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Tumor cells caught in the act of invading: their strategy for enhanced cell motility

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Invasion of neighboring extracellular matrix tissue, the lymphatic system and blood vessels is a key element of tumor cell metastasis in many epithelial tumors. Understanding the cell motility pathways that contribute to invasion can provide new approaches and targets for anticancer therapy. The recent convergence of technologies for expression profiling and intravital imaging has revealed the identities of some of the genes that contribute to motility and chemotaxis of cancer cells in tumors. In particular, the genes encoding a minimum motility machine are coordinately upregulated in tumor cells collected by an *in vivo* invasion assay. These results support a 'tumor microenvironment invasion model' and provide new target opportunities for cancer therapy.

Introduction

The ability of cancer cells to spread from primary tumors gives rise to a growing tumor burden that is distributed across several sites in the body, resulting in death for many individuals with cancer. Understanding the cellular steps that are used by cancer cells during spreading can form the basis for new ideas for diagnostic, prognostic and therapeutic approaches that might facilitate control over cancer metastasis (see Glossary).

Spreading to other organs relies on cell motility and the invasion of neighboring connective tissue, the lymphatic system and blood vessels. Cell motility has been implicated in the spreading of cancer cells [1–3] and is an essential step in metastasis [4–8]. The invasive process can be enhanced by chemotaxis, in which a gradient of extracellular compounds (often growth factors) is detected by intracellular signal processing pathways, which in turn coordinate cell movement along the gradient [9,10]. For amoeboid cells such as tumor cells, cell motility relies on the actin-based cytoskeleton for both generating protrusions and retracting the rear of the cell in order to generate a motility cycle that results in net translocation [8,11].

Although cell migration and chemotaxis have been studied intensively by *in vitro* assays, intravital imaging has been recently used to define the details of cancer cell migration *in vivo* and this technique has led to several new insights into differences in motility that cancer cells show in tumors as compared with their behavior in culture. The increase in speed and linearity of movement of a subset of tumor cells towards blood vessels *in vivo* is in marked contrast to the typically slow random movement of cells in culture [10]. In addition, tumor cells can use several modes of motility, such as fibroblastic or amoeboid movement, enabling them to invade surrounding tissues of varying matrix density [74]. These features of cancer cells indicate that the phenotype of cell motility is readily adaptable to the microenvironment in which the cells find themselves. The primary tumor is composed of several microenvironments and possibly only a few tumor cells are actively invading at any one time. Thus, bulk analyses of primary tumor properties might not adequately describe the characteristics of invading tumor cells.

The tremendous variation among human cancers implies that specific pathways or mechanisms of growth, apoptosis or metastasis are likely to be important for only a subset of tumor types. In this review, we describe our studies to identify the gene expression patterns that are

Glossary

Cell invasion: A term used in tumor biology to emphasize the pathological and inappropriate spread of tumor cells into neighboring tissues and extracellular spaces.
Cell motility: The active extension and retraction of cell processes, such as filopods and lamellipods, that results in a net movement of the cell body. In fibroblastic movement cells form focal adhesions and make use of matrix metalloproteases, whereas in amoeboid movement cells deform markedly and squeeze through pores in the matrix with relatively little degradation of the matrix.
Cell polarity: The presence of a preferred direction of movement and the polarized distribution of organelles, including microtubules and the actin cytoskeleton.
Chemotaxis: The directed movement of cells in response to a spatial gradient of a soluble compound. Cells are said to chemotax up or down a gradient of a chemoattractant (or repellant).
Intravital imaging: The imaging of cells, structures and tissues in living animals.
Metastasis: The process of tumor cells spreading away from the primary tumor. Metastasis (plural metastases) can also refer to a new growth of tumor cells far from the primary tumor.
Paracrine interaction: Signaling between two different types of cell via secreted molecules.

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present in invasive tumor cells as an approach to defining strategies to impede the spread of cancer cells from tumors. We have placed our emphasis on motility-related genes in order to focus on a potentially rich source of new targets specifically related to metastasis in the face of the overwhelming amount of data that is typically generated in microarray-based studies.

We first summarize the studies of other research groups that have analyzed the expression patterns of invasive and metastatic tumors and highlight the difficulties that are inherent in specifically analyzing invading tumor cells. We then discuss our studies that are based on an alternative approach to isolating invasive tumor cells – namely, the *in vivo* invasion assay. These studies reinforce the concept that changes in the expression status of pathways can be a valuable way in which to interpret variations in gene expression. We conclude with a description of the tumor microenvironment invasion model, which might help us to define how we think about tumor invasion.

