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MDA-9/syntenin: a positive gatekeeper of melanoma metastasis

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1. ABSTRACT

Melanoma differentiation associated gene-9 (MDA-9), synonymous with syntenin, is an adapter protein that provides a central role in regulating cell-cell and cell-matrix adhesion. MDA-9/syntenin transduces signals from the cell-surface to the interior through its interaction with a plethora of additional proteins and actively participates in intracellular trafficking and cell-surface targeting, synaptic transmission, and axonal outgrowth. Recent studies demarcate a seminal role of MDA-9/syntenin in cancer metastasis. In the context of melanoma, MDA-9/syntenin functions as a positive regulator of melanoma progression and metastasis through interactions with c-Src and promotes the formation of an active FAK/c-Src signaling complex leading to NF-κB and matrix metalloproteinase (MMP) activation. The present review provides a current perspective of our understanding of the important features of MDA-9/syntenin and its significant role in tumor cell metastasis with special focus on molecular mechanism of action.

2. INTRODUCTION

Cancer accounts for one in four deaths in the USA, which represents over 500,000 deaths in 2009, with more than 1,400,000 new cases diagnosed over the same time period in the USA alone (1). The primary cause of cancer death is metastasis, operating through a multifaceted and stringently orchestrated cascade (Figure 1) of events (2,3) including detachment of cells from the original tumor, invasion through the basement membrane, intravasation into the blood stream, and eventual extravasation from the blood stream at a distant site where implantation and tumor cell proliferation give rise to cancer metastases. Classical models of tumor invasion and metastasis implicate the progressive accumulation of genetic and epigenetic alterations in the generation of locally invasive and metastatic tumors and it is generally believed that metastatic conversion is a mutation-driven process. However, an expanding number of genes and gene products have been identified that positively or negatively affect the probability of established human tumor cell lines to

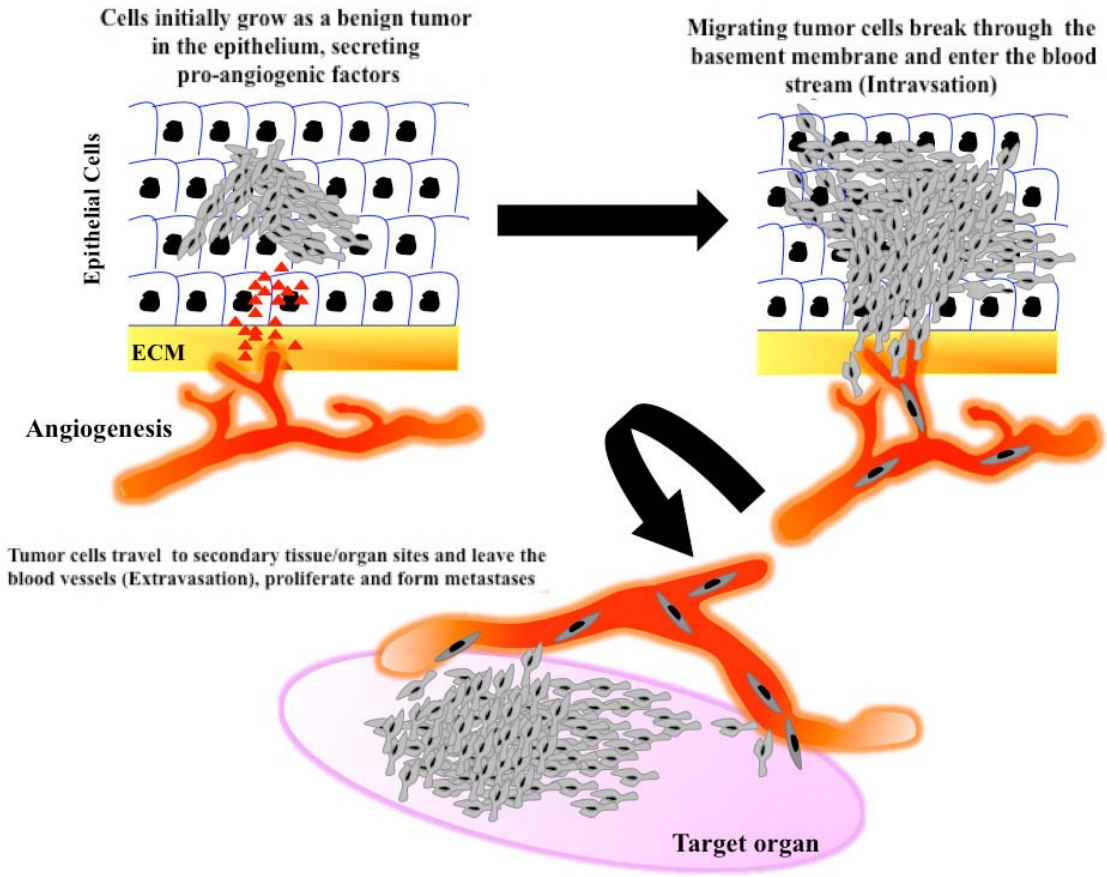

Figure 1. Model of the primary events involved in the metastatic cascade. The metastatic process is complex and involves numerous changes in cellular phenotype resulting from both genetic and epigenetic modification of the cancer genome. The process is initiated by the spread of cancer cells from a primary tumor site to other regions in the body. Cells initiate growth as primary tumors in the epithelium and with genetic and epigenetic modifications, subsets of tumor cells develop metastatic properties allowing them to degrade the basal layer and invade the blood stream (Intravasation). A small percentage of tumor cells escape into blood vessels (Extravasation) survive in the bloodstream, adhere to new target organ sites and ultimately form secondary tumors (metastases) in distant organ or tissue sites. A key component of both the primary and secondary expansion of the tumor and metastases is the development of a new supply of blood vessels, i.e., angiogenesis.

metastasize (4-6). Melanoma differentiation associated gene-9 (*mda*-9) also referred to as syntenin, is an important member of an expanding family of scaffolding PDZ domain containing proteins (an acronym representing three proteins- postsynaptic density protein PSD95/SAP90, drosophila tumor suppressor DLGA, and tight junction protein ZO-1). Our group cloned MDA-9/syntenin in the unique contexts of melanoma cells reprogrammed to terminally differentiate (7,8) and we have documented that MDA-9/syntenin functions as a positive regulator in melanoma metastasis (7-13). We presently review recent advances in our understanding of MDA-9/syntenin-mediated metastasis, in particular the molecular aspects involved in regulating metastasis.

