Abstract

Metastasis is a significant event in cancer progression and continues to pose the greatest challenge for a cancer cure. Defining genes that control metastasis in vivo may provide new targets for intervening in this process with profound therapeutic implications. Melanoma differentiation associated gene-9 (mda-9) was initially identified by subtraction hybridization as a novel gene displaying biphasic expression during terminal differentiation in human melanoma cells. Mda-9, also known as syntenin, is a PDZ-domain protein overexpressed in many types of human cancers, where it is believed to function in tumor progression. However, a functional role of mda-9/syntenin in tumor growth and metastasis and the signaling pathways involved in mediating these biological activities remain to be defined. Evidence is now provided, using weakly and highly metastatic isogenic melanoma variants, that mda-9/syntenin regulates metastasis. Expression of mda-9/syntenin correlates with advanced stages of melanoma progression. Regulating mda-9/syntenin expression using a replication-incompetent adenovirus expressing either sense or antisense mda-9/syntenin modifies the transformed phenotype and alters metastatic ability in immortal human melanocytes and metastatic melanoma cells in vitro and in vivo in newborn rats. A direct relationship is observed between mda-9/syntenin expression and increased phosphorylation of focal adhesion kinase, c-Jun-NH2-kinase, and p38. This study provides the first direct link between mda-9/syntenin expression and tumor cell dissemination in vivo and indicates that mda-9/syntenin expression activates specific signal transduction pathways, which may regulate melanoma tumor progression. Based on its ability to directly alter metastasis, mda-9/syntenin provides a promising new focus for melanoma cancer research with potential therapeutic applications for metastatic diseases.

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Introduction

Cancer cells frequently display plasticity relative to maintenance of the transformed state (1, 2). By appropriate manipulation, it is possible to revert specific tumor cells to a more normal phenotype, a process termed “differentiation therapy of cancer” (1, 2). We have investigated this process extensively in the context of human melanoma cells, in which treatment with a combination of fibroblast IFN-γ and the antileukemic compound mezerein results in a rapid and irreversible loss of proliferative potential, profound changes in surface antigens and gene expressions, and a reduction in oncogenic potential (3–5). By combining this experimental protocol with subtraction hybridization, a series of melanoma differentiation associated (mda) genes were cloned that display altered expression as a function of terminal differentiation (4–7). One such gene, mda-9, revealed early enhancement followed by decreased expression during the course of reversion of the cancer phenotype in melanoma cells, suggesting a potential role during tumor progression (8, 9). mda-9 has subsequently been identified in additional contexts, called syntenin (10), and is now recognized as an important member of the expanding family of scaffolding proteins that share sequence identity with PDZ domains (11). mda-9/syntenin is a widely distributed cytosolic protein that recruits a number of proteins into a complex through a tandem of PDZ domains of 83 and 80 amino acid residues (PDZ1 and PDZ2, respectively) specialized for binding of COOH termini in partner proteins (12). By virtue of its association with itself and with a large number of additional proteins, including class B ephrins, protransforming growth factor-α, phosphotyrosine phosphatase-1, neurofaschin, neurexin, schwannomin (also known as merlin), IL-5 receptor α, various glutamate receptor subtypes, and the syneccanal families of heparan sulfate proteoglycans, mda-9/syntenin has been implicated in diverse processes (11), including protein trafficking (13), activation of the transcription factor Sox4 (15), cytoskeleton-membrane organization, and cell adhesion (16, 17). Studies using an enhanced green fluorescent protein mda-9/syntenin fusion protein showed that endogenous mda-9/syntenin colocalized with the E-cadherin complex and syndecan-1 at adherens junctions as well as with focal adhesions and stress fibers at cell-substratum contact in fibroblastic and epithelial cells (18). These findings suggest that mda-9/syntenin might promote cytoskeletal organizational changes and intracellular signaling. The organization of these dissimilar focal contacts is complex but was shown not only to contain the appropriate integrin but also cytoskeletal proteins (vinculin, talin, and α-actinin) as well as several cytoplasmic protein tyrosine kinases, including members of the src family and focal adhesion kinase (FAK; ref. 19). Despite extensive research documenting an ability of mda-9/syntenin to form multivalent interactions, little is known about the role of mda-9/syntenin in cancer development.

Recent evidence suggests that mda-9/syntenin may function as an important determinant of the malignant phenotype in multiple cancers (20, 21). It was shown previously that advanced metastatic melanomas strongly express mda-9/syntenin when compared with both melanocytic nevi and primary melanomas (21). Although
these data suggest that development of metastatic capacity in melanoma cancer cells is associated with an increase in mda-9/ syntenin expression, they fall short of providing definitive proof for a causal relationship between elevated mda-9/syntenin expression and tumor cell invasion in vivo culminating in augmentation of the metastatic phenotype.

We have presently examined the functional role of mda-9/ syntenin in melanoma metastasis using a human melanoma model that closely mimics the early events of metastasis in humans, composed of weakly metastatic versus immunosuppressed newborn rat–selected highly metastatic variants (22–24). Using recombinant adenoviral vectors expressing either sense or antisense mda-9/syntenin, evidence is now provided indicating that altered mda-9/syntenin expression induces profound phenotypic changes in vitro, which correlates with a dramatic inhibition of the formation of spontaneous lung metastases in an orthotopic rat model in vivo. This study provides the first direct functional evidence that mda-9/syntenin can activate metastatic mechanisms and specific signal transduction pathways capable of modulating melanoma tumor progression. Based on these provocative findings, it is plausible that mda-9/syntenin may provide a promising new target for melanoma cancer therapy.