Expression analysis of invasive and metastatic tumors

A chief goal of microarray-based expression tumor analysis is to identify genes that are involved in metastasis and patterns of gene expression that will give an indication of the likelihood of metastasis [12]. An ability to predict whether a given primary tumor is likely to have metastasized would have direct effects on therapy design for an individual with cancer. Most studies have concentrated on the analysis of bulk tumor samples that incorporate other tissue elements, including supporting stroma, microvasculature and inflammatory cells (Figure 1a). A potential problem with this method is that primary tumors can show extensive variation: for example, different regions of the tumor might have different growth, histology and metastatic potential and only a few cells in the parental tumor population might be capable of metastasizing [13]. The array data derived from whole tumors inevitably result in an averaging of the expression of different types of cell from all of these diverse regions.

The altered expression of several motility-related genes has been detected in studies of whole tumor tissue (Table 1). The proteins encoded by these genes could be important for tumor cell invasion during metastasis. There is, however, the possibility that these expression patterns reflect the noninvasive cells that make up the bulk of the tumor mass and that the expression signature of invasive tumor cells – arguably the motile population essential for metastasis – might be masked. Under these circumstances, insight into the mechanisms of motility, invasion and metastasis is less likely. If a specific combination of gene expression patterns can be correlated with metastatic ability, however, then such analyses can be predictive of metastatic outcome without needing to understand the mechanism. For example, recent expression profiling studies of primary tumors have suggested that the metastatic potential can be indicated by the overall gene expression pattern of some tumors, enabling gene expression patterns to be used in prognosis [14].

Ideally, to understand motility, invasion and metastasis, the invasive tumor cell population must be isolated and studied directly. In this context, it is important to develop technologies to separate pure populations of invasive cancer cells for gene expression studies. The development of laser capture microdissection (LCM) has been an important advance in this field. LCM was developed at the National Cancer Institute at the National Institutes of Health (Bethesda, MD, USA) and was initially described by Emmert-Buck *et al.* [15]. LCM facilitates the precise isolation of individual cells of interest from a standard histological section of stained tissue by using a microscope to guide laser transfer of groups of cells to a plastic film for further study (Figure 1b). The identification of cells in the tumor is based on cell morphology and location.

