The Great Escape: When Cancer Cells Hijack the Genes for Chemotaxis and Motility

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Abstract

The combined use of the new technologies of multiphoton-based intravital imaging, the chemotaxis-mediated collection of invasive cells, and high sensitivity expression profiling has allowed the correlation of the behavior of invasive tumor cells in vivo with their gene expression patterns. New insights have resulted including a gene expression signature for invasive cells and the tumor microenvironment invasion model. This model proposes that tumor invasion and metastasis can be studied as a problem resembling normal morphogenesis. We discuss how these new insights may lead to a better understanding of the molecular basis of the invasive behavior of tumor cells in vivo, which may result in new strategies for the diagnosis and treatment of metastasis.

Contents

INTRODUCTION

The ability of tumor cells to spread from primary tumors (and metastatic tumors) is the major cause of death in cancer patients. Spreading of tumor cells relies upon cell motility, which results in the invasion of neighboring connective tissue and entry into lymphatics and blood vessels (intravasation) (Clark et al. 2000, Condeelis & Segall 2003, Woodhouse et al. 1997). We focus on tumor invasion and metastasis as a problem in cell motility. In this context, the motility behavior of tumor cells inside the tumor must be analyzed as carefully as the gene expression patterns displayed by invasive cancer cells. During motility, the microenvironment becomes a determinant in the success or failure of a cancer cell in its attempt to traverse the tumor and enter blood and lymphatic vessels (Liotta & Kohn 2001). Subtle changes in the cancer cell's interactions with extracellular matrix and gradients of growth factors and cytokines define whether a cell becomes invasive or remains stationary in the tumor mass. Only by understanding the basic biology of how the motility of cells inside the tumor is influenced by, and influences, the gene expression patterns of cancer cells and their microenvironment will it be possible to define strategies to impede the spread of cancer cells from tumors.

In this review three questions are considered: What motility behaviors contribute to invasion and intravasation? Is there an expression signature that correlates with these behaviors, that is, an invasion signature? How do the genes of the invasion signature contribute to invasion? Answering these questions to date has suggested a novel model for tumor invasion and metastasis, which is discussed at the end of the review.

WHAT MOTILITY BEHAVIORS CONTRIBUTE TO INVASION AND INTRAVASATION?

Intravital Imaging of Tumor Cell Behavior in Tumors In Vivo

An attempt to understand the behavior of tumor cells at single-cell resolution in vivo predates the introduction of green fluorescent protein (GFP) and its derivatives. Tumor cells were transiently labeled with vital dyes and observed with conventional transmitted and fluorescence microscopy (Chambers et al. 1995, Scherbarth & Orr 1997, Suzuki et al. 1996, Vajkoczy et al. 1999, Wood 1958, Yuan et al. 1995). This required the use of short-lived preparations in thin regions of tissue where light could pass efficiently and sometimes the use of viewing windows (Chambers et al. 1995, Wood 1958). These approaches

usually limited the analysis of tumors to artificial locations and introduced the potential for artifact resulting from the viewing method. A major step was the introduction of stable GFP expression, which allowed genetic labeling of cells in tumors with tissue- and cell-type specificity without rejection of the GFP-tagged cells (Chishima et al. 1997, Farina et al. 1998). Thus more clinically relevant tumor models were developed that could be imaged in the location in the animal where the tumor naturally forms and progresses to different stages (Ahmed et al. 2002, Brown et al. 2001, Yang et al. 2000).

The introduction of the laser-scanning confocal microscope was an essential advance that made optical sectioning and single-cell resolution possible, essential capabilities for relating cell behavior to mechanisms of invasion (Chantrain et al. 2004, Farina et al. 1998). However, conventional one-photon laser-scanning confocal microscopy is limited by the relatively poor optical depth of penetration of short wavelength excitation light, photobleaching, and phototoxic damage to the whole tissue and not just at the focal point. The recent introduction of multiphoton microscopy, which uses 800–900 nm light from a pulsed laser, has largely solved the problems of photobleaching and toxicity and extended the depth of penetration by 20-fold (Condeelis & Segall 2003, Helmchen & Denk 2002, Jain et al. 2002, Wang et al. 2002, Williams et al. 2001, Zipfel et al. 2003b). In addition, the multiphoton excitation of tissue causes second harmonic scattering of photons from α-helix-containing proteins, thus allowing the imaging of extracellular matrix proteins such as collagen without the need for fluorescent labeling of the tissue matrix (Campagnola et al. 2001, Condeelis & Segall 2003, Zipfel et al. 2003a). This benefit of multiphoton excitation can be used to analyze cell-extracellular matrix interactions and matrix remodeling directly in live tissue (Condeelis & Segall 2003, Masters et al. 1997). The application of gradient index (GRIN) lens technology to multiphoton imaging holds the promise of extending intravital imaging to any depth within live mice, making systemic analysis of tumor invasion and metastasis possible (Levene et al. 2004). The tumor cell behaviors in live tumors discussed in the next section are derived from studies using laser-scanning confocal and multiphoton imaging in rats and mice.

Motility Behaviors Contributing to Invasion and Intravasation

An understanding of the motility of cancer cells and its contribution to metastasis has begun to emerge from intravital imaging of cells, at single-cell resolution, inside tumors within living animals. An important outcome of studying tumor cells within their normal tumor environment is that the behavior observed is an indication of what tumor cells actually do in vivo, not what they can do as inferred from in vitro and ectopic models in vivo. That is, artificial models such as ectopic growth of tumor cells in tissues in which tumors do not normally form, e.g., growing breast tumor cells in dermis instead of mammary gland, can lead to the observation of cell behaviors that do not occur in real breast tumors. Tumor models where tumor cells are grown in tissue that is the natural site for the tumor, e.g., breast in breast, are called orthotopic models. The behaviors discussed next are those seen in orthotopic models.

A number of behaviors have been observed in orthotopic models in vivo that relate to metastatic potential. These form a pattern common to a variety of tumor types and provide insight into mechanisms of invasion and intravasation (Condeelis & Segall 2003, Farina et al. 1998, Friedl & Wolf 2003, Sahai et al. 2005, Wang et al. 2002, Wyckoff et al. 2000a).

Tumor cells in primary mammary tumors move as solitary cells at up to 10 times their velocity in vitro. Tumor cell motility is characterized as solitary amoeboid movement, but it can also occur as cell streams and linear files suggesting the use of common

paths on extracellular matrix (ECM) fibers (Farina et al. 1998, Friedl & Wolf 2003, Sahai et al. 2005, Wang et al. 2002, Wyckoff et al. 2000a). In fact, the highest velocities are observed for carcinoma cells in metastatic tumors that are moving along linear paths in association with ECM fibers, in particular, collagen fibers. These high-velocity linear excursions are unrestricted by networks of ECM in mammary tumors, except around blood vessels (Condeelis & Segall 2003).

Intravasation and invadopodia. Tumor cell motility is restricted at the basement membrane of blood vessels, where the cells must squeeze through small pores in the basement membrane/endothelium to gain access to the blood space. The degree to which the basement membrane of blood vessels represents a barrier has been documented by direct observations of cell behavior during intravasation. Carcinoma cells in nonmetastatic tumors are fragmented during intravasation as they squeeze across the basement membrane/endothelium indicating that the cell must be highly distended and under tension as it crosses. Remarkably, carcinoma cells in metastatic tumors cross this restriction as intact cells, possibly in large measure owing to the high levels of expression of cytokeratins in metastatic cells (Wang et al. 2002) and their ability to extend invadopodia (Condeelis & Segall 2003, Wang et al. 2002, Wyckoff et al. 2000a, Yamaguchi et al. 2005).