3. CLONING OF *MDA*-9/SYNTENIN

mda-9 was first discovered fifteen years ago by Lin *et al* (7) utilizing a subtraction hybridization screen for genes that were differentially expressed in human melanoma cells reprogrammed to terminally differentiate. A detailed discussion of the cloning of *mda*-9/syntenin has been previously described (12,13). Briefly, it is possible to reprogram tumor cells that display un-controlled growth and a less differentiated phenotype to undergo irreversible growth arrest and terminal differentiation by appropriate treatment(s) (15-22). In the context of human melanoma, exposure to a combination of fibroblast interferon (IFN-β) and the antileukemic agent mezerein (MEZ) results in an irreversible loss of proliferative capacity, changes in biochemical programs, alterations in surface antigen expression, modifications in cellular morphology, profound changes in gene expression, and induction of terminal differentiation (19-28). Subtraction hybridization between temporal libraries of normal and terminally differentiated human melanoma cells resulted in the identification of melanoma differentiation associated genes (*mda*). Interestingly, rather than exhibiting a sustained induction, a feature of *mda* genes regulating growth suppression during terminal differentiation, *mda*-9 mRNA expression showed

distinct biphasic kinetics peaking 8 to 12 hours after IFN-β + mezerein treatment with a return to basal level by 24 hours, indicating that modulation of *mda*-9 expression is disassociated from growth suppression (8).

The identical gene was subsequently cloned, and named syntenin, by yeast two-hybrid assay as an interacting partner of cell-surface heparan sulfate syndecans, involved in cell-cell and cell-matrix adhesion, signal transduction from the cell surface to the interior, and trafficking of lipoproteins and lipases, thus playing prominent roles in cell growth, development, and differentiation (29).

4. STRUCTURE AND REGULATION OF *MDA*-9/SYNTENIN

The *mda*-9/syntenin cDNA is ~2.1-kb with an open reading frame of 894-bp that codes for a protein of 298 amino acid (aa) residues with a predicted molecular mass of ~33-kDa (8,9,29). Cloning of mouse and rat *mda*-9/syntenins revealed that the molecule is highly homologous across species (30). The molecule has four domains: an N-terminal domain (aa 1-109) that shows no striking homology to any structural motifs, the first PDZ domain (PDZ-1; aa 110-193), the second PDZ domain (PDZ-2; aa 194-274) and a COOH-terminal domain. In comparison to the PDZ domains, the role of N- and C-terminal domains (NTD and CTD, respectively) are not well documented functionally, however, both of these domains influence the structure and stability of the full-length protein (31,32). Independently, the NTD domain can recruit the transcription factor SOX4 and eukaryotic translation initiation factor 4A (EIF4A) into signaling complexes, and is important for homo and heterodimerization with syntenin-2 (33-35). High-stringency analysis revealed that four conserved tyrosine residues are potential phosphorylation sites in NTD. Phosphorylation at tyrosine residues prevent the interaction of MDA-9/syntenin with the receptor type protein tyrosine phosphates (rPTPη) CD148, indicating that post-translational modifications of the NTD affect the interactions between the PDZ domains and their targets (36). The CTD probably contains structural elements that interact in tandem with the PDZ, as judged from NMR spectra (32). However, more studies are needed to elucidate exactly how the NTD and CTD contribute to MDA-9/syntenin function. Functionally, the PDZ domain is well described consisting of 80-100 residues that form globular, compact domains of 25-30 Å that usually comprise six β-strands (βA-βF) and two α-helices (αA and αB) and generally mediate the assembly of dynamic multi-protein complexes at the membrane by binding to the C-terminus of target proteins (34). A cooperative binding profile was observed between MDA-9/syntenin and syndecans, whereby neither of the two PDZ domains is sufficient by itself but where PDZ-2 functions as a major or high-affinity syndecan binding domain and PDZ-1 functions as an accessory or low affinity syndecan binding domain (37). PDZ domains are typically grouped into three classes depending on their target peptides: class I (-S/T-X-ϕ), class II (-ϕ-X-ϕ), and class III (-D/EX-ϕ), where ϕ is a hydrophobic residue. The PDZ domains of MDA-9/syntenin do not bind a unique sequence, but rather bind to multiple peptide motifs (class I, class II and other sequences (12,13,38) with low-to-medium affinity (39,40).

Based on database entries, two isoforms of MDA-9/syntenin, syntenin-2α and syntenin-2β, were cloned by PCR from a fetal human brain cDNA library (34). Syntenin-2α has a similar domain structure as MDA-9/syntenin and shares 70% identity over the PDZ domains. Syntenin-2β is a shorter isoform of syntenin-2α lacking 85 amino acid residues at the NH_2 terminal.

At present, the regulation of *mda*-9/syntenin has not been extensively studied. The promoter region of *mda*-9/syntenin has not been cloned. The only information relative to its regulation comes from our studies that *mda*-9/syntenin is an interferon-inducible gene (7,8) and from a second study which identified *mda*-9/syntenin as a TNF-α inducible gene in human umbilical arterial endothelial cells (41). *mda*-9/syntenin mRNA was induced by TNF-α as early as 10 min post-treatment indicating that TNF-α might directly regulate its transcription. Recently, Hwangbo and colleagues (42) documented that the Protein Kinase C alpha (PKCα), stimulated by fibronectin (FN), regulates *mda*-9/syntenin expression at a post-translational level. The expression of *mda*-9/syntenin reached a peak within one hour, and then this induction returned to basal level 8 hours after FN stimulation. Interestingly, the regulation is interdependent, that is, the inhibition of PKCα suppresses FN-induced and endogenous expression of *mda*-9/syntenin and inhibition of *mda*-9/syntenin impairs FN-induced activation of PKCα. Experimental evidences showed that syndecan-4, a proteoglycan coreceptor is essential for PKCα activation during adhesion to FN. The cytoplasmic domain of syndecan-4 binds both phosphatidylinositol 4,5-biphosphate (PIP2) and PKCα and may potentiate the activation of PKCα (43,44). MDA-9/syntenin can also bind with syndecan-4 (37), PIP2 (45) and PKCα (42) through its PDZ domains. Upon FN-stimulation, the association of MDA-9/syntenin and PKCα are significantly increased in the plasma membrane and knocking down of *mda*-9/Syntenin impaired the plasma membrane targeting of PKCα. Therefore, it is speculated that MDA-9/syntenin may facilitate the binding of PKCα to PIP2 through the formation of MDA-9/syntenin-synedecan-4/PKCα complexes at the plasma membrane after FN attachment. Since the inhibition of PKCα suppressed both endogenous and FN-induce expression of *mda*-9/syntenin, it is possible, that *mda*-9/syntenin is regulated by a positive feedback mechanism from PKCα activation (42). However, the detailed underlying mechanism of this phenomenon is still unknown.

The expression of MDA-9/syntenin was detected in all fetal and adult tissues of human origin (7,30). High expression of *mda*-9/syntenin was detected in fetal kidney, liver, lung, and brain, in adult placenta, spleen, and heart, and in a wide spectrum of cell lines of diverse tissue origins.

