EphA2 Reexpression Prompts Invasion of Melanoma Cells Shifting from Mesenchymal to Amoeboid-like Motility Style

Matteo Parri, Maria Letizia Taddei, Francesca Bianchini, Lido Calorini, and Paola Chiarugi

Abstract

Eph tyrosine kinases instruct cell for a repulsive behavior, regulating cell shape, adhesion, and motility. Beside its role during embryogenesis, neurogenesis, and angiogenesis, EphA2 kinase is frequently up-regulated in tumor cells of different histotypes, including prostate, breast, colon, and lung carcinoma, as well as melanoma. Although a function in both tumor onset and metastasis has been proposed, the role played by EphA2 is still debated. Here, we showed that EphA2 reexpression in B16 murine melanoma cells, which use a defined mesenchymal invasion strategy, converts their migration style from mesenchymal to amoeboid-like, conferring a plasticity in tumor cell invasiveness. Indeed, in response to reexpression and activation of EphA2, melanoma cells activate a nonproteolytic invasive program that proceeds through the activation of cytoskeleton motility, the retraction of cell protrusions, a Rho-mediated rounding of the cell body, and squeezing among three-dimensional matrix, giving rise to successful lung and peritoneal lymph node metastases. Our results suggest that, among the redundant mechanisms operating in tumor cells to penetrate the anatomic barriers of host tissues, EphA2 plays a pivotal role in the adaptive switch in migration pattern and mechanism, defining and distinguishing tumor cell invasion strategies. Thus, targeting EphA2 might represent a future approach for the therapy of cancer dissemination. [Cancer Res 2009;69(5):2072–81]

Introduction

Understanding the molecular mechanisms of metastatic spread of cancer cells is crucial for the design of novel strategies directed at preventing growth in a distant organ and its consequences to the patient. The early steps in the process that leads to metastasis are dependent on tumor cell invasiveness (e.g., the ability of tumor cells to invade surrounding tissues, cross anatomic barriers, and diffuse through the bloodstream or lymphatic system; ref. 1). Several in vitro and in vivo systems developed to study the mechanisms of invasiveness indicate that tumor cells migrate through extracellular matrix (ECM) and basement membrane components by multiple mechanisms. Among these mechanisms, matrix degradation by proteases, such as metalloproteases and serine proteases (plasminogen activator/plasmin system), was particularly considered (2). Proteolytic enzymes focused a degenerative cascade of molecular events close to the cell surface of tumor cells to clear a path for the invading tumor cells (2). Alongside, invasiveness of tumor cells may include loss of cell polarity, alteration of cell-cell and cell-matrix adhesions, independence from the anoikis program, as well as deregulated signaling for growth, adhesive, and motile factors (3). Together, these changes, evoking the ontogenetic communication between epithelial and mesenchymal cells during embryonic development, may support detachment, migration, and organ colonization. Hence, reemergence of embryonic key cell-cell communication or motility factors may strongly affect the behavior of cancer cells (1, 4).

Recently, several key advances have challenged the view of cancer cell motility indicating essentially two milestones: (a) the gene expression-based motile phenotype is determined very early during cancer development and (b) cancer cells may display different types of cell motility, including mesenchymal, collective, and amoeboid style. One of the main features of invasiveness is the ability of metastatic cancers to shift between modes of motility, eluding simple anticancer treatments, and represents a major challenge for developing strategies aimed at blocking the spread of cancer cells (1, 3, 5). Mesenchymal motility is characterized by an elongated and polarized cell morphology; it depends on ECM proteolysis of the moving cells, which, through production of matrix metalloproteinases (MMP), generates a “path.” This leads to activation of Rac1 at the leading edge of the cell, and to inhibition of RhoA GTPase (5). Amoeboid motility is a primitive form of cell migration that allows cells to glide through, rather than degrade, ECM barriers through weakened cell-ECM attachments. Conversely to mesenchymal motility, cells moving through an amoeboid mode show inhibition of Rac1 and strong activation of RhoA (5). In fibrosarcoma cells, the inhibition of integrin or MMP function leads to switch from mesenchymal to an amoeboid-like migration program, thereby rescuing motility by alternative mechanisms (6, 7). Although mesenchymal-amoeboid transition (MAT) in response to protease inhibitors or integrin antagonists has been proposed to be a key event in the dissemination of invasive cells, the identification of factors regulating this conversion is still at its infancy.

