

mda-9/Syntenin Regulates the Metastatic Phenotype in Human Melanoma Cells by Activating Nuclear Factor- κ B

Habib Boukerche,¹ Zao-zhong Su,¹ Luni Emdad,^{1,2} Devanand Sarkar,^{1,3} and Paul B. Fisher^{1,2,3}

Departments of ¹Urology, ²Neurosurgery, and ³Pathology, Herbert Irving Comprehensive Cancer Center, College of Physicians and Surgeons, Columbia University Medical Center, New York, New York

Abstract

mda-9/Syntenin is a scaffolding PDZ domain-containing protein overexpressed in multiple human cancers that functions as a positive regulator of melanoma metastasis. Using a normal immortal human melanocyte cell line and weakly and highly metastatic human melanoma cell lines, we presently show that *mda-9*/syntenin initiates a signaling cascade that activates nuclear factor- κ B (NF- κ B) in human melanoma cells. As a consequence of elevated *mda-9*/syntenin expression, tumor cell growth and motility, fundamental components of tumor cell invasion and metastatic spread of melanoma cells, are enhanced through focal adhesion kinase (FAK)-induced and p38 mitogen-activated protein kinase (MAPK)-induced activation of NF- κ B. Inhibiting *mda-9*/syntenin, using an adenovirus expressing antisense *mda-9*/syntenin, NF- κ B, using an adenovirus expressing a mutant superrepressor of κ B α , or FAK, and using a dominant-negative mutant of FAK (FRNK), blocks melanoma cell migration, anchorage-independent growth, and invasion. Downstream signaling changes mediated by *mda-9*/syntenin, which include activation of FAK, p38 MAPK, and NF- κ B, promote induction of membrane-type matrix metalloproteinase-1 that then activates pro-MMP-2—promoting migration and extracellular matrix invasion of melanoma cells. These results highlight the importance of *mda-9*/syntenin as a key component of melanoma metastasis providing a rational molecular target for potentially intervening in the metastatic process. [Cancer Res 2007;67(4):1812–22]

Introduction

Melanoma differentiation associated gene-9 (*mda-9*) was identified by subtractive hybridization as a novel gene displaying biphasic expression during terminal differentiation in human melanoma cells treated with a combination of fibroblast IFN (IFN- β) and the antileukemic compound mezerein (1, 2). *mda-9*, also called syntenin, is now recognized as a significant member of the expanding family of scaffolding proteins with highly potent and diverse biological activities (3, 4). A noticeable feature of *mda-9*/syntenin is the presence of tandem PDZ domains of 83 and 80 amino acid residues, respectively (PDZ1 and PDZ2), which

selectively bind to specific motifs at the COOH termini of partner proteins, including class B ephrins, pro-transforming growth factor- α , PTP- η , phosphatidylinositol 4,5-bisphosphate, neurofascin, neuexin, schwannomin (also known as merlin), interleukin-5 receptor α , lymphocyte receptor CD63, various glutamate receptor subtypes, and the syndecan family of heparan sulfate proteoglycans (3, 5, 6). This flexibility of interacting partners allows MDA-9/syntenin to participate in an assortment of biological functions, including receptor clustering (7), protein trafficking (8, 9), synaptic transmission (3), activation of the transcription factor Sox4 (10), syndecan recycling through endosomal compartments (5), and cytoskeleton-membrane organization (11, 12).

Based on its biphasic expression in terminally differentiating human melanoma cells, we postulated that *mda-9*/syntenin might display an inverse relationship with tumor progression (low-level expression in melanocytes versus elevated expression as melanocytes evolve into malignant melanoma). This hypothesis was tested recently and validated using a clinically relevant melanoma model (13, 14) providing direct evidence that *mda-9*/syntenin promotes melanoma progression and metastasis *in vivo* (15). The biological significance of *mda-9*/syntenin in melanoma progression is supported by immunohistochemical studies showing that MDA-9/syntenin is expressed by an increasing proportion of primary melanomas in the transition from the radial to vertical growth phase (15, 16). An intriguing property of *mda-9*/syntenin is its ability, when expressed by means of a replication-incompetent adenovirus (Ad.*mda-9*/S) in melanoma cells, to alter cytoskeletal organization, and increase focal adhesion kinase (FAK) phosphorylation and other signaling molecules, such as phospho-p38 and phospho-c-Jun NH₂-terminal kinase (JNK), resulting in increased motility and invasive potential of melanoma cells (15). In these contexts, by altering cytoskeletal organization and cell shape, signaling through *mda-9*/syntenin seems to be critical for regulating cell motility and invasion. However, it is unclear precisely how *mda-9*/syntenin regulates gene expression that culminates in these phenotypic changes (15, 17).