Materials and Methods

Antibodies and reagents. Anti-extracellular signal-regulated kinase (ERK), anti-phospho-ERK, and polyclonal antibody targeting the COOH-terminal region of FAK were from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphotyrosine-specific polyclonal antibodies were from Biosource (Camarillo, CA). Monoclonal antibodies to FAK and vinculin were from Transduction Laboratories (San Jose, CA) and Sigma (Saint Louis, MO), respectively. Polyclonal antibodies against anti-Akt, anti-phospho-Akt, anti- p38, and anti-phospho-p38 were from Cell Signaling (Beverly, MA). Antisense polyclonal antibody was from Alpha-Diagnostic International (San Antonio, TX). Polylsine and fibronectin were from Sigma. PD98059 and SB203580 were from Calbiochem (San Diego, CA).

Virus construction and plaque assays. Constructions of Ad.mda-9/S, expressing mda-9/syntenin, and Ad.mda-9/A5, expressing mda-9/syntenin in an antisense orientation, were done by cloning the transgene into a shuttle vector (p0f/TMV) and then performing homologous recombination of the shuttle vector with E1 and E3 region deleted parental adenoviral vector in Escherichia coli (25, 26). The Massey Cancer Center Virus Vector Shared Resource constructed these adenoviruses. A dominant-negative kinase-deficient mutant p38 mitogen-activated protein kinase (MAPK) expressing adenovirus was prepared by cloning the kinase-inactive p38α cDNA into an adenovirus transfection plasmid and made into a replication-incompetent Ad-5 (delE1, E3) virus (27). Adenovirus constructs encoding FAK COOH-terminal domain (FRNK) or a dominant-negative c-Jun-NH2-kinase (JNK) were generously provided by Dr. A.M. Samarel (Loyola University Chicago, Stritch School of Medicine, Maywood, IL) and Dr. R.A. Fine (Department of Medicine, Columbia University Medical Center, New York, NY), respectively.

Human melanoma cells and virus infection conditions. The M4Beu. melanoma cell line was established from a lymph node metastasis from a patient with malignant melanoma (22, 24). Highly metastatic 7GP and T1P26 melanoma cell lines derived from these parental cells were obtained by successive orthotopic transplantations of M4Beu. tumors or lymph node metastases into immunosuppressed newborn rats (22–24). FMS16-SV are normal human melanocytes immortalized by the SV-40 T-antigen gene, for the present studies a stable subclone of FMS16-SV was used (5). Cells were routinely cultured as monolayers and virus infections were done as described (7). The optimal multiplicity of infection was determined by infection with a replication-incompetent adenovirus expressing green fluorescence protein (AdGFP) in a range of 5 to 100 plaque-forming units (pfu)/cell and analyzed 24 hours later by flow cytometry for green fluorescence protein expression.

RNA isolation and Northern blot analysis. Total RNA was extracted from cells using the Qiagen RNAeasy mini kit according to the protocol of the manufacturer and was used for Northern blotting (24). The blots were probed with an α-32P[dCTP]-labeled, full-length human mda-9/syntenin cDNA probe (8, 9), then stripped and reprobed with an α-32P[dCTP]-labeled human glyceraldehyde-3-phosphate dehydrogenase probe.

Immunoblot analysis. Cells were lysed in 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 1% NP40, and 0.5% sodium deoxycholate containing protease inhibitor cocktail (Protease Arrest, Calbiochem, La Jolla, CA). Where indicated, serum-starved cells (16 hours at 37°C) were detached using 0.05% trypsin and 0.53 mmol/L EDTA, washed once with 0.5 mg/mL soybean trypsin inhibitor, and washed twice with serum-free medium. Cells were then replated on fibronectin or heat-denatured bovine serum albumin–coated dishes (10 μg/mL) and allowed to adhere for 30 minutes at 37°C. Cells were lysed in a modified radioimmunoprecipitation assay buffer [50 mmol/L HEPES (pH 7.4), 0.15 mol/L NaCl, 1% Triton X-100, 1 mmol/L MgCl2, and 1 mmol/L CaCl2] containing protease inhibitor cocktail, 25 mmol/L NaF, 2 mmol/L NaVO4, and 20 mmol/L Na3P04, and Western blots were done (22, 25, 27).

Invasion and migration assays. Invasion assays were done using modified Boyden Chambers with a polycarbonate nucleopore membrane (Collaborative Biomedical Products, Bedford, MA; refs. 20, 22). Cell migration assays were done with bovine serum albumin– or fibronectin-coated membranes (10 μg/mL; refs. 20, 22). Briefly, uninfected or infected cells (5 × 104 in 500 μL of serum-free medium) were seeded in triplicate on the upper part of each chamber. When indicated, cells were treated with SB203580 (a p38 inhibitor) or SP600125 (a JNK1/2/3 inhibitor). Chambers were incubated at 37°C for the indicated times, after which the filters were removed, fixed, and stained with Diff Quick Staining kit. Migration was determined by counting cells that had migrated to the lower side of the filter with a microscope at ×100 magnification. For wound-healing assays, cells were plated on slides coated with fibronectin and allowed to grow to confluence in complete medium. Then, a wound was introduced by scratching the confluent monolayer with a pipette tip (time 0). Cell migration into the wound (scraped) empty space was followed over 18 hours.