Analysis of the subcellular distribution of MDA-9/syntenin revealed its localization at areas of cell-cell contact in cells of epithelial origin in colocalization with F-actin, syndecan-1, E-cadherin, β-catenin, and α-catenin (30). In fibroblasts, MDA-9/syntenin localizes to focal adhesions and in stress fibers. Overexpression of *mda*-9/syntenin in different cells induces the formation of distinct plasma membrane structures, including ruffles, lamellipodia, fine extensions, and neurite-like structures, emphasizing its role in regulating the structure and function of the plasma membrane (30). Anchoring of MDA-9/syntenin in the plasma membrane is facilitated by its interaction with PIP2 and phospholipase Cγ (30). MDA-9/syntenin also localizes in the early secretory pathway, such as the endoplasmic reticulum, intermediate compartment, and cis-Golgi, as well as in apical endosomes facilitating trafficking of cell-surface located molecules (46).

5. MDA-9/SYNTENIN AS A POSITIVE REGULATOR OF TUMOR METASTASIS

A hallmark of malignant tumor cells is their ability to invade tissues and form metastatic foci at distant locations in the body. This complex multistep process requires tumor cell attachment to various matrix proteins, degradation of ECM mainly by matrix metalloproteinases (MMP) followed by migration into the surrounding stroma by tumor cells (47). Suppression subtractive hybridization between poorly invasive/non metastatic breast cancer cell line, MCF-7, and an invasive/metastatic breast cancer cell line, MDA-MB-435, which was shown to actually be derived from the M14 melanoma cell line as opposed to being a breast carcinoma cell line (48), identified *mda*-9/syntenin to be overexpressed in the MDA-MB-435 cells (49). Overexpression of *mda*-9/syntenin was also detected in a number of metastatic melanoma, breast and gastric cancer cell lines in comparison to their primary or poorly metastatic counterparts. Notably, forced expression of *mda*-9/syntenin resulted in increased migration by non-metastatic cancer cells, and correlated with a more polarized distribution of F-actin and increased pseudopodia formation (9,29,50). In the following section we focus on *mda*-9/syntenin-mediated metastasis in melanoma and discuss in detail recent investigations that have scrutinized and dissected the molecular mechanisms underlying this phenomenon.

5.1. MDA-9/syntenin and metastatic melanoma

A model of progression of melanoma suggests that it originates by conversion of a normal melanocyte into a benign nevi, subsequent transformation into a radial (RGP) and then a vertical (VGP) growth phase primary melanoma, and finally evolution into a metastatic melanoma. Immunoperoxidase staining of paraffin-embedded tissue from different lesions representative of specific stages of melanoma progression, including benign skin lesions, RGP primary melanomas, VGP primary melanomas and metastatic melanomas revealed a statistically significant gradual increase in *mda*-9/syntenin expression level during progression from acquired melanocytic nevi to primary melanoma without or with conversion to metastatic melanomas (9,51). Melanocytes in normal epidermis did not display MDA-9/syntenin positive staining. With respect to cellular localization, at higher magnification, distinct cytoplasmic and membrane staining was observed in metastatic melanoma cells. Moreover, biomarker analysis also identified MDA-9/syntenin in uveal melanoma cell secretomes of patients with metastatic melanoma (52). Contrary to these findings, *mda*-9/syntenin mRNA and protein expression were also detected in immortalized normal human melanocytes (FM516-SV) and in a poorly metastatic human melanoma cell line M4Beu. (9). However, the level of expression was significantly augmented in highly metastatic M4Beu. variant melanoma cell lines (7GP and T1P26) selected by injection of M4Beu. cells into nude rats (53), and in a series of human metastatic melanoma cell lines (9) suggesting that *mda*-9/syntenin expression correlates positively with melanoma metastasis.

5.2. MDA-9/synenin interacts with c-Src through PDZ domains

c-Src, a prototype of the nine-member family of structurally related Src family tyrosine kinases (SFKs), is implicated in various biological processes associated with cytoskeletal organization, including increased cell motility, invasiveness, and survival (54). Additionally, several studies implicate c-Src in the pathogenesis and progression of multiple cancers, including breast, pancreatic, prostate, and melanoma, underscoring the importance of c-Src in cancer biology (55-58). MDA-9/syntenin co-localizes with c-Src in highly metatstatic cells (10) (Figure 2), predominantly in areas that correspond to the peripheral focal adhesion area. It also localizes in a diffuse manner in the cytoplasm and nucleus. In addition, both proteins co-localize in discrete structures in specific areas in the periphery of the cells, indicating that these focal adhesions may differ in terms of their protein content and partners. Of potential interest, both proteins also co-localize in or near the cell nucleus, suggesting that these two proteins could interact and may promote transcriptional activities (12,13,59). An antisense strategy confirmed the physical interaction between MDA-9/syntenin and c-Src (10).

The interaction with c-Src is mediated through the PDZ domains of MDA-9/syntenin since deletion mutants (either one of the two PDZ domains) of MDA-9/syntenin strongly reduced the interaction with c-Src (11). Although it was evident that the activation of the transcription factor NF-κB requires (MDA-9/Syntenin-mediated NF-κB activation, discussed later in this review) the tandem PDZ domains of MDA-9/Syntenin, mutation of one of the key contact residues (G128E in PDZ-1 and G212E in PDZ-2) within each PDZ domain for its transmembrane partners (60,34) documented that the functional integrity of the carboxylate-binding loop of PDZ-2, but not of PDZ-1, is essential for c-Src binding and activation of the transcription factor NF-κB. These findings raise obvious questions about the role of PDZ-1 in MDA-9/Syntenin and c-Src interaction and its role in NF-κB activation. Increasing experimental evidence indicates that the PDZ domains in multi-PDZ domain-containing proteins are grouped into functional units and that tandemly arranged PDZ repeats are often necessary to mediate specifi

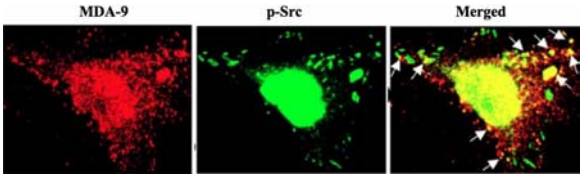

Figure 2. MDA-9/syntenin interacts with c-Src. The metastatic variant T1P26 cells were plated on fibronectin coated plate in serum-starved condition followed by immunostatining for MDA-9/syntenin (green) and phospho-c-Src (Red). Fluorescent confocal micrographs showing immunolocalization of phospho-c-Src protein and MDA-9/syntenin (arrows). Reproduced with permission from (10).