Transcriptional regulators, such as nuclear factor- κ B (NF- κ B), play central roles in regulating gene expression that promote cancer cell motility and invasion (18, 19). Cancer progression in multiple cancers, including melanoma, breast, prostate, colorectal, and ovarian cancer, and in specific forms of leukemia and lymphoma correlates with a significant elevation in NF- κ B expression (18, 20, 21). Based on this consideration, we have presently investigated the possible role of *mda-9*/syntenin in NF- κ B activation. In its classic form, NF- κ B consists of homodimer or heterodimer subunits of the Rel family, including p50 and p65 subunits, which are maintained in resting cells in an inactive state in the cytoplasm through binding to κ B α (19, 22). However, on stimulation with various stimuli, κ B α becomes phosphorylated, ubiquitinated, and subsequently degraded via the ubiquitin-proteasome pathway, thereby resulting in activation and migration

Note: This work was done by H. Boukerche as a visiting research scientist from Institut National de la Sante et de la Recherche Medicale.

Current address for H. Boukerche: Institut National de la Sante et de la Recherche Medicale Unit 590, 28 rue Laennec, Centre Léon Bérard, 69373, Lyon, Cedex 08, France.

Requests for reprints: Paul B. Fisher, Departments of Pathology and Urology, College of Physicians and Surgeons, Columbia University Medical Center, 630 West 168th Street, BB-1501, New York, NY 10032. Phone: 212-305-3642, -3966; Fax: 212-305-8177; E-mail: pbf1@columbia.edu.

©2007 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-06-3875

of NF- κ B proteins into the nucleus where this complex regulates expression of multiple target genes (23).

We presently uncover a novel role of *mda-9/syntenin* as an important scaffolding protein that can initiate a signaling cascade resulting in the induction of NF- κ B in human melanoma cells. Tumor cell growth and motility, which are primary contributors to invasion and metastatic spread of melanoma cells, are specifically enhanced by the functional cooperation of *mda-9/syntenin*-induced FAK and p38 mitogen-activated protein kinase (MAPK) activation through activation of NF- κ B. This increase in specific cell signaling pathways in our genetically manipulated melanoma cancer cells may represent an essential process that leads to induction of membrane-type matrix metalloproteinase-1 (MT1-MMP) subsequently activating pro-MMP-2, which in turn promotes migration and extracellular matrix (ECM) invasion by melanoma cells. These novel observations provide additional support for the hypothesis that *mda-9/syntenin* may provide an appropriate genetic target for melanoma cancer therapy.

Materials and Methods

Reagents and cell lines. Normal immortal human melanocyte (FM516-SV), M4Beu., a poorly metastatic human melanoma cell line, T1P26 and 7GP highly metastatic variants derived from M4Beu. cells, and MeWo and HO-1 metastatic melanoma cell lines isolated from patients were cultured as described (13, 15, 24). Rabbit polyclonal antibodies, anti-p50, anti-p65, and I κ B α were from Santa Cruz Biotechnology (Santa Cruz, CA). A β -galactosidase expression plasmid (pSV- β -gal) and Luciferase Reporter Gene Assay kit were from Promega Corp. (Madison, WI). Galacto-Light Plus kit was from Tropix (Bedford, MA).

Virus construction and infection protocol. The recombinant replication-incompetent adenoviruses expressing Ad.*mda-9/S* and Ad.*mda-9/AS* expressing *mda-9/syntenin* in an antisense orientation were created in two steps as described previously and plaque purified by standard procedures (15). Adenovirus constructs encoding FAK COOH-terminal domain (FRNK) or a dominant-negative JNK replication-defective adenovirus were provided by Drs. A.M. Samarel (Loyola University Chicago, Maywood, IL; ref. 25) and R.A. Fine (Department of Medicine, Columbia University Medical Center, New York, NY), respectively. Dominant-negative kinase-deficient mutant p38 MAPK (Ad.p38DN) and mt32I κ B α -superrepressor (Ad.I κ B α -mt32) adenoviruses were prepared as described and provided by Dr. K. Valerie (Virginia Commonwealth University, Richmond, VA; refs. 15, 26). Cells were infected with a multiplicity of infection (MOI) of 1 to 100 plaque-forming units (pfu) per cell of Ad.*null* (control replication-incompetent adenovirus lacking a gene insert) or Ad.*mda-9/S* and then plated on fibronectin in serum-free medium for 2 to 4 h as described previously (15).