Eph kinases have been extensively studied for their roles in embryonic development where they transduce key directional signals for neuronal growth cones, neuronal crest cells, as well as endothelial cells during vasculogenesis (8, 9). Emerging evidences also implicate Eph family proteins in cancer progression (8–11). In particular, beside breast and prostate carcinomas, EphA2 kinase was found to be overexpressed in several highly aggressive melanomas, whereas it was not detected in normal melanocytes (10–12). In addition, the level of EphA2 expression was significantly higher in metastatic cells than in cells from primary melanoma (11, 13). Nevertheless, its strong correlation with cancer progression, the real
function of EphA2 in invasive tumors, is far to be understood. One open question is if the implication of this molecule in carcinogenesis is really linked to its nature of motility factor.

This research work investigates the role of EphA2 in invasiveness of tumor cells, particularly its involvement as a motility factor. Previous findings of our laboratory indicate that, in the low metastatic F10-M3 melanoma cells, the proinflammatory cytokine IFN-γ increases their mesenchymal type of invasiveness through up-regulation of MMPs and urokinase plasminogen activator system (14, 15). F10-M3 melanoma cells represent a suitable model of tumor cells to investigate EphA2 role in cancer cell invasiveness in view of the finding that they do not express, even after inflammatory cytokine stimulation, EphA2 kinase. Data presented herein revealed that the reexpression of EphA2 in melanoma cells increases their capacity to migrate through Matrigel as well as to colonize host organs through a MMP-independent/RhoA-dependent amoeboid-like strategy.

Materials and Methods

Materials. Unless specified, all reagents were obtained from Sigma. Anti-phosphotyro sine (clone 4G10) and anti EphA2 antibodies were from Upstate Biotechnology, Inc.; anti-RhoA, anti-focal adhesion kinase (FAK), and Rac1 antibodies and collagen I were from Becton Dickinson; and anti-phospho-Y576/Y577-FAK was from Santa Cruz Biotechnology. Tumor necrosis factor α (TNFα) was from PeproTech. Biomastat and Y27632 were from Calbiochem. Cell-permeable C3 was from Cytoskeleton. DQ collagen I fluorescein conjugate and calcine-AM were from Molecular Probes.

Plasmid and protein overexpression. EphA2 coding sequence, subcloned into pTargetT vector (Promega) as in ref. 16, was transiently transfected using Lipofectamine 2000 (Invitrogen) using 4 μg plasmid DNA. Enhanced green fluorescent protein (EGFP) cotransfection (in a 1:10 ratio) was carried out. More than 85% of cells were positive to transfection, as revealed by cytofluorimetric analysis of EphA2-positive cells or EGFP-positive cells. At 48 h after transfection, the cells were recovered for analysis. For studies using soluble ephrinA1, cells in the logarithmic growth phase were always stimulated with 1 μg/mL.4 Fc or ephrinA1-Fc for the indicated times. Dominant-negative RhoA (dnRhoA-N19) was a kind gift of K. Defea (Division of Biomedical Sciences, University of California, Riverside, Riverside, CA).

Cell lines and culture conditions. In this study, we used a low metastatic clone of B16 (F10-M3) murine melanoma cells, the F10-M3 line (17). Cells were cultivated in DMEM supplemented with 10% FCS at 37°C in a 10% CO2 humidified atmosphere. Proinflammatory cytokine-treated murine melanoma cells were obtained by incubating tumor cells, for a period of 24 h, in medium supplemented with different doses of TNFα (25–100 ng/mL). For studies using soluble ephrinA1, cells were stimulated with 1 μg/mL.4 Fc or ephrinA1-Fc for the indicated times.

Immunoprecipitation and Western blot analysis. Cells (1 × 107) were lysed for 20 min on ice in 500 μL of complete radioligand precipitation assay (RIPA) lysis buffer [50 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 1% NP40, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin]. Lysates were clarified by centrifugation and were immunoprecipitated for 4 h at 4°C with 1 to 2 μg of the specific antibodies. Immune complexes were collected on protein A-Sepharose, separated by SDS-PAGE, and transferred onto nitrocellulose. The immunoblots were incubated in 3% bovine serum albumin, 100 mmol/L Tris–HCl (pH 7.5), 1 mmol/L EDTA, and 0.1% Tween 20 for 1 h at room temperature and were probed first with specific antibodies, and then with secondary antibodies. For chemiluminescence detection, we used a Gel Logic 2002 Kodak Imaging System, equipped with a charge-coupled device camera, which guarantees a high linearity, and Quantity One software (Bio-Rad) was used to obtain quantitative analyses.

Intraperitoneal colonization. F10-M3 melanoma cells were harvested by trypsinization, washed twice in PBS, and then suspended in serum-free medium at 1 × 106/mL. The suspension (0.2 mL) was injected into the peritoneal cavity of syngeneic C57BL/6 mice (Charles River). Animals were monitored, and 14 d after, they were sacrificed. The peritoneal cavities of injected animals were inspected with the aid of a dissecting microscope and the amount of tumor recovered in association with the lymphatic system was weighted.

