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Isaiah J. Fidler

Critical determinants of cancer metastasis: rationale for therapy

Abstract The major cause of death from cancer is metastases that are resistant to conventional therapies. Several reasons account for treatment failure in patients with metastases. First, neoplasms are biologically heterogeneous and contain subpopulations of cells with different angiogenic, invasive, and metastatic properties. Second, the process of metastasis selects a small subpopulation of cells that preexist within a parental neoplasm. Although metastases can have a clonal origin, genetic instability results in rapid biological diversification and the regeneration of heterogeneous subpopulations of cells. Third, and perhaps the most important principle for the design of new cancer therapies, is that the outcome of metastasis depends on multiple interactions (''cross-talk'') of metastatic cells with homeostatic mechanisms which the tumor cells usurp. The organ microenvironment can influence the biology of cancer growth, angiogenesis, and metastasis in several different ways. For example, the survival and growth of tumor cells are dependent on angiogenesis, which is mediated by an imbalance between positive and negative regulating molecules released by tumor cells, normal cells surrounding a tumor, and infiltrating lymphoid cells. Many cytokines that stimulate or inhibit angiogenesis are present in different tissues, and thus the organ environment profoundly influences this process. Moreover, the organ microenvironment can also influence the response of metastases to chemotherapy by regulating the expression of different drug resistance genes, such as *mdr*-1. The finding that the resistance of metastases to some chemotherapeutic agents can be mediated by epigenetic mechanisms has obvious implications for therapy. The identification of organ-specific cytokines that can upregulate expression of *mdr*-1 (or other resistant mechanisms) may suggest an approach to overcome the resistance of some metastases to particular chemotherapeutic agents. Therefore therapy of metastasis should be targeted not only against metastatic tumor cells, but also the homeostatic factors that are favorable to metastasis, growth, and survival of the metastatic cells.

Key words Metastasis · Homeostasis · Drug resistance · Angiogenesis

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I.J. Fidler
Professor and Chairman, Department of Cancer Biology,
Box 173, University of Texas M.D. Anderson Cancer Center,
1515 Holcombe Boulevard, Houston, Texas 77030, USA
Tel.: +1 713 792-8577; Fax: +1 713 792-8747

Role of the organ environment in metastasis

Despite improvements in diagnosis, surgical techniques, patient care, and adjuvant therapies, most deaths from cancer are due to metastases that are resistant to conventional therapies [18, 78, 89]. The major obstacle to effective treatment is tumor cell biologic heterogeneity. By the time of initial diagnosis, neoplasms contain multiple cell populations with characteristics including diverse growth rates, karyotypes, cell-surface properties, antigenicity, immunogenicity, marker enzymes, sensitivity to various cytotoxic drugs, and ability to invade and produce metastasis [2, 18, 63]. In a large number of patients with cancer, metastasis may have occurred by the time of diagnosis [18, 60, 78, 89]. The metastasis can be located in lymph nodes and different organs, and the specific organ environment can influence the biologic behavior of metastatic cells, including their response to systemic therapy.

Since most deaths from cancer are due to metastases that are resistant to conventional therapies, there is an urgent need to develop effective regimens against disseminated cancer. A better understanding of the molecular mechanisms that regulate the process of metastasis and of the complex interactions between the metastatic cells and host factors can provide a biological foundation for the design of more effective therapy for different cancers and new options for oncologists to deal with cancer metastasis.

Clinical observations of cancer patients and studies with experimental rodent tumors have revealed that certain tumors produce metastasis to specific organs independent of vascular anatomy, rate of blood flow, and number of tumor cells delivered to each organ. The distribution and fate of hematogenously disseminated, radiolabeled melanoma cells in experimental rodent systems demonstrate that tumor cells reach the microvasculature of many organs, but extravasation into the organ parenchyma and growth occur in only some organs [16, 17, 78, 89].

The search for the mechanisms that regulate the pattern of metastasis began a century ago. In 1889, Paget [58] asked whether the distribution of metastases was due to chance. He analyzed autopsy records of 735 women with breast cancer. The nonrandom pattern of visceral metastases suggested to Paget that the process was not due to chance but rather that certain tumor cells (the "seed") had a specific affinity for the milieu of certain organs (the "soil"). Metastases resulted only when the seed and soil were compatible [58].

