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Early stage cancer cell invasion: signaling, biomarkers and therapeutic targeting

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1. ABSTRACT

The process of primary cancer invasion of distant organs is multifactorial and multistep. Successful therapeutic management of invasive cancers remains hampered by the multitude of overlapping signaling pathways that initiate and drive cancer cell migration. A crucial early event by which cancer cells switch from localized to invasive states is initiated by the acquisition of autonomous motile properties; a process driven by dynamic assemblies and disassemblies of multiple focal adhesion, cytoskeleton and motor proteins. Several of the protein complexes involved are tightly regulated through posttranslational modifications and intermolecular collisions with partners that occur in a time- and space-dependent manner. These concerted mechanisms are essential for the regulation of cell shape, cell polarity, and cell motility and migration in response to chemotactic signals. This review summarizes the current knowledge in the field and potential clinical implications for molecular pathology and cancer therapeutics. It is not meant to be comprehensive; aspects related to basic signaling are not dealt with extensively in this review. However, the reader is referred to excellent reviews that provide coverage of these topics.

2. INTRODUCTION

Cancer remains a highly prevalent disease. Despite a significant improvement in the overall survival attributed in part to early detection and introduction of targeted therapy, many cancer patients at primary diagnosis of early disease experience recurrence of the cancer. Of these numerous scenarios, the development of distant metastases is the most life threatening and ultimately incurable, even with the best therapies available to date. The time course of distant recurrence is unpredictable; some patients sustain a recurrence within the first few months/years after diagnosis, while others suffer such an event years or decades after initial diagnosis. Paramount studies have led to the identification of potential prognostic markers (e.g. axillary lymph node involvement), predictive factors (e.g. amplification/overexpression of ErbB-2/Her-2 tyrosine kinase receptor and loss of estrogen receptor alpha for breast cancer), and transcriptional changes in gene clusters that may predict disease progression (1-4). Yet, none of the markers identified so far have revealed to be specific or sufficient enough to predict or efficiently target invasive cancer in the practice. In the case of breast cancer for instance, some patients free of axillary lymph-node metastases still display disseminated disease. Moreover, the

response of ErbB2-positive metastatic breast cancer to the anti-ErbB-2 humanized antibody herceptin (trastuzumab) is measured in terms of substantial improvement in survival with frequent relapses and no cure.

3. CANCER METASTASIS: AN EARLY EVENT (S)

Metastasis formation has long been recognized as the result of progressive and late genetic events during the multistep theory of carcinogenesis. However, increasing evidence supports the hypothesis that the dissemination of primary cancer can be initiated at early stages of cancer development from rare and genetically programmed cell variants with intrinsic invasive capacity (2, 5, 6). For instance, (**i**) disseminated circulating breast cancer cells can be detected at primary diagnosis and in the absence of clinical or pathological signs of metastases (5, 7); (**ii**) metastases can occur in patients with early stage and lymph node negative breast cancer, and in patients with unseen primary cancer (8-11), (**iii**) the concept of cancer dormancy where metastatic cells may colonize distant organs early on and stay viable and clinically dormant until undefined factors trigger their growth has been documented (12); and (**iv**) striking clinical reports describe rapid "explosion" of undetectable metastases observed in some patients after removal of pathologically classified early primary cancer (6). These observations support the theory that rare, invasive cancer cell variants, possibly stem cell-like cells, do exist in very early cancer; these cells have been proposed to be more resistant to standard chemotherapy compared to mature cancer cells.

4. CELL SIGNALING THAT INITIATE AND CONTROL CANCER CELL INVASION

Conceptually, the metastatic process can be categorized into (i) cancer invasion of neighboring tissue structures; (ii) motility and invasion of host stroma, and chemotaxis toward capillaries and thin-walled venules, a process referred to as 'intravasation"; (iii) survival and arrest in the capillary beds of distant organs; and (iv) extravasation and growth into the target organ parenchyma. During early stages of cancer cell invasion of neighboring tissue structures, cell migration, a mechanism by which part of the cell extends to reach a target, or the physical movement of an entire cell towards a target such as an "amoeba", can occur in at least three forms: (i) random (occurs in the absence of extracellular stimulus), (ii) kinesis (random motion that is influenced by chemical stimulus), and (iii) chemotaxis (directed motion towards a gradient of stimulus).

In general, invasive cancer cells are more sensitive to chemotactic signals compared to non-invasive cells or adult normal epithelial cells (13-16), and exhibit finger-like protrusions, known as "invadopodia", of the plasma membrane in response to chemotactic stimuli. This is followed by the formation of stable cell-matrix attachments near the leading edge of the protrusion that contact and degrade extracellular matrix (ECM) barriers, movement of the cell body forward, and release and retraction of the trailing edge or rear of the cell (17, 18).