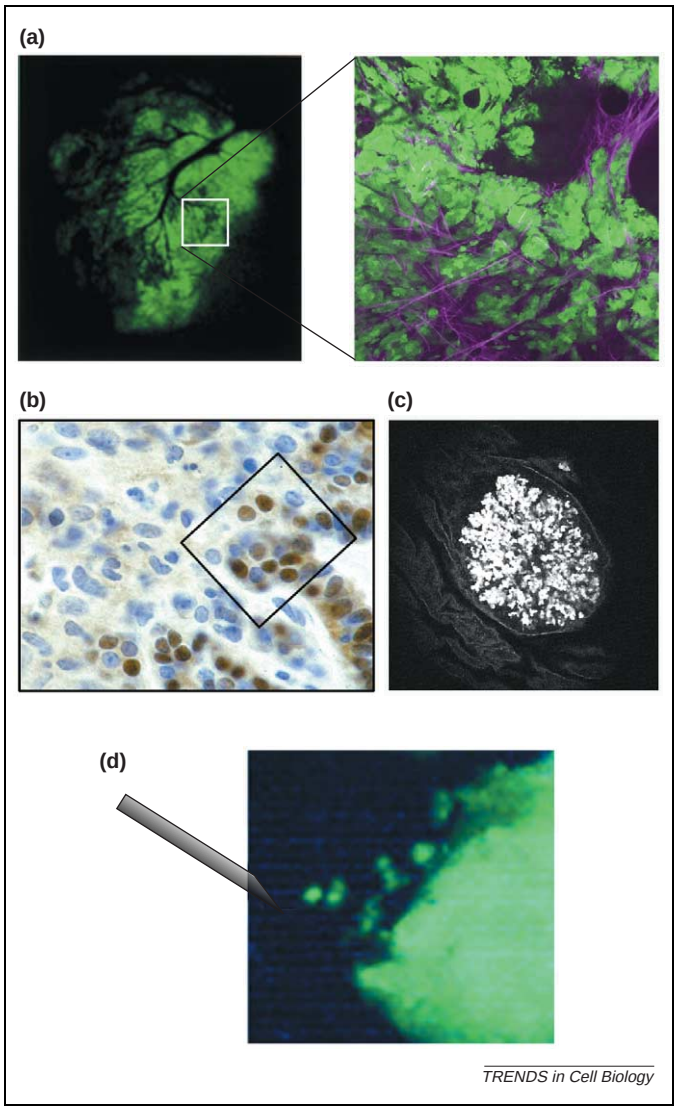

Figure 1. Methods of collecting cells for gene expression profiling. **(a)** Whole primary tumor, in which all types of cell are collected. Shown are low-magnification (left) and high-magnification (right) views of a breast tumor in rat containing carcinoma cells expressing green fluorescent protein (GFP). **(b)** LCM of cells at invasion fronts. The histological section shows an invading margin in a carcinoma and a region (boxed) from which cells would be collected by LCM. **(c)** Metastatic tumors. Shown is a lung metastatic tumor, derived from breast, containing carcinoma cells expressing GFP. **(d)** The *in vivo* invasion assay, in which only the invasive subpopulation of cells are collected by chemotaxis. The breast tumor contains carcinoma cells expressing GFP. The carcinoma cells migrate into the indwelling needle, which contains chemotactic ligands, and are collected.

Table 1. Motility genes associated with invasion and metastasis

Cell/tissue source	Deregulated motility genes
Carcinoma cell lines	Upregulated: AR16[a], AR21[a], EGFR[a] [11]; RhoA[a] [50,51]; CXCR4[a] [52]; Vimentin[a] [53]; ERK5, RhoGDIβ, PAK1 [54]; Mts1/S100[a] [55,56] Downregulated: ZBP1[a], Cttn, Pfn2, Pak1 [11]; RhoGDI2[a], MYRL2, Rac2 [57]
Whole tumors	Upregulated: AR16[a], AR21[a], Cappa1, ROCK2 [11]; Ezrin[a] [58]; PK428 [46]; N-WASP [59]; RhoA[a], RhoC[a], ROCK[a] [33]; Mena[a] [34]; Arp2/3[a] [35]; PAK1[a] [60]; FAK[a] [61]; CFL1, MSN [62]; Mts1 [55] Downregulated: Pfn2, Pak1, ZBP1[a] [11]; MYLK, MYH11 [14]; RhoGDI2[a] [57]
LCM of tumor	Upregulated: RhoC [63]; RhoA [64], 14–3-3σ[a] [65]; S100A4, EGFR[a] [66]; S100A6[a] [67]; HER2[a], HER3[a], HER4[a], FAK[a], MAPK[a], ERK[a] [68,69]; MIF[a] [70]; RhoGDI[a] [71]
Metastasis	Upregulated: CDC42, ROCK1, RAC1, CTNNB1, ACTB [72]; RhoC, Thymosin β4, α-actinin 1, calmodulin [4]
In vivo invasion assay	Upregulated: CAPZA1, Cdc42[a], CAPZA2, MSN, Arp2/3 p16[a], Rho interaction protein 3, LIMK1[a], Palladin, ZYX, CTNNB1, TPM1, RhoA[a], ROCK1[a], TEX9, PIP5K2A[a], EGFR[a], CAPG, ACTN3[a], ANXA5, CRIPT, PKC2[a], AR21, RAB25, VCL, KIF5B, CCT4, CCT3, TUBA4, ITGB1, CFL1[a], KTN1, BLR1, FGFR1 [8] Downregulated: ZBP1[a] [8]

[a]These results have been confirmed by alternative methods, such as quantitative polymerase chain reaction with reverse transcription, northern blotting, western blotting, immunohistochemistry or protein arrays.

Once cells have been successfully transferred, molecular analysis, including DNA and RNA extraction and gene expression analysis, is possible. This technique combines the histology of tumor cells with gene expression and has been used successfully for numerous studies in the field of prostate, breast, lymphoma and lung cancer research [16]. The results from these studies are potentially more informative with regard to the mechanism of invasion than is molecular profiling done on bulk tissue samples. Indeed, changes in the expression of motility-related genes associated with the invasive parts of tumors have been detected (Table 1). Because the cells in the tumor are identified on the basis of their morphology in fixed tissue rather than their behavior before fixation, however, classification of the collected cells as migratory and invasive is not certain.

An alternative approach involves collecting cells from metastatic tumors (Figure 1c). These cells must be expanded in culture either for further cycles of tumor growth [4] or to obtain sufficient cell mass for array analysis [17]. These approaches have identified some interesting candidate genes for invasion (Table 1). In addition, cell lines established from tumors have been used to correlate expression patterns with metastatic potential (Table 1). In all approaches that rely on culturing *in vitro*, however, there is concern that the gene expression patterns might change during culturing, partly owing to cellular responses to the *in vitro* culture conditions, which are likely to be irrelevant to invasion *in vivo*.

To avoid this problem, a direct approach has been developed for the qualitative gene expression profiling of cancer cells that have spread to red bone marrow and axillary lymph nodes. In this approach, carcinoma cells are enriched by immunomagnetic selection from bone marrow, axillary lymph nodes obtained from individuals with breast cancer, and the isolated cell populations are compared by differential display analysis [18]. These immunomagnetic approaches have not, however, identified changes in the expression of motility-related genes.