Chemotaxis to blood vessels. Carcinoma cells in metastatic tumors are attracted to blood vessels, where they form a layer of cells that are morphologically polarized toward the vessel. Chemotaxis to epidermal growth factor by carcinoma cells has been demonstrated in vivo (Wyckoff et al. 2004) and resembles that observed for tumor cells in vitro (Wyckoff et al. 2000b). Chemotaxis ability of tumor cells is highly correlated with their potential for invasion, intravasation, and metastasis and appears responsible for the attraction of carcinoma cells to blood vessels (Wyckoff et al. 2000a). Cell polarity toward blood vessels is correlated with increased intravasation and metastasis, indicating a local blood vessel associated source of chemoattractants (Pollard 2004, Wyckoff et al. 2004).

Blood vessel–associated macrophages are a source of EGF and other chemoattractants. An in vivo invasion assay (Wyckoff et al. 2000b) has been used to study the mechanism of chemotaxis in primary mammary tumors of rats and mice. These studies demonstrate that macrophages form a paracrine loop with invasive tumor cells (Wyckoff et al. 2004). Expression analysis of tumor cells and macrophages caught invading together indicates how these cells are attracted to each other and can invade jointly: Tumor cells express CSF-1, which stimulates macrophage chemotaxis, whereas macrophages express epidermal growth factor (EGF), which stimulates tumor cell chemotaxis (Wyckoff et al. 2004). Because metastatic mammary tumors contain large numbers of rapidly moving macrophages with many clustered near blood vessels (Condeelis & Segall 2003, Wyckoff et al. 2000a), they are a local source of chemotactic cytokines and chemotactic growth factors, such as EGF, within the tissue and near blood vessels (Lin et al. 2001, Pollard 2004, Wyckoff et al. 2004). The in vitro assay of invasive cell motility inside collagen matrices demonstrates that macrophages and tumor cells and the activity of their CSF-1 and EGF receptors, respectively, are necessary and sufficient for enhancement of invasion (Goswami et al. 2005).

IS THERE AN EXPRESSION SIGNATURE THAT CORRELATES WITH THESE BEHAVIORS?

The Concept of an Invasion Signature

Gene expression profiling has been used extensively in an attempt to sort tumors into

subtypes that might be diagnosed and treated more effectively (Ramaswamy et al. 2003, van 't Veer et al. 2002). In addition, expression profiling has been used in an attempt to identify invasion- and metastasis-specific genes that might predict the metastatic potential of tumors and to gain insight into the mechanisms of invasion and metastasis. In general, studies involving (*a*) entire primary tumors, (*b*) laser capture microdissection of fixed primary tumors, and (*c*) cells isolated from metastases of bone marrow, lymphatics and distant solid organs have identified candidate genes that might be important for tumor cell invasion (reviewed in Wang et al. 2005). However, such approaches have had limitations:

- Expression analysis of whole primary tumors provides bulk tumor expression patterns, in which case the specific patterns of expression typical of invasive cells might be diluted.
- Laser capture microdissection studies must rely on morphology and histological location, an uncertain exercise, to select cells that might have been invading, thereby making the relevance to invasion of expression profiles from such cells questionable.
- Isolating cells from metastases are likely to produce expression profiles that are relevant to successful growth at the new site but not necessarily profiles indicative of invasion potential from the primary tumor.

In an alternative approach, an in vivo invasion assay, based on the chemotaxis of tumor cells to blood vessels seen in vivo (Wyckoff et al. 2000b), was used to collect invasive cells from live primary tumors in mice and rats. Because the in vivo invasion assay employs microneedles containing chemoattractants such as EGF and extracellular matrix that mimic conditions around blood vessels that are involved in chemotaxis (Wyckoff et al. 2000b, 2004), the invasive cells collected are likely also to be the cells involved in intravasation. Invasive tumor cells collected by this method can then be interrogated directly relative to the tumor cells that remain behind in the primary tumor and, after subtraction of gene expression changes occurring in response to EGF and other collection conditions, this reveals the expression pattern unique to invasive tumor cells, an "invasion signature." The invasion signature shown in **Tables 1**, **2**, and **3** is derived from invasive cells collected in rat mammary tumors generated from carcinoma cell lines (Goswami et al. 2004; Wang et al. 2003, 2004). A similar invasion signature has been derived from mouse mammary tumors resulting from expression of the PyMT oncogene in situ (W. Wang, personal communication). This indicates that the invasion signature is common to mammary tumors in rats and mice regardless of the origins of the mammary tumor. The invasion signature shown in **Tables 1**, **2**, and **3** indicates that invasive cells are a population that is neither proliferating nor apoptotic but is highly motile (Goswami et al. 2004, Wang et al. 2004). The reduction in apoptosis is consistent with tumor cells having a survival advantage owing to suppression of apoptosis genes and up-regulation of prosurvival genes (**Table 2**). Furthermore, the pattern of expression of genes involved in proliferation suggests that invasive tumor cells are not proliferating (**Table 1**). This predicts that cancer treatments targeting cell growth may not be very effective at killing invasive tumor cells. This was tested by exposing invasive cells collected using the in vivo invasion assay to conventional chemotherapy that is directed at dividing cells. As predicted, the invasive cells survived better compared with non-invasive cells from the same tumor (Goswami et al. 2004).

Several of the genes of the invasion signature have been identified in clinical and conventional gene expression profiling studies. Clinical studies of bladder, breast,

Table 1 Genes of the cell proliferation part of the invasion signature[a]

Gene symbol	Gene description	Fold change[b]
Suppression of cell proliferation		
Psmc5	Protease (prosome, macropain) 26S subunit, ATPase 5	5.5
Rad9	Cell cycle checkpoint control protein (Rad9) mRNA	4.0
Hmg1	High mobility group protein 1	3.5
CKS2	Cyclin-dependent kinases regulatory subunit 2	3.4
Cks1	Cyclin-dependent kinase regulatory subunit 1	3.2
Fmo5	Flavin containing monooxygenase 5	3.0
GAS6	GAS 6 mRNA associated with growth arrest	2.8
Phb	Prohibitin	2.8
Mad2	Mitotic checkpoint component Mad2 mRNA	2.4
Madh3	MAD homolog 3	2.4
Hmg14	High mobility group protein 14	2.3
Enhancement of cell proliferation		
CGMC	Carcinoembryonic antigen CGM6 precursor	0.5
CPR2	Cell cycle progression 2 protein (CPR2)	0.4
Ask	Activator of S phase kinase	0.2

[a]To determine the significance of changes in gene expression in each of the functional categories of the genes represented in microarrays, the Student's t test, Chi-square, or SAM analysis were performed. The fold changes in gene expression of the invasion signature shown in **Tables 1–3** were found to be statistically significant in the invasive cells by Chi-square or SAM analysis. In addition, in all cases, $P < 0.05$. Random sets of equal numbers of genes did not generate the same pattern of up- and down-regulation, indicating that the pattern was not observed by chance ($P < 0.05$). Similarly, clustering the results from all genes of the general population in the same space of all genes on the microarray did not yield an outcome similar to the invasion signature. All results are from Goswami et al. 2004 and Wang et al. 2004.