Protrusions are morphologically diverse and can be large, broad lamellipodia, which are flat lamellar extensions, or spike-like filopodia, which are pointed protrusions. These protrusions are usually driven by synchronous assembly and disassembly of specific actin binding proteins responsible for the regulation of actin filament turnover (17-21), and are stabilized by adhering to the ECM or adjacent cells via focal adhesions (FAs) and/or transmembrane receptors linked to the actin cytoskeleton. Once formed, focal adhesions provide robust anchors to the ECM (sites at which force is applied to the ECM) and serve as traction sites for migration as the cell moves forward over them, and are simultaneously disassembled at the cell's rear, allowing the contractile actinomyosin system to pull the cell body and trailing edge forward and hence cell detachment and movement (22) Figure 1.

4.1. The focal adhesion signaling network

Cell adhesions are structures that couple the cell membrane to the actin cytoskeleton. They are termed focal adhesions or focal complexes (FCs) depending on their size, location, molecular composition, and regulation (23, 24). FCs represent precursor focal adhesions, which mature into focal adhesions, a process involving in part the activation of RhoA and/or by external mechanical signals. In general, FCs are small nascent adhesion sites found at membrane protrusions. In contrast to FC, FAs represent larger, more stable structures that extend underneath the cell body. They are regulated by a network of signaling molecules, including integrins, Src and focal adhesion kinase (FAK) and their partners such as Crk, Neural Wiskott-Aldrich syndrome protein (N-WASP), Crk-associated substrate (p130Cas) and its homologue NEDD9 (25-29), microtubules and actins, cytoskeleton-associated proteins such as paxillin, vinculin, and talin (21, 25, 30, 31), as well as the small GTPases of the Rho family (21, 32-34); these proteins act in a tightly regulated and concerted manner.

4.1.2. The dynamics of focal adhesion formation and turnover

Focal adhesions have many Src homology 2 (SH2)-domain containing proteins (such as c-Src, PI3K, SHP2), as well as many tyrosine phosphorylated molecules (such as focal adhesion kinase, paxillin, tensin, p130Cas, caveolin). Tyrosine phosphorylation of FA proteins is induced by clustering of integrins, activation of growth factor receptors such as ErbB tyrosine kinases (28, 35, 36). In the later case, we have shown that efficient ErbB-2 tyrosine kinase-induced cell transformation, cell protrusion formation and invasion required FAK expression in mouse embryonic fibroblasts, reinforcing the fact that ErbB-2, like other members of this receptor family, signals through FAK. Figure 2

The assembly of FA in migratory cells occurs at the leading edge of the cell. Some rapidly migrating cells such as leukocytes have few visible FA clusters, and thus, very small submicroscopic adhesions that are important for their migration, while in other cells, precursor small adhesions called focal complexes can be observed at the leading edge. It has been shown that some proteins of FA