In summary, studies involving whole primary tumors, subfractions of fixed primary tumors, or cells isolated from metastases have identified candidate genes that might be important in tumor cell invasion. However, such measurements have limitations: analyses of primary tumors are looking at bulk tumor expression, microdissection studies must rely on the uncertainty of morphology and location to select cells that might be invading, and metastases are likely to show properties that are crucial for successful growth in a new site but not necessarily for invasion and motility. Preferably, to determine the gene expression signature of invasion, we would like to catch primary tumor cells that are actually in the process of invading neighboring tissue.

Using chemotaxis to catch invasive cells in the act of spreading

Chemotaxis and the in vivo *invasion assay*

Our research group has approached the challenge of directly collecting tumor cells during the invasion step by making use of one of the properties that is likely to be important for invasion metastasis – namely, chemotaxis [19]. Chemotaxis to blood vessels facilitates the intravasation of cancer cells and their systemic spread. Chemotaxis and cell migration in response to epidermal growth factor (EGF) are correlated with invasion, intravasation and metastasis in animal models of breast cancer [19–21]. EGF is a physiologically relevant stimulus that can be used to mimic tumor cell invasion induced at the borders of tumors near blood vessels because overexpression of the EGF receptor and other members of this receptor family has been correlated with poor prognosis [22] and gradients of EGF receptor ligands can be generated by diffusion from the blood and from stromal cells in the tumor microenvironment [23,24].

Thus, on the basis of the principle of tumor cell chemotaxis to blood vessels, invasive cells from live primary tumors in mice and rats can be collected by using microneedles containing chemoattractants such as EGF and extracellular matrix to mimic chemotactic signals from blood vessels and surrounding tissue [20,25]. We have called this method for collecting invasive tumor cells during migration the '*in vivo* invasion assay' (Figure 1d).

The *in vivo* invasion assay has provided strong evidence for a paracrine interaction between macrophages and tumor cells that results in their co-migration *in vivo* in breast tumors and has suggested a mechanism for how macrophages might contribute to metastasis (Figure 2).

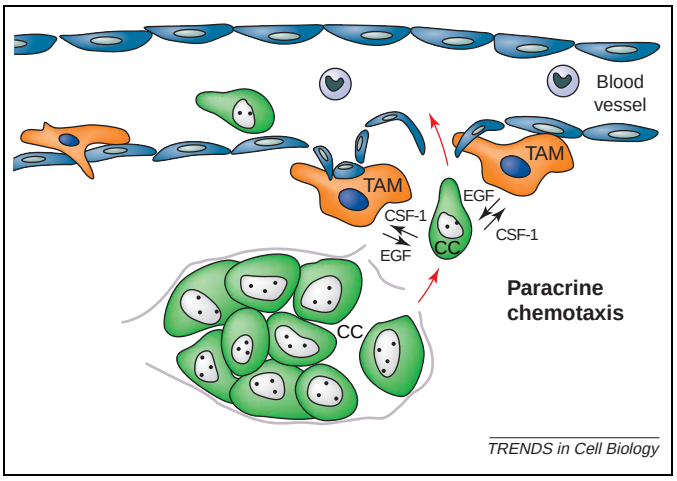

Figure 2. Model of the contribution of macrophages to metastasis. Tumor-associated macrophages (TAM) accumulate near blood vessels that are associated with carcinoma cells (CC) owing to the local secretion of CSF-1 by the carcinoma cells. Macrophages secrete EGF in response, which attracts the carcinoma cells to the vessels by chemotaxis, leading to intravasation. The recruitment of carcinoma cells and macrophages is predicted to be efficient over long distances, owing to the self-propagating paracrine interaction between these cells that leads to relayed chemotaxis.

Intravital imaging during the *in vivo* invasion assay shows that macrophages and tumor cells migrate towards microneedles containing either EGF or colony-stimulated factor 1 (CSF-1) and that the cells are locked together in a paracrine loop that requires signaling by the EGF receptor and the CSF-1 receptor [26]. This paracrine interaction between macrophages and carcinoma cells provides a mechanism for collecting carcinoma cells near blood vessels, where macrophages that are located in the vicinity help to direct carcinoma cells to the vessels (Figure 2) [26]. The existence of a paracrine interaction between cancer cells and macrophages that facilitates invasion has been confirmed by reconstituting paracrine invasion *in vitro* with purified cells [27] and supports other studies that have implicated macrophages in tumor progression [28,29].