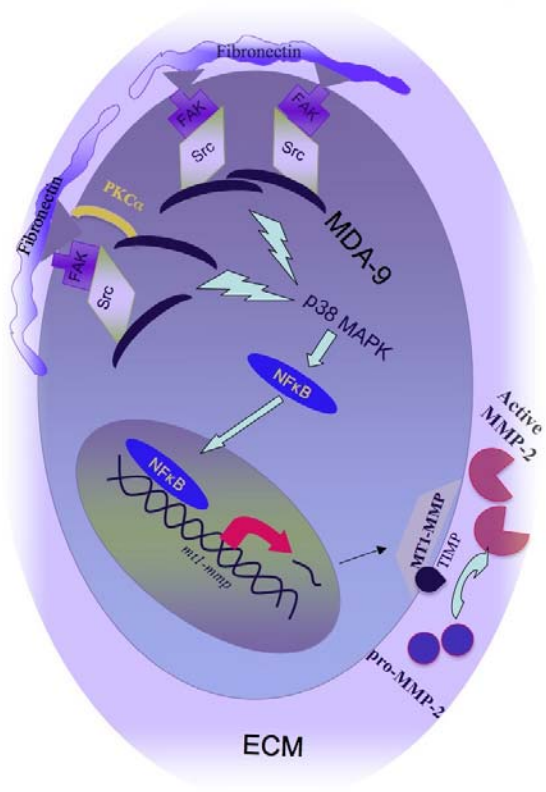

Figure 3. Hypothetical model of MDA-9/syntenin-augmented Src activation. Upon engagement with extracellular matrix (fibronectin), MDA-9/syntenin physically interacts with c-Src in a highly cooperative manner, with the PDZ2 being the dominant motif and then interacting with PDZ1 resulting in the assembly of MDA-9/syntenin into multimeric complexes and consequently a more stable functional unit (11). Fibronectin stimulation also increases the association of PKCα and MDA-9/syntenin in the plasma membrane and activates c-Src/FAK complex (42). Reproduced with permission from (11).

interactions with binding partners (12,37,61). Current structural data suggest that PDZ domains in these functional units mutually chaperone each other, enabling the tandem PDZ domains to interact with its targets (62). Indeed, it has been shown that PDZ4 and PDZ5 domains in glutamate receptor interacting protein mutually chaperone each other, enabling tandem PDZ domains to interact with GluR2 (62,63). The similar structural features of the paired MDA-9/syntenin PDZ domains (40) suggest that the PDZ-1 domain of MDA-9/syntenin may be essential for proper folding of PDZ-2. Indeed, the two PDZ domains of MDA-9/syntenin are structurally associated and undergo denaturation in a highly cooperative manner (40). In addition, structural integrity of MDA-9/syntenin is required for its self-association for promoting PDZ domain-mediated protein interactions (34). As a result, it is possible that following binding of c-Src to the MDA-9/syntenin PDZ-2 domain, PDZ-1 promotes the proper folding of PDZ-2 that assembles MDA-9/syntenin into a multimeric complex resulting in a more stable functional unit (Figure 3). In support of this conclusion, nuclear magnetic resonance (NMR) and single crystal X-ray diffraction indicate that cooperative binding of MDA-9/syntenin to syndecan results in improved binding affinity relative to single PDZ modules of MDA-9/syntenin (64).

5.3. Binding with c-Src, MDA-9/syntenin facilitates assembly of c-Src/FAK complexes

Upon integrin engagement, FAK together with c-Src form a dual-kinase complex that generates signals important for regulation of the migration machinery that promote tumor growth and metastasis (55,65). Metastatic melanoma cells, which express elevated levels of MDA-9/syntenin, displayed higher levels of molecular complexes containing both FAK and c-Src compared to weakly metastatic or normal melanocytes (9). The phosphorylation of FAK was also higher in metastatic melanoma cells. *In vitro* experiments demonstrated that the overexpression of MDA-9/syntenin augmented phosphorylated FAK levels in poorly metastatic cells (M4Beu.) while antisense *mda*-9/syntenin downregulated constitutively active phospho-FAK in highly metastatic cells (9-13). A recent study (42) demonstrated that MDA-9/syntenin and Protein Kinase C alpha (PKCα) are interdependently regulated during adhesion to fibronectin (FN) and could function as upstream regulators of FAK phosphorylation. Experimental evidence comes from treatment with either *mda*-9/syntenin siRNA or dominant negative PKCα that blocked FN-induced FAK phosphorylation in breast and melanoma metastatic cells. PKCα is essential for integrin-mediated adhesion, migration, and signaling events through participation in integrin-β1-associated protein complexes downstream of FN (65-67). In particular, binding of integrin $\alpha_5\beta_1$ to FN activates PKCα, and inhibition of PKCα suppresses focal adhesion formation and cell migration (66). MDA-9/syntenin physically interacts with PKCα and their association and localization in the membrane was significantly increased by FN-stimulation. Immunoprecipitition of β_1-integrins revealed that FN-stimulation increased FAK association with this integrin, c-Src and MDA-9/syntenin, which was blocked by using siRNA for *mda*-9/syntenin or overexpression of dominant negative PKCα. Collectively, MDA-9/syntenin could

facilitate the association of integrin-β_1/ FAK/c-Src signaling complexes during adhesion to FN, leading to FAK and c-Src activation (42) (Figure 3).

The role of FAK phosphorylation in *mda*-9/syntenin-mediated metastatic phenotypes in melanoma was confirmed using a dominant negative inhibitor of FAK (FRNK), which dramatically decreased *mda*-9/syntenin-induced migration in weakly metastatic melanoma M4Beu. cells plated on fibronectin (9). The phosphorylation of FAK creates a high-affinity binding site for SH2 domains of SFKs, and this interaction promotes Src kinase activity through a conformational change. Activated c-Src bound to phospho-FAK then phosphorylates additional sites on FAK, which promotes the assembly of distinct higher-order individual signaling complexes, thereby providing a mechanism for coordinating signaling through multiple pathways (68,69). Normally, inactive c-Src exists as a tight complex in which the SH2 domain interacts with phosphotyrosine at position 527 localized in the C-terminal region of the protein. Src activation results in the displacement of C termini from the SH2 domain proteins, promoting interactions with SH2 and/or SH3 ligands that prime c-Src for activation and thereby allow phosphorylation of the kinase on Tyr416 (70,71). In support of this observation, manipulation of *mda*-9/syntenin expression in different melanoma cells revealed that expression level is correlated with Src phosphorylation at Tyr416 upon adhesion to fibronectin and blocking of c-Src kinase activity either by pharmacological or genetic approaches causes reduction of FAK phosphorylation.