[b]The fold change indicates the level of expression in the invasive tumor cells compared with the general population of tumor cells of the primary tumor.

and colorectal cancers have implicated Rho A, Rock (Kamai et al. 2003), Mena (Di Modugno et al. 2004), and the Arp2 and 3 subunits of the Arp2/3 complex (Otsubo et al. 2004), respectively, as up-regulated in these cancers. Studies have suggested that the elevated expression of Arp2/3 complex by both neoplastic and stromal cells contributes to the increased motility of both cell types and thus provides suitable conditions for invasion (Otsubo et al. 2004). In addition, LIM-kinase 1 is up-regulated in metastatic breast and prostate tumors (Davila et al. 2003, Yoshioka et al. 2003). Hence, the pathways involved in actin polymerization at the leading edge are implicated in invasion by different approaches (**Figure 1**).

HOW DO THE GENES OF THE INVASION SIGNATURE CONTRIBUTE TO INVASION?

Coordinate Regulation of Motility Pathways in Invasion

An important insight into the special motility properties of invasive cells, the high speeds of locomotion, chemotaxis, and invadopod formation, comes from the motility pathways portion of the invasion signature (**Table 3**). That is, the finding that the genes coding for the key effectors of the minimum motility machine (Loisel et al. 1999), i.e., the cofilin, capping protein, and Arp2/3 pathways, that regulate β-actin polymerization at the leading edge, are dramatically up-regulated

Table 2 Genes of the apoptosis and survival part of the invasion signature[a]

Gene	Description	Fold change[b]
Anti-apoptotic genes		
Ier3	Immediate early response 3	4.9
Ubl1a2	Ubiquitin-like 1 (sentrin) activating enzyme subunit 2	4.7
Txn	Thioredoxin	3.7
Hsp105	Heat shock protein, 105 kDa	3.5
Odc	Ornithine decarboxylase, structural	3.0
Dad1	Defender against cell death 1	2.7
Trp53	Transformation related protein 53	2.5
Hsp60	Heat shock protein, 60 kDa	2.4
Api4	Apoptosis inhibitor 4	2.3
Cldn3	Claudin 3	2.3
Api5	Apoptosis inhibitor 5	2.3
Hsp86-1	Heat shock protein, 86 kDa 1	2.1
Api1	Apoptosis inhibitor 1	2.0
Adam17	A disintegrin and metalloproteinase domain 17	2.0
Pro-apoptotic genes		
Pdcd4	Programmed cell death 4	0.1
Fem1b	Feminization 1 b homolog (*C. elegans*)	0.4
Apaf1	Apoptotic protease activating factor 1	0.6
Pdcd8	Programmed cell death 8 (apoptosis inducing factor)	0.8
	Cellular apoptosis susceptibility protein	1.1
	ESTs, highly similar to apoptosis specific protein	1.1
	Apoptosis-associated speck-like protein containing CARD	1.2
AIF	Apoptosis-inducing factor AIF	1.3

[a]For details on methodology and results, please see footnote a to **Table 1**.
[b]For more details, please see footnote b to **Table 1**.

(**Figure 1**) (Wang et al. 2004). Furthermore, the genes of the motility portion of the invasion signature (**Table 3**) can be organized into a series of converging pathways based on the known functions of the proteins for which they code (**Figure 2**) (Wang et al. 2004). The functions of each of these pathways and how they may contribute to the behavior of tumor cells during invasion and intravasation is considered next.

Cofilin pathway. The cofilin family of proteins in vertebrates consists of cofilin/ADF. The cofilin pathway for invasive carcinoma cells is summarized in **Figure 2*a***, with the genes whose expression is altered in invasive tumor cells highlighted. The invasion signature indicates that the cofilin activity cycle has been impacted at several levels of regulation in invasive cells. Cofilin is the more abundant isoform of the family in carcinoma cells, and its expression is highly up-regulated in invasive cells (**Figure 2*a***).

Cofilin's severing and depolymerization activities are inhibited by phosphorylation, G-actin binding, and binding to phosphatidylinositol (4,5)-bisphosphate (PIP_2) (Bamburg 1999, DesMarais et al. 2004a, Paavilainen et al. 2004). Changes in pH can also regulate the level of activity of cofilin, but over the physiological range of pH found in vertebrate cells (6.6–7.4) (Bernstein et al. 2000), the activities of cofilin are only graded, not inactivated, suggesting that pH may act more like a

Table 3 Genes of the motility part of the invasion signature[a]

Accession	Gene description	Fold change[b]
AA414612	Capping protein α1[c]	4.00
AW556230	Cell division cycle 42[c]	3.96
AU015486	Capping protein α 2	3.89
C79581	Moesin[c]	3.67
C86972	Arp 2/3 complex subunit p16[c]	3.52
AW538432	Rho interactin protein 3[c]	3.33
AU015879	LIM-kinase 1[c]	3.24
AA285584	Palladin	3.12
AW555565	Zyxin	2.93
W10023	Catenin β	2.88
C76867	Tropomyosin α chain	2.86
AU023806	Rho-associated coiled-coil forming kinase 1[c]	2.71
AW536576	Testis expressed gene 9	2.67
AI324089	Phosphatidylinositol-4-phosphate 5-kinase type II α[c]	2.60
AI427644	Epidermal growth factor receptor[c]	2.59
AW541453	Capping protein (actin filament), gelsolin-like	2.53
C86107	Actinin α 3[c]	2.52
AW543636	Annexin A5	2.47
AA052404	CRIPT protein	2.32
AA014771	Protein kinase C, ζ[c]	2.30
AW546733	Arp 2/3 complex subunit p21[c]	2.22
AA538228	RAB25, member RAS oncogene family	2.19
AA275245	Vinculin	2.16
AA386680	Kinesin family member 5B	2.13
AW536843	Chaperonin subunit 4 (δ)	2.06
AW536183	Chaperonin subunit 3 (γ)	2.06
AI326287	Tubulin alpha-4 chain	2.05
AW553280	Integrin β 1 (fibronectin receptor β)	2.00
AW536098	Cofilin 1, nonmuscle[c]	2.00
AU017992	Kinectin 1	2.00
AW557123	Downstream of tyrosine kinase 1	2.00
AW549817	Burkitt lymphoma receptor 1	2.00
AA272097	Fibroblast growth factor receptor 1	0.54
AA073514	Zipcode-binding protein 1[c]	0.11

[a]For details on methodology and results, please see footnote a to **Table 1**.
[b]For more details, please see footnote b to **Table 1**.
[c]These results have been validated by quantitative real-time-PCR.

rheostat to regulate the amplitude of activity without acting like an on-off-switch.

The consequence of regulating cofilin by phosphorylation appears to differ by cell type. In some cell types, cofilin is almost 100% phosphorylated in resting cells and motility is stimulated by dephosphorylation (Kanamori et al. 1995, Okada et al. 1996). In carcinoma cells in serum, phospho-cofilin is less than half of the total cofilin at steady state (Zebda et al.

