the authors revealed three distinct subpopulations that shared genomic alterations. When analyzing single cells from a homogeneous breast tumor and matching liver metastasis samples, the authors found a single subpopulation of aneuploid cells, indicating that the metastasis formed from a single subpopulation with little further evolution.

These data provide compelling evidence that clonal selection occurs in metastatic progression. While this model gives important insight to the possible mechanisms for metastasis, some observations cannot be explained by clonal selection theory. If all metastases occurred in the manner described by this model, the metastasis should be entirely composed of only a subset of cells observed in the primary tumor; however it has been shown that metastatic tumors phenotypically resemble their cells of origin [21, 22]. Another inconsistency arises from reports showing that metastatic gene expression signatures can be derived from expression profiles of the primary tumors [16, 23]. If a minority of genetically divergent tumor cells gain metastatic ability, it is unlikely that their gene expression profile would be detectable by

expression profiling of the bulk tumor. Nonetheless, the clonal selection model was the first to show a potential mechanism through which metastasis occurs. These insights by Fidler and subsequent studies have filled major gaps in metastasis biology.

Metastatic Cancer Stem Cell Model

In addition to the clonal selection model, another theory has been proposed to explain the observation of cell heterogeneity in primary tumors. Based on the concept of stem cells, this theory has postulated the existence of a niche of cancer stem cells. Stem cells are specialized cells that self-renew by asymmetric division to produce two daughter cells. While one cell remains a stem cell and retains self-renewal capacity, the other becomes a progenitor cell that differentiates [24, 25]. With these characteristics, stem cells provide life-long cell growth for tissue homeostasis and provide regenerative capacity for tissue repair.

Cancer stem cells are thought to have similar properties that allow them to sustain constant tumor growth. Due to their properties of self-renewal and differentiation, cancer stem cells are thought to provide the heterogeneity observed in the primary tumor [21, 24, 26]. Further, it has been hypothesized that cancer stem cells are tumor-initiating cells capable of forming new tumors at distant sites [21, 27]. Similar to the clonal selection model, the cancer stem cells hypothesis states that only cancer stem cells can colonize distant organs: only a distinct subset of cells is thought to have the ability to successfully metastasize—the cancer stem cell population [28, 29]. In contrast to the clonal selection model, cancer stem cells are the cells intrinsically programmed to have this advantage rather than metastatic cells being stochastically selected in the context of genomic instability (Fig. 17.2b).

The first evidence for the cancer stem cell hypothesis was demonstrated in acute myeloid leukemia where it was observed that a small percentage of leukemia cells were capable of proliferating extensively [30]. Dick took this finding and isolated a small subpopulation of acute myeloid leukemia cells that resembled normal hematopoietic stem cells and introduced them into immunodeficient mice. Upon transplantation, the cells from that subpopulation were able to induce leukemia in the mice whereas other cells found in the acute myeloid leukemia cell population were not, suggesting that the cancer stem cell hypothesis could indeed be true, at least in the case of hematologic malignancies. Since this study, cancer stem cells have been reported in solid tumors including breast, pancreas, colon and prostate, although these findings are less clear and continue to be controversial [31–34].

Breast cancer was the first solid tumor to show the existence of cancer stem cells. Al-Hajj et al. identified and isolated a subgroup of breast cancer cells using specific cell surface markers and showed that a few of these cells were needed to initiate new tumor formation while thousands of cells of other subtypes did not [31]. These tumor-initiating cells were identified to be $CD44^{+}CD24^{-/low}$ lineage and resembled stem cells. It was demonstrated that subpopulations within the tumorigenic cells

upon serial transplantation into nude mice continually gave rise to the same subpopulations of cells in new tumors. These data led Al-Hajj et al. to conclude that the isolated subset of cells had stem cell capacity and were alone responsible for the initiation of these tumors.

The identification of cancer stem cells in solid tumors has given rise to the possibility of these cells being involved in the metastatic process. Different theories regarding metastasis and cancer stem cells have been proposed. One hypothesis proposes that tumor cells undergoing epithelial-to-mesenchymal transition (EMT) gain stem cell-like properties. EMT and its counterpart, MET, are processes dictated by distinct signaling pathways during embryonic development that allow cells to migrate to appropriate regions of the body and develop into various tissue types [35]. These processes are defined by the loss of epithelial markers such as E-cadherin, and the gain of mesenchymal features such as vimentin and myosin, which lead to reduced attachment to the extracellular matrix and increased in cellular motility [36]. Such temporary phenotypic shifts in progenitor cells play an important role in embryonic development.

EMT and MET have also been observed in tumor cells and some evidence exists that they may play a role in invasion and metastasis [26, 37, 38]. During EMT, cells undergo a transition in gene expression programs that alter cell morphology and behavior that endows tumor cells with invasive properties that enable metastatic progression to commence. Cells lose their adhesion to the basement membrane by downregulation of E-cadherin, and upregulation of vimentin allowing for reduced adhesion and increase in motility [39]. Further, upregulation of membrane-degrading genes, such as matrix metalloproteinases enables cancer cells to escape the primary tumor and disseminate [40]. Since cancer stem cells appear to be the tumor-initiating cells at the distant site, it is hypothesized that the EMT process could bestow cancer cells with stem cell-like properties in order for them to successfully metastasize. This was demonstrated in a study by Mani et al. in which the authors showed EMT-induced cells acquired stem cell-like phenotypes [37]. Data from this study indicates a potential link between cancer stem cells and EMT, a process that may initiate dissemination.