The formation of an active c-Src/FAK-signaling complex is essential to activate the NF-κB pathway. NF-κB is maintained in an inactive form with IκB proteins. Following induction, the activated IKK complex mediates the phosphorylation and degradation of IκBα, and the p50-p65 NF-κB translocates into the nucleus where it binds to consensus NF-κB sequences in the promoter of diverse target genes, thereby augmenting their transcription (72). c-Src can lead to NF-κB activation in several cell types through IKKβ and/or IκBα tyrosine phosphorylation (73-75) and SFKs have important signaling roles both upstream and downstream of MAP kinases (76-79). The phosphorylated form of p38 MAPK was significantly reduced in weakly metastatic melanoma M4Beu. cells by overexpression of different deletion mutants of MDA-9/syntenin (MDA-9/sytenin ΔPDZ2, MDA-9/syntenin ΔPDZ1 or MDA-9 ΔPDZ1+ ΔDPDZ2) compared with cells transfected with wild type MDA-9/syntenin or MDA-9 PDZ1+PDZ2 (11). Consistent with this finding, blocking p38 MAPK signaling using SB203580 or using a dominant negative p38 MAPK mutant construct had no effect on the activation state of c-Src in metastatic melanoma, whereas it markedly downregulated NF-κB activation. In contrast, siRNA knockdown of c-Src expression significantly inhibited p38 MAPK activation as well as NF-κB activation. These observations clearly show that recruitment of MDA-9/syntenin to focal contact sites is a defining step that allows c-Src bound to MDA-9/syntenin to activate the NF-κB pathway in a melanoma model (9-11) and p38 MAPK acts as a downstream target of c-Src in the signaling pathway.

5.4. MDA-9/syntenin stimulates activation of pro-MMP-2 in melanoma

Matrix metalloproteinase-2, a key MMP member of ECM-degrading enzymes, was increased in mRNA level as well as in the latent (72-kDa protein) and the active (64-kDa protein) forms of the protein with forced overexpression of *mda*-9/syntenin in FM516-SV or M4Beu. cells and this activation occurs through upregulation of MT1-MMP, a transmembrane metalloprotease that controls MMP-2 activity in participation with the tissue inhibitor of metalloproteinase-2 (TIMP-2). (Figure 4) This overall process is a result of NF-κB activation, controlled by physical interaction between PDZ domains of MDA-9/syntenin and c-Src with subsequent activation of p38 MAPK (9).

5.5. MDA-9/syntenin-mediated FAK/Src activation induces metastasis

Given the central role c-Src/FAK signaling pathways play in regulating cell motility in cancer cells, the functional consequence of on-target and off-target siRNAs on MDA-9/syntenin-induced FAK/c-Src complex formation was scrutinized through a series of experiments. Anchorage-independent growth assay can assist in distinguishing tumorigenic cells with the capacity to metastasize *in vivo* versus tumorigenic cells that lack this ability. Overexpression of *mda-9*/syntenin in non-metastatic/weakly metastatic cells resulted in more and larger colonies in agar compared to control cells (10). However, anchorage independence was significantly suppressed by knockdown of c-Src protein using specific siRNAs. Similarly, c-Src siRNAs also reduced the ability to form soft agar colonies of two highly metastatic cells signifying the potential involvement of MDA-9/syntenin in the activation of c-Src/FAK complexes in this process (10). The requirement for c-Src in maintaining the transformed phenotype was also confirmed by cell invasion/migration assays (10). The invasive ability, showing greater effects upon overexpression of *mda*-9/syntenin in normal or weakly metastatic cells and inhibited by c-Src siRNAs in highly metastatic cell lines, further supports an involvement of the Src/FAK signaling pathway in MDA-9/syntenin-enhanced migration and invasion. In parallel, the direct involvement of *mda*-9/syntenin-induced activation of the c-Src/FAK signal transduction pathway in tumor progression was also confirmed by evaluating lung metastases in newborn rat models. Metastatic lung lesions were significantly enhanced in animals receiving cells with forced overexpression of *mda*-9/syntenin and multiple injections of c-Src siRNAs from day 2 to day 18 resulted in a significant decrease in the average number of metastatic surface tumor nodules per lung lobe. Similarly, silencing c-Src in highly metastatic variants T1P26 and 7GP through multiple treatments with active siRNAs resulted in a significant decrease in the average number of metastatic surface tumor nodules per lung lobe. Consistently, deletion of the two PDZ domains significantly reduced lung metastasis compared to wild type full-length MDA-9/syntenin expressing cells. These notable *in vivo* studies