Preparation of cell extracts and electrophoretic mobility shift assay. Cells infected with either the Ad.*null* vector, Ad.*mda-9/S*, or Ad.*mda-9/AS* were washed in cold PBS (pH 7.2), and cytoplasm and nuclear extracts were prepared as described previously (26). Double-stranded oligonucleotides containing the consensus or mutated DNA-binding site for the NF- κ B proteins (5'-AGTTGAGGGGACTTCCAGGC-3' and 5'-AGTTGAGGC-GACTTCCAGGC-3', respectively; Santa Cruz Biotechnology) were end labeled using [γ -³²P]ATP according to the manufacturer's protocol (Promega) and purified using Sephadex G-25 column chromatography (Amersham BioSciences, Buckinghamshire, United Kingdom). The binding assay for the proteins was carried out as described previously (26).

Western blot analysis. Whole-cell lysates and nuclear or cytoplasmic extracts were prepared, and Western blot was carried out as described previously (15, 26). Blots were probed using rabbit polyclonal antibodies against p65, p50, or I κ B α according to the manufacturer's recommendation followed by sequential addition of a goat anti-rabbit IgG antibody conjugated to peroxidase and enhanced chemiluminescence development reagent.

Transfections and reporter assays. Briefly, cells (2×10^5) were infected with either the Ad.*null* vector, Ad.*mda-9/S*, Ad.*mda-9/AS*, or Ad.*mda-9/S/*

Ad.FRNK with the indicated MOI. Twelve hours later cells were transfected with a NF- κ B-responsive luciferase reporter construct with LipofectAMINE 2000. A total of 1.2 μ g plasmid DNAs, including pGL3Basic, 3 κ B-Luc, or 3 κ Bmut-Luc plasmids, provided by Dr. A. Baldwin (University of North Carolina in Chapel Hill, Chapel Hill, NC; ref. 26), were mixed with 0.3 μ g pSV- β -gal and transfected according to the manufacturer's protocols. Forty-eight hours after transfection, cells were trypsinized and plated on either poly-L-lysine- or fibronectin-coated surfaces (10 μ g/mL) in serum-free medium for 1 h. Cell lysates were harvested and luciferase activity was measured using a Luciferase Reporter Gene Assay kit according to the manufacturer's instructions. The β -galactosidase activity was determined using the Galacto-Light Plus kit. Luciferase activity was normalized by β -galactosidase activity and data represent the average of triplicates \pm SD.

Restrictive anchorage-independent growth assay. Colony formation in soft agar was determined as described previously using a modified assay that differentiates between tumorigenic and metastatic cells (15, 27). Briefly, cells (1×10^5) that had been infected with either the Ad.*null* vector, Ad.*mda-9/S*, Ad.I κ B α -mt32, or Ad.*mda-9/S/Ad.I κ B α -mt32* were plated in complete culture medium containing 0.6% agar on top of 0.6% agar in the same medium in 60-mm dishes. Macroscopic colonies were counted and scored after 2 weeks growth under a dissection microscope. Clonogenicity was determined using three independent experiments.

Invasion and migration assays. Tumor cell invasion assays were done using 24-well BioCoat cell culture inserts (Becton Dickinson Labware, Bedford, MA) with 8- μ m porosity polyethyleneterephthalate membranes coated with Matrigel as described previously (15). Briefly, uninfected or infected cells (5×10^4 in 500 μ L serum-free medium) were seeded in triplicate on the upper part of each chamber. Chambers were incubated at 37°C at the indicated times, after which the filters were removed, fixed, and stained with DiffQuick Staining kit. Cells on the upper surface of the filters were removed by wiping with a cotton swab, and migration was determined by counting cells that had migrated to the lower side of the filter with a microscope at $\times 100$ magnification. Experiments were done in triplicate, and at least eight fields were counted in each experiment. Wound healing scratch motility assays were done as described previously (15).