Lung colonization. F10-M3 melanoma cells were harvested from dishes by treatment of trypsin-EDTA solution. Cells were washed twice in PBS and then suspended in serum-free medium at 1 × 106/mL. The suspension (0.2 mL) was injected into the tail vein of syngeneic C57Bl/6 mice. Animals were monitored, and 21 d after, they were sacrificed. Lungs were inspected with the aid of a dissecting microscope and lung nodules were counted.

Wound-healing assay. Cells were cultured in 6-cm plates until confluence. The monolayer of F10-M3 cells was scratched using a fine sterile pipette tip. Pictures were taken before and 24 h after the addition of 2% serum using an inverted Leica microscope equipped with a Nikon digital camera. Wound closure was followed over 24 h by light microscopy, and the wound areas were imaged using a digital camera (Nikon Coolpix 900) for calculation of the percentage of wound closure using ImageJ software and percentage of wound closure at each time point was derived by the following formula: 1 – (current wound size/initial wound size) × 100.

Boydgen in vitro invasion assay. Transwell system of Costar, equipped with 8-μm pore polyvinylpyrrolidone-free polycarbonate filters, was used. Cells were loaded into the upper compartment (5 × 104 in 500 μL) in serum-free growth medium. The upper sides of the porous polycarbonate filters were coated with collagen I. The lower chamber containing the filter was placed into six well culture dishes containing 1 mL of complete growth medium. After 24 h of incubation at 37°C, noninvading cells were removed mechanically using cotton swabs, and the microporous membrane was stained with Diff-Quick solution. Chemotaxis was evaluated by counting the cells migrated to the lower surface of the polycarbonate filters (six randomly chosen fields, mean ± SD). For cells transfected with EphA2, always cotransfected with EGFP with more than 85% efficiency, migrated cells have been normalized for EGFP-expressing cells.

Cell migration in three-dimensional collagen matrices. Reconstruction by time-lapse video microscopy and confocal microscopy was performed on melanoma F10-M3 cells. Subconfluent cells transfected with EphA2 mutants were detached by EDTA (2 mmol/L), washed, incorporated into three-dimensional collagen lattice (1.67 mg/mL; native dermal bovine type 1 collagen; RD Systems), and monitored by time-lapse video microscopy according to Friedl and colleagues (18). We used HT1080 cells as model for a clear visualization of fiber breakdown in the process of proteolytic migration. For inhibition studies, a protease inhibitor cocktail (7) was added to the lattice before polymerization as well as to the supernatant. Protease inhibitor cocktail consisted of ilomastat, leupeptin, pepstatin A, E-64, and aprotonin. Protease inhibitor cocktail does not affect cell viability in collagen after 48 h, as assessed after cell release from the collagen lattice by collagenase digestion, propidium iodide staining, and flow cytometry, or on liquid culture after 72 h of exposure. For three-dimensional time-lapse confocal microscopy (Leica-SP5 system), cells within the lattice were labeled by calcine-AM (1 μmol/L), scanned at 3-min time intervals for simultaneous fluorescence and back scatter signal (reflection), and reconstructed. Three-dimensional motility of cells is shown by time lapse of xyt analysis (three-dimensional analysis during time). Movies are a two-dimensional projection (xy) of all stacks during a time course. Yx two-dimensional migration of cells has been excluded by analysis of yz axis movements during the same time course (Supplementary Fig. S1).

Collagen degradation assay. F10-M3 cell suspension was copolymerized with nonlabeled rat tail collagen (Becton Dickinson) containing 2% FITC-labeled collagen monomers (Molecular Probes). Migration was allowed for 40 h and solid-phase collagen containing the cells was pelleted, whereas FITC released into the supernatant was analyzed by spectrofluorometry. One hundred percent values were obtained by complete collagenase digestion of cell-free collagen lattices. Background fluorescence was obtained by pelleting nondigested cell-free lattices.

RhoA or Rac1 activity assay. Melanoma F10-M3 cells were directly lysed in RIPA buffer, the lysates were clarified by centrifugation, and...
RhoA-GTP or Rac-GTP was quantified. Briefly, lysates were incubated with 10 μg Rhotekin-glutathione S-transferase (GST) fusion protein (Upstate) or p21 activated kinase-GST fusion protein, both absorbed on glutathione-Sepharose beads for 1 h at 4°C. Immunoreactive RhoA or Rac1 was then quantified by Western blot analysis. Lysates were normalized for RhoA or Rac1 content by immunoblot.