In 1929, Ewing challenged Paget's seed and soil theory and hypothesized that metastatic dissemination occurs by purely mechanical factors that are a result of the anatomic structure of the vascular system [13]. These explanations have been evoked separately or together to explain the secondary site preference of certain types of neoplasms. In a review of clinical studies on secondary site preferences of malignant neoplasms, Sugarbaker concluded that common regional metastatic involvement could be attributed to anatomic or mechanical considerations such as efferent venous circulation or lymphatic drainage to regional lymph nodes, but that distant organ colonization by metastatic cells from numerous types of cancers had different patterns of site specificity [78].

Experimental data supporting the seed and soil hypothesis of Paget were derived from studies on the preferential invasion and growth of B16 melanoma metastases in specific organs [21, 38, 39]. Hart and Fidler injected B16 melanoma cells into the circulation of syngeneic C57BL/6 mice. Tumor growths developed in the lungs and in fragments of pulmonary or ovarian tissue implanted intramuscularly. In contrast, metastatic lesions did not develop in renal tissue implanted as a control or at the site of surgical trauma [39]. This study confirmed that sites of metastasis are determined not solely by the characteristics of the neoplastic cells but also by the microenvironment of the host tissue. In vitro experiments demonstrating organ-selective adhesion, invasion, and growth [62] also support Paget's hypothesis [58]. With the B16 melanoma system, cells that are selective for organ adhesion, invasion, and growth have been isolated. Moreover, experiments with organ tissue-derived soluble growth factors indicate that soil factors can have profound effects on certain tumor cell subpopulations [for a review, see 20].

There is no question that the circulatory anatomy influences the dissemination of many malignant cells [16, 44, 60, 62, 78, 89, 90]; however, it cannot, as Ewing proposed [13], fully explain the patterns of distribution of numerous tumors. Ethical considerations rule out the experimental analyses of cancer metastasis in patients similar to those performed in laboratory animals, by which either Paget or Ewing might be proved correct. The introduction of peritoneovenous shunts for palliation of malignant ascites has, however, provided an opportunity to study some of the factors affecting metastatic spread in humans.

Tarin and colleagues have described the outcome in patients with malignant ascites draining into the venous circulation via an implanted Levine shunt, with the resulting entry of viable tumor cells into the jugular veins [85, 86]. Good palliation with minimal complications was reported for 29 patients with different neoplasms. The autopsy findings in 15 patients substantiated the clinical observation that the shunts do not significantly increase the risk of metastasis, and despite the continuous entry of millions of tumor cells into the circulation, metastases in the lung (the first capillary bed encountered) were rare [85, 86]. These results provide compelling verification of the venerable seed and soil hypothesis.

An interesting demonstration of organ-specific metastasis comes from studies of brain metastasis. Schackert and Fidler described the development of a mouse model of cerebral metastasis after injection of syngeneic tumor cells into the internal carotid artery [65, 66]. Direct, intracranial injection of tumor cells was used to determine tumorigenicity. Injection of cells into the internal carotid artery of mice simulates the hematogenous spread of tumor emboli to the brain. Thus this technique can be used to examine the last steps of the metastatic process: release of tumor cells into the circulation, arrest of tumor cells in capillaries, penetration and extravasation of the tumor cells into the brain through the blood–brain barrier, and continuous growth of the cells in the tissue [67, 68, 94].

Two melanomas were studied and differed in their pattern of brain metastasis: the K-1735 melanoma produced lesions only in the brain parenchyma, whereas the B16 melanoma produced only meningeal growths [30, 66]. Similarly, different human melanomas [67] or carcinomas [68] injected into the internal carotid artery of nude mice produce unique patterns of brain metastasis. These results demonstrate specificity of metastatic growth in different regions within a single organ. The results from site distribution analysis of radiolabeled murine melanoma cells injected into the internal carotid artery ruled out the possibility that patterns of initial cell arrest in the microvasculature of the brain predict the eventual sites of growth. Thus an alternative explanation for the different sites of tumor growth involves interactions between the metastatic cells and the organ environment, possibly in terms of specific binding to endothelial cells and responses to local growth factors. In other words, organ-specific metastases are produced by tumor cells that are receptive to their new environment [62].