Restrictive anchorage-independent growth assay. Colony formation in soft agar was determined as previously described using a modified assay that differentiates between tumorigenic and metastatic cells (28). Briefly, cells (1 × 105) that had been infected with either the Ad.null vector (adenovirus vector lacking a gene insert), Ad.mda-9/S, or Ad.mda-9/AS were plated in complete culture medium containing 0.6% agar on top of 0.6% agar in the same medium in 60 mm dishes. All dishes were incubated at 37°C in a humidified atmosphere of 5% CO2. Macrosopic colonies were counted and scored after 2 weeks growth under a dissection microscope. Cloning efficiency was determined in three independent experiments.

Immunohistochemistry. Immunofluorescence with uninfected or infected cells plated on fibronectin for 15 to 20 minutes was done as described (18). Cells were incubated for 1 hour with the indicated primary antibody and/or rhodamine conjugated-Phalloidin (Sigma). Cells were then incubated with the relevant secondary antibody conjugated to FITC or Texas red (Molecular Probes, Eugene, OR). Staining was observed under a fluorescent microscope or by using a Zeiss confocal laser-scanning microscope. Surgical biopsy materials of malignant lesions of melanocyte origin were obtained from the Departments of Pathology and Dermatology at Hôpital de l’Hotel-Dieu, Lyon, France. Immunohistochemistry on paraffin-embedded tissue sections (5-6 μm) were done using a catalyzed amplification system (Dako, Carpinteria, CA; ref. 22).

Metastasis assay. Spontaneous metastatic ability in immunosuppressed newborn rats was determined as described (23). Briefly, adherent melanoma cells were treated with PBS (mock-infection condition) or infected with Ad.null, Ad.mda-9/S, or Ad.mda-9/AS at 100 pfu/cell in the presence of 1% fetal bovine serum/DMEM. Cells were detached 2 days after infection with trypsin-EDTA, washed with PBS, counted, and suspended in PBS. One million cells in 100 μL of PBS were then injected s.c. into the abdomen of
Wistar rats that were $<24$ hours old followed by a s.c. injection of an optimal dose of antithymocyte serum in the dorsum on days 0, 2, and 6. Three weeks later, metastatic potential was determined and lung invasion was evaluated by directly counting pulmonary nodules under a dissecting microscope.

**Results**

**Mda-9/syntenin is overexpressed in human melanoma cell lines and patient-derived tumor samples but not in normal melanocytes.** Expression of mda-9/syntenin protein by HO-1 cells treated with the combination of IFN-$\beta$ + mezerein displayed a biphasic induction pattern, consisting of a rapid increase in expression at 8 to 12 hours posttreatment followed by a decrease below the basal level at 48 hours (Fig. 1A). Interestingly, these changes in protein expression by HO-1 cells treated for the same period of time with the combination of IFN-$\beta$ + mezerein paralleled alterations in mRNA expression as originally reported (8). The biphasic expression of this gene product suggested the intriguing possibility that mda-9/syntenin expression might display an inverse relationship with tumor progression (i.e., low-level expression in melanocytes with elevated expression as melanocytes evolve into malignant melanoma cells). Considering this possibility, we evaluated the relationship between progression in human melanoma cells and mda-9/syntenin expression. Using Northern and Western blotting analyses, mda-9/syntenin was expressed at significantly reduced levels in normal immortalized melanocytes FM516-SV, a nonmetastatic radial growth phase (RGP) primary melanoma cell line, WM35, and a weakly metastatic human melanoma cell line, M4Beu. (Fig. 1B and C). In contrast, expression was significantly enhanced in metastatic cells, including vertical growth phase (VGP) primary melanoma (WM278) and highly metastatic melanoma variants (T1P26, 7GP) as well as cell lines derived from patients with metastatic melanomas.

To expand on the *in vitro* cell culture data, immunoperoxidase staining of paraffin-embedded tissue from different lesions representative of specific stages of melanoma progression was done. Cells from nevi and RGP lesions were rarely stained with anti-mda-9/syntenin polyclonal antibody (Fig. 1D; Table 1). In marked contrast, VGP melanoma cells and metastatic melanoma cells stained strongly positive with this antibody (Fig. 1D; Table 1). The mda-9/syntenin polyclonal antibody in each lesion stained ~30% to ~50% of the melanoma cells. At higher magnification, distinct cytoplasmic and membrane staining could be observed in

**Table 1. Immunoperoxidase staining of nevus and melanoma paraffin-embedded tissue sections with anti-mda-9/syntenin antibody**

<table>
<thead>
<tr>
<th>Type of lesion</th>
<th>Mda-9/syntenin positive/total</th>
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<tbody>
<tr>
<td>Benign</td>
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<tr>
<td>Normal skin</td>
<td>0/9</td>
</tr>
<tr>
<td>Dermal nevi</td>
<td>1/15</td>
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<tr>
<td>Compound nevi</td>
<td>1/6</td>
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<tr>
<td>Junctional nevi</td>
<td>0/2</td>
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<tr>
<td>Primary melanoma</td>
<td></td>
</tr>
<tr>
<td>RGP</td>
<td>3/20</td>
</tr>
<tr>
<td>VGP</td>
<td>24/30</td>
</tr>
<tr>
<td>Metastatic melanoma</td>
<td></td>
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<tr>
<td>Lymph nodes</td>
<td>8/12</td>
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![Image](https://www.aacrjournals.org)
metastatic melanoma cells (Fig. 1D). Melanocytes in normal epidermis did not display mda-9/syntenin–positive staining, whereas some staining of keratinocytes was evident. Taken together, these data further support an association between mda-9/syntenin expression and acquisition of an aggressive phenotype by evolving melanoma cells.