Results

High constitutive NF- κ B (RelA/p65) activation is observed in melanoma cells overexpressing *mda-9/syntenin*. *mda-9/Syntenin* expression is elevated in metastatic melanoma cells versus normal melanocytes and it regulates the ability of melanoma cells to migrate toward fibronectin and invade a reconstituted basement membrane (Matrigel; ref. 15). Because NF- κ B is required for specific aspects of tumor progression, including cell invasion/migration (19, 28, 29), we investigated the possible relationship between NF- κ B and *mda-9/syntenin* in melanoma cells. NF- κ B electrophoretic mobility shift assays (EMSA) were done using an oligonucleotide probe containing a canonical NF- κ B binding site (Fig. 1A). When plated on poly-L-lysine-coated plates, normal immortal melanocytes (FM516-SV) and poorly metastatic melanoma cells (M4Beu.) contain low levels of two NF- κ B protein complexes, suggesting that NF- κ B is constitutively active in these cells. The level of these protein complexes slightly increased in FM516-SV and M4Beu. cells when plated on fibronectin. In contrast, when plated on fibronectin, the intensity of these complexes was greater in metastatic cells, including metastatic melanoma variants, T1P26 and 7GP, as well as in cell lines derived from patients with metastatic melanomas, MeWo and HO-1, which expressed high levels of *mda-9/syntenin*. In addition, when a reporter construct containing three NF- κ B consensus binding sites upstream of the luciferase gene (3 κ B-Luc) was transiently transfected into melanoma cells, constitutive NF- κ B promoter activity was significantly higher in the metastatic variants (T1P26