Another theory hypothesizes that metastatic cancer stem cells directly derive from cancer stem cells. Data from a study led by Hermann et al. demonstrated the existence of metastatic cancer stem cells in pancreatic tumors [32]. First, the investigators analyzed pancreatic cancer samples and found a distinct population of cancer stem cells. Upon further analysis they discovered a second population of stem cell-like cells at the invasive front of the tumors. These tumors were found to be $CXCR4^{+}$, which is a specific receptor for SDF-1, a mediator of cell migration. Inhibition of CXCR4 with a receptor-specific inhibitor reduced the metastatic capability of these cells, indicating that this distinct population of cancer stem cells is important in cell dissemination. These data therefore demonstrate that metastatic stem cells may derive from cancer stem cells.

The identification of cancer stem cells in primary tumors sets forth the possibility that they are the drivers of tumorigenesis and metastasis. Evidence for cancer stem cells in solid tumors has provided a large body of knowledge for potential

mechanism of tumor initiation, progression, and metastasis. Whether cancer stem cells can explain all aspects of the invasion–metastasis cascade requires further elucidation but sufficient data suggest that they may play a role in metastasis in at least some solid tumors.

Transient Compartment Model

The transient compartment model is an extension of the dynamic heterogeneity model and was first proposed by Weiss [41]. This theory attempts to explain the observation that secondary tumors, although having successfully metastasized, do not necessarily have an increase in metastatic capacity over primary tumor cells. Having observed this in a number of experimental systems (reviewed in ref. 41), Weiss proposed that all cells within the primary tumor have the ability to metastasize. However, due to spatial or epigenetic factors only a few cells will successfully disseminate. Therefore, as depicted in Fig. 17.3, only cells that have a positional advantage (i.e., have adequate blood supply) will gain the required capability to metastasize. Similarly, throughout the steps to successful metastasis, cells may undergo transient epigenetic changes that provide them further advantage to disseminate. Furthermore, it is thought that when cells from the secondary tumor are injected into the primary site, they revert to their original phenotype, indicating that the disseminated cells do not permanently acquire an enhanced metastatic ability. Unlike the clonal selection or cancer stem cell model, in which only a certain cell population gains advantage through somatic mutations or stem cell characteristic, respectively, the changes in the transient compartment model is temporary and may affect any cell in the primary tumor.

The hypothesis that epigenetics plays a role in the transient compartment model is supported by studies demonstrating that methylation inhibitors modulate the metastatic capacity of cell lines [42–46]. Data by Kerbel et al., for example, demonstrated that when non-metastatic cell lines were treated with a demethylating reagent, they obtained small, unstable tumor clones with enhanced metastatic capability [46]. However, while global demethylation may mimic some of the proposed epigenetic events, these agents also cause chromosomal aberrations [47], opening the possibility that the modulation of metastatic capacity was due to mutational rather than epigenetic events.

While the transient compartment model accounts for the observation that not all metastatic cells are consistently more metastatic, this phenomenon is also explained by other models. The inability of cells isolated from metastases to be consistently more metastatic than the primary tumor could be explained by the tumor microenvironment which has been shown to play a significant role in metastasis (reviewed below). Furthermore, the transient compartment model does not explain the clonal nature of metastases [47–49]. Studies have shown that primary tumors are heterogeneous [50, 51] and, therefore, if metastatic capability was only modulated by transient epigenetic events, then it is less likely that significant proportions of secondary tumors would appear to be of clonal origin [12, 17].

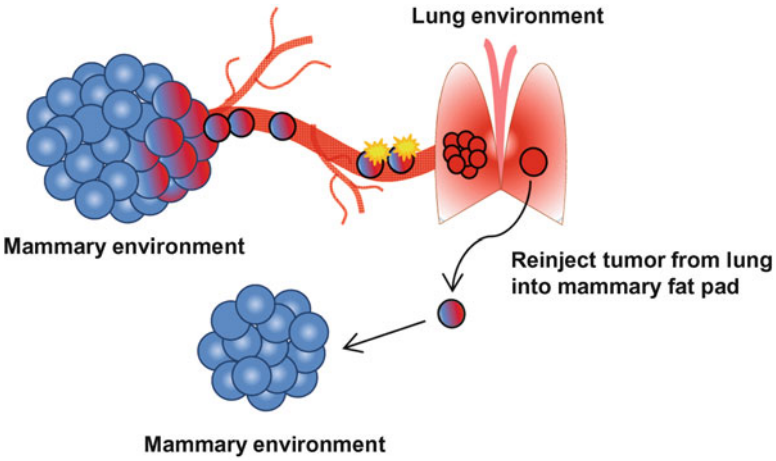

Fig. 17.3 Transient compartment model. Transient compartment model suggests that epigenetic or microenvironmental factors allow cells to become metastatic. All viable cells (*blue*) in the tumor acquire metastatic capacity, but due to positional (*red*/*blue*) and/or random epigenetic (*yellow star*) events only a small fraction is capable of completing the process at a given moment (as depicted by the change from *blue* to *red*)