**Forced expression or inhibition of expression of mda-9/syntenin by adenovirus transduction regulates invasiveness and motility of human melanoma cells.** To assess the contribution of mda-9/syntenin in tumor metastasis, we focused on the poorly metastatic parental human melanoma cell line M4Beu. and its stable nude rat–derived variant T1P26, which generates a high frequency of spontaneous metastasis in immunosuppressed neonatal rats (22, 23, 29). Replication-defective recombinant adenoviruses were engineered in which the full-length mda-9/syntenin cDNA was placed under the control of the cytomegalovirus early gene promoter and cloned into an E1 region–deleted, replication-deficient human type 5 adenovirus in either the sense (Ad.mda-9/S) or antisense (Ad.mda-9/AS) orientation. As shown in Fig. 2A, mda-9/syntenin protein expression in normal immortal melanocytes or in the poorly metastatic parental melanoma cell line M4Beu. infected with 100 pfu/cell of Ad.mda-9/S was increased ~2- and ~3-fold, respectively, compared with Ad.null-infected controls. Similarly, the highly metastatic variant T1P26 infected with 100 pfu/cell of Ad.mda-9/AS displayed an ~3-fold reduction in MDA-9/syntenin protein levels when compared with Ad.null-infected control cells. It is worth noting that adenovirus transduction of mda-9/syntenin (100 pfu/cell) resulted in a comparable level of MDA-9/syntenin protein in FM516-SV and M4Beu. cells as observed in control (data not shown) or T1P26 cells infected with Ad.null (100 pfu/cell). This finding is significant and indicates that the level of MDA-9/syntenin protein produced by adenovirus infection falls within the physiologic range of expression of this protein, increasing the significance of the experimental results obtained using adenoviruses to express sense or antisense mda-9/syntenin.

To determine if a cause and effect relationship existed between the observed increase or reduction in mda-9/syntenin expression in

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**Figure 2.** Phenotypic alterations in immortal melanocytes and melanoma cell lines infected with Ad.mda-9/S or Ad.mda-9/AS. A, lysates of infected FM516-SV and melanoma cells were analyzed by Western immunoblotting for MDA-9/syntenin protein expression. moi, multiplicity of infection. B, top, migration/invasion of immortal melanocytes and melanoma cells infected with Ad.null, Ad.mda-9/S, or Ad.mda-9/AS. Bottom, columns, assays were done as described in Materials and Methods. Bars, SD of triplicate samples from three independent experiments. C, migration of FM516-SV and melanoma cells infected with Ad.null, Ad.mda-9/S, or Ad.mda-9/AS following wounding of cell monolayers. Phase contrast images were taken 18 hours after wounding to assay cell migration. D, anchorage-independent growth of FM516-SV and melanoma cells infected with Ad.null, Ad.mda-9/S, or Ad.mda-9/AS. Top, photomicrographs of Ad.null-, Ad.mda-9/S-, or Ad.mda-9/AS-infected cells. Photomicrographs were taken 2 weeks after seeding in agar. Magnification, ×20. Bottom, columns, mean of triplicates from at least three different experiments; bars, SD.
melanoma cells and cellular phenotype, in vitro assays associated with the metastatic phenotype (i.e., cell migration/invasion) were used. As shown in Fig. 2B, migration/invasion of Ad.mda-9/S-infected melanocytes or poorly metastatic M4Beu. cells into the lower chamber was significantly increased up to ~4- and ~8-fold, respectively, compared with either uninfected cells (data not shown) or cells infected with Ad.null. In contrast, Ad.mda-9/AS infection of the highly metastatic variant T1P26 decreased migration/invasion by almost 85% relative to controls (uninfected or Ad.null-infected cells; Fig. 2B). In addition to Transwell assays, migration of Ad.mda-9/S-infected melanoma cells was also significantly altered in "wound healing" assays that examine cells migrating away from a confluent monolayer into an open area. Healing (cell migration) was inhibited in Ad.mda-9/AS-infected T1P26 cells by ~70%, whereas Ad.mda-9/S-infected normal immortal melanocytes or M4Beu. cells exhibited higher levels of wound closure activity (migration) compared with the uninfected cells (data not shown) or cells infected with Ad.null (Fig. 2C).

Adenovirus-mediated mda-9/syntenin expression regulates restricted anchorage-independent growth of melanoma cells. A modified restrictive agar colony formation assay (28) was used to measure anchorage-independent cell growth. This assay has been used previously to distinguish between tumorigenic cells with the capacity to metastasize in vivo versus tumorigenic cells that lack this ability. As shown in Fig. 2D, when normal immortal melanocytes were seeded in a more restricted semisolid medium (containing 0.6% Noble agar), they failed to grow or form discrete colonies. M4Beu. cells, with low metastatic potential, formed small colonies in 0.6% agar. In sharp contrast, T1P26 variant cells with high metastatic potential grew faster and formed larger colonies by day 14 after plating. As previously shown, this difference could not be attributed to variations in cell doubling times (24, 29). An ~2- to 3-fold overexpression of mda-9/syntenin in normal immortal melanocytes or M4Beu. cells following Ad.mda-9/S infection significantly increased the number and the size of colonies, with a more dramatic enhancement observed in M4Beu. cells than in FM516-SV cells. In contrast, the number of colonies in Ad.mda-9/AS-infected T1P26 cells decreased to ~80% of Ad.null-infected T1P26 cells (Fig. 2D). Additionally, in the Ad.mda-9/AS-infected T1P26 cells, inhibition in colony formation was accompanied by a frequent decrease in the size of developing colonies.