Zymography for MMPs. F10-M3 melanoma cells were used for the determination of MMP activity. Aliquots from medium conditioned by tumor cells were electrophoresed on 8% SDS-polyacrylamide gels copolymerized with 0.1% (w/v) type A gelatin. After electrophoresis, the gels were washed in 2.5% (v/v) Triton X-100 for 30 min to remove SDS. Gelatin substrate gels were then incubated in 50 mmol/L Tris-HCl (pH 7.4), 200 mmol/L NaCl, and 5 mmol/L CaCl₂ for 24 h at 37°C. After incubation, the gels were stained with 0.1% Coomassie brilliant blue in acetic acid, methanol, and distilled water at a volume ratio of 1:2:3, respectively, for 60 min at room temperature. After destaining, the gels were immersed in distilled water and scanned immediately with Quantity One Image analysis software (Bio-Rad). The gelatinolytic activities of HT1080 human fibrosarcoma cells were used as markers of molecular weight.

Results
Metastatic F10-M3 melanoma cells: a mesenchymal type of invasion. Cell migration can be classified into several motility...
styles, including amoeboid, mesenchymal, or collective migration. These molecular programs are associated with a characteristic structure of the actin cytoskeleton, use of integrins and/or MMPs, as well as signaling toward the cytoskeleton carried out by Rho and Rac GTPases (1, 5, 6, 19, 20). F10-M3 melanoma cells exposed to three-dimensional matrices of type I collagen lattice seem to move in response to 2% serum essentially through a spindle-shaped mesenchymal and proteolytic movement (see Fig. 1A and movie 1, confocal reflection microscopy invasion assay). Exposure of F10-M3 moving cells to a protease cocktail inhibitor is strongly efficient in blocking their ability to proteolytically degrade the three-dimensional matrix, although their effective motility is not eliminated. In keeping with previously reported data (7), we observe that inhibition of collagenolysis causes a shift of invasive cell migration to a round-shape squeezing movement across collagen fibers, without matrix degradation (Fig. 1B; movie 2). The broad-range MMP inhibitor ilomastat gives rise to similar effects (Fig. 1C; movie 3), suggesting a major contribution of MMPs to the degradation of collagen by F10-M3 moving cells. This MMP-independent motility style has been referred as amoeboid migration and strongly correlated with plasticity of cell motility of several invasive cancers (1, 3, 6). The strong inhibitory effect on cell-dependent collagenolysis was confirmed by a quantitative three-dimensional fluorometric FITC release assay, identifying the degradation of FITC-labeled...
collagen fibers (Fig. 1D). On migration within FITC-collagen, melanoma cells release 80% of the total FITC content, confirming the observed macroscopic collagenolysis, whereas the treatment with protease cocktail inhibitor dramatically inhibits melanoma-mediated FITC release.

Up-regulation of MMP-dependent motility leads to increased invasiveness of melanoma cells. The proinflammatory tumor microenvironment has already been causally linked to a MMP-dependent increase in melanoma invasiveness (21–23). To confirm the key role of MMPs in melanoma invasion, we treated them with TNFα and investigate MMP expression and Matrigel invasion (Fig. 2A and B). The results indicate that TNFα strongly increases the expression of pro-MMP-9, thus causing a 3-fold increase in Matrigel invasion of melanoma cells. Treatment of cells with ilomastat strongly inhibits the increase in invasion induced by TNFα, confirming a major contribution of TNFα-induced MMPs to melanoma invasiveness (Fig. 2B). MMPs are therefore useful tools for melanoma malignancy, as finally confirmed by the ability of TNFα to increase both in vivo lymph node and lung colonization of tumor cells (Fig. 2C and D).