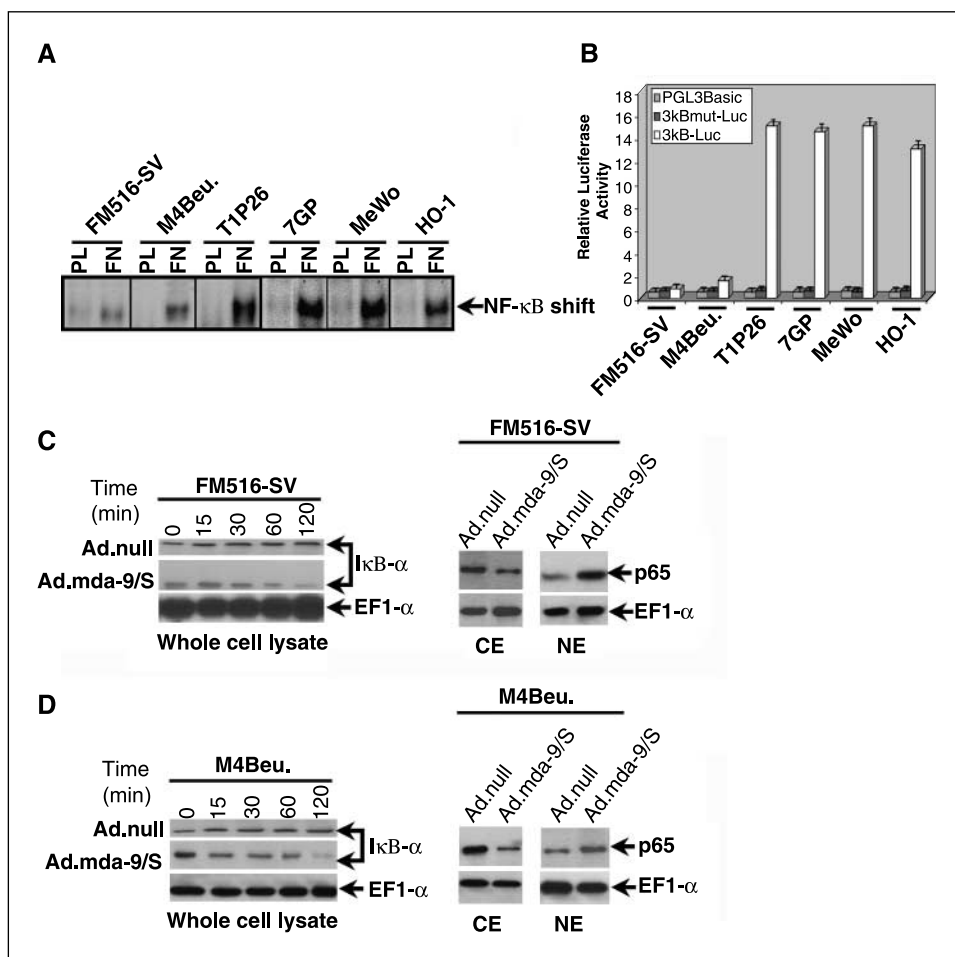


Figure 1. NF- κ B activity in normal immortal human melanocytes and human melanoma cell lines derived from tumors at different stages of progression and patient-derived tumors. **A**, constitutive NF- κ B DNA binding activity in FM516-SV, M4Beu. (weakly metastatic), T1P26 and 7GP (highly metastatic), and additional metastatic human melanoma cell lines. Nuclear extracts from cells plated on poly-L-lysine (PL) or fibronectin (FN) in serum-free medium for 2 to 4 h were assayed by EMSA with a 32 P-labeled oligonucleotide probe containing a NF- κ B consensus binding site. **B**, constitutive NF- κ B promoter activity. Cells transfected with the indicated reporter constructs were plated on fibronectin-coated plates in serum-free medium. Luciferase activity was measured as described in Materials and Methods. *Columns*, mean of triplicate samples from three independent experiments; *bars*, SD. **C** and **D**, I κ B α is degraded in a time-dependent manner and p65 is translocated from the cytoplasm into the nucleus following infection of FM516-SV (**C**) or M4Beu. (**D**) cells with Ad.*mda-9/S*. Cells were infected with Ad.*null* or Ad.*mda-9/S* at a MOI of 100 pfu/cell. After 48 h, cells were plated on fibronectin-coated plates in serum-free medium. At the indicated times, whole-cell lysates were analyzed by Western blot (left). Expression of p65 protein in cytoplasmic (CE) and nuclear (NE) extracts were analyzed by Western blot (right).

and 7GP) as well as in the metastatic melanoma cell lines (MeWo and HO-1) in comparison with FM516-SV or M4Beu. cells (Fig. 1B).

Forced expression or inhibition of expression of *mda-9/syntenin* by adenovirus transduction regulates NF- κ B binding activity in human melanoma cells. To determine whether *mda-9/syntenin* regulates NF- κ B activity that may underlie the ability of this gene to promote melanoma cell invasion and migration, we manipulated the expression levels of *mda-9/syntenin* in M4Beu., with low metastatic potential, and T1P26, which generates a high frequency of spontaneous metastasis in immunosuppressed neonatal rats (15). Previous studies showed that the level of MDA-9/syntenin protein produced by adenovirus infection of FM516-SV or M4Beu. cells was comparable with the constitutive level found in T1P26 cells (15). As an early indicator of NF- κ B activation, we assessed the cytoplasmic levels of I κ B α protein by Western blot analysis. Infection of FM516-SV or M4Beu. cells with Ad.*mda-9/S* induced a time-dependent degradation of I κ B α , which became evident within 15 to 30 min and decreased further at 2 h after plating cells on fibronectin in comparison with uninfected or Ad.*null*-infected control cultures (Fig. 1C and D). Because NF- κ B activation requires nuclear translocation of the p65 subunit of NF- κ B, we measured the level of p65 in the cytoplasm and nucleus. As predicted, the level of the p65 subunit of NF- κ B decreased in the cytoplasm of Ad.*mda-9/S*-infected FM516-SV and M4Beu. cells (Fig. 1C and D) and concomitantly increased in the nucleus when compared with Ad.*null*-infected cells (Fig. 1C and D).

NF- κ B EMSAs were done to further assess the ability of *mda-9/syntenin* to modulate the levels of NF- κ B activity in melanoma cells. Nuclear extracts prepared from control or uninfected FM516-SV or M4Beu. cells plated in serum-free medium on fibronectin contained low levels of two protein complexes (Fig. 2A and B). Infection of FM516-SV or M4Beu. cells with Ad.*mda-9/S* resulted in a pronounced dose-dependent increase of NF- κ B consensus oligomer-binding activity compared with nuclear extracts prepared from control cells or Ad.*null*-infected cells (Fig. 2A and B). The specificity of the DNA-protein complex formed in infected cells was shown by the ability of increasing concentrations of unlabeled NF- κ B oligonucleotides (25- to 100-fold molar excess) to significantly decrease the binding of the 32 P-labeled oligonucleotides, whereas the addition of control oligonucleotides containing a mutated NF- κ B binding site did not affect the binding of the labeled probe to the same extent (Fig. 2A and B). To establish the identity and composition of these complexes, EMSAs were done using p65 and p50 polyclonal antibodies. Incubating nuclear extracts with the combination of anti-p50 and anti-p65 resulted in the appearance of two supershifted bands in M4Beu.- and FM516-SV-infected cells with a concomitant loss of the unshifted NF- κ B complexes (Fig. 2B; data not shown).