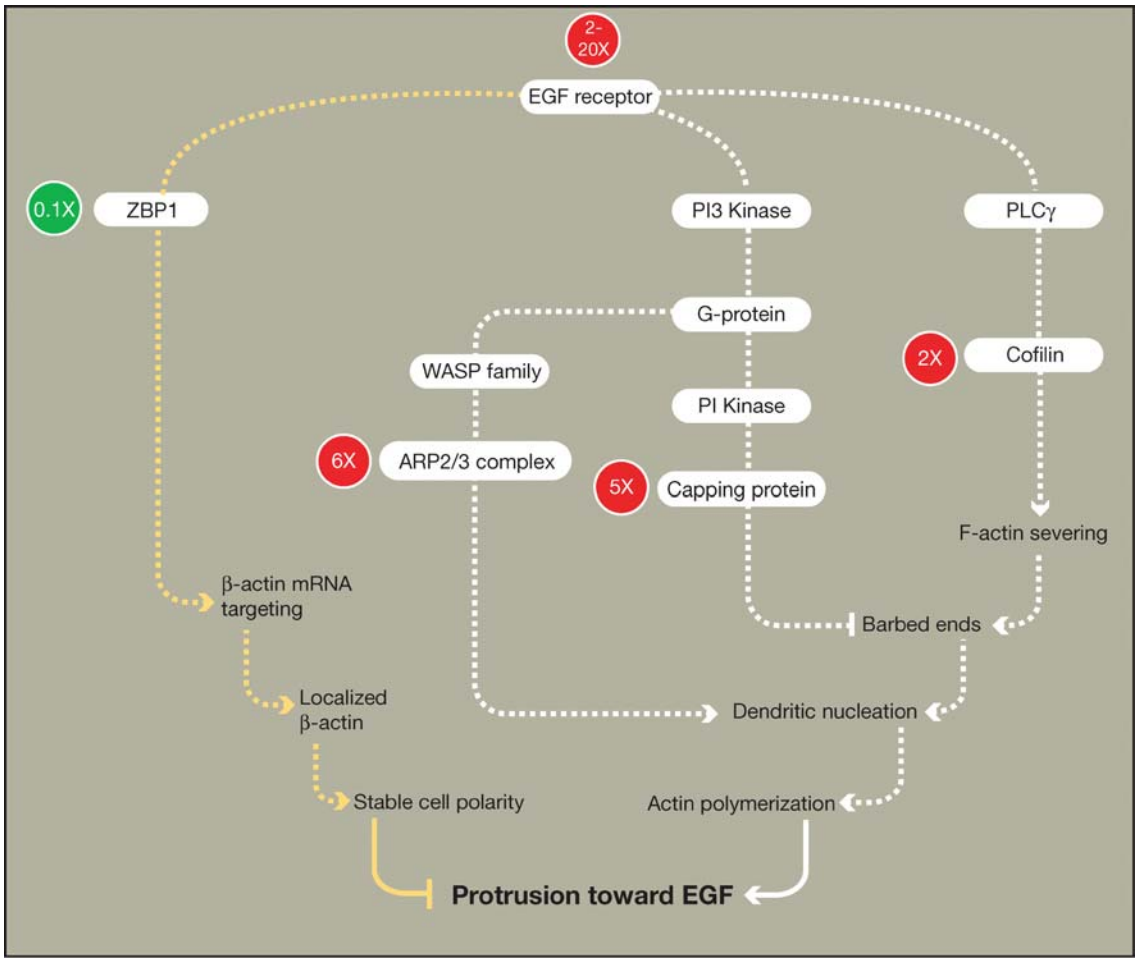

Figure 1

The four pathways leading to β-actin polymerization at the leading edge of carcinoma cells in response to EGF. The major effectors responsible for leading-edge polymerization are cofilin, capping protein, and the Arp2/3 complex. Zip-code-binding protein 1 (ZBP1) regulates chemotaxis to EGF through β-actin mRNA targeting. The fold changes in gene expression in this and **Figure 2** were determined by quantitative real-time PCR and are indicated as (*n*x). Cofilin and Arp2/3 complex are synergistic in the production of free barbed ends leading to dendritic nucleation and protrusive force. Capping protein funnels the available G-actin onto productive elongating barbed ends by capping nonproductive barbed ends. The four pathways therefore coordinately generate protrusions that act to steer the cells during chemotaxis and invasion.

2000), and in serum-starved cells, phosphocofilin is as little as 10% of the total cofilin (X. Song & R. Eddy, personal communication). Even so, cofilin in both cases is mostly inactive (Chan et al. 2000), indicating that a mechanism other than phosphorylation must be at work to inhibit cofilin activity in carcinoma cells.

Another function of phosphorylation of cofilin in carcinoma cells is the recycling of cofilin from G-actin. Cofilin binds to G-actin with submicromolar affinity and the heterodimer is inactive in both severing and depolymerization (Bamburg 1999, Paavilainen et al. 2004). The release of cofilin from this heterodimer is crucial to the

recycling of cofilin activity. Cyclase-associated protein (CAP) is capable of releasing cofilin from the heterodimer through a direct interaction with actin (Bertling et al. 2004, Paavilainen et al. 2004). In addition, because phospho-cofilin cannot bind to actin, LIM-kinase may also be involved in breaking the G-actin-cofilin heterodimer in vivo. Phosphorylation may also function to put limits on the amplitude, location, and duration of cofilin activity after its activation by EGF. Hence, while the phosphorylation/dephosphorylation cycle of cofilin may not be directly involved in the activation of cofilin in carcinoma cells by EGF, LIM-kinase, along with CAP, may be crucial in regulating the localization and recycling of cofilin activity.

Four different kinases that appear to be downstream of the Rho-family GTPases have been shown to phosphorylate cofilin, LIM-kinase 1 and 2, and TES-kinase 1 and 2 (Arber et al. 1998, Dan et al. 2001, Rosok et al. 1999, Toshima et al. 2001, Yang et al. 1998). In invasive carcinoma cells, LIM-kinase 1 is

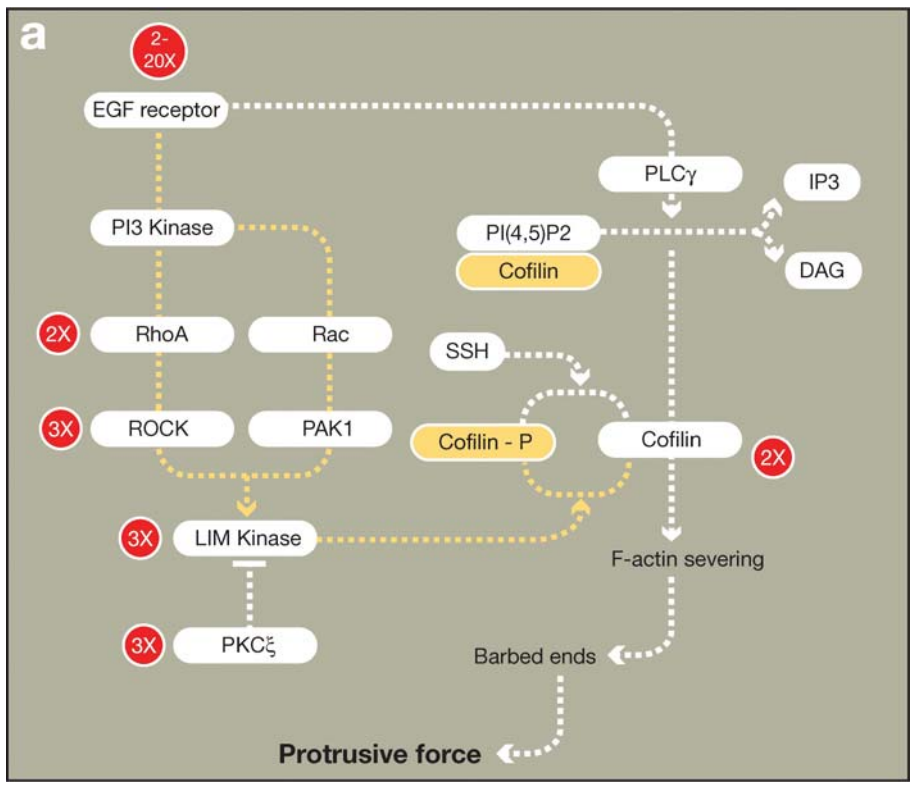

Figure 2

The pathways to barbed end generation and protrusive force. The fold changes in gene expression are indicated as (*n*x). (*a*) The cofilin pathway leading to barbed end production in response to EGF. Gene for both inhibitory (PAK, ROCK, LIM kinase) and stimulatory (PLC and PKCζ) inputs to cofilin are more highly expressed in invasive cells; these regulate the location, timing, and sharpness of cofilin-dependent actin polymerization transients that are required for chemotaxis. For *a*, the inhibitory parts are in yellow and the stimulatory parts in white. (*b*) The capping protein pathway leading to barbed end capping. Genes for both inhibition (Mena and PI5K) and stimulation (capping protein) of the capping activity of this pathway are more highly expressed in invasive cells. (*c*) The Arp2/3 complex pathway leading to dendritic nucleation in response to EGF. Genes coding for Arp2/3 complex subunits and upstream activators are more highly expressed in invasive cells.