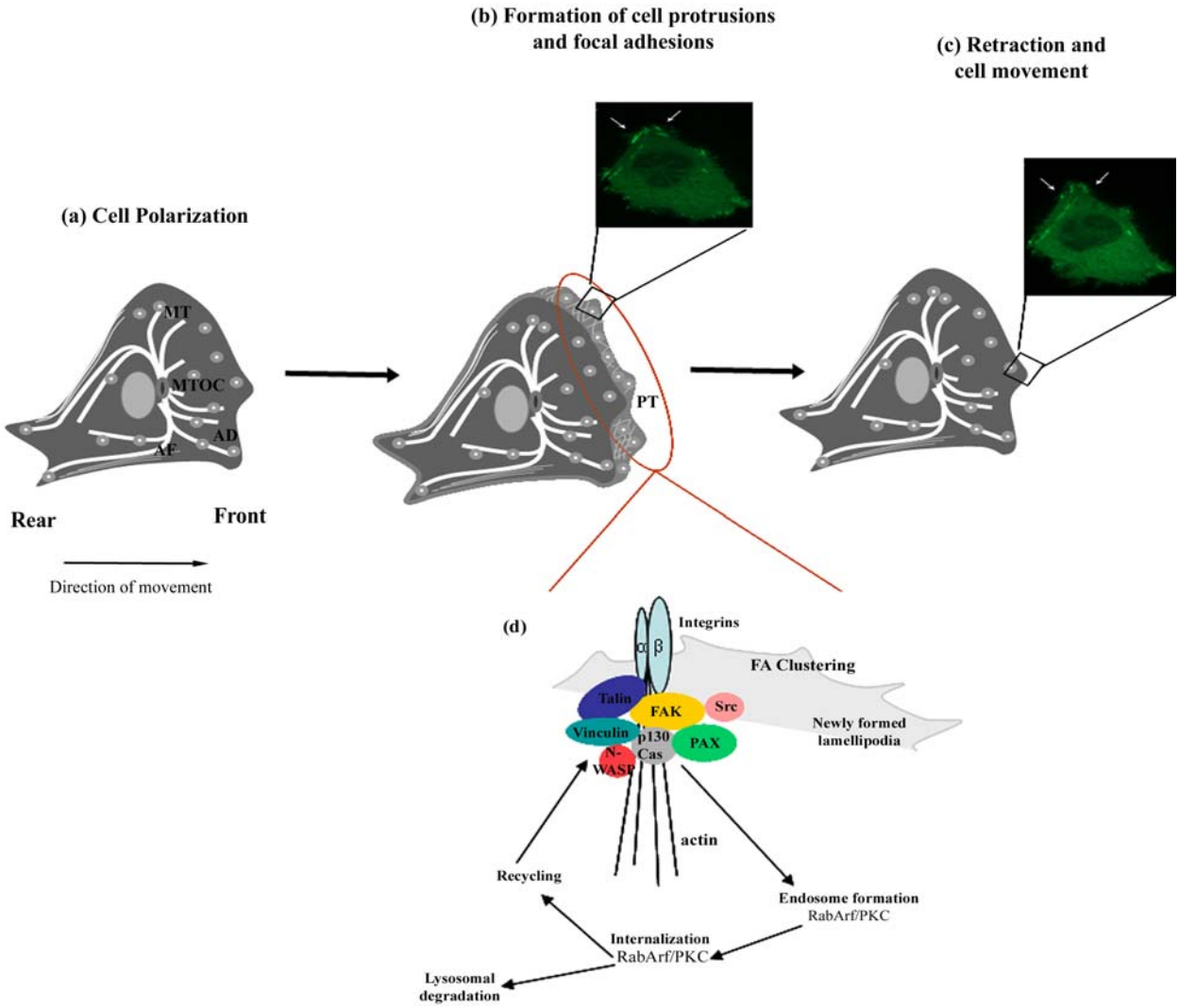

Figure 1. Model for FA formation and turnover during cell migration**.** Directional cell movement and migration requires the integration of several mechanisms, including: (a) defined cell polarization associated with differential localization of FA proteins at two poles of a moving cell; this can be an intrinsic property of an invasive cancer cell in response to chemotactic signals. Cell polarization is associated with directed vesicle trafficking toward the leading edge, reorganization of microtubules, and localization of the MTOC and Golgi apparatus in front of the nucleus (reviewed in (21)). (b) The migration cycle is initiated by the formation of a protrusion. Actin polymerization is induced via proteins that regulate actin monomers such as profilin and depolymerizing proteins such as ADP/cofilin, as well as several capping and severing proteins. Protrusions are stabilized by the formation and stabilization of adhesions, which rely on focal adhesion turnover, actin polymerization, and tractional forces facilitated by microtubule dynamics and myosin II (21). (d) FA formation and turnover is modulated by integrin signaling via FAK and other mediators. These trigger the recruitment of several signaling and adaptor molecules, including binding of the SH2-domain of Src family kinases to activated FAK. The FAK-Src complex subsequently induces tyrosine phosphorylation of additional sites on FAK and of FAK partners p130Cas and paxillin. Activated FAK also promotes the recruitment of other SH2-containing proteins such as PI-3K, PLC-y, Grb7, Grb2-SOS (128). The trafficking of these various FA proteins from the rear to the front (FA assembly and disassembly) occurs via several alternative pathways, including Rab-GTPases. (c) Cell retraction at the rear of the moving cell is associated with adhesion disassembly (21). Inserts in (b) and (c) show real time FA formation and removal, respectively, in the same live cell expressing GFP-paxillin as determined by a Quorum Spinning disk confocal microscope with time-lapse imaging capability (arrows show FA formation and removal in the same cell). Adhesions (AD), actin filaments (AF), microtubules (MT), microtubule organisation center (MTOC), protrusions (PT).

are recruited to sites of adhesion with similar kinetics, suggesting that they exist in preformed cytoplasmic complexes (37), while other components may enter adhesions with very distinct kinetics, which supports a tight regulatory event that initiates the serial addition of different proteins. Paxillin, for example, is present in nascent