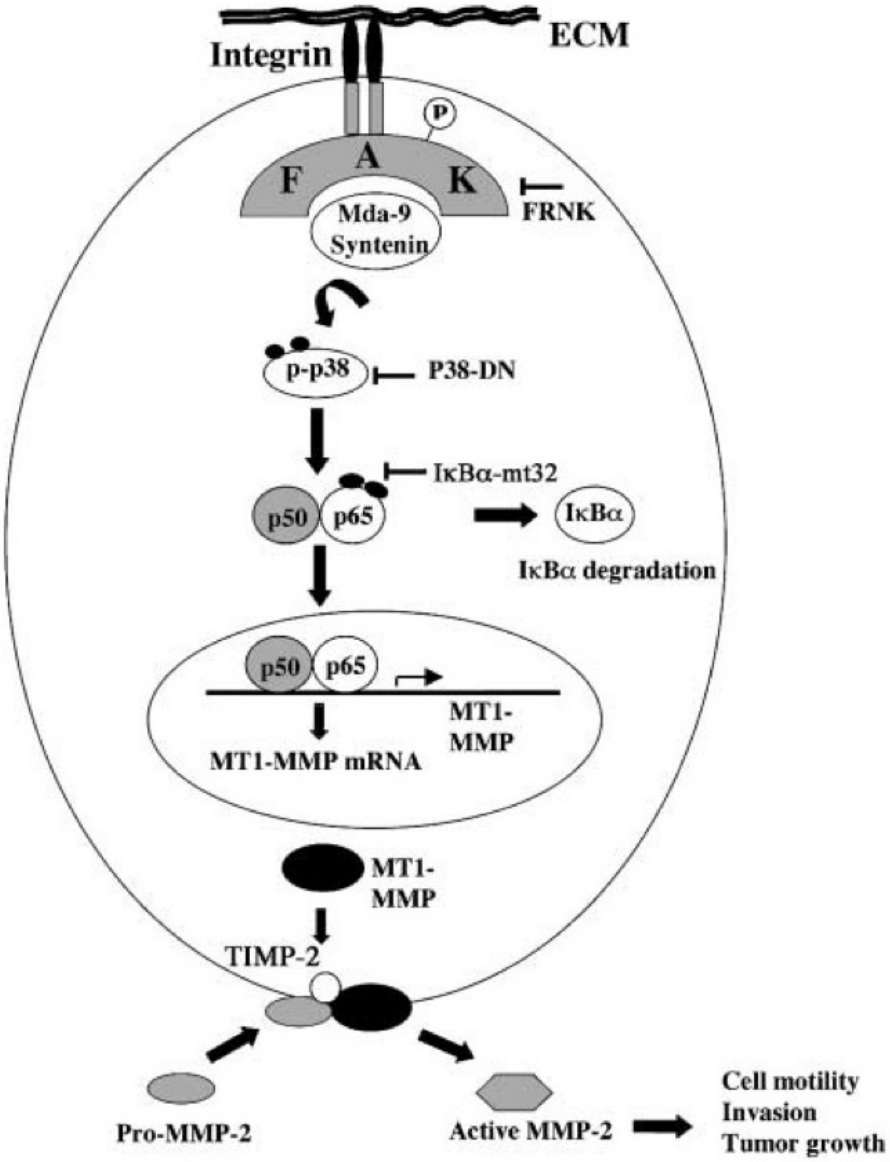

Figure 4. Schematic diagram for *mda-9*/syntenin mediated NFκB activation. Upon interaction with ECM (fibronectin), MDA-9/syntenin activates the p38MAPK by augmenting FAK phosphorylation. This results in degradation of IκBα and movement of p65 from the cytoplasm where interaction with p50 results in binding to target genes (MT1-MMP) resulting in enhanced production of MT1-MMP, which interacts with TIMP-2 activating pro-MMP-2 to produce active MMP-2. This product then enhances cell motility, invasion, and cancer cell growth. *mda*-9/Syntenin activates the NF-kB pathway. Reproduced with permission from (9).

confirm a cause-and-effect relationship between *mda*-9/syntenin-induced Src activation and metastatic competence in human melanoma cells.

5.6. MDA-9/syntenin and metastasis in the context of other malignancies

Although the role of MDA-9/syntenin in cell migration, invasion, and metastasis has been confirmed in different malignancies other than melanoma, e.g., breast and gastric carcinoma, several discrepant findings have been observed depending on the cell type studied and in different environmental contexts. In melanoma, NF-κB activation by *mda*-9/syntenin resulted in induction of MT1-MMP that lead to activation of MMP-2 (9). However, in breast cancer cells, *mda*-9/syntenin did not induce MMP-2 or MMP-9. Deletion mutation analysis revealed that the presence of both PDZ domains of MDA-9/syntenin is necessary to facilitate collagen I invasion of HEK 293T cells (61), which is in contrast to the findings in breast cancer cells showing that the PDZ-2 domain plays a major role in conferring MDA-9/syntenin function (49). This discrepancy might be explained by the cell type–specific

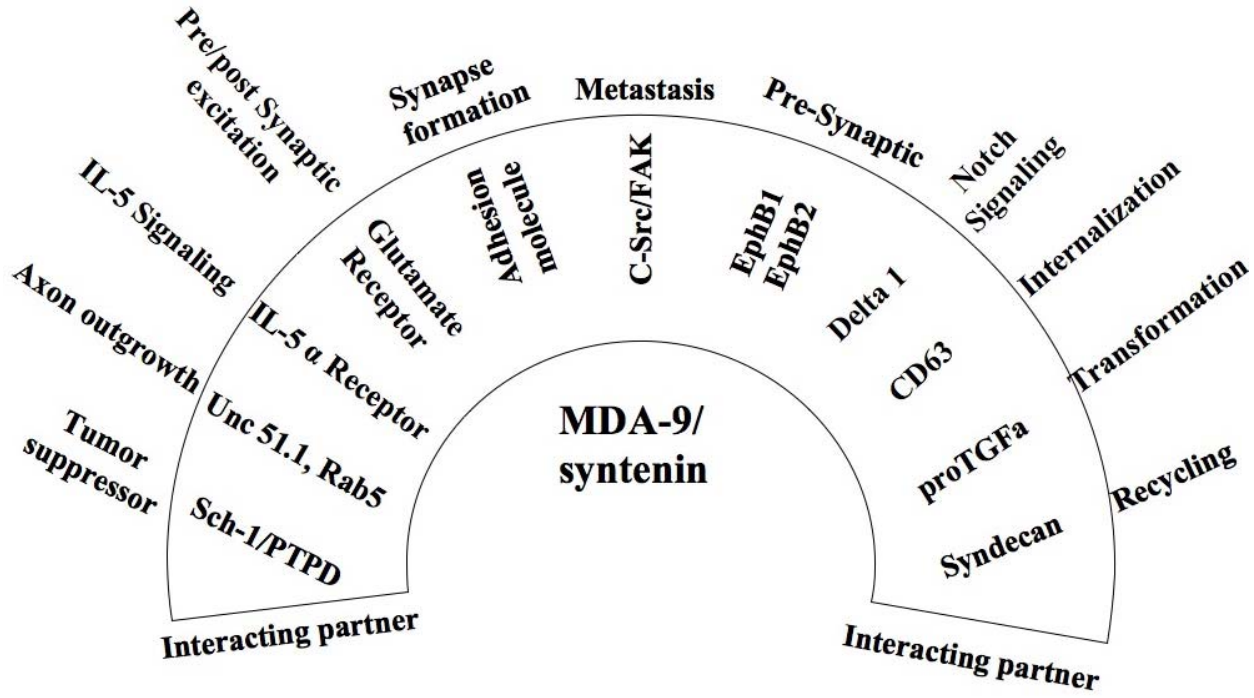

Figure 5. Diversity of potential MDA-9/syntenin interacting partners and their putative functions. As an adaptor protein, MDA-9/syntenin interacts with multiple partners through its PDZ domains and regulates a plethora of molecular events. See text for details.

action of MDA-9/syntenin as well as by ECM-induced signaling because in melanoma cells, fibronectin engagement was shown to be important for induction of MDA-9/syntenin signaling that involves FAK, p38 MAPK, and NF-κB, whereas in HEK 293T cells, Ras, Rho, Rac, PI3K/Akt, and MAPK signaling were shown to mediate MDA-9/syntenin function (9,10,61). Thus, it remains to be determined which key regulatory molecules MDA-9/syntenin interacts with to augment invasion and metastasis in different cancer types.