A current definition of the seed and soil hypothesis consists of three principles. First, neoplasms are biologically heterogeneous and contain subpopulations of cells with different angiogenic, invasive, and metastatic properties [2, 18, 23, 63, 84]. Second, the process of metastasis is selective for cells that succeed in invasion, embolization, survival in the circulation, arrest in a distant capillary bed, and extravasation into and multiplication within the organ parenchyma [17, 22, 24, 82]. Although some of the steps in this process contain stochastic elements, as a whole metastasis favors the survival and growth of a few subpopulations of cells that preexist within the parent neoplasm [22, 83]. Thus metastases can have a clonal origin, and different metastases can originate from the proliferation of different single cells [24, 41, 57, 83]. Third, and perhaps the most important principle for the design of new cancer therapies, is that the outcome of metastasis depends on multiple interactions (cross-talk) of metastatic cells with homeostatic mechanisms, which the tumor cells can usurp [19, 20]. Therefore therapy for metastasis should be targeted not only against tumor cells but also against the homeostatic factors that are favorable to tumor metastasis, growth, and survival.

Influence of the organ environment on the invasive phenotype

The influence of organ environment on the invasive and metastatic potential of human colon cancer (HCC) cells was examined using four cell lines with different metastatic potentials. When any of the HCC cells were injected subcutaneously, they did not produce visceral metastases. In contrast, metastatic cells metastasized from the cecum to regional mesenteric lymph nodes and the liver [19, 32, 53, 55]. A degradation assay of type IV collagen demonstrated significant differences in the levels of secreted type IV collagenases between HCC cells growing subcutaneously and those growing in the cecum. These results suggest that a factor(s) in the cecum's environment may stimulate the production of type IV collagenases in colon cancer cells. In the subcutis, another factor may suppress production of this enzyme by the colon cancer cells.

Since the interaction of stromal fibroblasts can influence the tumorigenicity and biological behavior of tumor cells, we determined whether organ-specific fibroblasts could directly influence the invasive ability of tumor cells [15]. Primary cultures of nude mouse fibroblasts, lung, and colon were established. Invasive and metastatic colon cancer cells were cultured alone or with the fibroblasts. The growth and invasive properties of the colon cancer cells were evaluated, as was gelatinase activity. Colon cancer cells grew on monolayers of all three fibroblast cultures but did not invade through skin fibroblasts. Colon cancer cells growing on plastic and on colon or lung fibroblasts produced significant levels of latent and active forms of type IV collagenase, whereas colon cancer cells cocultivated with nude mouse skin fibroblasts did not. Incubation of the cells in serum-free medium containing recombinant human interferon (IFN)-beta (fibroblast IFN) significantly reduced gelatinase activity. Since IFN-β is produced by skin fibroblasts but not by colon or lung fibroblasts, the results support in vivo data indicating that organ-specific factors can influence the invasive and metastatic properties of tumor cells [15, 33, 34].

Modulation of cancer cell response to chemotherapy by the microenvironment

Clinical observations have suggested that the organ environment can influence the response of tumors to chemotherapy. For example, in women with breast cancer, lymph node and skin metastases respond better than lung or bone metastases [75]. Experimental systems suggested a basis for this observation [10, 61, 62, 75, 76]. Studies from the author's laboratory have shown that a mouse fibrosarcoma growing subcutaneously in syngeneic mice is sensitive to systemic administration of doxorubicin (DOX), whereas lung metastases are not [77]. Similar results were obtained with the CT-26 murine colon cancer: subcutaneous tumors were sensitive to DOX, whereas metastases growing in the liver or lung were not [91, 92].

Several intrinsic properties of tumor cells can render them resistant to chemotherapeutic drugs, including increased expression of the *mdr* genes [4, 5, 31, 87], leading to overproduction of the transmembrane transport protein P-glycoprotein (P-gp) [4, 5, 43, 87]. Increased levels of P-gp can be induced by selecting tumor cells for resistance to natural product amphiphilic anticancer drugs [4, 5, 87]. Elevated expression of P-gp accompanied by development of the multidrug resistance (MDR) phenotype has also been found in many solid tumors of the colon, kidney, and liver that had not been exposed to chemotherapy [8, 88]. Since the development of MDR in tumor cells is a major obstacle to cancer therapy, understanding the mechanisms that control this process has been the goal of many researchers. Most of the present knowledge about drug resistance in tumor cells is derived from examining tumor cells growing in culture, but the relevance of these findings to in vivo growing tumors has been uncertain.