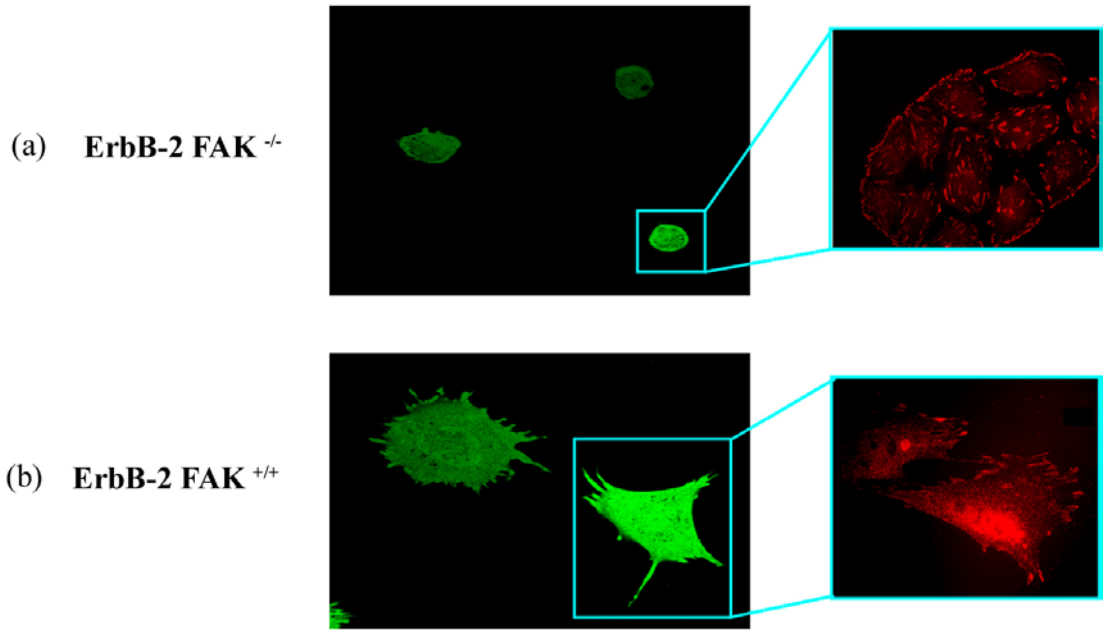

Figure 2. FAK is an important downstream target for ErbB-2 tyrosine kinase receptor-induced cell invasion. FAK-deficient fibroblasts overexpressing the ErbB-2 receptor are poorly oncogenic and invasive. Restoration of FAK in these ErbB-FAK-deficient cells restores cell protrusion formation, and cell invasion in vitro and in vivo. ErbB-2 FAK$^{-/-}$ cells (a) and ErbB-2 FAK $^{+/+}$ cells (b) stained with GFP (green) and with immunofluorescence labeling for paxillin (red) (35).

adhesions, whereas alpha-actinin appears to be more prominent in "older" adhesions (37). In contrast to FA assembly, FA disassembly occurs both at the leading edge, where it accompanies the formation of new protrusions and at the cell rear, where it promotes tail retraction. At the front of migrating cells, adhesions at the base of a protrusion disassemble as new adhesions form at the leading edge (38). In general, cells with stable and large FA are tightly adherent and are either non-migratory or with reduced motility. Most recent evidence support that FA disassembly is controlled by several pathways, including Src-FAK complex, Rho-GTPase signaling, and calpains. The later are a family of calcium-dependent cysteine proteases that cleave several FA-containing proteins, including Src, FAK, and talin (reviewed in (39)).

Current models support integrin engagement and FAK autophosphorylation at tyrosine 397 (Y397) as initial events leading to FA formation and turnover. This initiates focal adhesion assembly by promoting intermolecular SH2-phosphotyrosine interactions (40). In particular, activated FAK Y397 recruits Src through its SH2 domain and then the Src-FAK complex subsequently phosphorylates additional phospho-sites of FAK and FAK partners, including paxillin and p130Cas (41-43). Tyrosine phosphorylation in focal adhesion structures is believed to precede focal adhesion assembly (44). Not surprising, inhibitors of tyrosine phosphorylation block adhesion complex formation and the recruitment of a large subset of focal adhesion components (45, 46).

In addition to the above interactions, FAK is able to recruit and bind to cytoskeletal proteins such as alpha-actinin, vinculin and talin (46-48), and to activate Rho GTPases through FAK binding proteins like p130Cas, which link to Rac through the interaction of Crk with Dock 80 (49); these events are defective in FAK-null cells (26). FAK interaction with N-WASP can lead to activation of Cdc42, engagement of Arp2/3 and actin cytoskeletal contraction to push the lamellipodia forward. Interaction of FAK with p190RhoGEF can lead to Rho activation, the formation of stress fibers and maturation of FA, and stabilization of nascent adhesions. These events are dependent on actin polymerization and actinomyosin contractility (25-27) and seem to be needed for FA turnover at both the cell front and cell rear (26).