Fusion Model

Many models have been proposed to explain the mechanism of metastasis, most of which attempt to explain discrepancies between experimental observations and shed light on specific aspects of metastasis. For metastasis to successfully occur, cells must enter and survive in the circulation, then invade and form tumors at a secondary site. The clonal expansion model suggests that somatic mutations contribute to the heterogeneity in the primary tumor while the cancer stem cell model requires dedifferentiation of cells into a more embryonic phenotype, and both genomic instability and anaplasticity are attributes of highly invasive cells [7, 37]. While these phenomena are not characteristic of normal epithelial cells, they are to cells that originate from lymphoid tissue. Cells from myeloid origin are capable of dedifferentiation, migration throughout the body, and survival in many tissue environments [52]. Using features of epithelial and myeloid cells, the fusion model has been proposed to explain the dedifferentiated phenotype observed in primary tumor cells [53, 54]. The fusion model hypothesizes that epithelial cells in the primary tumor fuse with myeloid cells, resulting in the fusion of both cells' nuclei (Fig. 17.4). Lymphoid cells are known to migrate throughout the body and therefore such fusion might allow tumor cells to obtain the necessary characteristics to successfully metastasize.

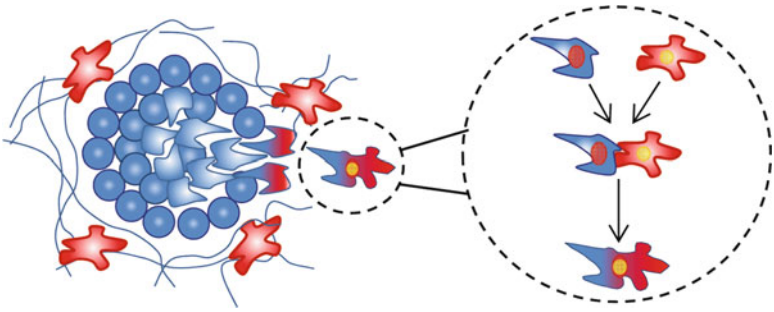

Fig. 17.4 Fusion model. Metastatic potential is achieved by fusion of a primary tumor cell with a lymphoid cell. Nuclear fusion of these two cell types endows tumor cells with gene expression programs of lymphoid cells, which enhances their metastatic potential

The idea that fusion of cells results in genomic instability is not a new one as demonstrated by early studies of fertilization by Boveri and Aichel. It was observed in the early 1900s that eggs experimentally fertilized with multiple spermatozoa underwent abnormal mitosis, which suggested that chromosomal imbalance might result in oncogenesis (reviewed in ref. [52]). Upon this observation, Aichel and Boveri proposed that the mechanism of metastasis could stem from the fusion and hybridization of cells. They hypothesized that this imbalance led to "qualitative differences" in chromosomes and resulted in metastasis.

Many in vivo experiments have supported the notion of cancer cell fusion: animal studies demonstrated that cancer cells have the ability to fuse with epithelial cells, stromal cells and endothelial cells. While the first fusion studies only observed enhanced tumorigenicity, Goldenberg et al. made the first connection between cell fusion and metastasis in 1974 [55]. These investigators injected human astrocytic tumor cells into the cheeks of hamsters and observed the formation of lethal metastases. Upon dissection and analysis of these cells, they found them to be hybrids containing human and hamster cells. Similarly, data from a study by Larizza et al. showed that the fusion of low-metastatic T-cell lymphoma cells with host macrophages resulted in hybrids that were more metastatic in nature than the tumor cells alone [56]. They observed that the hybrid cells expressed the macrophage-specific antigen Mac-1, which was not found in the T-cell lymphoma line or any other tumor cell line except for a macrophage tumor cell line. The investigators concluded therefore that the fusion of the tumor cells with the host macrophages could be a mechanism for genetic alterations leading to metastasis. Furthermore, recent evidence by Carloni et al. suggests that cellular fusion also plays a role in chemoresistance in colon cancer [57]. The authors showed a mechanism by which the expression of ADAM10 on colon cancer cells drives cellular fusion and this, in turn, leads to the development of chemoresistance to 5-fluoro-uracil and oxaliplatin. It is known that metastatic cells are highly resistant to chemotherapy and, therefore, these data could have important implications in understanding therapeutic resistance in metastatic colon cancer.

It is known that metastatic tumor cells target specific tissues, and the fusion model has attempted to explain this phenomenon of organotropism. In a study dating back to 1984, De Baetselier et al. demonstrated that fusion of tumor cells with a particular lymphocyte resulted in differential organ metastasis: the fusion of myeloma cells with B cell lymphocytes led to metastases to the spleen and liver whereas the fusion of plasmacytoma with a macrophage gave rise to lung metastases. While this study did not implicate cell fusion to enhancing metastatic capability, it linked cell fusion to metastatic organotropism [58].