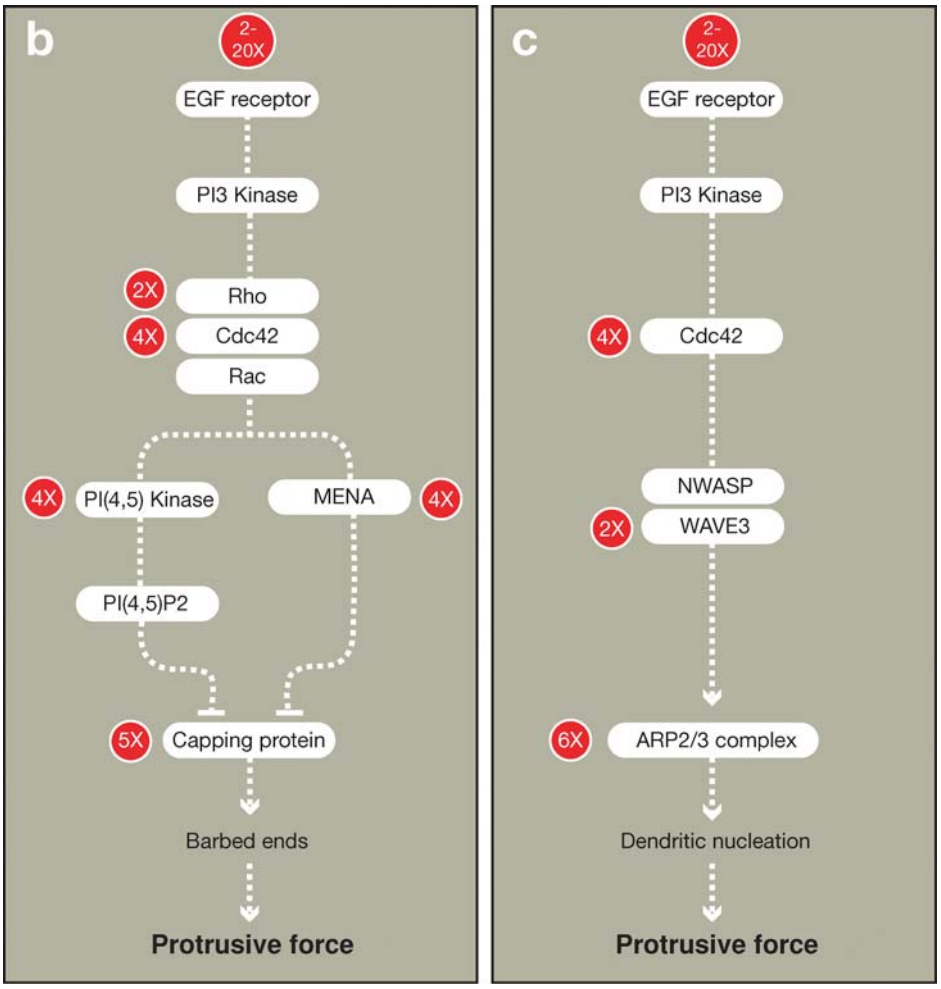

Figure 2
(*Continued*)

most prominently expressed, and its expression is up-regulated in invasive cells (Wang et al. 2004) (**Figure 2*a***). Furthermore, the activation of LIM-kinase 1 occurs through the PI3K-induced activation of Rho-family G-proteins, which activate PAK and ROCK. Rho is highly expressed in invasive cells. Either PAK (Edwards et al. 1999) or ROCK (Ohashi et al. 2000) can phosphorylate LIM-kinase at threonine 508 thereby activating it to increase cofilin phosphorylation. Both kinases are also up-regulated in invasive cells (**Figure 2*a***).

Inhibition of LIM-kinase activity is PKC dependent, and this involves one of the atypical PKC isoforms (Djafarzadeh & Niggli 1997, Kuroda et al. 1996). LIM-kinase and PKCζ tightly associate via the interaction through the second LIM domain of LIM-kinase, which indicates direct phosphorylation of LIM-kinase (Kuroda et al. 1996). Additional studies have implicated the δ isoform of PKC as a negative regulator of LIM-kinase (Martiny-Baron et al. 1993). The expression of PKCζ is up-regulated in invasive cells (**Figure 2*a***).

The general pattern of regulation in the cofilin pathway indicates that genes coding for proteins that both increase and decrease the activity of cofilin are coordinately up-regulated along with cofilin itself. This pattern may result from the toxicity of elevated cofilin expression (reviewed in Ghosh

et al. 2004), where expression of inhibitory genes is essential to maintain higher levels of cofilin. Alternatively, the significance of this paradoxical pattern may be understood when one considers that the cofilin pathway is directly involved in sensing during chemotaxis of carcinoma cells to EGF (Mouneimne et al. 2004), and cofilin is sufficient to set the direction of cell movement (Ghosh et al. 2004). Directional sensing of EGF requires an early transient of free, actin filament barbed ends resulting from cofilin severing that causes localized actin polymerization (Chan et al. 2000, Mouneimne et al. 2004). If the free barbed ends of the early transient are either inhibited or sustained, then directional protrusion in response to EGF fails (Chan et al. 2000, Mouneimne et al. 2004, Zebda et al. 2000). That is, it is the generation of a transient of free barbed ends that is essential in directional sensing, not sustained polymerization. The up-regulation of genes that both increase and decrease cofilin severing activity, as seen in **Figure 2*a***, is consistent with the enhanced ability of invasive cells to generate an early transient that is essential for chemotaxis. In addition, the localization and timing of the stimulatory and inhibitory branches of the cofilin pathway are believed to determine the precise location and duration of cofilin activity and its recycling to compartments where cofilin is inhibited in resting cells (DesMarais et al. 2004a).

Capping protein pathway. Capping protein binds to the growing barbed ends of actin filaments to prevent further elongation and regulate filament length. The patterns of regulation of genes of the capping protein pathway exhibit the same antagonistic relationships as seen in the cofilin pathway where expression of stimulatory and inhibitory branches are up-regulated together. Expression of both the α and β-subunits of capping protein is dramatically increased, suggesting higher capping protein activity in the pathway. However, the expression of genes that code for proteins that are inhibitory to capping protein activity, the type II-α isoform of PI4, 5 kinase (Cooper & Schafer 2000) and Mena (Bear et al. 2002), are also up-regulated (**Figure 2*b***). Capping protein, like cofilin, is essential for viability, and large changes in its expression level may not be tolerated by cells over time (Cooper & Schafer 2000). Therefore, a more interesting interpretation of these results is that the amplitude and sharpness of capping protein activity as a transient is increased in invasive cells because of this antagonistic pattern of expression. The combination of heightened transient capping protein activity and changes in its timing and location could synergize with the barbed end generating activities of the cofilin and Arp2/3 pathways (**Figure 1**) to cause intense focal bursts of actin polymerization, as observed in in vitro experiments with purified proteins (Carlier 1998, Loisel et al. 1999).