Among these multiple interactions, the Src-FAK complex has a principal role in promoting the turnover rather than the assembly of focal adhesions since deficiencies in either FAK or Src do not prevent focal adhesion formation; the number and size of adhesions was increased in FAK-/- and Src-/- cells compared to wild type mouse embryonic fibroblasts (MEF) (26). Furthermore, the number of adhesions turned over in protrusive regions of FAK- and Src-deficient cells is markedly reduced compared to wild type MEF cells. In a similar manner, paxillin-deficient fibroblasts show defects in the cortical cytoskeleton, cell spreading, and cell migration (50). Paxillin is readily detected on FA sites in retracted FAK-deficient cell but is seldom seen in FA sites of wild type MEF cells. When FAK was expressed in paxillin-null cells, the disassembly of FAK was markedly slower than in control cells (23). However, whether these mechanisms account for FA turnover in *in-vivo* conditions remains to be established.

4.2.2. Trafficking of focal adhesion proteins during cell migration

As noted above, directed cell polarization, FA clustering at sites of cell protrusion, attachment to the extracellular matrix, and their removal from these sites all depend on the correct trafficking and recycling of FA proteins between intracellular compartments and membrane cell protrusions. Recycling of intracellular proteins has been described in numerous ways and wide speculations have surfaced on the mechanisms governing plasma membrane fusion and membrane topology during secretory and endocytic processes. Previous studies on exocytosis, carried-out mostly on immune and inflammatory cells, pinpointed important regulatory proteins, including Rab (which associates with secretory vesicles and then tethers the vesicles to actin filaments) (51), kinesin (known as a molecular motor protein able to actively transport vesicles towards the plus end of microtubules, *i.e.* towards the plasma membrane), SNARE (soluble NSF attachment protein receptors) and SNAP (soluble NSF attachment protein) proteins (52). In addition, tyrosine kinases such as Src, MAP kinases (53-55) and protein kinase C (56) have been reported to contribute to the regulation of exocytosis. These processes are dependent on actin and microtubule organization (57-59).

Several recent studies have proposed a model for integrin trafficking via Rab GTPases during cell migration. These studies support a model where integrin exocytosis at the advancing leading edge assists cell locomotion by providing fresh adhesion receptors and that these trafficking receptors are constantly internalized by endocytosis at the retracting end of the cell. The function of this internalization is believed to recycle rather than degrade the FA sites and most of these studies pinpoint the Rab proteins as key regulators of integrin trafficking (60-65). The Rab GTPase family comprises of more than 30 members and is a key regulator of the structure and dynamics of intracellular membranes (51, 66), in particular they function by tethering and fusing membrane vesicles, as well as transporting them and their associated effector (cargo) proteins through direct or indirect interactions with microtubules or actin-based motor proteins such as myosins. However, each member of the family is involved in a distinct function in this process. For instance, Rab5 acts as a sensor for endocytic membrane fusion (67), while Rab11 regulates the recycling of endosomes to and from trans-Golgi network to plasma membrane, as well as plasma membrane recycling of proteins such as alpha-1 integrins and other molecules (68, 69). On the other hand, Rab27 generally promotes membrane trafficking through interaction of the GTP-bound form of Rab with specific Rab effector molecule (s) (70). Rab 27 is involved in the docking step of dense-core vesicles to the plasma membrane during exocytosis. In particular, Rab27a-deficient *ashen* mice (a model for human Griscelli syndrome caused by a mutation in Rab27A) have a defect in the vesicle-docking step in exocytosis of secretory cells, including cytotoxic T-lymphocytes (71, 72). Rab7 is involved in microtubule-based transport of late endosomes/lysosomes (73, 74). Specifically, the minus-end of the microtubule-based motor complex dynein-dynactin is recruited to the Rab7-containing compartments through its effector Rab7-interacting lysosomal protein (RILP), and both Rab7 and Rab27A colocalize to melanosomal membranes (75, 76). The manner in which Rab signalling regulates FA in invasive cells have recently begun to be delineated, and some Rab proteins such as Rab 25 have been correlated to aggressiveness of ovarian and breast cancers (77).