Most of the data for the fusion model so far has occurred in vitro. However evidence for spontaneous cellular fusion in solid tumors, although rare, has been shown in humans. The occurrence of renal cell carcinoma in bone marrow transplant recipients has been described in which the tumor cells contained markers from both donor and recipient [59, 60].

Evidence exists to support the mechanism of cellular fusion in tumorigenesis. Data have shown that hybrid cells exist with features of both tumor cells and macrophage-specific phenotypes. However, there is no clear pathway or evidence suggesting cell fusion to be mechanistically linked to metastatic progression. The role of the tumor microenvironment and bone marrow-derived cells in metastasis has been studied (reviewed below) and shown to be significant in the dissemination of primary tumor cells. Whether this occurs via the fusion of these two cell types requires elucidation in vivo models or from patient samples.

Gene Transfer Model

Similar to the fusion model, a related hypothesis regarding metastatic capacity has been proposed. The gene transfer model is based on a theory observed among nineteenth century physicians who debated whether primary tumors could release unknown substances that then influence normal cells at secondary sites. Years later, in 1965, Bendich et al. revisited these original observations and demonstrated that DNA, indeed, can be found in the circulation of tumor mice [61]. Similarly, a study by Leon et al. demonstrated levels of free DNA in patients with and without tumors. While the levels of DNA did not correlate with the size of a primary tumor in cancer patients, the authors did see a significant correlation in those with metastatic disease as compared to those with no metastases [62]. These studies gave rise to the model of genometastasis which hypothesizes that secreted DNA from primary tumors could be horizontally transferred to susceptible cells in a distant site and therefore give rise to a secondary tumor (Fig. 17.5). To demonstrate this theory, experimental data showed that plasma from tumor-injected animals can transfect cells with DNA in culture [61]. Furthermore, it has been proposed that the circulating DNA can be taken up by stem cells at secondary sites [63]. Thus metastatic lesions may not derive directly from primary tumor cells but rather circulating DNA from primary tumor cells may mediate horizontal gene transfer that may induce tumorigenesis at the distant site.

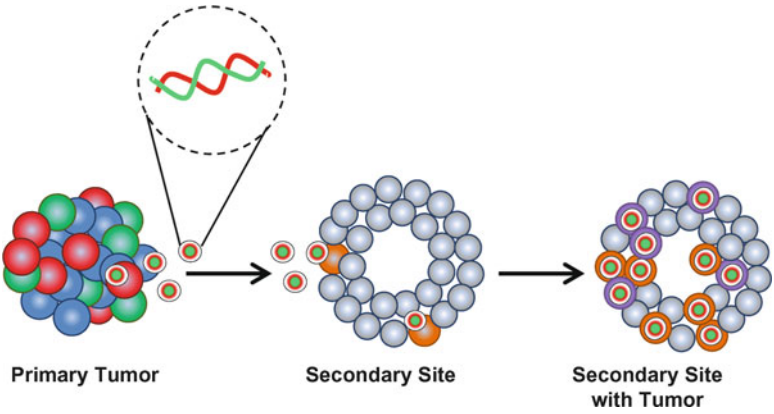

Fig. 17.5 Gene transfer model. The primary tumor secretes DNA (*small green/red circles*) into the bloodstream and is taken up by stem cells (*orange cell*) in the distant organ. This horizontal gene transfer enables stem cells to develop into tumor cells at the secondary site (*orange* and *purple cells*)

Even though experimental data exists to support the validity of this hypothesis, a few caveats must be taken into account. It has been well observed and studied that cancers exhibit specific organ preference for dissemination and colonization [4, 5]. If primary tumors do release oncogenic DNA into circulation that then has access to all tissues in the body, how does it transform only specific cells at specific distant sites? If the genometastasis theory held true, then the DNA would have to contain markers which only certain tissue sites could recognize. While this may be theoretically possible, no in vivo data exist to support this phenomenon. Furthermore, if DNA in plasma is the basis for metastasis, sufficient uptake of the genetic information of the primary tumor by the cells at the distant sites has to occur for reprogramming of the cells to resemble the primary tumor cells. Enough evidence exists for the presence of tumor DNA in blood plasma, however thus far it has only been suggested to be used as a biomarker for disease [64, 65] and has not yet been shown to be mechanistically involved in metastasis promotion.

MicroRNAs and Metastasis

MicroRNAs (miRNAs) are short nucleotide sequences (17–20 nt) of noncoding RNAs each of which are capable of sequence specific binding of numerous mRNA targets. miRNAs regulate mRNA transcript abundance or expression by targeting mRNA for degradation or interfering with its translation, respectively. As such, miRNAs are capable of regulating cellular functions such as development, proliferation, apoptosis, and cell cycle progression, functions critical to cellular homeostasis, tumorigenesis, and metastasis [66–68].