6. MDA-9/SYNTENIN ACTS AS A MULTIFUNCTIONAL INTRACELLULAR ADAPTER PROTEIN

One of the most challenging issues currently facing cell biologists is how signal specificity and compartmentalization are achieved, thereby allowing extracellular stimulation to result in a unique and predefined intracellular outcome. For this to occur, intracellular components must be correctly positioned in both space and time. Adaptor molecules, which contain protein-protein interaction domains, are often involved in the assembly of multimeric complexes that play an essential role in modulating signal transduction from the extracellular environment to the intracellular milieu by virtue of their association with key regulatory molecules (80). PDZ domain–containing molecules are a family of proteins that control diverse and central physiologic processes (81) and MDA-9/syntenin, belonging to PDZ domain family proteins, has a surprising variety and diversity of interacting partners and through its specific localization, controls a plethora of molecular events (Figure 5). Here we assimilate and discuss current data that support a role for *mda*-9/syntenin in processes other than cancer metastasis, including transmembrane-receptor trafficking and neuronal-synapse function.

6.1. MDA-9/syntenin regulates syndecan recycling

The association of MDA-9/syntenin with syndecans, an abundant type-I membrane proteins that bear heparin sulphate (HS) side chains on their extracellular domains was the first reported functional interaction for this adaptor protein (29). MDA-9/syntenin mutants that are defective for PIP2 binding trap syndecans in perinuclear recycling endosomes, suggesting an important role for MDA-9/syntenin in syndecan trafficking (81). Experiments have revealed that MDA-9/syntenin-syndecan interaction is not responsible for internalization, but rather for syndecan recycling that traffics back to the plasma membrane. In addition, fibroblast growth factor receptor (FGFR) accumulates in syndecan-MDA-9/Syntenin-PIP2-endosomes in an FGF-dependent manner supporting the hypothesis that Syndecan regulates the activity of several transmembrane receptors through its HS chain by the syntenin-regulated recycling pathway (82).

6.2. Syntenin and pro-TGFα transport

Evidence demonstrating a role for MDA-9/syntenin in targeting integral membrane proteins to the

cell surface has come from a study of the secretory pathway of pro-transforming growth factor α (proTGFα) (46), a functional membrane bound ligand for epithelial growth factor (EGF) receptor. The subcellular distribution is controlled by different determinants in the cytoplasmic tail of proTGFα, and MDA-9/syntenin was found to bind to one of these determinants, the C-terminal amino acid valine (46). Disruption of binding by point mutation is sufficient to result in retention of these mutants in the perinuclear area that probably coincides with the endoplasmic reticulum, and not at the cell surface suggesting that MDA-9/syntenin is also involved in internalization of transmembrane proteins at the cell surface.

6.3. MDA-9/syntenin and Delta 1: impact in Notch signaling

Delta1, ligand of Notch, stimulates adjacent cells to differentiate and promotes stem cell clustering. Following binding with Delta1 or other receptors (e.g. Jagged), Notch undergoes cleavage of its intracellular domain and activates transcription by translocation to the nucleus. A conserved PDZ domain-binding motif was located in the C-terminus of Delta1 and point mutation of the C-terminal valine (Delta1VA) dramatically increased Notch transcriptional activation and epidermal differentiation (82). A yeast two-hybrid screen confirmed that MDA-9/syntenin and Delta1 physically interact and localize to cell-cell borders in cultured keratinocytes. In human interfollicular epidermis both proteins are upregulated and knocking down of *mda*-9/syntenin or overexpressing Delta1 with a mutated PDZ-binding domain have similar effects in the context of Notch signaling and epidermal differentiation. RNA interference of *mda*-9/syntenin also results in decreased Delta1 plasma-membrane expression (82) indicating the critical role of this adaptor protein in maintaining Delta1 cell-surface expression by internalization or some other unidentified mechanisms.

6.4. MDA-9/syntenin: role in tetraspanins internalization

Tetraspanins, a large family of proteins, are clustered in specific microdomains (named tetraspanin-enriched microdomains, or TERM) in the plasma membrane that regulate maturation and processing of associated transmembrane receptors, including integrins and receptor tyrosine kinases as well as their cell-surface activity and internalization (32). A biochemical and heteronuclear magnetic resonance spectroscopy (NMR) approaches showed that MDA-9/syntenin interacts with the cytoplasmic tail of CD63, a tetraspanin ubiquitously expressed and localized both in late endocytic organelles and on the plasma membrane through PDZ domains (32). Interaction was abolished by a deletion mutant of MDA-9/syntenin (last 17 amino acids from C terminus) and demonstrated a stabilizing role of C terminus of MDA-9/syntenin. Elevated expression of MDA-9/syntenin slows down rapid constitutive internalization of the CD63 and the internalization was completely blocked by overexpressing mutant MDA-9/syntenin lacking the first 100 amino acids. AP-2 complex and clathrin-dependent endocytosis (83) are involved in CD63 internalization and MDA-9/syntenin can counteract the AP-2-dependent internalization through a competitive inhibition of the CD63-AP-2 association. Since tetraspanins are involved in regulating the membrane dynamics of their transmembrane partners, the association with MDA-9/syntenin may represent a key determinant of this activity.

6.5. MDA-9/syntenin and regulation of the nervous system through control of synaptic integrity

Neurons communicate through specialized asymmetrical cell-contact sites called synapses that have machinery for neurotransmitter release at the pre-snyaptic membrane and multiple signaling molecules at the post-synaptic membrane. Emerging evidence reveals that deregulation of synaptic functions is associated with various neurodegenerative diseases (e.g., Alzheimer's disease) and psychiatric disorders (e.g., schizophrenia) (84). An electron dense protein matrix was identified underneath these membranes containing cytoskeletal elements, scaffolod proteins including MDA-9/syntenin and homo and heterotypic adhesion molecules. Adhesion molecules involved in the formation of the synapse move towards the periphery of the contact site, allowing the sorting of signal-transducing units towards the center. MDA-9/syntenin, by interacting with several adhesion molecules such as SynCAM, neurexin and neurofascin, contributes to the establishment and maintenance of proper synaptic structures (34,37,85-87). A recent study also showed that MDA-9/syntenin forms large multimeric complexes by interacting with a cell surface molecule ERC2 (also known as CAST1) (88) and stabilizes the asymmetric protein composition at the synaptic membrane and recruits a multitude of intracellular regulators.

6.6. MDA-9/syntenin and ephrins: involvement in presynaptic development

Ephrin receptors and their cognate ligand ephrins are critical regulators of neuronal development. Based on the extracellular sequence homology and ligand-binding properties, Eph receptors can be classified into two groups: EphA receptors (EphA1–10) that preferentially bind to ephrin-A ligands and are anchored to cell membrane via a glycosyl-phosphatidylinositol linkage, and EphB receptors (EphB1–6) that exhibit high affinity to ephrin-B ligands and possess a transmembrane region and a short intracellular domain. The best characterized role of ephrin/Eph is their action as repulsive cues in retinotectal topographic mapping and axon guidance (89,90), in organizing the movements of neural crest cells during development, in directing fusion of epithelial sheets in closure of the palate, and in angiogenesis (91-96). In recent years, the roles of ephrin/Eph signaling in synapse development are also beginning to be unraveled. Upon activation, Eph receptors recruit a number of adaptors and signaling molecules for transduction of downstream signaling, such as SFKs, guanine nucleotide exchange factors (GEFs) and PDZ proteins, which are important players in neuronal synapses (97).