Altering mda-9/syntenin expression regulates focal adhesion kinase activity and p38 and c-Jun-NH2-kinase mitogen-activated protein kinase levels. Modifying mda-9/syntenin expression in normal immortal or melanoma cells altered their morphology. Although Ad.mda-9/S or Ad.mda-9/AS did not have an effect on cell attachment to uncoated or fibronectin, type I collagen, or laminin-coated surfaces (data not shown), the shape of infected cells was significantly modified. The well-organized actin stress fiber network of mock-infected or Ad.null-infected cells, detected by Phalloidin staining, was substantially modified in T1P26-cells (Fig. 3A). Additionally, in the Ad.mda-9/AS-infected T1P26 cells, inhibition in colony formation was accompanied by a frequent decrease in the size of developing colonies.

Figure 3. Effect of Ad.mda-9/S and Ad.mda-9/AS on cytoskeletal organization and phosphorylation of FAK, ERK, JNK, and p38 in immortal melanocytes and melanoma cells. A, polymerization of F-actin in Ad.mda-9/S or Ad.mda-9/AS-infected cells. Infected cells were allowed to adhere to a fibronectin-coated surface in the absence of serum and stained with Phalloidin for detection of F-actin filaments. B, fluorescent confocal micrographs of the highly metastatic variant T1P26 plated onto fibronectin showing immunolocalization of focal adhesion phospho-FAK protein and mda-9/syntenin. C, effect of Ad.mda-9/S and Ad.mda-9/AS on the phosphorylation and expression levels of selected protein kinases. Uninfected or infected cells (100 pfu/cell) were plated on fibronectin in the absence of serum for 1 hour at 37°C and protein samples were analyzed by Western blotting and stained with phosphospecific antibodies to FAK Tyr397, ERK, JNK, and p38 MAPK. Membranes were reprobed with specific antibodies directed against total enzyme.
and de-adhesion events take place, whereas other fractions localized in a diffuse manner in the cytoplasm and nucleus (Fig. 3B).

Experiments were done to determine if mda-9/syntenin expression affected FAK phosphorylation at Tyr397 in melanoma cells plated on fibronectin under serum-free conditions. As shown in Fig. 3C, the level of tyrosine phosphorylation of FAK between M4Beu. and T1P26 was comparable. A similar level of tyrosine phosphorylation of FAK was also evident in FM516-SV cells (data not shown). In contrast, infection of normal FM516-SV immortal melanocytes (data not shown) or poorly metastatic M4Beu. cells with 100 pfu/cell of Ad.mda-9/S displayed greater FAK phosphorylation (~3-fold increase) upon adhesion to fibronectin than cells infected with the same multiplicity of infection of Ad.null (Fig. 3C). In contrast, Ad.mda-9/AS-infected T1P26 cells displayed a significant reduction in FAK phosphorylation (~3-fold reduc-

Figure 4. Inhibitory effect of FRNK on mda-9/syntenin promotion of melanoma cell migration. A and B, expression of Ad.FRNK suppresses phosphorylation of FAK Tyr397 and melanoma cell motility of TIP26 cells. A, TIP26 cells infected with 100 pfu/cell of Ad.null or Ad.FRNK were serum-starved and plated on fibronectin and analyzed by Western blot with phosphospecific antibodies to FAK Tyr397 or antibodies specific to FRNK directed against the COOH terminus of FAK. B, TIP26 cells infected with 100 pfu/cell of Ad.null or Ad.FRNK were serum-starved and plated onto bovine serum albumin or fibronectin-coated filters and allowed to migrate over a period of 5 hours in migration medium (DMEM with 0.5% bovine serum albumin) as described in Materials and Methods. Bars, SD of triplicate samples from three independent experiments. C, fluorescent confocal micrographs of TIP26 cells infected with 100 pfu/cell of Ad.null or Ad.FRNK, plated on fibronectin, and stained with phosphospecific antibodies to FAK Tyr397. Ad.null-infected cells display a clear punctate and focal distribution at the cell margins, which are significantly reduced in Ad.FRNK-infected melanoma cells. Bar, 10 μm. D and E, Ad.FRNK is a negative regulator of mda-9/syntenin-induced cell migration. D, poorly metastatic M4Beu. cells were coinfected with Ad.null, Ad.mda-9/S or Ad.FRNK at the indicated pfu/cell and plated onto fibronectin in the absence of serum and cell lysates were analyzed by immunoblotting with phosphospecific antibodies to FAK Tyr397. E, M4Beu. cells infected with the indicated virus were serum-starved and seeded onto bovine serum albumin or fibronectin-coated filters and allowed to adhere for 30 minutes or migrate over a period of 5 hours. Bars, SD of triplicate samples from three independent experiments.

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migratory ability of Ad.FRNK-infected T1P26 cells on fibronectin was also decreased by >75% when compared with controls (Fig. 4B). The inhibitory effect of FRNK is believed to be due to its ability to compete with and displace FAK from focal contacts (30–32). Consistent with this mechanism, T1P26 cells expressing FRNK showed a substantial decrease in the total number (∼50-60% reduction) and size of the focal adhesions in comparison with controls as determined by staining with phospha-FAK (Fig. 4C).

