CANCER GENOMICS & PROTEOMICS *4*: 211-222 (2007)

Review

Molecular Targets in Metastasis: Lessons from Genomic Approaches

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Abstract. *Microarray studies have yielded valuable information that can be used to determine a cancer patient's prognosis and allow for optimum treatment choices. Tumor profiling has also changed our perception of metastatic propensity. Genomic analyses clearly showed that a metastasis signature is encoded within the genome so that when a cancer develops, the likelihood of metastasis is high, whereas other cancers which do not have this genotype metastasize as a result of random mutations. It is certain however, that cells other than tumor cells contribute to the development of metastasis through their production of various pro-metastatic proteins. Here, we review the published metastasis profiling studies and the role of the host in metastasis. Collagen type I, CXCR4, CSF-1, OPN and RhoC are metastasis-associated genes for which evidence exists for a causal contribution to elements of the metastatic process. These genes are discussed in detail and represent excellent drug targets for anti-metastasis therapies.*

Metastasis is a term that encompasses multiple cellular processes. The literal meaning of the Greek roots 'meta' and 'stasis' define the end result of these cellular processes, a tumor that is now beyond its original place. For many years, physicians and scientists have sought to reduce the complex metastatic cascade to its component events and determine the molecular controllers of these events. Hence we have gained an understanding of cell invasion and proteolysis, of cell migration, of adhesion molecules and how they are down-regulated, of embolization and other methods by which tumor cells survive in vessels and, to a lesser degree, of the molecules needed to facilitate growth at secondary sites. Such a reductionist approach has taught us much of the biology of metastasis but has not yet led to significant advances in therapy. More recently, the technological developments that have spawned methods for global gene analysis have been harnessed to investigate whether metastasis is a predictable outcome for any particular cancer. These exciting profiling studies have opened up new areas of exploration in metastasis research. Here we will review some of these studies and the innovative investigations that have been initiated as a result.

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Key Words: Microarray, profile, host, collagen, rhoC, CXCR4, CSF-1, osteopontin, review.

Profiling Metastasis

Microarray studies using both animal models and human patient samples have identified multiple signatures associated with the metastatic phenotype. Initial studies used samples obtained from patients' primary tumors to investigate whether prognosis could be predicted based on genes expressed. Despite prevailing theories of how genetic instability would allow only rare cells in the primary mass to gain the necessary genetic changes for them to progress to metastatically-competent cells, microarray analysis indeed found signatures of genetic changes that segregated with prognosis (1, 2). Since rare genetic changes are unlikely to be detected by such bulk profiling methods, these results suggest that the majority of a tumor carries the signature associated with prognosis. One of the best known of these studies came from the Dutch group of Rene Bernards in collaboration with Rosetta Inpharmatics (3). These researchers used cDNA obtained from the tumors of young breast cancer patients with sporadic, lymph node-negative disease (3, 4). Existing strategies for treating such patients usually involves adjuvant chemotherapy even though the majority of the patients will not progress to more advanced disease. These non-progressive patients are therefore needlessly exposed to toxic chemotherapeutic drugs that carry their own risks. van't Veer and colleagues showed that using primary tumor material obtained at time of resection, they could identify a subgroup of patients with a poor prognosis *i.e.* that were highly likely to develop invasive,

metastatic disease (4). The investigators developed a 70 gene set that could be used to discriminate these 'poor prognosis' patients. Genes within this set include those that encode cyclin E2, MMP-9, VEGFr1 (Flt-1) and RAB6B, however this initial work was focused on generating an expression signature that would allow patient stratification rather than on identifying the genetic controllers of the metastatic process.

Other profiling papers have shown that a metastatic lesion in any patient is more similar to the primary tumor rather than metastases from other patients (5, 6). These results would suggest that there is not a dominant broad range genetic change or reprogramming that must occur to effect metastasis. Additionally, the results further the idea that individualized approaches to therapy are likely to be more efficacious than a 'one-size-fits-all' approach. A landmark study from the lab of Todd Golub established that metastasis to various different sites from primary adenocarcinomas of different organs could be predicted based on a common gene signature present in the primary tumors (7). These researchers established a 17-gene signature to enable prognosis. So, once again, 'metastatic potential' is a property that is apparently present in the primary tumor and can be identified through bulk profiling methods implying that rare metastatic variants are not responsible.