Arp2/3 complex pathway. Both the cofilin and capping protein pathways converge on the Arp2/3 complex. Because the expression of key components of both pathways is up-regulated, it is interesting that the expression of several subunits of the Arp2/3 complex are also greatly up-regulated in invasive cells, as is the expression of upstream stimulators of the Arp2/3 complex, WAVE 3 and Cdc42 (**Figure 2*c***).

Cofilin and Arp2/3 complex synergistically contribute to the nucleation of a dendritic array both in vitro (Ichetovkin et al. 2002) and in vivo (DesMarais et al. 2004b). This synergy results from the amplification of the Arp2/3 complex's nucleation activity by cofilin's severing activity, which creates barbed ends that elongate to form newly polymerized actin filaments (Ichetovkin et al. 2002). The newly polymerized filaments are the preferred filament type for Arp2/3 complex-mediated branching (DesMarais et al. 2004a,b; Ichetovkin et al. 2002). This synergistic amplification of the Arp2/3 complex activity has been proposed to explain the ability of cofilin to determine sites of protrusion and cell direction in uncaging

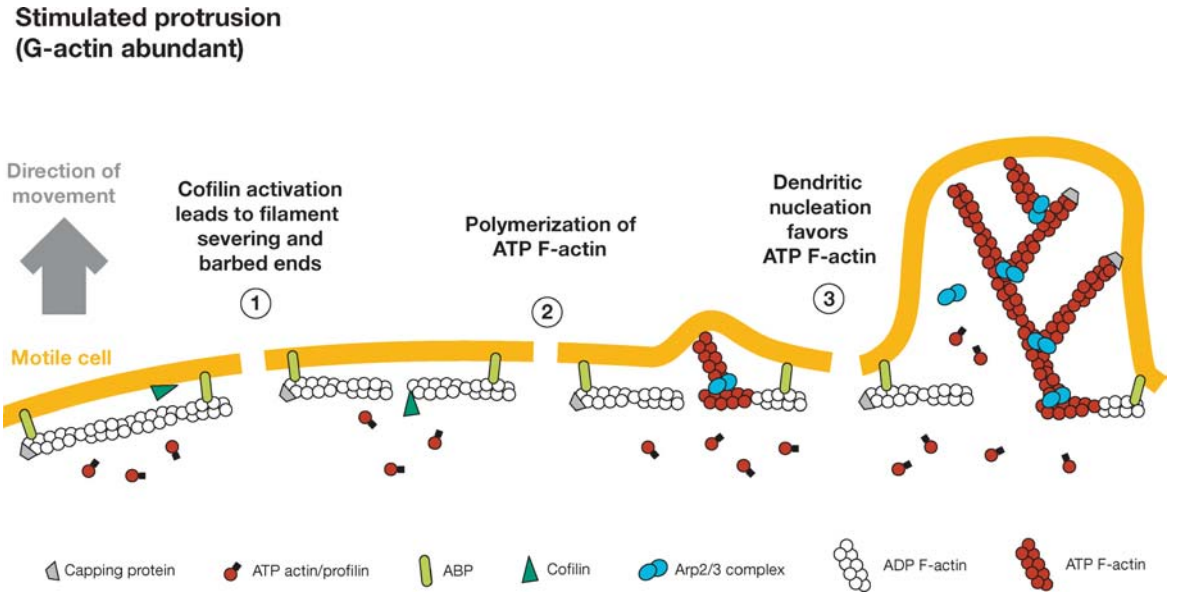

Figure 3

The stimulated protrusion model showing the role of cofilin severing in determining the site of dendritic nucleation, protrusion, and cell direction. Severing of actin filaments in the cortical actin cytoskeleton by cofilin creates free barbed ends that bias the location and the amount of dendritic nucleation by the Arp2/3 complex. Polymerization proceeds from a pool of pre-existing actin monomers, allowing the initiation of polymerization to occur without being tightly coupled to depolymerization. Redrawn from DesMarais et al. (2004a).

experiments (DesMarais et al. 2004a, Ghosh et al. 2004) (**Figure 3**).

Capping protein funnels actin monomers onto newly created free barbed ends by capping older filaments, thereby enhancing the formation of the short, branched filaments characteristic of Arp2/3 complex-nucleated dendritic arrays (Carlier 1998).

WAVE 3 is believed to activate the Arp2/3 complex, as do its relatives WAVEs 1 and 2 (Takenawa & Miki 2001). Both WAVEs 1 and 2 are regulated by Rac 1, which regulates their interaction with Arp2/3 complex to cause stimulation of the Arp2/3 complex's nucleation activity (Eden et al. 2002, Miki et al. 2000, Steffen et al. 2004). This in turn causes lateral (WAVE 2-dependent) and dorsal (WAVE 1-dependent) protrusions (Suetsugu et al. 2003). However, the molecular mechanism of regulation of WAVE 3 is unknown, as are the phenotypic consequences on cell behavior of stimulating WAVE 3. More work will need to be done on the consequences of WAVE 3 expression and activation in carcinoma cells to understand its significance for tumor cell invasion.

An interesting finding is the coordinated up-regulation of expression of genes for several subunits of the Arp2/3 complex and Cdc42. Cdc42 regulates N-WASP, a ubiquitous member of the WASP family, which induces actin polymerization by activating Arp2/3 complex (Ho et al. 2004). Regulation of the activity of N-WASP involves an intramolecular interaction by which the VCA (verproline/cofilin/acidic) domain, the active site that binds to Arp2/3 complex, is masked by the N-terminal regulatory region of N-WASP (Kim et al. 2000, Rohatgi et al. 2000). The known regulators of N-WASP activity appear to operate by either stabilizing or destabilizing this autoinhibitory

conformation (Ho et al. 2004). Elevated expression of Cdc42, as observed in invasive cells, in combination with the elevated expression of Arp2/3 complex (**Figure 2*c***), would be expected to enhance the activity of the N-WASP-Arp2/3 complex pathway, thereby leading to increased invadopod production and cell invasion (Mizutani et al. 2002, Yamaguchi et al. 2005). Cofilin also has been found to amplify and stabilize N-WASP generated invadopods, suggesting that the synergistic interaction between the cofilin and Arp2/3 complex pathways described above is at work during invasion (Ghosh et al. 2004, Yamaguchi et al. 2005).

An additional consequence of increased Cdc42 expression might be its effect on the ability of tumor cells to acquire polarity to blood vessels, as observed during intravasation. Carcinoma cells in metastatic tumors are attracted to blood vessels, where they form a layer of cells that are morphologically polarized toward the vessel. This vessel-directed polarization is believed to be important for intravasation (Condeelis & Segall 2003, Wyckoff et al. 2000a). Chemotaxis undoubtedly contributes to the accumulation of cells around the vessels, but the acquisition of vessel-directed polarity might require additional steps in the reorganization of the cytoskeleton.

A consensus has been building that Cdc42 is involved in determining the direction of cell movement and cell polarity. Inhibition of Cdc42 prevents macrophage migration toward a chemotactic signal (Allen et al. 1998) and directional migration in astrocytes (Etienne-Manneville & Hall 2001). The development of stable cell polarity in astrocytes involves Cdc42, the orientation of the microtubule organizing center, and depends on microtubule dynamics (nocodazole-sensitive) but not on actin polymerization (Etienne-Manneville & Hall 2001, Gundersen et al. 2004). In microtubule-dependent cell polarization in astrocytes, Cdc42 activation involves recruitment of a GEF, FGD-1 and appears to operate through Par6 to recruit PKCζ to inactivate GSK3b (Etienne-Manneville & Hall 2003). This pathway requires stimulation of integrins because arginine-glycine-aspartate (RGD) peptides inhibit the activation of Cdc42, PKC, and protrusion (Etienne-Manneville & Hall 2001).