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Thus, the *in vivo* invasion assay provides an opportunity to collect primary tumor cells that are actively in the process of invasion. The *in vivo* invasion assay has been combined with array-based gene expression analysis to investigate the gene expression patterns of invasive carcinoma cells during invasion [25]. By comparing gene expression patterns of invasive cells to those of the general population of carcinoma cells in the same primary tumor, patterns of gene expression unique to the invasive subpopulation of cells have been identified [8,30]. The results indicate that the invasive cells constitute a population that is neither proliferating nor apoptotic but is highly motile [8,30]. The lack of proliferation suggests that treatments that target growth pathways might not be very effective at killing invasive tumor cells. The reduction in apoptosis is consistent with the idea that tumor cells have a survival advantage – an idea that is emphasized by the ability of invasive cells to survive conventional chemotherapy as compared with cells that are noninvasive [30].

Pathways involved in tumor cell migration

Of particular relevance to the migratory behavior of invasive cells is the finding that the genes involved in the pathways that support the minimum motility machine – namely, the cofilin, capping protein and Arp2/3 pathways that regulate β-actin polymerization at the leading edge and cell protrusion – are markedly upregulated (Figure 3) [8]. These pathways might account for the enhanced migratory and chemotactic behavior of invasive cells. Several of the genes identified by the *in vivo* invasion assay have been implicated by the other methods shown in Figure 1 and Table 1. Taken together, the results of all approaches have begun to yield insights into the mechanisms of invasion.

When cells crawl, the initial step is the formation of a protrusion. Depending on the cell type and/or location, these protrusions can be long filopodia, broad and flat lamellipodia or tubular pseudopods. Because the site of protrusion sets the cell direction and thus can define chemotaxis, this step in the motility cycle might be central to determining invasive potential. As seen in Figure 3, genes encoding many components of the pathways that cause protrusion, including both stimulatory and inhibitory parts of each pathway, are coordinately upregulated in invading cells. The upregulation of both positive and negative regulatory elements of protrusion pathways might reflect the cyclic nature of cell motility: both protrusion and retraction are necessary for net translocation. Highly motile cells might require an increase in expression of the full motility apparatus, thereby facilitating several repetitions of transients of actin polymerization during chemotaxis [31,32] and enabling cells to adjust rapidly and move according to directional signals [32].

Among the genes upregulated in the EGF receptor signaling pathways that regulate actin polymerization (Figure 3), several have been implicated in invasion and metastasis in other studies. Clinical studies of bladder cancer, breast cancer and colorectal cancer have indicated that RhoA and the Rho kinase ROCK [33], Mena [34] and the Arp2 and Arp3 subunits of the Arp2/3 complex [35], respectively, are upregulated in these cancers. Studies have suggested that formation of the Arp2/3 complex by both neoplastic and stromal cells contributes to an increase in the motility of both types of cell and thus provides suitable conditions for invasion [35].

In previous studies, LIM kinase 1 has been shown to be overexpressed in metastatic breast and prostate tumors [5,36]. For example, overexpression of LIM kinase 1 in tumor cell lines increases their motility and invasiveness *in vitro* [36] and *in vivo* [5]; in addition, reduction of the expression of LIM kinase 1 in metastatic prostate cell lines decreases invasiveness in matrigel invasion assays [36]. These results are consistent with those from the microarray analysis of invasive cells collected by the *in vivo* invasion assay, which has shown that LIM kinase 1 is more highly expressed [8]. This increase in LIM kinase 1, coupled with higher expression levels of cofilin, might lead