An interesting set of experiments that have been reported from the laboratory of Joan Massagué have shed further light on some of these processes (8-10). In a series of elegant studies that revolve around understanding the ability of metastatic lesions to preferentially colonize particular secondary sites, these researchers have used a human breast cancer cell line, MDA-MB-231, originally isolated from the pulmonary effusion of a patient who had relapsed with metastatic disease years after surgical resection of the primary breast tumor. Analysis of the cell line and various clones isolated from it showed that it harbored the genetic fingerprint of 'poor prognosis' originally identified by van't Veer *et al.* (8) However additional genetic changes that corresponded to clonal isolates with abilities to grow in specific metastatic sites indicated that, on top of the poor prognosis gene set, an additional selection is made for expression of genes that enable metastatic growth at particular sites. Hence, there are several distinguishable signatures that can be thought of as equivalent to metastatic propensity ('poor prognosis') as well as ability to establish and grow at secondary site(s). When looking at clones that preferentially colonized lung as a secondary site, these researchers further broke down this second layer of genes into "lung metastagenicity" and aggressiveness signatures (9). Using combinations of overexpression and knockdown techniques, the causal relationship between gene expression and ability to grow at secondary sites was established. Interestingly no single gene was robustly able to confer ability to grow at particular sites. Instead combinations of three or more of the various genes identified were necessary to allow efficient growth in bone (8), adrenal glands (10) or lungs (9). The bone colonization signature identified in the Massagué lab includes genes such as IL-11, CTGF, CXCR-4 and MMP-1 (8) while the lung colonization signature includes Id-1, VCAM-1, MMP-1, COX-2 and epiregulin (9). We will further discuss the likely contributions of specific members of these genetic profiles and their potential usefulness as metastasis drug targets later in the article. In both bone and lung, a large subset of the identified genes encodes protein products that are secreted or expressed on the cell surface. This is not surprising as it has been recognized for some time that site-specific metastatic growth must involve interactions between the tumor cells and the host organ [(11) & refs therein].

The Host in Metastasis

An underlying assumption of the various profiling studies was that the gene expression signatures seen were primarily due to somatic mutations arising in the tumor cells. However, there is clear evidence from these profiles that more than mutated tumor cells are responsible for the expression profiles associated with metastatic propensity. Indeed Ramaswamy *et al.*, who identified the 17-gene multiple solid tumor metastasis profile, point out that genes in their 17-gene as well as an original larger 128-gene profile are expressed by stromal cells rather that tumor cells (7). Prominent amongst these are genes encoding type I collagen, *Col1a1* and *Col1b1*. It is possible that expression of particular genes is upregulated in stromal cells as a result of signals initiated by mutated tumor cells so that, indirectly, the expression profile is still related to cancer-associated mutations in tumor cells. An alternative theory, however, is that gene expression profiles are more reflective of polymorphisms within an individual's genome (12). This would certainly explain why the bulk of a primary tumor carries the signature and why this signature is so similar between the metastasis and primary tumor of a specific patient. The primary and most persuasive arguments for this line of thinking come from the studies of Kent Hunter and his laboratory.

An early focus for the Hunter laboratory was determining whether host genetic background had a role in tumor progression as it does in many other complex pathological events. To explore this question, they used a panel of inbred mouse lines crossed to the MMTV-PyMT transgenic mouse. *FVB/N-Tg (MMTV-PyVT)^{634Mul}* is a model of mammary cancer developed by Bill Muller and colleagues in 1992 (13). The animal carries a copy of the potent oncogene polyomavirus middle T antigen (PyMT) under the control of the murine mammary tumor virus (MMTV) thus targeting