However, chemotaxis by tumor cells requires actin polymerization and is unaffected by concentrations of nocodazole (100 nM) sufficient to block microtubule dynamics and inhibit cell polarity in astrocytes (Segall et al. 1996). In addition, N-WASP, a major effector of Cdc42, is required for invadopod formation (Yamaguchi et al. 2005) and chemotaxis in tumor cells (L. Soon, personal communication), indicating that cell polarization toward EGF in these cells is dependent on Cdc42 through N-WASP and actin polymerization. During N-WASP- and actin-dependent events at the leading edge of lamellipods, the Cdc42 GEF, intersectin 1, binds to and is activated by N-WASP (Hussain et al. 2001). Therefore, N-WASP may recruit, through intersectin 1, GDP-Cdc42 and activate it locally at the leading edge, making N-WASP function in the polarization to EGF of tumor cells analogous to that of Par6 in cell polarity during wounding. Furthermore, PKCζ may be involved in the regulation of LIM-kinase activity, which may regulate the amount of active cofilin at the leading edge and its synergy with N-WASP/Arp2/3 complex–mediated protrusion activity (**Figure 1**). These results illustrate parallels between how cells polarize (microtubule-dependent) and chemotax (actin-dependent) and suggest that N-WASP is involved in assembly of a compartment at the leading edge, analogous to the polarity complex in polarizing cells (Etienne-Manneville & Hall 2001), that is required for cell polarity during chemotaxis.

ZBP1 pathway. A gene whose expression is strongly down-regulated in invasive cells is ZBP1 (**Figure 1**). ZBP1 is a member of a family of RNA-binding proteins that contain four C-terminal hnRNP-K homology domains

and two N-terminal RNA recognition motifs (Yaniv & Yisraeli 2002). ZBP1 is a 68-kDa RNA-binding protein that binds to the mRNA zip-code of β-actin mRNA and functions to localize β-actin mRNA to the leading edge of crawling cells. Because β-actin is the preferred isoform of actin for the polymerization of filaments at the leading edge of cells, it is acted on by the cofilin, capping protein, and Arp2/3 pathways (Shestakova et al. 2001). β-actin mRNA localization is required for the maintenance of stable cell polarity as observed in the absence of exogenous signals such as that seen in normal primary fibroblasts, epithelial cells, and tumor cells with differing metastatic potential in which actin polymerization is nucleated at only one pole of the cell in normal and nonmetastatic tumor cells (Shestakova et al. 1999). Disruption of ZBP1-mediated β-actin mRNA targeting in cultured cells leads to cells without cell polarity that are able to nucleate actin polymerization globally and exhibit amoeboid movement (Shestakova et al. 2001). Therefore, ZBP1 may determine the sites in cells where the Arp2/3 complex, capping protein, and cofilin pathways converge to determine the leading edge and cell polarity by controlling the sites of targeting of β-actin mRNA and the location of β-actin protein that is the common downstream effector of these pathways.

Tests of Function of Genes of the Invasion Signature in Chemotaxis, Invasion, and Metastasis

The genes of the motility part of the invasion signature can be organized into three converging pathways based on the known functions of the proteins for which they code (**Figure 2**). The functions of key gene products in these pathways and how they affect chemotaxis, invasion, and metastasis by carcinoma cells have been tested. The results of these tests are described next.

Cofilin and LIM-kinase. Direct tests of cofilin function are complicated by the fact that cofilin is required for viability, which makes genetic approaches in carcinoma cells difficult to interpret. However, the acute inhibition of cofilin activity in carcinoma cells inhibits the generation of barbed ends and actin polymerization at the leading edge in response to EGF (Chan et al. 2000, DesMarais et al. 2004b). Inhibition of cofilin activity, through either the inhibition of PLCγ or direct inhibition using acute siRNA suppression of cofilin expression and cofilin function blocking antibodies, inhibits the early barbed end transient that is essential for the chemotaxis of carcinoma cells to EGF (Mouneimne et al. 2004). Furthermore, cofilin is required for the formation of the stable invadopods by carcinoma cells that are important in the invasion of dense extracellular matrix (Mullins et al. 1998, Yamaguchi et al. 2005), particularly that found around blood vessels (Condeelis & Segall 2003). Finally, the local activation of cofilin in carcinoma cells is sufficient to generate protrusive activity and determine cell direction (Ghosh et al. 2004). All these results indicate that cofilin is essential for the chemotaxis and invasion of mammary carcinoma cells to EGF through a mechanism involving the localized generation of barbed ends that causes the localized protrusion, which defines cell direction (**Figure 3**).

The effects of altering LIM-kinase expression have been studied in tumor cells by several groups, who have shown that overexpression of LIM-kinase 1 in tumor cell lines increases their motility and invasiveness in vitro (Davila et al. 2003, Yoshioka et al. 2003). Experimental reduction in the expression of LIM-kinase 1 in metastatic prostate cell lines decreased invasiveness in matrigel invasion assays. To study the effect of LIM-kinase 1 on metastasis in vivo, an experimental mestastasis model was used where cells were injected directly into the left ventricle of mice (Yoshioka et al. 2003). In this case, the ability of cancer cells to survive in the blood, extravasate from blood vessels, and grow at metastatic sites all contribute to the metastasis score, so it is not clear how these

results relate to invasion in the primary tumor. In general, these results are consistent with the observed overexpression of LIM-kinase 1 in invasive cells in mammary tumors and their invasion signature (**Table 3**, **Figure 2*a***).

In a separate set of studies, the overexpression of either full-length-regulated LIM-kinase 1 or its constitutively active kinase domain has been reported to inhibit cofilin activity in vivo by phosphorylation. Overexpression also inhibits EGF-induced barbed end production, in particular the early barbed end transient, and lamellipod extension in culture (W. Wang, G. Mouneimne, J. Wyckoff, X. Chen, M. Sidani, and J. Condeelis, unpublished data; Zebda et al. 2000). Furthermore, the overexpression of full-length LIM-kinase 1 in carcinoma cells without altering cofilin expression is correlated with the inhibition of chemotaxis, invasion, intravasation, and metastasis of tumor cells in mammary tumors prepared from these carcinoma cells (W. Wang, G. Mouneimne, J. Wyckoff, X. Chen, M. Sidani, and J. Condeelis, unpublished data). Although these results appear contradictory to those described above, in fact they are consistent with the invasion signature associated with the cofilin pathway (**Figure 2*a***). That is, highly invasive cells up-regulate LIM-kinase 1, cofilin, and their stimulatory and inhibitory effectors together (**Figure 2*a***), consistent with the hypothesis that the up-regulation of both inhibitory and stimulatory branches of the cofilin pathway increases the amplitude and sharpness of cofilin-dependent actin polymerization transients that are essential for chemotaxis and invasion in carcinoma cells (Mouneimne et al. 2004; W. Wang, G. Mouneimne, J. Wyckoff, X. Chen, M. Sidani, and J. Condeelis, unpublished data). Therefore, to compare studies in which the expression of LIM-kinase, cofilin, or other members of this pathway are experimentally altered, it is essential to measure the output of the cofilin pathway as the timing and amplitude of cofilin-dependent barbed end production during chemotaxis. Manipulations that increase the cofilin-dependent barbed end production of the early transient during chemotaxis are predicted to increase invasiveness, and this predicts that studies in which cells are more invasive and metastatic after overexpression of LIM-kinase have associated compensatory increases in the expression of other members of the cofilin pathway so as to increase barbed end production in response to EGF. Additional work will be required to investigate this possibility.