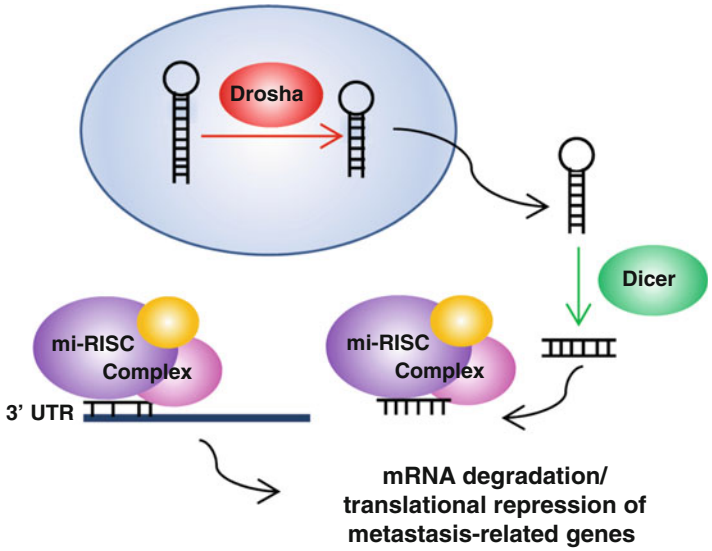

Fig. 17.6 miRNA maturation and function in metastasis

miRNAs are initially transcribed in the nucleus and processed by Drosha into precursor-miRNA structures which are then shuttled into the cytoplasm where they become further processed by Dicer. Upon maturation the miRNA, along with the multiprotein RNA-induced silencing complex (mi-RISC), bind to sequences on the 3′ untranslated region of target genes. Depending on the degree of complementarity of the seed sequence and the target mRNA, miRNA binding leads to the degradation or translational repression of its target transcripts (Fig. 17.6). The seed sequences vary from 2 to 8 nucleotides and, because complete complementarity binding to these sequences is not required for gene regulation, miRNAs can bind to more than one target. Due to this lack of perfect complementarity, it is thought that a single miRNA can influence the expression of hundreds of genes [66, 67]. Their function in gene regulation plays a significant role in cellular physiology and homeostasis but, when aberrantly expressed, they can also be involved in disease progression. Therefore, since their discovery, miRNAs have been a major focus in tumorigenesis and metastasis.

Evidence from miRNA studies has demonstrated that these small RNAs are involved in the suppression or progression of cancer pathways leading to metastasis [69, 70]. From regulation of cellular proliferation to epithelial-to-mesenchymal transition (EMT), one of the initial steps in the distant dissemination process, miRNAs have shown to play crucial roles as oncogenes and tumor suppressors. As an activator of metastasis, miR-21 has been well studied. First discovered in glioblastoma [71], this miRNA has since been shown to regulate gene function in a variety of solid tumors such as breast, colon, lung and prostate cancer [72–75]. In glioblastoma, miR-21 was demonstrated to function as an anti-apoptotic factor by

downregulating genes important in apoptosis. Since then, miR-21 has been shown to play many more roles in tumorigenesis and metastasis. In breast cancer, this miRNA was shown to target tumor suppressor tropomyosin 1 (TPM1), an actin-binding protein that suppresses anchorage-independent cell growth. Upon overexpression of miR-21, TPM1 levels are knocked down, leading to aberrant tumor growth. In metastasis-specific studies miR-21 was demonstrated to increase metastatic capacity by regulating the expression of genes important for cell invasion, such as matrix metalloproteinases (MMPs) [76].

Other metastasis-activating miRNA including miR-10b, a miRNA discovered by the group of Weinberg, can have their expression regulated by a gene involved in EMT, such as TWIST [77]. Overexpression of TWIST induces EMT and subsequent cellular invasion of tumor cells, upstream steps of the invasion–metastasis cascade. TWIST-mediated activation of miR-10b leads to the downregulation of HOXD10, initiating the transcription of various pro-invasion genes including the derepression of RhoC which enhances cellular motility. Identifying miR-10b as a target of TWIST regulation, in addition to other studies, demonstrated that miRNAs are significant contributors to the initiation of metastasis.

Since the discovery of miR-21 and miR-10b, several other miRNAs have been shown to be involved in tumorigenesis and metastasis [72, 74, 78]. While these small RNAs have added another layer to understanding molecular mechanisms of tumor progression, they have also opened a door to many new and interesting questions. Since each miRNA has the potential to target dozens if not hundreds of targets, it is currently difficult to discern the significance of any one particular target in tumor progression. A recent study shows that a miR-126-mediated regulon—a set of transcripts regulated by a single miRNA—non-tumor cell autonomously regulates endothelial cell recruitment to metastatic breast cancer cells [79]. With the recent advent of novel miRNA-based therapeutics, a more complete understanding of the roles of miRNA in tumor progression and metastasis may provide another avenue to clinically target metastasis.