oncogene expression to the mammary epithelium. Polyoma virus middle T antigen induces phosphatidylinositol-3 kinase (PI3K) signaling and activates src kinase, properties thought to be largely responsible for its potent transforming ability (13). MMTV-PyMT mice on the original FVB/n background in which they were made, begin developing mammary tumors in all ten mammary glands at approximately seven weeks of age (13). The tumors spread to the lung and by 100 days of age almost all animals have evidence of pulmonary metastasis. Since tumor development is directly related to expression of the oncogene, all animals irrespective of what strain they were crossed to, should have similar likelihood of developing tumors and presumably metastasis unless there is a modifying influence of genetic background. The results from the analyses carried out in the Hunter lab showed that indeed genetic background could significantly influence tumor development in this model (14). Even allowing for differential tumor latency and hence primary tumor burden among the different strains at time of sacrifice, a significant effect on lung metastasis was also apparent. Using markers of known polymorphisms represented by the various strains, Hunter and colleagues were able to identify regions of the genome that were likely regulators of metastasis (15). A polymorphism in the gene *Sipa1* present at one of these loci correlates strongly with metastatic propensity in mice (16). This gene encodes a GTPase-activating protein and, in the mouse, the polymorphism changes the amino acid sequence such that GAP activity is increased. By using murine mammary tumor cells in which *Sipa1* expression was either augmented or reduced, the investigators could show that metastatic ability was correlated to cellular Sipa1 levels and activity (16).

But do these findings in a transgenic mouse model have any bearing on human cancer? The MMTV-PyMT mouse has been used by a number of researchers and there is now extensive characterization of the tumor development process in this model. Several years ago, Jeff Pollard and colleagues presented a thorough examination of tumor development through different stages in the MMTV-PyMT mouse and were able to conclude that both in terms of histological appearance and expression of various biomarkers, mammary tumors produced in this mouse model are very similar to human breast cancer (17). Expression of the polyoma virus oncogene is not associated with development of human breast cancer, nevertheless, the genetic signature of MMTV-PyMT tumors overlaps considerably with those of mammary tumors from mice carrying activating mutations in the *Her2/neu* or *ras* oncogenes (18). Thus there is reason to believe that all 3 murine models recapitulate the oncogenic signature related to *HER2/NEU* amplification, a frequent event in human breast cancer. In addition, using metastasis development as a feature to correlate with gene expression amongst MMTV-PyMT mice crossed to different strains, investigators were able to show that, with one exception, the same 17 gene signature that can predict metastatic propensity in human solid tumors can also be predictive in this mouse model (19). Taking all these pieces of evidence together, the MMTV-PyMT transgenic model appears highly relevant to human breast cancer. Perhaps the most exciting demonstration of the power of the MMTV-PyMT mouse as a relevant model for human breast cancer also comes from the Hunter lab. Having established that a polymorphism in the *Sipa1* gene strongly correlated with metastatic propensity seen in various mouse strains, this team of scientists then turned to human patients and showed that polymorphisms also exist at the human *SIPA1* gene locus (20). Although the human polymorphisms are not the same as that in the mouse, they appear to have similar effects ultimately changing GAP activity in SIPA1. Notably, human *SIPA1* gene polymorphisms were found to strongly correlate with poor prognosis in breast cancer patients, associating either with lymph node involvement or with estrogen receptor negative tumors. Since the *SIPA1* polymorphisms were detected in genomic DNA isolated from peripheral leukocytes rather than tumor tissue, these findings strongly support the assertion that host genetics affects cancer aggressiveness.

A final piece of evidence, also from the Hunter lab, supporting the existence of a metastasis susceptibility signature encoded within the host genome comes from analysis of non-tumor bearing mice (21). RNA extracted from normal mammary glands or lungs of strains representing either high or low metastatic efficiency was analyzed using RT-PCR for expression of genes represented in the Ramaswamy 17-gene metastasis signature. Although extent of expression varied between the tissues, as might be expected given the differing cell types present, the majority of the genes analyzed could be detected and showed differential expression according to strain. Furthermore, using the completely different technique of mass spectroscopic analysis of peptides in saliva, that also segregates samples according to genotype, a new prediction profile was generated that accurately predicted extent of lung metastasis in approximately 80% of mice of mixed genetic backgrounds (21). In summary then, using multiple techniques and data collected from both a mouse model and a human patient cohort, it appears that there is strong evidence for a contribution of host genotype to metastasis susceptibility in breast cancer.