N-WASP. N-WASP has been implicated in invasion of extracellular matrix in a number of studies. The invasion of Madin-Darby canine kidney cells during tubulogenesis in collagen gels is inhibited by expression of dominant-negative N-WASP (Yamaguchi et al. 2002). Furthermore, N-WASP, in cooperation with cofilin, is required for the formation of invadopods (Yamaguchi et al. 2005), and its activity is localized to nascent invadopods during the invasion of fibronectin gels (Lorenz et al. 2004). In particular, the depletion of N-WASP or the p34arc subunit of Arp2/3 complex by siRNA interference suppresses invadopod formation. In addition, siRNA interference and dominant-negative mutant expression analyses revealed that cofilin and the N-WASP regulators, Nck1, Cdc42, and WIP, but not Grb2 and WISH, are necessary for invadopod formation (Yamaguchi et al. 2005). EGF receptor kinase inhibitors block the formation of invadopods by carcinoma cells in the presence of serum, and EGF stimulation of serum-starved cells induces invadopod formation. These results indicate that EGF receptor-activated N-WASP and cofilin are required for the formation of invadopods and that Nck1 and Cdc42 mediate the signaling pathway.

A phenomenon that may be related to chemotaxis, invadopod formation, and pathfinding is the observation that the localized stimulation of the EGF receptor on carcinoma cells using EGF-bound beads results in localized actin polymerization and protrusion

(Kempiak et al. 2003). This highly focal actin polymerization requires the activation of the Arp2/3 complex by N-WASP and cofilin and is regulated by Grb2 and Nck2 (Kempiak et al. 2005). This phenomenon may be relevant to how EGF receptor ligands, which can bind to extracellular matrix, stimulate focal protrusions, invadopod formation, and adhesion in vivo (Kempiak et al. 2005). Additional work will be required to determine the effects of altering N-WASP activity on invasion, intravasation, and metastasis in vivo.

ZBP1. The targeting of β-actin mRNA to the leading lamella is essential for stable cell polarity during locomotion, and ZBP1 is required for mRNA targeting (Condeelis & Singer 2005). Highly metastatic cells lines have reduced levels of ZBP1, and this is consistent with the reduction in ZBP1 expression seen in invasive cells (Wang et al. 2004). Decreased β-actin mRNA targeting seen in cells with reduced ZBP1 is correlated with the loss of cell polarity and increased amoeboid movement in metastatic carcinoma cell lines in vitro and in vivo (Shestakova et al. 1999, Wang et al. 2002) and increased chemotaxis (Wang et al. 2004). Increasing the level of expression of ZBP1 in invasive carcinoma cells rescues the localization of β-actin mRNA to one pole of the cell and results in the inhibition of chemotaxis to EGF both in vitro and in vivo in tumors. In addition, tumors prepared from cells re-expressing ZBP1 are significantly less invasive and metastatic than their parental cell–generated counterparts (Wang et al. 2004). However, tumor growth is not significantly affected by increasing the expression of ZBP1. This suggests that the suppression of invasion and metastasis by ZBP1 is not related to growth of the tumor. These results are consistent with the observation that mouse mammary tumors that overexpress the ZBP1 homologue CRD-BP are not metastatic (Tessier et al. 2004).

The invasion and metastasis suppression activity of ZBP1 may result from its ability to suppress 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 (Iijima et al. 2002, Parent & Devreotes 1999). This may account for the enhanced ability of invasive carcinoma cells to chemotax to blood vessels (Condeelis & Segall 2003, Wyckoff et al. 2000a). It also suggests that the generation of polarity in carcinoma cells that occurs around blood vessels is independent of ZBP1 activity, as discussed above.

NEW INSIGHTS INTO TUMOR INVASION AND METASTASIS

The identification of an invasion signature for mammary tumors that implicates the coordinate regulation of genes involved in functionally related activities presents a rich collection of targets for chemotherapy not previously detected in conventional expression profiling of whole tumors. The fact that the pathways are coordinately regulated in invasive cells suggests that combinations of therapeutics may be particularly effective.

An additional insight resulting from the study of invasive cells and their invasion signature comes from the comparison of expression profiles obtained from invasive cells with the conventional expression profiles of whole tumors. Gene expression profiles of whole tumors have shown promise in prognosis by identifying patterns of expression that are correlated with metastasis (Ramaswamy et al. 2003, van't Veer et al. 2002). However, unlike the invasion signature described for invasive cells, these patterns of expression appear as random sets of genes with unrelated functions and thus are difficult to interpret in terms of mechanisms of invasion and metastasis. This suggests that the invasion signature is either averaged out when interrogating the whole tumor because invasive cells are rare or that the changes in gene expression

that represent the invasion signature are largely transient. Therefore, it is interesting that the expression profiles of whole tumors demonstrate 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 (Ramaswamy et al. 2003, van 't Veer et al. 2002). These results suggest that metastasis could occur early in tumor progression and that most cells in the tumor are potentially metastatic, thus favoring a "transient expression" model rather than an "averaged-out model" to explain the discordance between expression profile results. This conclusion is surprising because the traditional view of tumor progression is that tumors develop through a succession of stable genetic changes acquired through selection pressures, a process analogous to Darwinian evolution. According to the traditional view of tumor progression, the cells selected to be metastatic are very rare, and metastases arise from progressive genetic changes in these rare cells within a primary tumor delaying metastasis to late stages of tumor progression (Bernards & Weinberg 2002, Hanahan & Weinberg 2000).

The Tumor Microenvironment Invasion Model

A new model, the tumor microenvironment invasion model (TMIM), has been proposed to explain the relationship between the expression pattern of invasive cells and expression patterns of whole tumors and how these relate to the traditional view of tumor progression (Wang et al. 2005). In this model, the transient changes in gene expression leading to invasion (the invasion signature) result from microenvironments in the tumor that are defined by stable genetic changes in both stromal and tumor cells. That is, tumor progression, as described by traditional models (Hanahan & Weinberg 2000), leads to the development of microenvironments encoded within the tumor, which elicit the transient gene expression patterns that support invasion. In this context, invasion is similar to a morphogenetic program involving the transient expression of genes that lead to a change in the location of cells, a program that can occur repeatedly during tumor development and in any location in the tumor that has the microenvironment that elicits the morphogenetic program. The expression of genes that are synergistic for inducing microenvironments causing invasion could lead to the random appearance, in time and location, of these microenvironments during tumor progression leading to repeated episodes of invasion and metastasis throughout tumor progression.

TMIM is consistent with the finding that genes encoding the tumor microenvironment for invasion and metastasis appear to be expressed throughout the bulk of the tumor. It is also consistent with the 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 microenvironments inducing invasion, as claimed (Wang et al. 2004, Wyckoff et al. 2004). Furthermore, the TMIM hypothesis is supported by intravital imaging of experimental tumors where only a small proportion of tumor cells are motile, and moving cells are not uniformly distributed but are observed in localized areas of the tumor (Condeelis & Segall 2003, Wang et al. 2002), and the observation that micrometastases are often genetically heterogeneous, suggesting that invasive behavior is not stably specified (Klein 2002). Finally, the TMIM hypothesis is consistent with our current understanding of how the tumor microenvironment contributes to invasion and metastasis (Bissell & Radisky 2001).

The exciting new technologies reviewed here have brought us to the point where tumor invasion and metastasis can be studied as a problem in morphogenesis. The future will reveal if the new insights that are emerging will lead to new strategies for the diagnosis and treatment of metastasis.

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