4.3. Cell cytoskeleton remodeling and associated proteins

The repeated directional extension and stabilization of protrusions, which represent one of the key mechanisms by which cells move over a substrate, is dependent on cell cytoskeleton remodelling and microtubules (31). Cell cytoskeleton is represented mainly by actins, microtubules and intermediate filaments, generally differentiated by their rigidity (19). Forward cell locomotion is associated with cytoskeletal force-generating mechanisms, insertion of membrane at the leading edge, and the coordinated establishment and disruption of cell-substrate adhesive contacts (78). In response to extracellular cues, the actin cytoskeleton undergoes synchronous actin assembly and disassembly mediated by specific actin binding proteins responsible for the crosslinking, disassembly and formation of actin filaments. Vectorial actin disassembly at the rear of the cell and assembly at the leading edge may contribute to the directionality of lamellar extension and cell movement (79). Actin filaments are semi-flexible polymers that exhibit distinct functional polarity: they contain both a fast (plus) and a slow (minus) growing end that contribute to the dynamic characteristic of actin filaments. Actin filaments grow asymmetrically by polymerization such that the length of filaments remain roughly constant while individualized polymerized monomers transfer momentum forward, a process known as treadmilling. This phenomenon is crucial in mediating cell movement and motility as localized actin polymerization at the leading edge of the cell pushes the membrane forward in finger-like filopodial and sheet-like lamellipodial structures, generating the locomotive forces in migrating cells (80).

In a similar manner to actins, microtubules (MTs) are important regulators of cell protrusions, and have been implicated in FA turnover and sensing of FA tension. Microtubules are polymers of alpha/beta tubulin heterodimers, arranged head to tail to form long, stiff hollow tubes. They form well-organized networks that display similar dynamics to actin filaments such as asymmetrical polymerization and treadmilling, important for directional locomotion (30). Moreover, MTs and F-actin are co-regulated during cell migration, e.g. MTs exhibit interactions with F-actin bundles and meshworks that guide the movement and organization of MTs during cell motility process. Experiments in which fluorescent actin was injected into the cells show that lamellipodia are the primary sites of actin incorporation (81). Alongside their protrusive activity, lamellipodia are involved in the development of adhesion to the substrate, and as ruffles, serve in macropinocytosis and phagocytosis. More recent progress in characterizing others molecules which are

involved in cell motility has come in part from the use of green fluorescent protein (GFP) to tag putative components, combined with live-cell microscopy to localize them *in vivo* (82).

Microtubules function in locomotion has been shown to be correlated with the presence of focal adhesions. Cells that form focal complexes but do not subsequently transform them to focal adhesions can migrate independently of microtubules. In contrast, cells that demonstrate conversion from focal complexes to focal adhesions use the specific phases of microtubule dynamics, in coordination with Rac1 and Rho GTPase activity to promote cell migration (83). Thus, selective targeting of specific cytoskeleton proteins has the potential not only for therapeutic but also as means for understanding cellular functions of many cytoskeleton proteins still unclear today.

4.4. Myosin "motor" and related proteins and cancer cell movement

In addition to FAs, invasive cancer cells express a large number of motor proteins, which control cell tension, contractility, and movement (25, 31). In particular, the force transmitted to sites of adhesions derives primarily from the interaction of myosins with actin filaments that attach to these sites. As myosin motors bind to actin filaments and continually repeat the bind, power stroke, unbind process, they generate a contractile force necessary to pull the cell bulk forward during cell movement and migration (80). Myosin organization consists of a conserved N-terminal actin binding and ATPase domain (motor or head domain), a neck region containing IQ motifs that bind to myosin light chains, and a C-terminal tail domain for specific cargo binding; myosins act as actin-dependent M, $^{2+}$ ATPases that use the energy derived from ATP hydrolysis to move along the actin filaments within the cell. Among the 18 classes that constitute the myosin superfamily, 4 classes of myosins (I, II, V and VI) have been implicated in F-actin mediated functions such as cell motility, vesicular trafficking, and intracellular transport of macromolecules (84). In addition to myosins, cancer cells express other motor proteins such as kinesins and dyneins (85, 86). Like myosins, these proteins utilize the energy liberated by the hydrolysis of ATP to move their cargos along the cytoskeleton, microtubules, and actin networks; this process involves several signaling molecules, including Rab-GTPases (51, 85-87), which we found to regulate FAs in invasive cells.

5. HUMAN STUDIES ON THE POTENTIAL OF FOCAL ADHESION PROTEINS AS BIOMARKERS FOR INVASIVE CANCERS

The remarkable progress in the understanding of the signaling pathways that initiate and drive cell motility and cell migration implies that some of the signaling molecules involved may serve as biomarkers of pathological states during disease progression. The expression of several regulators of cell invasion has been tested in human cancer tissue using immunohistochemistry. In particular, the Src and FAK kinases have been extensively studied. Elevated Src expression/activity has been reported early in the course of colon cancer development such as early colonic polyps and adenomas, but also in several advanced carcinomas, including colorectal, breast, and pancreatic carcinomas (88). Elevated Src activity in cancer has been linked to activating mutations, e.g. in codon 531, or via other mechanisms (89, 90). Moreover, FAK levels were shown to be elevated in a subset of early stage cancers such as ductal carcinoma *in situ* (DCIS) of breast (91, 92), advanced breast cancer and metastatic nodules (93, 94). However, other studies failed to observe such a correlation between FAK levels and the invasive phenotype in breast cancer (95). Interestingly, a study reported that co-overexpression of active forms of c-Src and FAK in breast tumors can predict aggressiveness, tamoxifen resistance and/or risk of recurrence in estrogen-receptor-positive breast cancer (96).