Another tumor type showing a contribution of host genotype to aggressiveness and metastasis is hepatocellular carcinoma. Hepatocellular carcinoma (HCC) is a common cancer in Asia and Africa but currently occurs with relatively low frequency in Europe and North America (22). However, this is an extremely aggressive cancer with dismal

survival rates. In addition, according to statistics from the American Cancer Society, it is one of the few tumor types that increased in prevalence up to 1999 (23). By far the strongest risk factor for HCC is infection with hepatitis virus although other chronic inflammatory pathologies of the liver including those associated with alcohol consumption or obesity are also linked to HCC development (22). While metastasis development in HCC is common, an unusual feature is that the most frequent site of metastasis is the liver itself. Although surgery is the most effective treatment for HCC, long-term survival rates are not greatly enhanced in surgically treated versus untreated patient groups. This is due to the high rate of recurrence within the liver, likely representative of micro-metastases invisible at the time of curative resection. Thus this is a tumor type where a prognostic signature indicative of the likely presence of metastasis would be highly informative. Interestingly, the 17-gene adenocarcinoma signature identified by Ramaswamy *et al.* as being a predictor of metastasis in multiple solid tumors has no predictive power in HCC (24). Hence a number of groups have worked on HCC-specific gene signatures. Using a cohort of hepatitis B-positive HCC samples, an international group of scientists from the US and China were able to identify a molecular signature that could classify metastasis-positive and negative HCC patients (25). As in previous studies in other tumors, these researchers found that matched primary lesions and metastatic foci were very similar to each other thus suggesting that the genes associated with metastatic propensity were inherent in the primary tumors. Only tumors that had not metastasized were genetically distinguishable. Amongst the top 30 genes associated with metastasis, the authors of this study identified the secreted phosphoprotein osteopontin (SPP1). This gene, also identified by the Massagué group as being important for breast cancer metastasis to bone (8), will be discussed further in a subsequent section.

While the HCC metastasis signature identified by Ye *et al.* was a valuable tool for determining prognosis, it was not perfect with an overall accuracy rate of approximately 78% (25). Since HCC is a cancer intensely associated with the host response mechanisms of fibrosis and leukocyte influx seen in chronic inflammation, Budhu *et al.* reasoned that the gene expression profile of the non-tumor inflamed liver may be an important contributory factor to HCC progression (26). In an extensive study, these researchers analyzed gene expression in non-tumor sections of liver tissue from HCC patients. Surprisingly, they could identify a 454 gene metastasis-associated signature that had significant clusters of genes linked to inflammation and immune response. They could reduce this large signature to a "refined" 17-gene signature that could easily be analyzed by qRT-PCR and which could be used to accurately predict metastasis with a correct classification rate of 93%. In comparison to all routinely used clinical parameters, this refined 17-gene expression analysis is a vastly superior predictor of outcome for metastasis/recurrence in HCC patients (26). Since this signature was generated using samples of liver tissue that did not contain histologically-visible tumor, it is clearly host-derived. One gene within this signature, CSF-1, was further characterized as to its causal role in metastasis and will be discussed in the next section.

Gene Products Contributing to the Metastatic Phenotype

As has been alluded to in previous paragraphs, several of the various profiling studies have identified genes the protein products of which can be demonstrated to have causal roles in metastasis. As space limitations preclude a complete discussion of all the possible gene products involved, this discussion will be limited to 5 gene products identified in different profiling studies but which have also been more fully explored in other studies.