**EphA2 expression increases melanoma cell motility and invasion.** Plasticity in cell migration on variation of tumor microenvironment is a specific hallmark of cancer cells. Acknowledged mechanisms driving cancer cells to MAT are the weakening of integrin-ECM interactions, the inhibition of extracellular proteolysis using protease inhibitors, or the strengthening of small GTPase Rho signal (7, 19, 24, 25). Reexpression of EphA2 kinase in a large variety of human cancers, including melanoma, has been correlated with a more aggressive and metastatic tumor phenotype, mainly directing cell movements and positioning during invasion (10, 11, 16). In addition, EphA2 kinase activation leads to a strong up-regulation of RhoA and consequently to cell body contraction, as well as to inhibition of cell-ECM and cell-cell constraints (26, 27). To investigate the possibility that up-regulation of EphA2 expression leads melanoma cells to acquire a different strategy of invasion by converting their motility from mesenchymal to amoeboid, we overexpressed EphA2 in melanoma cells. We first observed that F10-M3 cells do not express detectable levels of endogenous EphA2 and that the treatment with TNFα cannot induce its expression (Fig. 3A). Nevertheless, F10-M3 cells do not express ephrinA1 (i.e., the natural ligand for EphA2; data not shown), and ectopic expression of EphA2 leads to its ligand-independent activation (Fig. 3B) as well as activation of EphA2 downstream signal via activation of FAK (Fig. 3C). As expected, EphA2 expression increases two-dimensional migration of melanoma cells, as indicated by wound-healing assay (Fig. 4A). Consistent with its effect on two-dimensional motility, EphA2 expression increases three-dimensional matrix invasion of melanoma cells, as revealed by Boyden-Matrigel assay (Fig. 4B). Treatment with ilomastat is almost ineffective in reducing the EphA2-mediated up-regulation of invasion. In keeping, EphA2-expressing cells do not modify their MMP-9 expression, as indicated by gelatin zymography. Furthermore, we excluded that EphA2 expression affects membrane-type MMP-1, which is not detectable by gelatin zymography (Supplementary Fig. S2). In addition, expression of EphA2 does not alter the up-regulation of MMP-9 promoted by TNFα (Fig. 4C). Finally, analysis of experimental peritoneal or lung metastasis, respectively, induced by i.p. or tail vein injection of EphA2-overexpressing F10-M3 cells confirms a prometastatic effect of expression of EphA2 in melanoma cells (Fig. 4D, left and right). In keeping with these data, EphA2 expression level is inversely correlated with MMP expression in A375 and HS29-4T human melanoma cell lines (Supplementary Fig. S3A and B).

**EphA2 induces a metalloproteinase-independent motility shift toward an amoeboid style.** To characterize the motility spur induced by EphA2 expression in melanoma cells, we analyzed the activation of two acknowledged regulators of cytoskeleton, the small GTPases RhoA and Rac1, as well as regulation of proteolytic enzymes. Indeed, mesenchymal motility has been causally linked to activation of Rac and inhibition of Rho GTPases, whereas amoeboid motility has been associated with the opposite phenotype (1, 3, 5). Expression of EphA2 in melanoma cells induces activation of RhoA and inhibition of Rac1 (Fig. 5A and B), in keeping with an induced shift of these cells toward an amoeboid motility style. Therefore, we investigated the effect of blocking RhoA using either a synthetic inhibitor or a dominant-negative mutant (dnRhoA), as well as its downstream regulator RhoA-activated kinase (ROCK) and the MMPs, on cell morphology of EphA2-expressing cells in threedimensional collagen lattice (Fig. 5C). Whereas EphA2-expressing...
cells acquire a rounded morphology within tridimensional lattice, the blockade of RhoA or of its downstream regulator ROCK, respectively, using the cell-permeable Tat C3 or the dnRhoA and Y27632 restores predominant cell morphology to a more elongated shape, more typical of mesenchymal melanoma cells. In keeping with our hypothesis of a MMP-independent function of EphA2, the treatment with ilomastat does not affect the rounded morphology of EphA2-expressing cells (Fig. 5C). The analysis of Matrigel invasion of these cells during inhibition of RhoA, ROCK, or MMPs confirms that the motility spur conferred to melanoma cells by EphA2 expression is Rho dependent and MMP independent (Fig. 5D). It is therefore likely that RhoA/ROCK signaling contributes to the amoeboid transition of F10-M3 cells owing to EphA2 expression.

To further confirm that EphA2-induced invasion is nonproteinolytic and independent from MMP activity, a quantitative analysis of FITC release from collagen has been carried out. Again, our observations sustain that the motility spur induced by EphA2