The Tumor Microenvironment and Metastasis

While historically most work on tumor progression has focused on the role of tumor-cell-autonomous mechanisms, it is now widely accepted that the tumor non-autonomous microenvironment also plays a significant role in progression and metastasis. The tumor microenvironment consists of the untransformed cell types in the immediate surroundings of tumor cells including myoepithelial cells, endothelial cells, lymphocytes, myeloid cells, and fibroblasts. It also includes noncellular components such as the extracellular matrix, as tumor-stroma interactions have been shown to alter the composition of extracellular matrix deposition [80, 81]. It has been known that epithelial cells communicate with the surrounding stroma to maintain tissue homeostasis [81]. Conversely, the stromal cellular environment secretes factors that modulate epithelial behavior such as proliferation. In addition

to the cells' own regulation, factors from cells in the microenvironment can send stimuli to further regulate homeostasis [82]. This concept can also be applied to the tumor environment, such as the stromal cells and primary cancer cells. During tumorigenesis, therefore, aberrant signaling from these cells can stimulate tumor cells to disseminate as well as prepare the secondary site for successful colonization by the disseminated tumor cells.

An important factor in the metastatic process is the extracellular matrix (ECM), which can regulate cell behavior. This matrix can act as a physical barrier which, when degraded, allows cells to leave their surroundings. It is also a repository for growth factors and cytokines that stimulate growth and modify cellular behavior. During tumorigenesis, cancer cells can degrade the proteins that normally allow them to stay in place so that they can leave their primary organ and enter the blood stream, which are the initial steps of dissemination.

Evidence for the interplay between tumor cells and the microenvironment initially came from studies showing that teratoma cells injected into blastocysts of a different cohort of mice gave rise to genetically normal mice [83]. The data from this study suggested that a non-tumorigenic cell microenvironment could suppress and reverse the cancerous phenotype of the injected cells. Since then, many other investigators have applied this concept to their research. Olumi and others have demonstrated that cancerous cells can reverse their aggressive tumor phenotype when cocultured with the ECM of normal cells. When added to a tumorous microenvironment, however, cancer cells can become more aggressive, leading to an increase in migration and invasion [84, 85]. Studies like these lead to the hypothesis that the interaction of tumor cells with their surrounding environment can activate or repress metastasis.

Recently, a set of observations have led to the concept of the premetastatic niche, which proposes that primary tumors produce factors that remodel the microenvironment of the secondary site to make it more amenable for colonization prior to the arrival of metastatic cells. The first study inspecting the premetastatic niche suggested that bone marrow-derived hematopoietic progenitor cells positive for vascular endothelial growth factor 1 ($VEGFR1^+$) arrive at the secondary site and alter the tissue microenvironment by upregulating integrins and cytokines. Interestingly, subsequent to the implantation of tumor but prior to its colonization by ($VEGFR1^+$) hematopoietic progenitor cells, an upregulation of fibronectin was observed at the distant site, suggesting that the primary tumor somehow communicated with the distant site to alter its gene expression to allow for the arrival of metastasis-promoting ($VEGFR1^+$) hematopoietic progenitor cells. Importantly, the functional role of ($VEGFR1^+$) hematopoietic progenitor cells in promoting metastasis was directly queried by demonstrating that the metastatic potential of tumor cells was abrogated by treating of ($VEGFR1^+$) hematopoietic progenitor cells with anti-VEFGR1 prior to implantation into irradiated mice [86].

To further add to the role of the microenvironment, one of the most recent findings is the theory of priming bone marrow-derived cells (BMDCs) towards a metastatic phenotype via exosomes. Exosomes are vesicles that are secreted from a variety of cells. These structures mainly carry cellular cargo such as proteins, mRNAs, and miRNA which can be transported from one cell to another [87, 88]. Such transfer of information may be a type of intercellular communication.

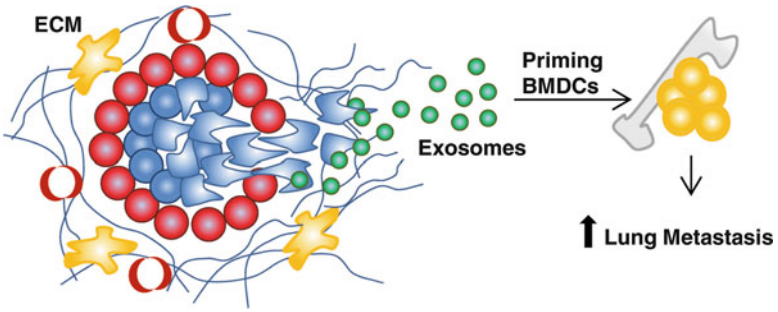

Fig. 17.7 The role of exosomes in metastasis. Exosomes released by metastatic cells prime bone marrow-derived cells (BMDCs) to potentiate lung metastasis

As stated above, BMDCs have been shown to play a significant role in adjusting the microenvironment to be more suitable for successful dissemination and it is possible that tumor cells communicate with distant sites to form an amenable premetastatic niche by secreting such exosomes.

Integrating the above findings, data by Peinado et al. showed that melanoma-derived exosomes have the ability to prime BMDCs to develop a pro-metastatic environment (Fig. 17.7). The authors of this study showed the interplay of tumor cells and their microenvironment at a molecular level [89]. First, the investigators determined the significance of exosomes by measuring their levels in various clinically staged melanoma patients and found that a positive correlation between tumor stage and exosome protein levels. They then demonstrated that introducing exosomes from highly metastatic melanoma cells into naïve mice resulted in exosomes localizing to sites at which metastasis is commonly observed. These initial experiments indicated that exosomes could play a role in metastasis. Furthermore, the authors showed that these exosomes carry proteins important in the formation of a pre-metastatic environment. Such proteins included the Met oncoprotein, heat shock protein 90 (HSP90) and tyrosinase-related protein 2 (TYRP2). This study therefore demonstrated the importance of primary tumor communication with cells needed for metastasis. By secreting exosomes that contained pro-metastatic proteins, tumor cells could prime the environment of metastatic sites before dissemination occurred.