An antisense strategy also confirmed the association between *mda-9/syntenin* and NF- κ B. After infection of T1P26 cells with Ad.*mda-9/AS* (50 and 100 pfu/cell) and plating on fibronectin in serum-free medium, a dose-dependent decrease in NF- κ B activity

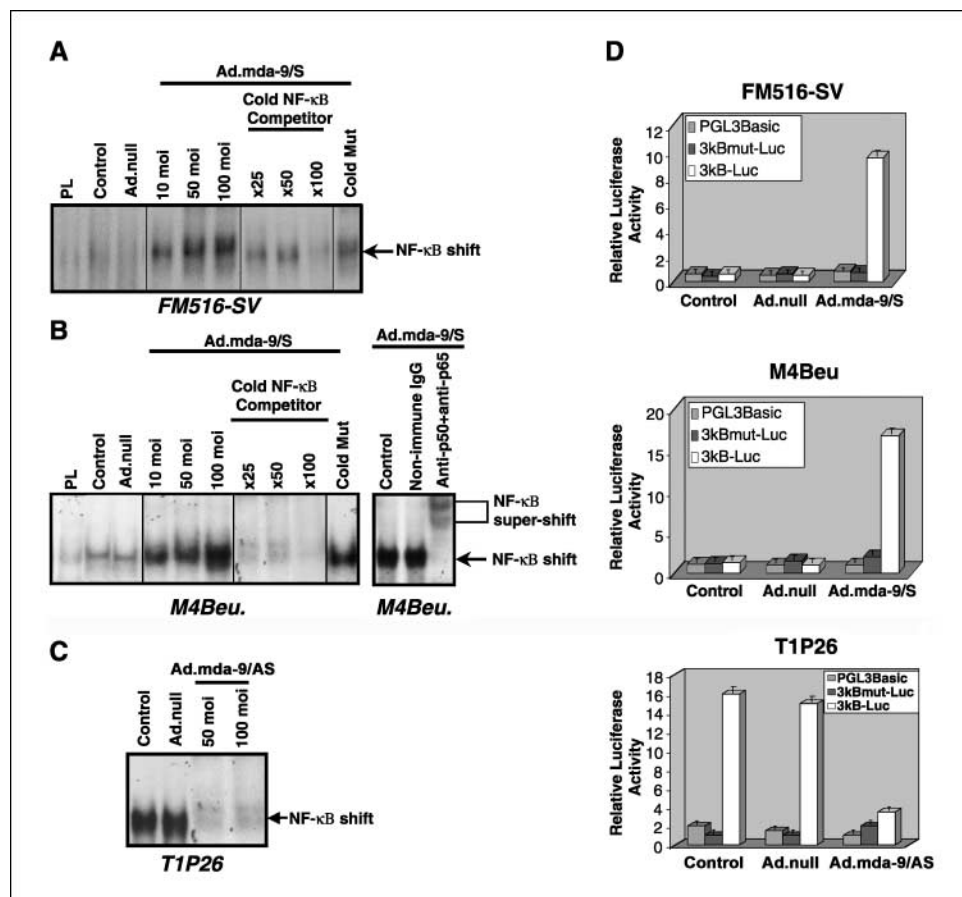
(up to 80% reduction) was observed (Fig. 2C). Treatment of additional metastatic human melanoma cell lines, including SH-1, 3S5, C8161, and FO-1, with Ad.mda-9/AS resulted in a similar reduction in NF-κB DNA binding activity (data not shown). These results suggest a direct correlation between elevated *mda-9/syntenin* and NF-κB expression and melanoma progression. Additionally, these experiments showed that *mda-9/syntenin* is able to induce an increase in NF-κB DNA binding activity that was mediated by the translocation of the p50/p65 NF-κB heterodimer into the nucleus of melanoma cells.