RhoC. In one of the original gene profile studies, melanoma cell lines that had been selected by multiple rounds of *in vivo* passaging for metastatic efficiency were compared with the original non-selected parental cell lines for changes in gene expression (27). The cell lines represented melanomas, of human (A375P) and murine (B16F0) origin. Metastatic variants grew similarly to parental lines when injected subcutaneously indicating that tumor growth characteristics were unchanged by the selection process. A set of approximately 30 genes was found to be upregulated in the metastatic variants. Two major groups within this gene set encode extracellular matrix proteins such as fibronectin and actin-binding proteins. Of this latter group, the most prominent was RhoC. RhoC is one of the Rho GTPase family of proteins that, amongst other functions, can control organization of the cytoskeleton (28). Overexpression of RhoC in the parental lines increased *in vitro* invasive and migratory abilities whereas expression of a dominant negative RhoC construct in metastatic variants significantly reduced invasive and migratory properties (27). These changes in the *in vitro* properties were also reflected by alterations in the *in vivo* metastatic abilities. Together the data support a causal role for invasion and migration, essential features of metastasis, in melanoma.

A number of studies have investigated roles for RhoC in other tumor types. For example, expression of RhoC is strongly correlated with lymph node metastasis in squamous cell carcinomas of the head and neck (29). In a complementary study, Faried *et al.* showed using functional assays in nude mice that expression of RhoC in esophageal squamous cell carcinoma lines induced lung metastasis (30).

Other researchers have documented functional roles in invasion and metastasis in hepatoma cells (31), prostate cells (32) and breast carcinoma cells (33, 34) while evidence for RhoC expression as a marker of poor prognosis has been collected in hepatocellular carcinoma (35), colon carcinoma (36) and breast carcinoma (37). The mechanisms by which RhoC directs invasive and metastatic behavior are beginning to be understood. In melanoma cells, overexpression of RhoC has been reported to activate the PI3K/ AKT pathway, which can be responsible for some of the pro-invasive activites of RhoC (38) and may also contribute to survival of metastatic cells (39).

Osteopontin (OPN). The secreted phosphoprotein OPN (gene name *SPP1*) has been studied by cancer researchers for some time (40, 41), however the results of several profiling studies identifying it as a gene linked to the metastatic phenotype have increased interest in it as a potential target for anti-metastasis drug development (8, 25). OPN exerts its effects through interactions with cell surface receptors including integrins and CD44, which then leads to activation of various downstream signaling pathways including PI3K (42) and NF-κB (43). Trans-activation of the EGF receptor has also been implicated in OPN function (44). Several recent reviews describe the biology of OPN and its role in tumor progression (45-47) and the reader is referred to these for more detailed information. OPN levels have been measured using a number of techniques and correlated with significantly poorer prognosis in various cancer types including prostate (48), squamous cell carcinoma of the tongue (49) and of the esophagus (50), clear renal cell carcinoma (51), uterine cervical cancers (52), uveal melanoma (53), breast (54) and colon (55). In hepatitis-B-associated HCC, where *SPP1* was one of the genes comprising a metastasis signature (25), there are several independent demonstrations of the prognostic value of measuring OPN levels either at the RNA (25, 56) or protein (24, 25, 57) levels.

To test whether OPN contributes directly to the metastatic process, researchers have used different OPN-modulating approaches. Ye *et al.* used an OPN-neutralizing antibody that significantly blocked the invasive abilities of at least 5 HCC cell lines *in vitro* (25). Using a spontaneous metastasis assay, these authors also showed that treatment with the OPN-neutralizing antibody significantly attenuated pulmonary metastasis. A short hairpin RNA to knockdown expression of OPN in an esophageal squamous carcinoma cell line significantly reduced *in vitro* cell motility and invasion as well as lymph node metastasis *in vivo* (50). Similar results on invasion and migration were reported for the human breast carcinoma line MDA-MB-435 in which OPN expression was knocked down using siRNA (58). In addition, the knockdown cells showed reduced ability to grow in soft agar and suppression of tumor take *in vivo* indicating that OPN may contribute to cell survival. In their study of genes responsible for bone-specific metastasis, Kang *et al.* did not list *Spp-1* since, although it was highly expressed in the metastatic variants of MDA-MB-231 cells compared to parental cells, it did not show site specificity but was also over-expressed in tumor cells that metastasized to the adrenal medulla (8). However, one of the other gene products identified as being specifically associated with bone metastasis, IL-11, collaborated with OPN to increase bone metastasis. These researchers showed that over-expression of IL-11 and OPN could significantly increase the metastatic ability of the parental MDA-MB-231 cells. When combined with another gene product identified in the bone metastasis signature, either CTGF or CXCR4, OPN and IL-11 expression was sufficient to fully recapitulate the robust metastatic phenotype. Thus, by either augmenting or reducing expression of OPN in wide range of cell types, it has been demonstrated that OPN is a potent regulator of attributes of the metastatic process including invasion, motility and survival.