Among many others, the aforementioned studies showed that an intertwined network of communications mechanisms exists between tumor cells, the primary tumor microenvironment, and the microenvironment at the distant secondary site. In the context of physiological function one can imagine that distant and disparate organs concertedly regulate homeostasis. In the context of cancer these same mechanisms can be exploited by the tumor to promote its own dissemination and virulence. While the exact mechanisms of bone marrow cell-derived education by tumors remain to be worked out, studies like these highlight the importance of understanding tumor biology on a scope beyond tumor cell intrinsic mechanisms. And though these studies cast an unanticipated layer of complexity to tumor progression, they also suggest an entirely novel set of molecular and cellular targets for the development of therapeutics.

Genetic Susceptibility

While the previous models propose that somatic mutations drive metastasis, our laboratory focuses on the genetic susceptibility to metastasis encoded within the germ line. Germ line polymorphisms contribute to defining each person as an individual. Differences such as eye color, height, or responses to drugs can be explained by polymorphisms within the germ line. Similarly, this concept also appears to hold true to the susceptibility of an individual to develop metastasis [90]. Studies in our laboratory, among others, have demonstrated that germ line polymorphisms modify cellular properties leading to tumorigenesis, invasion, and metastasis [91–93].

This concept was first developed with the observation that inbred mice of distinct genetic backgrounds showed differential susceptibilities to lung metastasis (Fig. 17.8). The initial experiment was conducted by crossing female mice of various inbred strains (such as FVB, NZB, C58BL/6, AKR, DBA, etc.) to male FVB mice transgenic for the mouse mammary tumor virus promoter driving mammary tissue-specific expression of the polyoma middle T antigen (MMTV-PyMT) oncogene. All female transgene positive F1 progeny acquired mammary tumors; however progeny of different maternal genetic backgrounds showed distinct pulmonary metastatic burdens. Since the oncogenic driver and paternal genotype were constant in all mice, this study clearly demonstrated that, in mice, polymorphisms in the maternal germ line contribute to metastatic susceptibility [94]. Extending this observation to humans led to the hypothesis that the genetic make-up of an individual can predispose him or her to be more vulnerable to metastatic progression upon tumor initiation and has opened the door to epidemiological studies to support it. In mice, the idea that metastatic susceptibility is a quantitative (polygenic) heritable trait has given way to quantitative trait loci (QTL) mapping, crossing mice with significantly different metastatic susceptibilities and tracking genotype and phenotype, to identify regions of the genome associated with—and therefore likely containing elements regulating—metastasis.

Data from our laboratory first identified a candidate polymorphic gene whose differential expression resulted in modulation of metastatic capability of murine tumor cells and that could be used to successfully stratify patients into poor and good survival groups. Since metastasis is the primary determinant of survival, it can be inferred that this polymorphism modified metastatic potential in patients [95]. By mapping the loci potentially responsible for differences in metastasis, we found *Sipa1* on the *Mtes1* locus. Ectopic expression of *Sipa1* was shown to enhance metastasis while knockdown of *Sipa1* reduced the metastatic capacity of tumor cells in a mouse model of metastasis. Furthermore, the same polymorphism was identified in a cohort of human breast cancer samples and, as predicted, it was a marker of poor outcome in estrogen receptor-positive (ER^+) breast cancer. These data were particularly exciting as this was one of the first studies to show that genetic background can influence susceptibility to metastasis in humans.

With recent advances in global transcript analysis, further investigations of gene networks and their role in metastatic progression became possible [96]. A study from

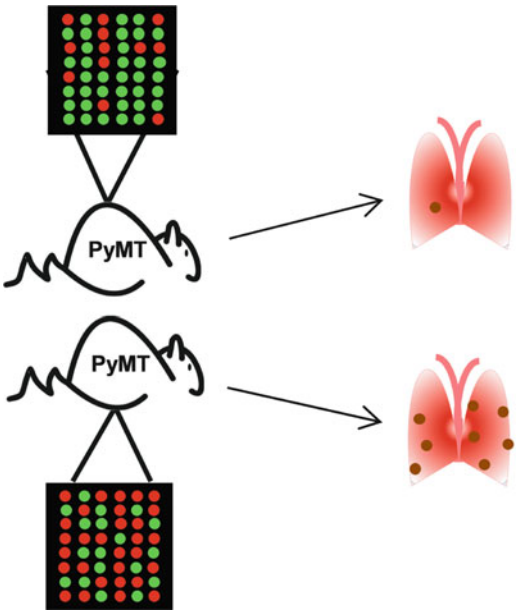

Fig. 17.8 Genetic susceptibility to metastasis. Variability in the germ line genome and in gene expression networks contribute to determining an individual's metastatic susceptibility

our laboratory demonstrated that the global transcript network analysis of human as well as mouse samples identified co-expressed gene networks capable of predicting metastasis-free survival in independent human breast cancer cohorts. Interestingly, these networks also suggested that the differences in breast cancer subtypes were either due to tumor-cell-autonomous behavior or the microenvironment. Estrogen receptor-positive (ER^+) breast cancers, for example, were shown to be tumor-driven while estrogen receptor-negative (ER^-) breast cancers appeared to be influenced by the host-derived stroma [97].