Figure 4. EphA2 expression increases melanoma cell motility and invasion. A, wound-healing migration assay. Confluent F10-M3 melanoma cells expressing EphA2 were serum starved for 24 h and scratched with a tip, and a photograph was taken (T0). Medium with 2% serum was added to induce migration of cells in the wound, and after 24 h, photographs were taken (T16h). Wound area has been analyzed using ImageJ software and reported above each time point. B, Boyden cell invasion assay. F10-M3 melanoma cells expressing EphA2, after 24 h of serum starvation, were seeded into the upper chamber of Boyden chambers. Cells were allowed to migrate for 24 h toward the lower chamber filled with complete growth medium in the presence or not of the broad-range MMP inhibitor ilomastat. Cell invasion was evaluated after crystal violet staining by counting cells in six randomly chosen fields. The results are representative of four experiments. *, P < 0.001, mock/ilomastat versus mock; EphA2 versus mock. C, analysis of MMP activity. F10-M3 melanoma cells expressing EphA2 were serum deprived for 24 h and treated with TNFα (100 ng/mL) for 24 h. The growth medium was collected and analyzed by gelatin zymography. The clear bands represent areas of gelatinase activity. The results are representative of four experiments. D, left, i.p. lymph node colonization. F10-M3 melanoma cells expressing EphA2 were injected into the peritoneal cavity of syngeneic C57Bl/6 mice. Animals were monitored, and 14 d after, they were sacrificed. The amount of tumor recovered (g) in association with the lymphatic system was weighted. Right, lung colonization. F10-M3 melanoma cells expressing EphA2 were injected into the tail vein of syngeneic C57Bl/6 mice. Animals were monitored, and 21 d after, they were sacrificed. Lungs were inspected with the aid of a dissecting microscope and lung nodules were counted. Pictures of i.p. and lung metastases are representative of a group of six mice. The results are representative of four experiments. *, P < 0.001, samples versus untreated.
expression is independent from pericellular proteolysis (Fig. 6 A). Melanoma EphA2-expressing cells exposed to three-dimensional type I collagen lattice seem to move in response to 2% serum essentially through a rounded/ellipsoid-shaped amoeboid movement (see Fig. 6B and movie 4, confocal reflection microscopy invasion assay). Again, we confirm the independence of EphA2-expressing cells from MMPs by means of ilomastat or cocktail protease inhibitor treatments during the confocal reflection microscopy invasion assay (Fig. 6D; movie 5). In keeping with these observations, the proteolytic activity of human melanoma cell lines, as well as the sensitivity of their invasiveness to MMP inhibition, is inversely correlated to EphA2 expression (Supplementary Fig. S3C and D).

Hence, these results suggest that expression of EphA2 causes a shift of invasive melanoma cell migration to a round-shape squeezing movement across collagen, driving melanoma-EphA2 cells to use a different motility strategy, avoiding using MMPs, and exploiting the plasticity of cytoskeleton architecture conferred by activation of EphA2 to move forward.

Discussion

The reemergence of embryonic growth factors or motility cues is one of the common features of cancer genetic alterations, strongly affecting the behavior of neoplastic cells (28–30). EphA2 kinase, which plays a pivotal role in embryonic development transducing directional motile signals during neurogenesis and angiogenesis, is frequently overexpressed and often functionally deregulated in advanced cancers, where it contributes to multiple aspects of malignant character (8, 10, 11, 31). Moreover, the highest degree of EphA2 expression and activation through phosphorylation is observed in metastatic lesions (10, 12, 30, 32, 33). Data presented herein show evidence that reexpression of EphA2 kinase in invasive F10-M3 melanoma cells converts their motility style from mesenchymal to amoeboid like, conferring them a strong invasive advantage both in vitro and in vivo.

Amoeboid motility, originally described for the amoeba Dictyostelium discoideum, has been reported for several eukaryotic cells needing rapid movements through flexible and weak adhesion sites, resulting in cell motion depending more on the rate of cytoplasmic contraction and protrusion (3, 34). Beside leukocytes (35, 36), proteolytic-independent and rounded-shape movements have been identified also in cancer cells and have been included among the different strategies used by neoplastic cells to evade the primary site of growth (37). Adenocarcinoma cells seem to move in response to EGF gradient in a MMP-independent manner. In these cells, adhesion complexes are diffused and not focalized, actin is polymerized in cortical ring, and cells squeeze between gaps in the ECM without degrading it (1, 37, 38). Other examples of cancer cells moving by amoeboid motility are V2 kidney carcinoma cells, Ras-transformed mammary carcinoma cells, as well as small cell carcinomas of the lung and the prostate (39, 40).