Colony-stimulating factor-1 (CSF-1). One of the most intriguing results from the various microarray studies discussed previously was the finding that the levels of the cytokine colony stimulating factor-1 (CSF-1), also called macrophage-colony stimulating factor (M-CSF) appear to be a major determinant of metastasis or recurrence in HCC patients (26). In these studies, CSF-1 was identified as one of a number of inflammation-associated genes whose expression in non-tumor sections of liver could predict recurrence. Further investigation of CSF-1 showed that serum levels were increased in patients with metastatic HCC and that exposure of CD3-positive T cells to this cytokine is sufficient to cause a switch in the cytokine profile from the inflammation-associated Th-1 subgroup to the tumor-associated Th2 subgroup (26).

CSF-1 interacts with its receptor CSF-1R, encoded by the *cfms* protooncogene, on macrophages and their precursors to regulate proliferation, differentiation and survival (59). However, recognition that higher levels of CSF-1 could be detected in patients with invasive breast cancers (60, 61) spurred interest in pursuing it as a cancer-related molecule. Other cancer types also show increased CSF-1 levels associated with malignancy and metastasis (62, 63) although this may be dependent on whether the CSF-1 is tumor cell or stromal cell-derived (64). Studies from the labs of Jeff Pollard and John Condeelis have been especially informative regarding mechanisms of action of CSF-1 in mouse models of breast cancer. The availability of a mouse carrying mutations in both alleles of the *Csf-1* gene, $Csf1^{op}/Csf1^{op}$, has facilitated these investigations (65, 66). Lin *et al.* used the MMTV-PyMT breast cancer model described previously and

crossed it with the *Csf1^{op}/Csf1^{op}* mouse specifically to examine how CSF-1 might be important for any aspect of tumor development (67). There was no significant impact on mammary tumor development or growth, however metastasis to the lungs was significantly attenuated. This correlated with a striking reduction in the number of mammary gland tumors that had progressed to frank carcinomas and with a reduction in the number of macrophages that were recruited to the mammary tumors. The tumor malignancy and associated metastasis phenotype could be rescued in the *Csf1^{op}/Csf1^{op}* mice by over-expression of CSF-1 specifically in the mammary gland. Similarly, mammary-specific over-expression of CSF-1 could accelerate progression to malignancy and enhance metastasis in MMTV-PyMT mice with normal systemic levels of CSF-1. Using state-of-the-art *in vivo* imaging techniques, Wyckoff *et al.* have clarified potential contributions of macrophages to the malignancy of breast cancer (68). A reciprocal interaction exists between the macrophages and tumor cells whereby the tumor cells produce CSF-1 and express the receptor for EGF while macrophages produce EGF and express the receptor for CSF-1. The suggested model is that tumor production of CSF-1 attracts macrophages which in turn contribute to tumor growth and migration by providing a gradient of EGF. Other researchers have used anti-sense and siRNA toward CSF-1 or its receptor to demonstrate its role in growth of breast cancer xenografts (69). As a complementary experiment, Okazaki *et al.* provided CSF-1 to mice inoculated with solid tumors (70). The result was increased systemic levels of vascular endothelial growth factor (VEGF) and concordant increases in tumor angiogenesis. Since CSF-1/M-CSF has been used to treat myelosuppression in cancer patients undergoing chemotherapy, these data suggest that such treatment could be counter-productive.