Recently McClelland *et al.* (98) documented that two members of ephrin-B family (ephrin B1 and ephrin B2) function to mediate EphB-dependent presynaptic

development via PDZ-binding domain-dependent interactions with MDA-9/syntenin. Identical PDZ-binding domains found on ephrin-B1 and ephrin-B2 suggests that they can both bind MDA-9/syntenin with equal affinity. Physiologically, ephrin–B1, ephrin–B2 and MDA-9/syntenin are clustered together with presynaptic markers and knockdown of ephrin-B1, ephrin-B2, or MDA-9/syntenin prevents EphB-dependent presynaptic development. More work is needed to resolve the precise role of *mda*-9/syntenin in synaptic development.

6.7. Regulation of glutamate signaling

Glutamate is the main excitatory neurotransmitter in the central nervous system that mediates pre- or postsynaptic excitation by binding to ion-channel associated (ionotropic) and G-protein-coupled (metabotropic) receptors (99). MDA-9/syntenin interacts with a variety of receptors at the synaptic cleft including glutamate receptors, the glycine transporter subtype 2, AMPA (an artificial glutamate analogue)-type glutamate receptors GluR1-GluR4, kainate receptors $GluR5_{2b}$, $GluR_{2c}$ and GluR6, and metabotropic receptors mGluR4a, mGLuR6, mGluR7a and mGluR7b (86,50). GluR5 and GluR6 are two kainate receptors, present at both pre- and post-synaptic membranes, that colocalized with MDA-9/syntenin in growth cones (50). Ectopic expression of MDA-9/syntenin increases the number of dendritic protrusions in young and mature neurons supporting the role of this adaptor protein as an initiator of glutamate-induced membrane protrusions that promote connections in the developing brain (50). Further studies, however, are required to critically evaluate whether there is a role for MDA-9/syntenin in maintaining the subcellular locations of these receptors and in synaptic integrity.

6.8. MDA-9/syntenin and axon outgrowth

The *Caenorhabditis elegans unc-51* gene is involved in axon elongation and Unc51.1, the mouse homologue 1 is a serine/threonine kinase that is important for neurite extension/parallel fiber formation in cerebellar granule neurons. MDA-9/syntenin interacts with Unc51.1 and Rab5, a member of the Ras-like small GTPases that is a marker of early endosomes and is essential for endocytic membrane fusion and trafficking (100). Interestingly, this interaction creates a scaffold for Unc51.1 and the endocytic machinery that plays a crucial role in regulating axon extension (100). Studies designed to inhibit *mda*-9/syntenin expression will provide a better understanding of the consequence of this scaffold in controlling axon growth.

6.9. MDA-9/syntenin and Interleukin-5 (IL-5) signaling

Interleukin-5 (IL-5) acts through the IL-5 receptor, which consists of an IL-5Rα and a signal-transducing β-subunit (βc) shared with the IL-3 and GM-CSF receptors (101). MDA-9/syntenin interacts with interleukin-5 (IL-5) receptor α and the transcription factor Sox4, a member of the family of transcription factors containing the high mobility group (HMG)-box DNA binding domain (102) and thus mediates IL-5–induced Sox4 activation (103). IL-5 and Sox4 regulate B-cell development and differentiation, implicating the involvement of MDA-9/syntenin in these processes.

7. MDA-9/SYNTENIN AS A TUMOR SUPPRESSOR

Although a majoriy of studies provide evidence for the role of *mda*-9/syntenin in tumor progression and metastasis studies have also implicated *mda*-9/syntenin as a tumor suppressor. Schwannomin-1 (sch-1), a product of *Neurofibromatosis type 2 (NF2)* gene, functions as a molecular adaptor linking integral membrane proteins to the cytoskeleton and suppresses growth when overexpressed. Yeast-two hybrid assays with sch-1 COOH terminus as bait identified MDA-9/syntenin as an interacting molecule (86) that colocalized in the cytoplasmic face of the plasma membrane. Inhibition of *mda*-9/syntenin by an antisense approach inhibited the plasma membrane localization of sch-1, indicating that MDA-9/syntenin-sch-1 interaction plays a role in the targeting of sch-1 to the plasma membrane. It has not been tested whether inhibition of MDA-9/syntenin interferes with the growth-suppressing function of sch-1. In addition to sch-1, MDA-9/syntenin interacts with another tumor suppressor protein, r-PTPD, a receptor-type tyrosine phosphatase (102). The human homologue of r-PTPD also shows decreased expression in thyroid carcinomas in comparison with normal thyroid tissues. Although MDA-9/syntenin was phosphorylated at tyrosine residues, it was observed that r-PTPD did not dephosphorylate it, indicating that MDA-9/syntenin is not a substrate of r-PTPD although there is an interaction. The biological implication of this interaction is not yet resolved.

Considering that MDA-9/syntenin plays an active role in mediating tumor progression and metastasis, the functional significance of its interaction with tumor suppressor proteins needs to be scrutinized in detail. It might be possible that by sequestering these proteins to the plasma membrane, MDA-9/syntenin might actually inhibit their function.

8. CONCLUSIONS AND FUTURE PERSPECTIVES

Since its discovery in a subtraction hybridization screen for genes involved in melanoma differentiation, MDA-9/syntenin has been frequently identified with surprising diversity of interacting partners suggesting its flexible cell-type-specific roles, forming unique scaffolds that are dependent on the intracellular environment or compartment in which they are localized. The role of MDA-9/syntenin in regulating cell migration, invasion, and metastasis has been well documented by multiple studies. As a positive regulator of metastasis, transgenic mice expressing *mda*-9/syntenin as well as conditional targeted *mda*-9/syntenin knockout mice would prove extremely valuable for analyzing tumor progression *in vivo*. Moreover, crossing these animals with other tumor models to determine potential cross-talk between *mda*-9/syntenin and other tumor-promoting pathways would also prove very informative. Angiogenesis is an integral component of metastasis and considering its interaction with ephrins, and their receptors and Src or other unidentified molecules *mda*-9/syntenin might regulate angiogenesis, another important parameter of tumor progression. Indeed, our initial findings indicate that *mda-9*/syntenin augments

tumor angiogenesis in a non-autonomous manner through upregulating several pro-angiogenic factors. Unraveling these diverse findings would help comprehend the function of *mda*-9/syntenin, thereby providing a better perspective in developing anti-metastatic strategies based on the targeted disruption of *mda*-9/syntenin or its regulated pathways.

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