We investigated whether different organ environments influence the response of tumor cells to chemotherapy and whether this response is regulated by the level of P-gp expression in tumor cells. In this study, murine CT-26 colon cancer cells growing in the lung of syngeneic mice were refractory to systemic administration of DOX, whereas the same cells growing subcutaneously were sensitive to the drug. CT-26 cells harvested from lung metastases exhibited increased resistance to DOX compared with cells harvested from subcutaneous tumors or parental cells maintained in culture. The resistance to DOX was reversed by the addition of

verapamil. All the CT-26 cells were sensitive to the antiproliferative effects of 5-fluorouracil (5-FU). The increased DOX resistance of the CT-26 cells directly correlated with levels of expression of *mdr*-1 mRNA transcripts and P-gp. The drug resistance and accompanying elevated expression of *mdr*-1 found for cells growing in the lung were dependent upon interaction with the specific organ environment. Once removed from the lung, the cells reverted to a sensitive phenotype similar to that of parental cells [11].

The increased DOX resistance of CT-26 cells in lung metastases was not due to selection of resistant subpopulations. We base this conclusion on the results of the crossover experiment. Once implanted into the subcutis of syngeneic mice, CT-26 cells from lung metastases produced tumors that were sensitive to DOX. In parallel studies, DXR-sensitive CT-26 cells from subcutaneous tumors became resistant to the drug when they were inoculated intravenously and grew in the lung parenchyma as metastases. P-gp levels directly correlated with the drug resistance phenotype in these experiments. Moreover, the increased DOX resistance and elevated levels of *mdr*-1 mRNA and P-gp were all transient in CT-26 cells growing in the lung. Subsequent to growth in culture for >7 days, *mdr*-1 mRNA and P-gp reverted to the baseline levels of CT-26 parental cells [11].

The findings of an organ-specific response to DOX are not restricted to CT-26 cells. UV-2237 mouse fibrosarcoma cells [77], human KM12 colon carcinoma cells [92], and murine B16 melanoma cells [93] also showed significant differences in DOX (but not 5-FU) resistance between subcutaneous tumors (sensitive) and lung or liver metastases (resistant). In patients with colon carcinoma, high-level P-gp expression is found on the invasive edge of the primary tumor (growing in the colon) and in lymph node, lung, and liver metastases [45, 46, 88].

Collectively, these data demonstrate that the organ environment can induce the P-gp-associated MDR phenotype in tumor cells. The expression of P-gp is transient: once removed from the environment (lung), the cells revert to the sensitive parent cell phenotype. This environmental regulation of the MDR phenotype may partly explain the polarized expression of *mdr*-1 in colon carcinomas [52] and the discrepancy between in vitro and in vivo expression levels of the MDR phenotype [4, 5, 43, 54].

Regulation of angiogenesis by the microenvironment

A crucial step in continuous growth of tumors and development of metastasis is the recruitment of new blood vessels in and around tumors [1, 25, 27, 28]. A tumor mass that is <1 mm in diameter can receive oxygen and nutrients by diffusion. Any increase in tumor mass requires angiogenesis, ie, the proliferation and morphogenesis of vascular endothelial cells [1, 27, 28, 42, 50]. The process of angiogenesis consists of multiple, sequential, and interdependent steps. It begins with local degradation of the basement membrane surrounding capillaries, followed by invasion of the surrounding stroma by the underlying endothelial cells in the direction of the angiogenic stimulus. Endothelial cell migration is accompanied by the proliferation of endothelial cells at the leading edge of the migrating column. Endothelial cells then organize into three-dimensional structures to form new capillary tubes. The onset of angiogenesis involves a change in the local equilibrium between positive and negative regulatory molecules [3, 27, 28, 50]. Some of the major angiogenic factors include basic fibroblast growth factor (bFGF), vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), interleukin 8 (IL-8), platelet-derived endothelial cell growth factor (PD-ECGF), platelet-derived growth factor (PDGF), and hepatocyte growth factor (HGF) [12, 14, 25, 29, 37].

In many healthy tissues, factors which inhibit angiogenesis predominate [1, 12, 37]. The switch from an angiogenesis-inhibiting to an angiogenesis-stimulating phenotype has been studied in different models. For example, in cultured fibroblasts the loss of the wild-type allele of the p53 tumor suppressor gene coincides with the acquisition of the angiogenic phenotype and is the result of reduced production of thrombospondin-1 [9].