To explore whether expression of FAK signaling might play a causal role in melanoma cell migration, M4Beu. cells were coinfected with Ad.mda-9/S and Ad.FRNK at different multiplicities of infection. As shown in Fig. 4D, a dose-dependent decrease of FAK phosphorylation at Tyr397 was detected in the M4Beu. coinfected with Ad.mda-9/S and Ad.FRNK (10 and 50 pfu/cell) in comparison with Ad.mda-9/S-infected or Ad.mda-9/S plus Ad.null-infected cells. FRNK expression did not alter M4Beu. cell adhesion to fibronectin, whereas it did inhibit cell migration on fibronectin (Fig. 4E). These studies suggest that mda-9/syntenin expression is accompanied by a specific tyrosine phosphorylation of FAK and prompted us to explore the nature of the signaling pathways connecting mda-9/syntenin to cell migration. Because FAK can activate the MAPK pathway in response to integrin engagement (19, 30), we determined whether activation of FAK by mda-9/syntenin could up-regulate ERK, AKT, JNK, and/or p38 activity. Surprisingly, ERK and AKT activity did not change following infection of poorly metastatic cells with Ad.mda-9/S compared with uninfected or Ad.null-infected control cultures (Fig. 3C). However, Ad.mda-9/AS infection of highly metastatic cells induced an ∼3- and ∼4-fold decrease in JNK and p38 MAPK phosphorylation, respectively, when compared with uninfected or Ad.null-infected control cultures (Fig. 3C).

To study the putative roles of p38 MAPK and JNK pathways in melanoma cell migration induced by mda-9/syntenin, we first used pharmacologic inhibitors. Poorly metastatic M4Beu. cells were infected with Ad.mda-9/S and then untreated or treated for 30 minutes with either 2.5 μmol/L SB203580, a pyridinyl imidazole inhibitor of p38α and p38β MAPK isoforms, or 40 μmol/L of SP600125, a JNK-specific inhibitor. As shown in Fig. 5A and B, cellular migration of M4Beu. cells on fibronectin was significantly enhanced upon infection with Ad.mda-9/S, whereas this enhancement was significantly inhibited when cells were treated with either the JNK-specific inhibitor (SP600125; Fig. 5A) or the p38-specific inhibitor (SB203580; Fig. 5B). To further show a specific role of JNK and p38 MAPKs, we used adenovirus vectors to overexpress either a catalytically inactive dominant-negative JNK1 construct (Ad.JNK.DN) or a dominant-negative mutant of p38 (Ad.p38α.DN) in Ad.mda-9/S-infected M4Beu. cells and tested whether changes induced by these molecules would affect fibroinectin-driven migration. As shown in Fig. 5A and B, cell migration was significantly inhibited by Ad.JNK.DN and Ad.p38α.DN in comparison with control Ad.null-infected cells, which was consistent with the results obtained with SP600125- or SB203580-treated cells. These findings support a role of JNK and p38 MAPK pathway activations in mediating mda-9/syntenin–enhanced migration of poorly metastatic melanoma cells.

**Mda-9/syntenin expression regulates spontaneous melanoma metastasis in vivo.** To confirm a direct involvement of mda-9/syntenin in the metastatic process, cells were treated as indicated in Fig. 6A, inoculated s.c., and metastatic spread was evaluated after 3 weeks. Uninfected or Ad.null-infected FM516-SV cells did not form tumors or metastases during the 3-week experimental procedure (Fig. 6A; data not shown). However, infection of FM516-SV cells with Ad.mda-9/S resulted in biphasic tumor growth (i.e., tumors formed within 6–12 days, but by 10 days there was a gradual decrease in tumor size in most animals; complete disappearance of tumors was seen by 21 days). In animals receiving Ad.mda-9/S-infected FM516-SV cells, lung metastases developed at a rate that was similar but slightly reduced from that observed with animals injected with uninfected or Ad.null-infected weakly metastatic M4Beu. cells. Ad.mda-9/S infection of M4Beu. cells or Ad.mda-9/AS infection of T1P26 cells did not affect the rate of primary tumor growth compared with mock- and Ad.null-infected cells (data not shown). However, when M4Beu. cells were infected with Ad.mda-9/S, the cells acquired an enhanced ability to spontaneously metastasize to the lungs of antithymocyte serum-treated newborn rats forming an average of 55 ± 12 lung lesions.
Arrows, localization of mda-9/syntenin in sites of cell-to-cell contact.

Figure 6. Mda-9/syntenin regulates spontaneous metastasis development in immortal melanocytes and melanoma cells injected s.c. into immunosuppressed newborn rats. A, FM516-SV, M4Beu., or T1P26 cells were infected with Ad.null, Ad.mda-9/AS, or Ad.mda-9/AS virus (100 pfu/cell) and then injected s.c. into newborns. The mean ± SD of metastatic lung nodules were determined after 3 weeks using a dissecting microscope. Experiments were done twice with 10 newborn rats per experimental condition. B, immunohistochemical localization of mda-9/syntenin–positive melanoma cells in cryostat sections of lung metastases generated by s.c. inoculation of Ad.mda-9/S-infected M4Beu.cells in newborns. Sections were stained with anti-mda-9/syntenin polyclonal antibody or irrelevant antibody followed by biotinylated anti-rabbit and streptavidin-peroxidase complex. Bound antibody was detected with AEC.