CXCR4. CXCR4 is the cell surface receptor for the chemokine CXCL12, also known as SDF-1. This ligand/receptor pair has become a hot topic in cancer research, and further confirmation of its importance was provided by the finding of Kang *et al.* that expression of CXCR4 is one of the members of the genetic signature associated with breast-to-bone metastasis (8). One reason for much excitement about CXCR4 as a metastasis regulator is that there are a number of therapeutic approaches already in development since interaction between CXCR4 and its ligand is also an important determinant of disease progression in other diseases including HIV infection (71-73).

In metastasis research, the CXCR4/CXCL12 pair was identified as a critical determinant of site specific metastasis. Muller *et al.* showed that chemokine *i.e.* CXCL12 expression in specific organs appeared to act as a "homing signal" for tumor cells expressing the receptor CXCR4 (74). Furthermore, using a neutralizing antibody to CXCR4, they showed that metastasis of breast cancer cells to regional lymph nodes and to lung was dependent on the receptor/ligand interaction. Similar studies have since been reported for other tumor types including renal cell carcinoma (75), HCC (76), and prostate cancer (77). In their breast-to-bone metastasis signature study, Kang *et al.* demonstrated that expression of CXCR4, when combined with IL-11 and OPN, was sufficient to confer a robust metastasis phenotype on low metastatic efficiency MDA-MB-231 cells (8). Production of CXCL12 by tumor-associated fibroblasts has also been demonstrated to contribute to angiogenesis and tumor growth in a breast cancer xenograft model since expression of CXCL12 siRNA in fibroblasts or treatment with an CXCL12 neutralizing antibody limited endothelial cell recruitment to tumors while expression of CXCR4 siRNA in tumor cells profoundly attenuated tumor growth (78). In a study of colon tumor cell lines, Ottaiano *et al.* demonstrated that CXCL12 induced clonogenic growth which could be inhibited by administration of the drug AMD3100, a non-competitive antagonist of CXCR4. Invasive activity of intrahepatic cholangiocarcinoma has also been shown to be regulated by interaction between CXCL12 produced by fibroblasts and CXCR4 expressed by the tumor cells (79). Again, use of the antagonist AMD3100 attenuated the invasive activity. This drug was also used in a model of peritoneal carcinomatosis where it significantly inhibited tumor growth and ascites production by gastric cancer cell lines (80). In gastric cancer patients, high levels of CXCR4 strongly correlated with the development of peritoneal carcinomatosis suggesting that targeting of this receptor may be therapeutically beneficial for gastric cancer (80). In fact, there are multiple studies where high CXCR-4 levels correlate with disease progression, poor prognosis or reduced survival. Relevant diseases include esophageal cancer (81), oral squamous cell carcinoma (82), pancreatic cancer (83), HCC (76), non-small cell lung cancer (84), colorectal cancer (85, 86) and breast cancer (87-89). Interestingly, in melanoma patients, high CXCR4 levels did not correlate with liver metastasis in contrast to colorectal cancer patients where a strong correlation was evident (85). Nevertheless, high CXCR4 expression in melanoma does correlate with reduced disease-free and overall survival (90). Overall then, there is strong evidence that CXCR4 expression by tumor cells contributes to their metastatic propensity through interactions with the ligand CXCL12. Ligand production by tumor-associated fibroblasts contributes to tumor growth, angiogenesis, clonogenic survival and migration while SDF-1 production in distant organ sites can act to attract metastasizing cells to specific organ sites. Antagonists of CXCR4 are currently in development for cancer as well as other diseases and presumably will be of significant therapeutic benefit.*Collagen*

type I. Several microarray studies have reported upregulation of transcripts representing extracellular matrix components such as fibronectin or various collagens in metastatically-inclined samples (3, 7, 19, 27). The 17-gene solid tumor metastasis signature identified by Ramaswamy *et al.* contains two collagen genes *COL1A1* and *COL1A2*, both of which encode components of type I collagen, a fibrillar collagen that is the most widely expressed protein in the body (7). These associations of metastatic competence with collagen production appear paradoxical since we consider collagens to be structural barriers to invading cells that must be breached for metastasis to occur. While it is inherent in the definition of 'malignant' that there is breakdown of extracellular matrix, collagens are more than merely structural barriers and can influence cellular behavior (91). Matrix molecules can bind and signal to cells *via* the cell surface receptors of the integrin family (92) as well as the discoidin domain receptors (93). The field of mechanotransduction, which describes mechanisms through which mechanical signals can be converted to biochemical signals (94) has dramatically changed how we view matrix molecules. A groundbreaking study from the lab of Valerie Weaver showed how increasing the concentration of collagen changed the "stiffness" in a given environment (95). Cells sensed this stiffer environment though integrins and changed the activation status of various signaling molecules including MAP kinase and Rho. Activation of these signals resulted in development of a malignant phenotype by nonmalignant breast epithelial cells.