As an example of this concept, a recent study from our laboratory identified *Cadm1*, a gene whose over- and under-expression influenced metastatic outcome of breast cancer cells. This gene was identified after analyzing the quantitative trait loci (QTLs)—regions of the genome that segregate with the phenotype of interest subsequent to introduction of genetic and phenotypic diversity by breeding genetically and phenotypically distinct mice—of NZB and FVB, mice with significantly different susceptibilities to pulmonary metastasis, and then searching for polymorphic genes that were also differentially expressed in tumor tissue. Although *Cadm1* was differentially expressed in both tumor and untransformed tissue between NZB and FVB, it showed no coding-level polymorphisms between the two strains. Similar to the network analyses above which queried samples based on differential expression, it appeared in this case that differential expression of *Cadm1* was the significant factor. This was confirmed by showing that overexpression of this candidate metastasis modifier gene resulted in the suppression of lung metastases while knocking down *Cadm1* increased the ability of breast cancer cells to colonize the lungs. Furthermore, this study showed that this difference in metastatic

susceptibility, though resulting from tumor-cell autonomous differential expression of *Cadm1*, had a tumor nonautonomous component as the metastasis suppressive effects of high *Cadm1* expression was lost in mice lacking functional T-cell-mediated immunity. This study therefore showed that polymorphisms in germ lines can not only predict the susceptibility to metastasis but that such genes can also play a role in tumor-nonautonomous factors [98]. Importantly, because T-cell-mediated immunity was essential to *Cadm1*-mediated effects on metastasis, the use of metastatic human tumor cell lines in athymic mice would have been incapable of detecting *Cadm1* as a metastasis suppressor. In this regard, the study by Faraji et al. underlines the essential role mouse mammary tumor cell lines in immune-competent mice play in modeling metastasis biology.

To emphasize the differences in gene network and breast cancer outcome based on subtype, another of our studies recently showed that tumor-autonomous genes influence metastasis. QTL analysis of PyMT crossed with the AKXD panel of recombinant inbred mice identified another metastasis susceptibility gene that influenced the dissemination of breast cancer cells to the lungs. We showed that polymorphisms in *Arib4b* on AKR/J and DBA/2J alleles had different metastatic phenotypes. Furthermore, while analyzing gene networks, we discovered that this gene regulated many genes of the *Tpx* network, which previously showed to be useful in predicting metastasis-free survival in ER^+ breast cancers. As expected, the levels of ARID4b predicted of ER^+ breast cancers. This data provided further evidence that germ line polymorphisms in tumor-autonomous genes play a role in predicting metastasis progression in specific subsets of breast cancer [99].

Taken together, these studies demonstrate that a germ line component exists that influences a tumor's ability to successfully form metastatic lesions. Additionally, polymorphisms in genes that regulate or are within the global gene networks can predict metastasis-free survival in subsets of breast cancer. While these data are promising and have been supported by epidemiological studies, additional studies could further confirm that this concept directly applies mechanisms of metastasis in humans. In contrast to the clonal selection model which bases the ability of metastasis on somatic mutations, genetic susceptibility to distant dissemination of cancer cells is inherited. This not only provides a novel approach to dissect molecular pathways involved in metastasis to find novel therapeutic targets, it also provides insights into predicting patient outcomes using gene expression signatures based on metastasis susceptibility-specific markers.

Conclusion

The invasion–metastasis cascade continues to be poorly understood, particularly with regard to therapeutically targeting metastatic lesions. Over the past century, clinical and anatomical insights into metastasis coupled to technical advances in cellular and molecular biology and animal modeling have shed light onto the mechanisms of metastasis. Yet cellular determinants and gene expression programs

mediating tumor cell dissemination continue to be incompletely understood, as evidenced by the largely refractory nature of metastatic lesions to classical and targeted chemotherapeutics. In this review, we have summarized the leading models and recent conceptual advances that provide a framework for our understanding of the invasion–metastasis cascade. The fact that several disparate models, each illuminating one layer of biology involved, have been proposed to describe key aspects of metastasis is a testament to the complexity of the metastatic process. The emerging challenge is now to link the relevant aspect of each model to identify rate-limiting steps of the invasion–metastasis cascade, which may reside at conceptual interfaces between models, for therapeutic targeting. The development of successful therapeutics against metastatic disease necessitates elucidating clear links between intracellular protein and RNA signaling pathways in tumor cells and in non-neoplastic components of the microenvironment while considering the vast genetic heterogeneity within the tumor. Such knowledge will pave a path for the development and strategies for implementation of a arsenal of novel therapeutics against the largely untreatable and final stage of cancer.

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