Figure 5. EphA2-induced invasion in melanoma cells is Rho dependent. A, F10-M3 melanoma cells expressing EphA2 were serum starved for 24 h. RhoA activity assay was performed. The total amount of RhoA was then quantified by anti-RhoA immunoblot and the bar graph obtained from densitometry analysis of triplicate experiments is shown. *, P < 0.001, EphA2 versus mock. B, F10-M3 melanoma cells were treated as in A and a Rac1-GTP was analyzed by a pull-down assay. The total amount of Rac1 was then quantified by anti-Rac1 immunoblot and the bar graph obtained from densitometry analysis of triplicate experiments is shown. *, P < 0.001, EphA2 versus mock. C, calcein-AM–loaded F10-M3 melanoma cells overexpressing EphA2 were incorporated into three-dimensional collagen I lattices in the presence of the Rho kinase inhibitor Y27632 (10 µmol/L), the cell-permeable Rho inhibitor C3 (50 µmol/L), and the broad-range MMP inhibitor ilomastat and monitored by confocal fluorescence-reflection microscopy. dnRhoA cells have been cotransfected with either EphA2 and dnRhoA-N19. Bar, 10 µm. D, Boyden cell invasion assay. F10-M3 melanoma cells expressing EphA2, after 24 h of serum starvation, were seeded into the upper chamber of Boyden chambers precoated with a Matrigel. Cells were allowed to migrate for 24 h toward the lower chamber filled with complete growth medium in the presence of the Rho kinase inhibitor Y27632 (10 µmol/L), the cell-permeable Rho inhibitor C3 (50 µmol/L), and the broad-range MMP inhibitor ilomastat. Cell invasion was evaluated after crystal violet staining by counting cells in six randomly chosen fields. The results are representative of four experiments. *, P < 0.001, EphA2 and EphA2/ilomastat versus mock.
In response to particular environmental cues, cancer cells can *de novo* acquire an amoeboid-like motility, thus undergoing to what has been termed MAT. MAT can be induced both by tumor microenvironment cues, as local inhibition of MMPs, and by epigenetic expression of regulating factors. Indeed, HT1080 cells, which move by a clear mesenchymal motility using both secreted and membrane-type MMPs in the presence of a cocktail of a broad-spectrum protease inhibitors, convert their motility style from protease based to protease independent, thus undergoing MAT (7). HT1080 cells have been also reported to undergo MAT during forced activation of stathmin, a known microtubule cytoskeleton regulator (41). Breast carcinoma cells undergo MAT during inhibition of the E3-ubiquitin ligase for RhoA Smurf1, thus underlining a key role of RhoA small GTPase in this phenomenon (19). Finally, loss of p53 promotes RhoA/ROCK-dependent cell migration and invasion in three-dimensional matrices for human melanoma cells (42).

Data presented herein allow including activation of EphA2 in melanoma cells as one of the natural occurrence driving cancer cells toward a conversion of their mesenchymal motility style to an amoeboid like. Indeed, the invasive F10-M3 melanoma cells use a proteolytic MMP-based motility strategy to invade and metastasize. In response to reexpression and activation of EphA2, these cells activate a nonproteolytic invasive program, which proceeds through activation of cytoskeleton motility, cytoplasmic contraction, and retraction of cell protrusions, Rho-mediated rounding of the cell body, and squeezing among three-dimensional matrix, finally giving rise to successful lung and lymph node peritoneal metastases. In agreement with these findings, we observed that in human melanoma cell lines HS29-4T and A375, the expression level of EphA2 and the use of proteolytic degradation of three-dimensional matrices for invasion are inversely correlated. In addition, MAT is associated with weakened integrin-dependent adhesions and to abolishment of proteolytic focal adhesion turnover (24). In keeping with this report, EphA2 expression has been reported to decrease integrin function and downstream signaling (26, 43). FAK has been described to be a key player of these effects on integrin-mediated adhesion (43). In addition, we recently reported that the role of EphA2 in the regulation of cell motility in prostate tumor cells is strongly reliant on FAK activation, likely accounting for a RhoA-GTPase exchange factor activation (26, 27). Our present data agree with these previous

**Figure 6.** EphA2 induces a MMP-independent motility shift toward an amoeboid style. A, migration-associated collagenolysis caused by F10-M3 melanoma cells transiently transfected with EphA2 within three-dimensional FITC-collagen lattices was quantified from the FITC release after 40 h of migration in the presence or absence of the broad-range MMP inhibitor ilomastat. $P < 0.001$, EphA2 EphA2/ilomastat versus mock. Bar, 15 μm. B, green calcein-loaded F10-M3 melanoma cells overexpressing EphA2 were incorporated into three-dimensional collagen I lattices and monitored by confocal fluorescence-reflection video microscopy. Arrowheads, round-shape squeezing movement of one cell across collagen I fibers. Bar, 10 μm. C, cells were treated with the broad-range MMP inhibitor ilomastat, which was added to the lattice before polymerization as well as to the supernatant. Bar, 10 μm.
reports. Indeed, we observed an activation of FAK in EphA2-expressing cells, accompanied by a strong activation of RhoA. This finding suggests that the FAK/RhoA signaling pathway is mainly responsible for driving the EphA2-mediated achievement of an amoeboid motility style. Consistently with this, Rho activation has already been correlated with amoeboid motility (7, 19, 25, 42), whereas FAK has been associated with promotion of an aggressive phenotype and in vascular mimicry of melanoma cells (44, 45), although a link with amoeboid motility has not been investigated.