Discussion

Cancer is a complex process involving multiple genetic and epigenetic changes culminating in temporally acquired altered phenotypes as a cell evolves from normalcy to malignancy (1, 28). Recent evidences support a potential role for mda-9/syntenin expression in cancer development and evolution (11, 20, 21). However, despite these intriguing observations, no mechanistic or functional data is currently available indicating how or indeed if mda-9/syntenin may mediate tumor metastasis in melanoma or other cancers.

Experimental evidence is now provided indicating that mda-9/syntenin functions as an important molecule in the activation of signal transduction pathways that promote spontaneous melanoma cell invasion and dissemination in vivo. (a) In vitro studies show that expression of mda-9/syntenin correlates with metastatic potential in malignant melanoma cells that recapitulate the clinical state. (b) Mda-9/syntenin is expressed at significantly elevated levels in patient-derived VGP primary melanomas as well as in metastatic lesions but not in melanocytes, nevi, or the majority of RGP primary melanomas. (c) When expression is elevated by means of a replication-incompetent adenovirus expressing mda-9/syntenin (Ad.mda-9/S), immortalized human melanocytes displayed a transformed phenotype and poorly metastatic melanoma cells exhibited a more aggressive transformed phenotype than unmodified or Ad.null-infected (a control virus lacking the mda-9/syntenin gene) parental cells, as documented by induced/enhanced restrictive anchorage-independent growth, and increased invasive ability and migratory capability in a monolayer “wounding assay.” Conversely, down-regulation of endogenous mda-9/syntenin expression using an adenovirus expressing an antisense of the mda-9/syntenin gene (Ad.mda-9/AS) rendered highly metastatic variants poorly aggressive. (d) A significant increase in FAK phosphorylation levels and other signaling components, such as phospho-p38 and phospho-JNK, were observed in Ad.mda-9/AS-infected immortalized human melanocytes or poorly metastatic cells, whereas highly metastatic variants infected with Ad.mda-9/AS displayed decreased phosphorylation of FAK and JNK and p38 MAPKs. (e) Poorly metastatic cells overexpressing mda-9/syntenin acquired a highly metastatic phenotype in vivo and readily metastasized to the lungs of immunosuppressed newborn rats, whereas highly metastatic variants displayed reduced metastatic potential, these nodules were found to be composed of nest cells that were intensely labeled by anti-mda-9/syntenin polyclonal antibody. Staining was prominent at the cell membrane, whereas some cells showed perinuclear staining close to the nucleus, presumably in the area of the Golgi bodies. In contrast, Ad.mda-9/AS-infected T1P26 cells displayed a significant decrease in the average number of metastatic surface tumor nodules per lung lobe (28 ± 6 versus 85 ± 15 in mock and 82 ± 14 in Ad.null-infected) approaching (although somewhat higher) than seen in mock-infected (15 ± 5) or Ad.null-infected (18 ± 4) M4Beu. cells. Qualitatively similar results were obtained in an additional independent study using 10 animals per experimental condition. These noteworthy studies confirm a cause and effect relationship between expression of mda-9/syntenin and metastatic competence in human melanoma cells.

potential when infected with Ad/mda-9/AS. In total, these data (i.e., the gain or loss of function with sense or antisense gene-delivery strategies, respectively) provide the first direct functional evidence for an involvement of mda-9/syntenin in promoting metastasis of human melanoma cells. Moreover, our data suggest that the specific signaling pathways we have identified as being regulated by mda-9/syntenin may underlie the ability of this gene to promote tumor cell metastasis in vivo.

The abilities of tumor cells to migrate from the site of a primary tumor and to invade surrounding tissue are prerequisites for metastasis (33). Although numerous factors determine metastatic potential of cancer cells (22, 24, 33, 34), our present results suggest that mda-9/syntenin represents an additional and potentially important genetic factor contributing in two crucial junctures of metastasis. One by virtue of its ability to confer anchorage-independent growth under in vitro conditions, selectively supporting growth of metastatic cells and the other by promoting cell motility and invasion, thereby regulating dissemination of malignant tumor cells. The findings of the present study together with a previous report (21) have direct clinical relevance because primary melanoma tumors that invade vertically into human dermis often metastasize, whereas those in the RGP and confined to the epidermis do not display metastatic competence (35).

It is intriguing that although forced expression of mda-9/syntenin in immortalized human melanocytes, FM516-SV, induced a modest but significant transformed phenotype as evidenced by anchorage-independent growth in restrictive soft agar and enhanced cell migration/invasion, it caused only transient tumor formation in vivo while retaining the ability to induce metastasis to the lungs. Because FM516-SV cells are not completely normal melanocytes (i.e., they contain the SV40 T-antigen resulting in immortality), the induction of a quasi-transformed state by mda-9/syntenin may reflect cooperativity between expression of the SV40 T-antigen and mda-9/syntenin in this modified melanocyte background. It is also possible that although overexpression of mda-9/syntenin by itself induces a spectrum of phenotypic (transformation-associated) alterations in FM516-SV cells in vitro, these cellular modifications can only initiate tumor development, without providing angiogenic factors, such as basic fibroblast growth factor 2 and vascular endothelial growth factor, required to support tumorigenicity of melanoma cell lines (33, 35). Nevertheless, the fact that mda-9/syntenin expression occurs predominantly in the VGP of primary melanomas but not in the RGP stage suggests that mda-9/syntenin may play a relevant role in this particular phase of tumor evolution but not in the progression of normal melanocytes to malignant melanoma. In these contexts, it seems that the biological functions and consequences of mda-9/syntenin expression could depend on the cellular background and/or a given stage of melanoma development.