The production of angiogenic molecules is regulated in part by cell-to-cell contact, ie, cell density [74]. Human renal cell carcinoma (HRCC) cells express low levels of bFGF (both at the mRNA and protein levels) under dense culture conditions compared to sparse cultures. Similar data were obtained for endothelial cells [74]. In contrast to the inverse correlation of cell density and bFGF expression, expression of VEGF is directly correlated with cell density in HCC cell lines [47].

Our laboratory has shown that expression of bFGF by tumor cells is also dependent on the site of implantation. When HRCC cells were implanted in different organ microenvironments in nude mice, the expression of bFGF was 10–20-fold higher in those tumors implanted in the kidney than in those implanted in subcutaneous tissues [71]. The kidney tumors were more highly vascularized than tumors implanted in the subcutis. In contrast, the expression of IFN-β was high in epithelial cells and fibroblasts surrounding the subcutaneous tumors, whereas no IFN-β was found in or around HRCC tumors growing in the kidney. The parental cell line and metastatic clone also differed in bFGF expression. This alteration in bFGF levels by the site of implantation was due to adaptation to the organ microenvironment, as was demonstrated when the cells were reestablished in culture and bFGF levels returned to the previous in vitro concentration after 4 weeks [71].

Expression of bFGF in HRCC is cell density dependent [74]. By in situ mRNA hybridization (ISH) and Northern blot analysis, we found an inverse correlation between increasing cell density and bFGF expression [74]. Fluorescence-activated cell sorting, immunohisto-

chemistry, and enzyme-linked immunoabsorbent assay confirmed this finding at the protein level. Tumor cells harvested from dense cultures (low bFGF expression) and then plated under sparse conditions expressed high levels of bFGF. Similar data were obtained using endothelial cells. The effect was not mediated by soluble factors released into the culture medium. The in vivo manifestation of cell density-dependent regulation is likely to be differences in gene expression in the center of a tumor versus the periphery or leading edge [45, 46].

The expression of bFGF in surgical HCC specimens was determined using ISH and Northern blot analysis. ISH analysis revealed that bFGF levels were significantly higher in Dukes' stage C or D tumors than in Dukes' stage B tumors. Northern blot hybridization did not detect mRNA transcripts for bFGF. However, analysis by ISH revealed that bFGF was overexpressed at the periphery of the tumor (leading edge), where cells were rapidly dividing. This observation confirms that tumors are heterogeneous for cells with varying degrees of expression of invasion- and metastasis-related genes and that a subpopulation of cells within a tumor can give rise to distant metastasis [45]. In a follow-up study in colon cancer patients, bFGF expression was found to be highest in primary tumors from patients who presented with metastatic disease [46]. This study identified patients who appeared to be free of metastasis at the time of initial surgery (Dukes' stage B) yet developed distant metastasis at a later date; these patients had relatively high bFGF expression along with increased expression of other metastasis-related genes especially at the invasive edge of the tumor [45, 46].

The production of angiogenic molecules, eg, VEGF, bFGF, and IL-8, by melanoma cells is regulated by complex interactions with keratinocytes in the skin [40]. Recent reports from the author's laboratory show that IL-8 is an important molecule in melanoma growth and progression. Constitutive expression of IL-8 directly correlates with the metastatic potential of human melanoma cells. Furthermore, IL-8 induces proliferation, migration, and invasion of endothelial cells, and hence neovascularization [72, 73].

Several organ-derived cytokines (produced by inflammatory cells) are known to induce expression of IL-8 in normal and transformed cells [40]. Since IL-8 expression in melanocytes and melanoma cells can be induced by inflammatory signals, the question of whether specific organ microenvironments could influence IL-8 expression was analyzed. Melanoma cells were implanted into the subcutis and spleen (to produce liver metastasis), and injected intravenously (to produce lung metastasis) into athymic nude mice. Subcutaneous tumors, lung lesions, and liver lesions expressed high, intermediate, and no IL-8 mRNA and protein, respectively [36]. Melanoma cells established from the tumors growing in vivo exhibited similar levels of IL-8 mRNA as continuously cultured cells, thus demonstrating that the differential expression of IL-8 was not due to selection of a subpopulation of cells [36].