Beside its dependence from proteolytic path generation, mesenchymal motility has been associated with activation of Rac-mediated cell protrusions (5, 6). Conversely, amoeboid motility has been reported to be independent from the formation of holes within ECM but strongly reliant on a Rho-mediated cell body contraction and ROCK (Rho-associated coiled-coil-containing kinase)–driven generation of myosin contractile force (5, 6). ROCK, by means of regulating myosin light chain, leads to contraction of the cell cortex, enabling the cell to squeeze between collagen fibers and generating hydrostatic pressure that may aid cell protrusion (3). In keeping with our proposal of EphA2 functional activation as a driving force for mesenchymal to amoeboid conversion, in EphA2-expressing melanoma cells, we observed a down-regulation of Rac1 and an up-regulation of RhoA small GTPases. In addition, achievement of a rounded shape in three-dimensional matrices is strongly dependent from RhoA and ROCK activation, thus confirming the key role of Rho signaling in EphA2-induced motility style conversion.

Several evidence indicates that melanoma cells use a mesenchymal style to invade. Indeed, several proteases have been described to be up-regulated in invasive F10-M3 melanoma cells by humoral inflammatory mediators. In keeping with the idea that mesenchymal proteolytic invasion of melanoma is a successful strategy, we herein report that TNFα gives rise to a further increase of melanoma in vivo and in vitro invasiveness. Nevertheless, F10-M3 melanoma cells get a great benefit, in terms of invasive potential, by MMP-based motility and its up-regulation by TNFα, and they undergo MAT on EphA2 expression. This event leads to a further up-regulation of in vitro and in vivo invasiveness, conferring cytoskeleton plasticity and allowing MAT. Fascinatingly, this MAT is likely a reversible phenomenon because the treatment with TNFα restores the ability of cells to up-regulate MMPs, thereby responding to proinflammatory tumor microenvironment with a new shift toward mesenchymal motility. The plasticity of several melanomas, a widely documented phenomenon and a key obstacle for therapeutic approaches, can involve epithelial mesenchymal transition (46), as well as the ability of melanoma cells to mimic the vasculature (13, 47). Our data now include MAT as a further program, which melanoma cells can undergo, useful to increase their plasticity to finely adapt to environmental changes. In keeping with our engagement of EphA2 in MAT, the kinase has already been correlated with melanoma plasticity, being strongly involved in vasculogenic mimicry of melanoma cells (48, 49). In this light, EphA2-expressing melanoma cells could on one side enhance the formation of auxiliary vascular structures and on the other side help in inducing a MMP-independent invasiveness. In addition, we could speculate that EphA2-induced tumor cell plasticity can be useful to exploit different pathways of invasiveness. Considering that amoeboid motility is reminiscent of lymphocyte motility (36), and that our data correlate EphA2 expression with increased lymph node metastases, we speculate that EphA2-mediated MAT is correlated with preferential lymphatic dissemination of melanoma cells. In keeping with this hypothesis, the MMP inhibitor MM270 inhibits hematogenic but not lymphatic metastasis of B16 melanoma cells in both experimental and spontaneous metastasis models (50).

Nevertheless, the relationship between the epigenetic programs leading cells toward amoeboid or mesenchymal transition needs further and deeper investigations; we can speculate that aggressive melanomas can exploit a dual invasive strategy depending on the surrounding microenvironment and their genetic alterations. By this way, they can either exploit the proinflammatory milieu to increase their mesenchymal motility or undergo an amoeboid conversion, which can be extremely beneficial during local inhibition of proteolytic activity. It would be therefore stimulating to test EphA2 targeting in tumor therapy by the use of antibody-mediated or small interfering RNA-mediated down-regulation of EphA2 expression (51, 52) in preclinical models of cancer invasion and metastasis, monitoring their effects on particular tumors invaded by mesenchymal or amoeboid strategies by in vivo tumor cell imaging.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

Received 5/15/2008; revised 11/27/2008; accepted 12/10/2008; published OnlineFirst 02/24/2009.

**Grant support**: Italian Association for Cancer Research, Interuniversity Biotechnology Consortium, Ente Cassa di Risparmio di Firenze and Fondazione Cassa di Risparmio di Lucca, and Tuscany Regional Project TRESOR. M.L. Taddei is supported by a Fondazione Italiana per la Ricerca sul Cancro fellowship.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**References**

Parri M, Buricchi F, Giannoni E, et al. EphrinA1
27.
26.
Carragher NO, Walker SM, Scott Carragher LA, et al. EphrinA1 embryonic and tumorigenic signaling pathways to
Seftor RE, Hendrix MJ. Exploiting the convergence of
EphA2 Reexpression Prompts Invasion of Melanoma Cells Shifting from Mesenchymal to Amoeboid-like Motility Style

Matteo Parri, Maria Letizia Taddei, Francesca Bianchini, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-08-1845

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2009/02/12/0008-5472.CAN-08-1845.DC1

Cited articles
This article cites 52 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/69/5/2072.full.html#ref-list-1

Citing articles
This article has been cited by 15 HighWire-hosted articles. Access the articles at:
/content/69/5/2072.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.