In a similar manner, elevated expression of the FAK homologue Pyk2 was reported to correlate with the progression of hepatocellular carcinoma (97), gastric carcinoma (98) and astrocytomas (99). In contrast, other studies reported rather decreased Pyk2 expression in osteosacroma (100) and in high grade prostate cancer compared to normal epithelial prostate tissue and benign prostatic hyperplasia (101). Our own work using a large tissue array from breast cancer patients revealed that most focal adhesion-associated proteins are upregulated in both early and advanced stages of breast cancer progression compared to benign or normal tissues Figure 3 (unpublished data), suggesting a role in early cancer progression. In addition to Src and FAK, histochemical studies on human tissues have also revealed changes in the expression of other FA-associated proteins such as integrins (102); paxillin (103), Rho-GTPases (104), cofilin (105), and fascin (106).

Together these histochemical studies support an implication of the FA-associated proteins in cancer development and/or progression. However, more specific studies are needed to sustain this. Studies in knockout mice have been limited by the fact that most of these proteins are essential for survival. However, conditional knockout technology can certainly contribute to establish the importance of these molecules in human cancer.

6. STATUS OF PRECLINICAL AND CLINICAL STUDIES WITH SMALL MOLECULE INHIBITORS OF CELL INVASION SIGNALING

Insights gained from basic studies on the manipulation of cell signalling molecules associated with cell invasion have helped to foresee the potential clinical implications of targeting the rate limiting molecules. In particular, RNA interference studies have allowed the identification of proteins whose inhibition can impact on the process of cell invasion. Inhibition of proteins such as FAK, Src, fascin, cofilin, and paxillin support the therapeutic utility of these targets to prevent cancer cell invasion in vitro and/or in preclinical models (107, 108). Chemical inhibitors for some regulators of cell migration signaling already exist although these are of limited clinical utility due to toxicity or pharmacological and non-selective

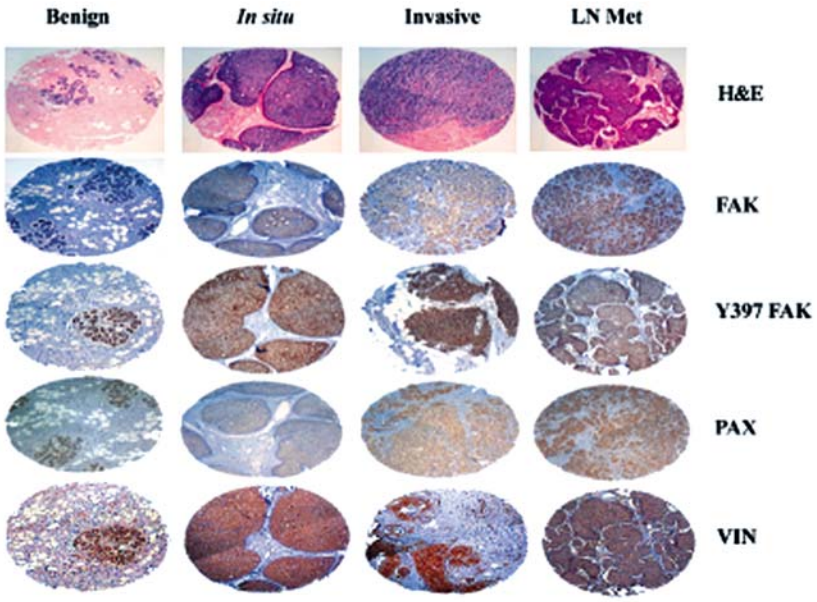

Figure 3. A representative expression pattern of selected focal adhesion and cytoskeleton proteins in human breast cancer tissues. Tissue microarrays (TMA) from breast cancer patient cohort (>400 cases) were assembled using a manual tissue arrayer. All TMA cores were assigned a diagnosis (i.e., benign, ductal carcinoma in-situ (*in situ*), invasive and lymph node metastatic (LN Met) by the study pathologist. Three to five replicate tissue cores on average were sampled from each patient for the tissue microarray. After tissue array construction, 4 µm sections were cut and stained with hematoxylin and eosin (H&E) and immunostaining using appropriate antibodies. Protein expression was assessed using a 4 tiered system (0, negative; 1 weak; 2 moderate; 3 high expression). The results indicate that many FA proteins are upregulated in >50% of early stage DCIS (*in situ*) and advanced breast cancer as compared to benign or normal breast tissues (unpublished).

issues. These include cytochalasin D, which stops protrusion formation by inhibiting the polymerization of actin-free barbed ends. Blebbistatin which inhibit the ATPase activity of non-muscle myosin II motor protein and ROCK inhibitor Y27632 or the MLCK inhibitor ML7 (inhibit myosin II activation) which inhibit cancer cell contractility and cell migration.