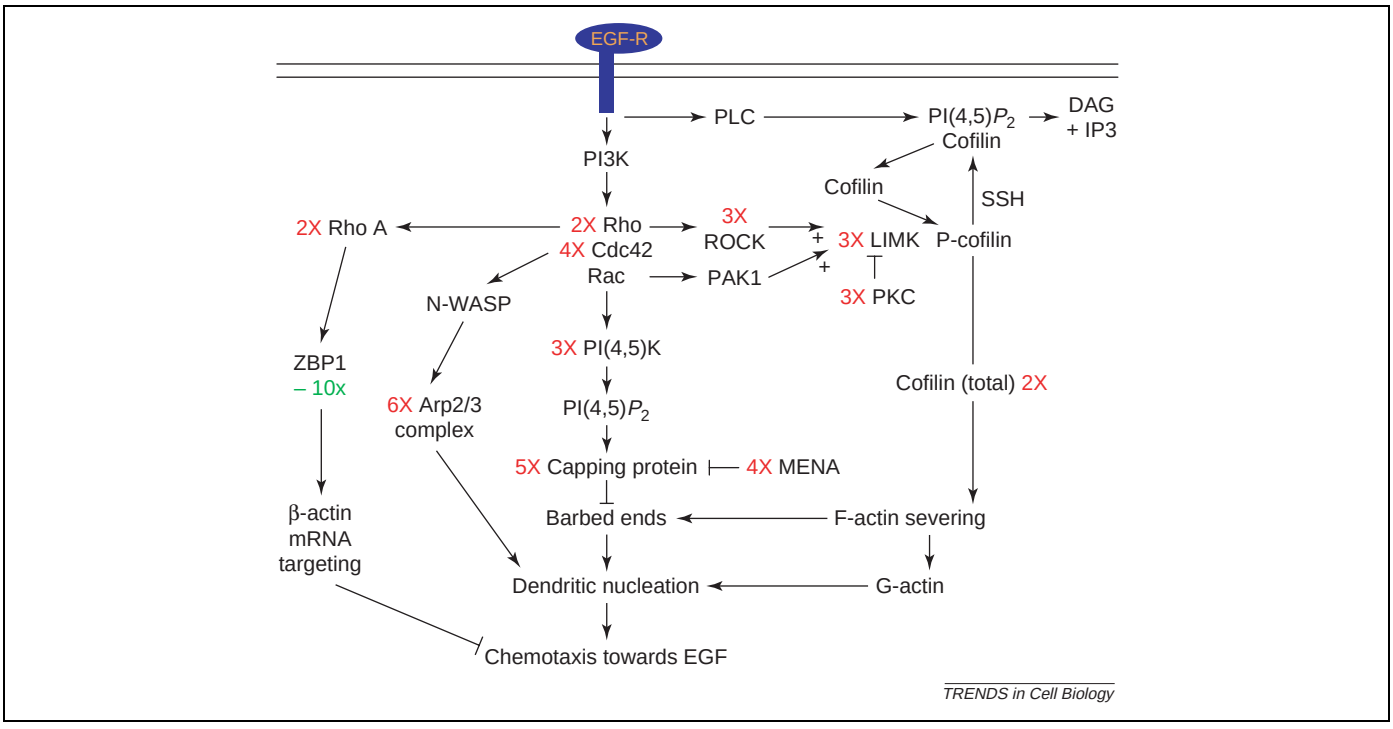

Figure 3. Upregulation of the minimum motility machine pathways in invasive cells collected from tumors by the *in vivo* invasion assay. Shown are the pathways leading from activation of the EGF receptor (EGF-R) to regulation of actin polymerization. In general, genes encoding proteins in the pathways that support the minimum motility machine – namely, the cofilin, capping protein and Arp2/3 pathways that regulate polymerization of β-actin at the leading edge and cell protrusion – are markedly upregulated in invasive cells. Increases (red) or decreases (green) in the mRNA encoding specific proteins in the pathway in invasive tumor cells as compared with their average expression level in the primary tumor are indicated in parentheses. The functions of the affected pathways are understood. Activation of the EGF receptor leads to changes in phospholipase Cγ (PLCγ) and phosphatidylinositol 3-kinase (PI3K), resulting in the activation of cofilin and Rho family proteins. Activated cofilin severs pre-existing actin filaments, triggering the formation of new free barbed ends that initiate cell protrusion and determine cell direction during crawling. LIM kinase (LIMK) phosphorylates cofilin and suppresses its severing activity. In turn, the Rho kinase ROCK, p21-activated kinase 1 (PAK1) and protein kinase Cζ (PKCζ) regulate LIM kinase. Cdc42 can regulate neural Wiskott–Aldrich syndrome protein (N-WASP) and Arp2/3 complex to stimulate invasion [73]. Rac-dependent phosphatidylinositol (4,5)bisphosphate kinase [PI(4,5)K] regulates the ability of capping protein to cap free barbed ends. ZBP1 is required for the targeting of β-actin mRNA, resulting in stable cell polarity. Expression of ZBP1 is downregulated in invasive cells, thereby reducing stable cell polarity and enabling cells to show amoeboid movement and to chemotax. Abbreviations: DAG, diacylglycerol; IP_3, inositol (1,4,5)-trisphosphate; PI(4,5)P_2, phosphatidylinositol (4,5)-bisphosphate; SSH, slingshot.

to local, transient activation of cofilin, resulting in actin polymerization, transient-generated protrusion and cell motility, as has been observed experimentally [37]. Cofilin can also act as a steering wheel during chemotaxis [32] and would increase invasion dependent on chemotactic signals.

In summary, by using chemotactic responses to EGF, we can directly collect the invasive cell subpopulation from primary tumors. Microarray analysis of the gene expression patterns of these cells identifies pathways that regulate protrusion and include subsets of genes that have been identified in other studies of human tumors. Such genes are prime candidates for further studies to evaluate their importance in tumor cell invasion and metastasis.