In a study of prostate cancer cells, Hall and colleagues demonstrated that expression of the receptor for type I collagen, alpha2 beta1 integrin, is limited to cells that form bone metastases as opposed to visceral metastases (96). The bone microenvironment is particularly rich in type I collagen so it is not surprising that its receptor is present on cells that grow there. When a prostate tumor line was serially passaged on type I collagen, a variant with a high propensity to metastasize to bone *in vivo* resulted (96) These cells also had increased levels of the motility and metastasis-associated protein RhoC (see above). Thus culturing cells on collagen I was sufficient to induce a metastatic phenotype in prostate cancer cells. Pancreatic cancer is a disease that is strongly associated with fibrosis or excessive deposition of collagen matrix (97). Several research groups have now demonstrated that contact with collagen changes the behavior of pancreatic tumor cells rendering them more proliferative (98, 99), motile (100), invasive (98) and resistant to chemotherapeutic agents (99). Together, the results of all these studies show that increased expression of type I collagen can indeed result in malignant behavior by tumor cells that could lead to metastasis.

Since deposition of collagen or fibrosis is a clinical problem in many ways, there has been impetus to develop inhibitors of collagen. One such drug is halofuginone (tempostatin), which is an approved agent for treatment of scleroderma. Halofuginone inhibits collagen alpha I synthesis at the transcriptional level and has demonstrated efficacy in a number of animal models of fibrosis (101). More recently, it has been tested in various cancer models and has been shown to also reduce expression of another metastasis-associated gene, matrix metalloproteinase 2 (*MMP2*) (102-104). In bladder cancer cell lines, halofuginone treatment reduced MMP-2 levels by 50% which correlated with decreased invasive ability by *in vitro* assays (103). Halofuginone treatment of mice in which the bladder cells were injected intravenously caused an 80-90% reduction in metastatic foci in the lungs (103). Several researchers have focused specifically on effects of halofuginone on angiogenesis which could be mediated both by inhibition of collagen synthesis and of MMP-2 (102, 105). Xenograft models of HCC (106) and glioma (107) also showed marked reductions in tumor growth and progression following halofuginone treatment. A significant reduction in lung metastasis was also observed in a chemically-induced HCC model in rats (104), which was thought to be strongly related to halofuginone's effect on MMP-2 production. Results from a phase I clinical trial of halofuginone in patients with advanced solid tumors were recently reported (108). Dose-limiting toxicities were identified as nausea, vomiting and fatigue although some bleeding complications were also noted. However, the trial did identify a dose for phase II studies which should proceed rapidly.

Conclusion

Microarray studies have yielded valuable information that can be used immediately to determine patient prognosis and allow for optimum treatment choices. In addition, the data have changed our perception of metastatic propensity as being a characteristic acquired only by a few, rare cells within the mass of a primary tumor to a more widespread program within tumors. A strong possibility is that some individuals have metastatic propensity encoded within the genome so that when a cancer develops, the likelihood of metastasis is high whereas others do not have this genotype and so metastasis may be solely a consequence of random mutations as has been proposed for many years. Certainly cells other than tumor cells contribute to the development of metastasis through their production of various pro-metastatic proteins. Here we have discussed 5 metastasis-associated genes for which evidence exists from multiple studies for a causal contribution to elements of the metastatic process. Thus these gene products represent excellent drug targets for anti-metastasis therapies.

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Received February 9, 2007
Accepted February 20, 2007