Availability of the crystal structure information of many cell invasion proteins, e.g. Src, FAK, paxillin, talin, vinculin, combined with highthroughput chemistry has been successfully exploited to identify specific small molecule inhibitors. The most recent example targets the FAK protein, where specific small molecule inhibitors with potential efficacy in preclinical models were identified and at least one molecule is being subject to phase I clinical trials (109-111). Similarly to FAK, several Src inhibitors (reviewed in (112)), Rho-GTPase inhibitors ((113, 114), myosin II inhibitors (115, 116) and calpain inhibitors (117, 118) are undergoing preclinical and/or clinical trials as these may prove to be useful to tackle invasive cancers.

As noted above, the rate of FA assembly and disassembly is dependent on microtubule dynamics, myosin II, which regulate the force transmitted to sites of adhesions via interaction with actin filaments. Therefore, inhibitors of actin and microtubules can perturb the cell invasion machinery. Several actin inhibitors were isolated from the Red Sea sponge *Latrunculia magnifica (*119, 120). Among these are the actin destabilizing agents latrunculin A (121, 122), misakinolide A, swinholide A, and mycalolide B (these agents act primarily by severing filamentous actin and binding actin monomers and induces rapid depolymerization of assembled polymers) (123). Additional inhibitors include phalloidin, which disrupts actin dynamics by shifting the equilibrium towards the assembled actin polymer (124); Jasplakinolide, which is an actin filament stabilizer and potent inducer of actin polymerization (125); chondramides, which function by accelerating actin polymerization and disrupting the cytoskeleton (119); and dolastatins, a family of cytotoxins that demonstrate similar but more potent actin stabilizing activity than jasplakinolides and phalloidin but do not compete with phalloidin for actin binding (119, 126). As for actins, several microtubule destabilizing agents are available, including nocodazole, myoseverin; taxanes, epothilone B, eleutherobin, discodermolide, laulimalide. Although most of these agents revealed efficacy on preventing tumor growth in preclinical models and in the clinic, e.g. taxanes, their selectivity toward cell migration signaling and prevention of early cancer invasion remains to be established in relevant in-vivo models .

7. PERSPECTIVES

Even with the remarkable progress in the understanding of cell signaling that control early cell invasion, several additional questions remain to be answered in order to establish the clinical implications of targeting the cell invasion signaling network in cancer. For

example, how the spatially segregated FA components are temporally and spatially integrated and removed across the cell protrusions in invasive compared to non-invasive cells remain a challenging question; what are the factors controlling FA protein trafficking; how does protein phosphorylation impact these processes. Furthermore, molecular understanding of the multiple feedback loops, cooperative signaling, and intracellular compartmentalization is essential to link specific molecules all the way through to a biological response, and hence identify rate limiting pathways that must be disrupted to achieve a meaningful inhibition of cancer cell invasion. Moreover, discovery of inhibitors of FA signaling pathways remains of limited clinical values, in part because of the shortcomings of targeting a single protein, often using assay systems that may not reflect the native conformation and activity of the target in its physiological context such as in respect to protein folding, splicing, post-translational modifications, and crosstalk regulatory loops.

Another important aspect is the impact of the multicellular tissue microenvironment on intracellular cell invasion signaling. Tissue microenvironment is a complex milieu with relevance to cancer invasion; among its salient characteristics is the occurrence of dynamic heterotypic cell-cell and cell-stroma interactions and the propensity for rapid inflammatory response. This can lead to increased neovascularization, increased vascular permeability, and extravasation of diverse inflammatory cells, e.g. macrophages, owing to their capacity to release cytokines and other soluble factors that can impact on several aspects of cancer invasion, including serving as a source of chemotactic/motility-inducing factors (127). Therefore, a clearer understanding of cell signaling that regulates early cancer cell invasion signaling must respect this relevant context of cancer tissue microenvironment. Thus, the need for *in vivo* model systems and technology to study the dynamics of FA turnover and cell migration *in vivo* is increasingly needed; such models will be useful for preclinical testing of selective inhibitors of cell invasion signaling and may better predict successful activity in human clinical trials.

8. ACKNOWLEDGMENTS

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Abbreviations: FAK: focal adhesion kinase; ECM: extracellular matrix; MT: microtubules; FA: focal adhesion; FC: focal complex

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