The importance of pathways that regulate protrusion in cancer invasion and metastasis

The hypothesis that genes that are differentially regulated in invasive cells contribute to the invasive and metastatic phenotype has been examined. For example, a gene that is strongly downregulated in invasive cells is zipcode-binding protein 1 (ZBP1), an RNA-binding protein of 68 kDa that binds to the mRNA zipcode of β-actin mRNA and functions to localize the mRNA to the leading edge of crawling cells. β-actin is the preferred isoform of actin for the polymerization of filaments at the leading edge of cells and therefore is acted upon by the cofilin, capping protein and Arp2/3 pathways [38]. ZBP1 could determine the sites in cells where the Arp2/3 complex, capping protein and cofilin pathways converge by controlling both the sites to which β-actin mRNA is targeted and the location of β-actin protein, which is the common downstream effector of these pathways (Figure 3).

Correct localization of β-actin mRNA is required to generate the intrinsic and stable cell polarity that is characteristic of normal primary fibroblasts and epithelial cells [38–41]. Disruption of ZBP1-mediated β-actin mRNA targeting in cultured cells leads to cells without intrinsic cell polarity that are more amoeboid [38]. Loss of β-actin mRNA targeting is correlated with a loss of intrinsic stable cell polarity and an increase in amoeboid movement in metastatic carcinoma cell lines *in vitro* and *in vivo* [11,41], as well as with an increase in chemotaxis [8]. In addition, both *in vitro* and *in vivo* chemotaxis in tumors is inhibited in cells expressing ZBP1, and tumors prepared from these cells are significantly less invasive and metastatic, although they are unaltered in growth [8].

Thus, ZBP1 is a 'metastasis suppressor' and might act by suppressing the chemotaxis of cancer cells by maintaining them in a polarized epithelial-cell-like state. Cells that lack an intrinsic and stable polarity are more chemotactic to exogenous gradients, presumably because there is no intrinsic polarity to be overcome by the exogenous chemotactic signal and the cell can turn in

any direction to respond to the gradient [42,43]. In carcinoma cells, a gradient of EGF generates transients of actin polymerization that lead to transient cell polarity towards the source of the gradient [32]. In tumors, therefore, cells that have proceeded through the epithelial mesenchymal transition to the point where all remnants of the intrinsic and stable cell polarity of the original epithelium are lost are predicted be more efficient at responding to external chemotactic signals. This might account for the enhanced ability of invasive carcinoma cells to chemotax to blood vessels and to intravasate in metastatic primary tumors [19,21].

These results also suggest that the pathways leading to the minimum motility machine present a rich collection of targets for chemotherapy that have not been previously detected in conventional expression profiling data. The fact that the pathways are coordinately regulated in invasive cells suggests that combinations of therapeutics might be particularly effective. It is difficult to foresee whether targeting such pathways will be more or less toxic than current treatments. For example, inhibitors of ROCK are reasonably well tolerated [44,45], but microtubule-based inhibitors such as taxol and its derivatives are toxic in higher doses (although this might reflect the effect of such inhibitors on cell division process more than their effects on motility).

The tumor microenvironment invasion model

Gene expression profiling of whole tumors with microarrays has shown promise in prognosis by identifying patterns of expression that are correlated with metastasis [14,46]. These patterns of expression do not provide clues about the mechanisms of invasion and metastasis, however, and can appear as random sets of genes with unrelated functions. A particularly surprising feature of such profiling studies is that they support the notion that the invasive and metastatic potential of the primary tumor can be encoded early in the development of the tumor and throughout the bulk of the tumor including the stroma [14,46]. This notion is surprising because the traditional view of tumor progression is that tumors develop through a succession of stable genetic changes in the tumor cells that are selected by growth and survival pressures (Figure 4a). The process has been compared to Darwinian evolution, whereby the units of selection are individual tumor cells, the cells selected to be metastatic are very rare, and the metastases that arise from progressive genetic changes in these rare cells within a primary tumor cause metastasis late during tumor progression [47,48].