IL-8 expression can be upregulated by coculturing melanoma cells with keratinocytes (skin) and inhibited by coculturing melanoma cells with hepatocytes (liver). We also investigated the effects of two cytokines produced by keratinocytes (IL-1 and IFN-β) and two cytokines produced by hepatocytes (tumor growth factor [TGF] alpha and TGF-β) on the regulation of IL-8 in human melanoma cells. IL-1 upregulated the expression of IL-8 in human melanoma cells at both the mRNA and protein levels in a dose- and time-dependent manner in the presence of de novo protein synthesis [73]. IFN-β did not affect constitutive IL-8 mRNA and protein production in human melanoma cells, but it did block the induction of IL-8 by IL-1 [73]. TGF-β inhibited the expression of IL-8, while TGF-α had no effect on IL-8 expression.

The expression of the common angiogenic molecule VEGF is increased in necrotic areas of human tumor as shown by ISH [7]. In vitro studies have confirmed that VEGF expression is increased in response to hypoxia, probably due to increased transcription and to increased mRNA stability [70]. Treatment of cells with IL-1, IL-6, IL-8, TGF-β, PDGF, HGF, and bFGF can increase expression of VEGF [49]. VEGF expression is also regulated by certain oncogenes (*src* and *ras*) and tumor suppressor genes like p53 [64].

The organ microenvironment also influences the expression of VEGF/VPF. Human gastric cancer cells were implanted into orthotopic (stomach) and ectopic (subcutaneous) organs of nude mice. Tumors in the stomach were highly vascularized, expressed high levels of VEGF, and grew more rapidly than the subcutaneous tumors. In addition, metastasis occurred only from the tumors implanted in the stomach [80, 81].

Lymphoid-mediated angiogenesis

Angiogenesis is essential to homeostasis and its regulation by lymphoid cells, such as T lymphocytes, macrophages, and mast cells, is well recognized [6, 26, 48, 51, 59, 69, 79]. A local inflammatory reaction consisting of T lymphocytes and macrophages is often associated with invasive cutaneous melanoma, and an intense inflammatory reaction is often associated with increased risk of metastasis, suggesting that angiogenesis induced by inflammation may contribute to melanoma progression and metastasis [56].

Immunological mechanisms involved in physiological angiogenesis are active subsequent to wound healing [79]. Systemic chemotherapy has been shown to retard the process of wound healing, possibly by decreasing the immune response; whether this is mediated by inhibition of angiogenesis is not clear [56]. The author and colleagues have investigated the role of tumor vascularization and its effect on tumor growth in immunosuppressed mice. The growth of weakly immunogenic B16 melanoma was retarded in myelosuppressed mice compared with control mice [35]. Further

evidence implicating myelosuppression in the retardation of tumor growth and vascularity was obtained from DOX-pretreated animals injected with normal spleen cells one day before tumor challenge. Tumor growth in these mice was comparable to that in control mice [35]. Similar results were obtained in athymic mice, suggesting that the tumor vascularization observed in DOX-treated mice reconstituted with normal splenocytes was not mediated solely by T lymphocytes. Since reconstitution with spleen cells enhanced vascularization of the B16 tumors, the results suggest that myelosuppressive chemotherapeutic drugs, eg, DOX, can inhibit host-mediated vascularization and support the concept that developing tumors can usurp homeostatic mechanisms to their advantage [20].

The role of infiltrating cells in the angiogenesis of HCC has recently been reported [80]. High expression of PD-ECGF was found in infiltrating cells, mostly macrophages and lymphocytes, and very little expression was found in the cancer epithelium. The intensity of staining for PD-ECGF in infiltrating cells correlated with vessel counts, suggesting the involvement of these cells in the angiogenesis of HCC.

Conclusions

The pathogenesis of metastasis depends on multiple favorable interactions of metastatic cells with host homeostatic mechanisms. Interruption of one or more of these interactions can lead to the inhibition or eradication of cancer metastasis. For many years, all of our efforts to treat cancer have concentrated on the inhibition or destruction of tumor cells. Strategies both to treat the tumor cell, eg, chemotherapy and immunotherapy, and to modulate the host microenvironment, eg, tumor vasculature, could provide an additional approach for cancer treatment. The recent advances in our understanding of the biological basis of cancer metastasis present unprecedented possibilities for translating basic research into the clinical reality of cancer treatment.

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