Experiments using adenovirus gene delivery of FRNK, a dominant-negative inhibitor of FAK, support the view that mda-9/syntenin is a proximal mediator of FAK function. Blocking FAK activation by infecting poorly metastatic melanoma cells with Ad/mda-9/S and an adenovirus expressing the COOH-terminal noncatalytic domain of FAK, FRNK (Ad-FRNK; ref. 36) resulted in significant inhibition of phosphorylation of FAK at Tyr297. This raises the intriguing question as to the mechanism by which mda-9/syntenin regulates the formation of focal adhesions and subsequent tumor progression, thereby promoting metastasis. FAK, a tyrosine kinase that is activated by tyrosine phosphorylation in response to integrin-matrix interactions, has been implicated in the regulation of a multitude of biological responses that are central to cell proliferation, survival, and migration (19). Of import, elevated FAK expression correlates with enhanced invasiveness (37) and motility of human tumor cells (36). In this report, we provide several lines of evidence that the recruitment of mda-9/syntenin to focal contact sites can affect the FAK transduction pathway, leading to melanoma cell invasion/migration. First, high levels of expression of Ad-FRNK block the migratory properties of highly metastatic variants containing endogenous mda-9/syntenin with no obvious effect on cell adhesion on a fibronectin substrate. Second, mda-9/syntenin significantly enhanced their migratory properties when expressed in poorly metastatic cells, which was blocked by FRNK, suggesting that mda-9/syntenin is a downstream target of FAK and is required for cell migration. In support of this possibility, fibronectin-mediated migration of Chinese hamster ovary cells is increased by overexpression of FAK (38) and conversely blocking FAK activation by overexpression of FRNK prevents their migration into a wound (39). Taken together, these findings provide support for a possible causal role of mda-9/syntenin in promoting cell motility and invasion by enhancing the activity of FAK thereby leading to an increase in tumor cell dissemination in vivo.

Cell migration is viewed as a dynamic multistep process, basically governed by autophosphorylation of FAK as well as its interaction with other intracellular signaling molecules (19). Because FAK is already constitutively activated in the poorly metastatic M4Beu, melanoma cells and in RGP WM35 melanoma cells (data not shown) as confirmed in this study and as shown previously (40), the present findings raise the intriguing possibility that an important regulatory protein, such as mda-9/syntenin (11), may be a functional amplifier that potentiates FAK-triggered activation of signaling pathways.

Signaling molecules that have PDZ domains are known to associate with other functional modules to form macromolecular signaling complexes (11, 41, 42). Mda-9/syntenin may also be important for the assembly and regulation of macromolecular protein signaling complexes at the plasma membrane. These multimeric protein complexes may influence actin dynamics at the plasma membrane, which is required for cell migration by virtue of its localization in actin stress fibers (17, 20). How mda-9/syntenin holds multiprotein-signaling complexes together remains unknown. In preliminary studies, we were unable to detect any direct interaction of mda-9/syntenin with FAK as assessed by coimmunoprecipitation experiments (data not shown). Therefore, mda-9/syntenin might regulate the function of any of the intracellular constituents of focal contacts, such as Rho-GEF or LARGE (19). Theses possibilities are currently under investigation.

Evidence is also provided that two members of the MAPKs, JNK and p38, known to regulate actin dynamics and cell migration (19, 43, 44), once activated also lead to increased cell migration of poorly metastatic melanoma cells in vitro. Indeed, specific inhibitors of these two kinases or either a dominant-negative JNK or p38a adenovirus construct consistently inhibited migration of tumor cells. These data suggest that activation of the JNK and p38 MAPK pathways by mda-9/syntenin regulate melanoma cell migration and may contribute to tumor cell dissemination in vivo. It is interesting to note that of the GTPases, Cdc42 has been shown to activate both p38 and JNK.
pathways and Rac1 has been found to activate p38, leading to integrin-mediated cell motility and invasiveness (45–47). Because JNK and p38 are enriched in focal adhesion complexes and lamellipodia, respectively (44, 48), it is possible that one or more components of focal adhesion sites, such as mda-9/ symlinkin, acts as a scaffold to organize elements of the cytoplasmic MAPK cascade in a manner that manifest differential signaling events leading either to the formation or turnover of focal adhesion complexes (19). These data and previous work (49) suggest that high levels of p38 activity induced by mda-9/ symlinkin in melanoma may provide a selective advantage that favor the ability of melanoma cells to metastasize to the lung.

In summary, we now document for the first time that mda-9/ symlinkin is a critical tumor-modulating gene that potentially activates metastatic mechanisms in a model of human melanoma. Moreover, our results suggest that a specific increase in FAK tyrosine phosphorylation and activation of JNK and p38 pathways in our melanoma model may represent an essential mechanism that causes acceleration of the spread of mda-9/ symlinkin--overexpressing tumor cells. Based on the importance of these molecules and the biochemical pathways they regulate, mda-9/ symlinkin may significantly affect melanoma progression and potentially progression of other cancers in the clinical setting. Directly inhibiting this gene, using antisense or small interfering RNA approaches or with small molecule inhibitors, could provide a means of intervening in a decisive step in cancer progression; metastatic tumor spread.

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References


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