A solution to this apparent inconsistency between expression profiling results and the traditional view of tumor progression might be to consider the idea that expression profiling studies of whole tumors of different metastatic potential are looking at a stable average expression pattern. Regions of the tumor that are in the act of invading are not selectively sampled by these methods, as discussed above. By contrast, expression profiling studies of invasive carcinoma cells that are caught in the act of invading inside tumors reveal gene expression patterns that are grouped into pathways of

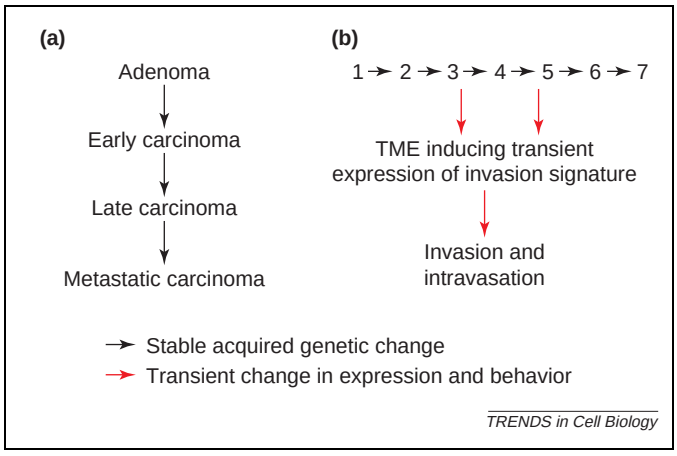

Figure 4. Comparison of the traditional model of tumor invasion and metastasis and the tumor microenvironment invasion model. **(a)** The traditional model of cancer (adenoma) progression to metastasis proposes a series of stable genetic changes that progressively build up a subpopulation of cells with high metastatic potential. This mechanism predicts that metastatic cells arise late, are rare, and remain rare within the primary tumor. Thus, they are unlikely to be represented on expression profiling of the whole tumor mass. **(b)** The tumor microenvironment invasion model of acquisition of the metastatic phenotype. Sequential stable genetic changes (1–7) can give rise to a tumor microenvironment (TME), shown here at changes 3 and 5, that induces transient alterations in gene expression, which in turn drive marked but transient cell migration and intravasation, leading to metastases. According to this model, invasion and micrometastasis can occur early and repetitively. Because the expression patterns giving rise to invasion are transient, they are unlikely to be represented on expression profiling of the whole tumor mass; however, the stable genetic changes that give rise to the microenvironments for invasion will be represented.

functionality and mechanism (Table 1). The patterns of gene expression of invasive cells are usually not seen in their entirety in the expression profiles derived from whole tumors, suggesting that invasion and the patterns of gene expression that support invasion are at least partly transitory. An explanation for the relationship between the stable gene expression patterns seen in whole tumors and cell lines and the transitory patterns of actively invading cells might be that the transient changes that lead to invasion result from the microenvironment of the tumor, which is defined by the stable genetic changes in both stromal and tumor cells detected by whole tumor expression profiling.

We therefore propose a model of invasion called the 'tumor microenvironment invasion model', in which tumor progression leads to the development of microenvironments encoded within the tumor that elicit the transient gene expression patterns that support invasion (Figure 4b). In this model, invasion is proposed to be similar to morphogenesis, whereby the transient expression of genes leads to a change in the position and proliferation status of cells, and stable gene expression, as sampled by whole tumor profiling, supplies the context for where and when invasion occurs. For example, changes in blood supply, oxygen tension and pH are examples of microenvironments that can be generated in different ways by events that are unrelated to specific functional pathways. Such microenvironments might lead to the expression of genes that lead to invasion and metastasis. In turn, the expression of genes that are synergistic for inducing microenvironments that cause invasion could lead to the random appearance, in time and location, of these microenvironments during tumor

progression, leading to repeated episodes of invasion and micrometastasis (Figure 4b).

The tumor microenvironment invasion model hypothesis is consistent with our ability to collect invasive cells by chemotaxis using needles that are placed in random locations in tumors if the growth factors inside the needles mimic the microenvironments that induced invasion as claimed [8,26]. Furthermore, the tumor microenvironment invasion model is supported by intravital imaging of experimental tumors in which only a few tumor cells are motile and these moving cells are not distributed uniformly but are observed in localized areas of the tumor such as near blood vessels [10,11]. In addition, the tumor microenvironment invasion model is consistent with the observation that micrometastases are often genetically heterogeneous, suggesting that invasive behavior is not stably specified [49].

Concluding remarks

Further work on the behavior of invasive cells *in vivo*, and what supports their motility *in vivo*, will be required to test such new models of how tumors spread. A crucial prediction of the tumor microenvironment invasion model is that specific, localized patterns of gene expression in tumor cells (and potentially in stromal cells) will occur in areas of invasion. New methods of *in situ* hybridization that evaluate several genes simultaneously in the same cell will help to test the tumor microenvironment invasion model. In preclinical studies, the functions of proteins that show specific alterations in gene expression in invading cells will need to be measured directly, because gene expression patterns do not evaluate the functional state of the proteins that they encode. Direct inhibition of the expression or function of such proteins will then determine their importance for the invasion process and, consequently, their potential as therapeutic targets.

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