Adenovirus-mediated *mda-9/syntenin* expression regulates NF-κB-dependent gene expression in human melanoma cells. To confirm transcriptional activity of the NF-κB complexes, FM516-SV and melanoma cells were infected with Ad.null, Ad.mda-9/S, and/or Ad.mda-9/AS and transiently transfected with a luciferase reporter construct containing tandem NF-κB binding sites (3κB-Luc). A 6- and 8-fold induction in relative luciferase activity, respectively, was observed in Ad.mda-9/S-infected FM516-SV or M4Beu. cells plated on fibronectin compared with Ad.null-infected cells (Fig. 2D). In contrast, when Ad.mda-9/S-infected cells were transfected with a reporter plasmid containing a mutated (3κBmut-Luc) or lacking NF-κB sites (pGL3Basic), no induction in luciferase activity was seen. In a similar set of experiments, we tested whether down-regulation of *mda-9/syntenin* expression in T1P26 cells by Ad.mda-9/AS could affect NF-κB transcriptional activity. In this experimental protocol, the transcriptional activity of the NF-κB-responsive promoter was decreased (up to 6-fold) in Ad.mda-9/AS-infected T1P26 cells. In contrast, no reporter

induction was seen in control-, Ad.null-, or Ad.mda-9/AS-infected cells that were transfected with either pGL3Basic or the 3κBmut-Luc constructs (Fig. 2D). Taken together, these data confirm a direct relationship between the expression of *mda-9/syntenin* and the transcriptional activation of NF-κB in immortal melanocytes and melanoma cells.

FRNK functions as a dominant-negative of *mda-9/syntenin*-mediated NF-κB activation. *mda-9/Syntenin* plays a pivotal role in the regulation of cell invasion and migration of melanoma cells by virtue of its ability to increase phosphorylation of FAK (15). Given the central role FAK plays in the regulation of these processes and because both FAK expression and NF-κB activation associate with melanoma cancer progression (20, 30, 31), we hypothesized that *mda-9/syntenin* expression might affect the NF-κB pathway in melanoma. To address this possibility, we tested whether a dominant-negative acting splice variant of FAK, FRNK, could function as a competitive inhibitor of NF-κB. FM516-SV or M4Beu. cells were coinfecting with Ad.mda-9/S at a MOI of 50 pfu/cell and Ad.FRNK at a MOI of 10 to 100 pfu/cell, nuclear proteins were extracted, and EMSA was done. As shown in Fig. 3A and B, inhibition of NF-κB DNA binding activity was dose dependent and reached a maximum level of inhibition when infected with 50 pfu/cell of Ad.FRNK and Ad.mda-9/S compared with cells infected with Ad.mda-9/S alone. Significant inhibition of DNA binding activity was observed following infection with 10 pfu/cell of Ad.FRNK in combination with 50 pfu/cell of Ad.mda-9/S (approximately 80–85% inhibition of NF-κB DNA binding activity).

Figure 2. The NF-κB signaling pathway is activated by Ad.mda-9/S. FM516-SV (A) and (B) M4Beu. cells were infected with Ad.null or Ad.mda-9/S at the indicated pfu per cell and plated on fibronectin in serum-free medium for 2 to 4 h, and nuclear NF-κB binding activity was then assessed by EMSA. Adding a 100-fold excess of unlabeled consensus NF-κB oligonucleotide competitor inhibited binding, and a 100-fold excess of unlabeled mutated NF-κB probe had a minimal effect on binding. B, nuclear extracts from infected M4Beu. cells (100 pfu/cell) plated on fibronectin in serum-free medium were incubated with a double-stranded ³²P-labeled consensus NF-κB oligonucleotide followed by incubation with polyclonal antibody against the p65 and p50 NF-κB subunits or with control rabbit IgG. Arrows, supershifted bands. C, T1P26 cells were infected with Ad.null or Ad.mda-9/AS at the indicated pfu per cell and plated on fibronectin, and nuclear NF-κB binding activity was analyzed by EMSA. D, FM516-SV and M4Beu. cells were either uninfected or infected with Ad.null or Ad.mda-9/S at 100 pfu/cell. T1P26 cells were infected with Ad.null or Ad.mda-9/AS at 100 pfu/cell. Twelve hours later, cells were transfected with the indicated reporter constructs and then seeded on fibronectin-coated plates in serum-free medium. Luciferase activity was measured as described in Materials and Methods.



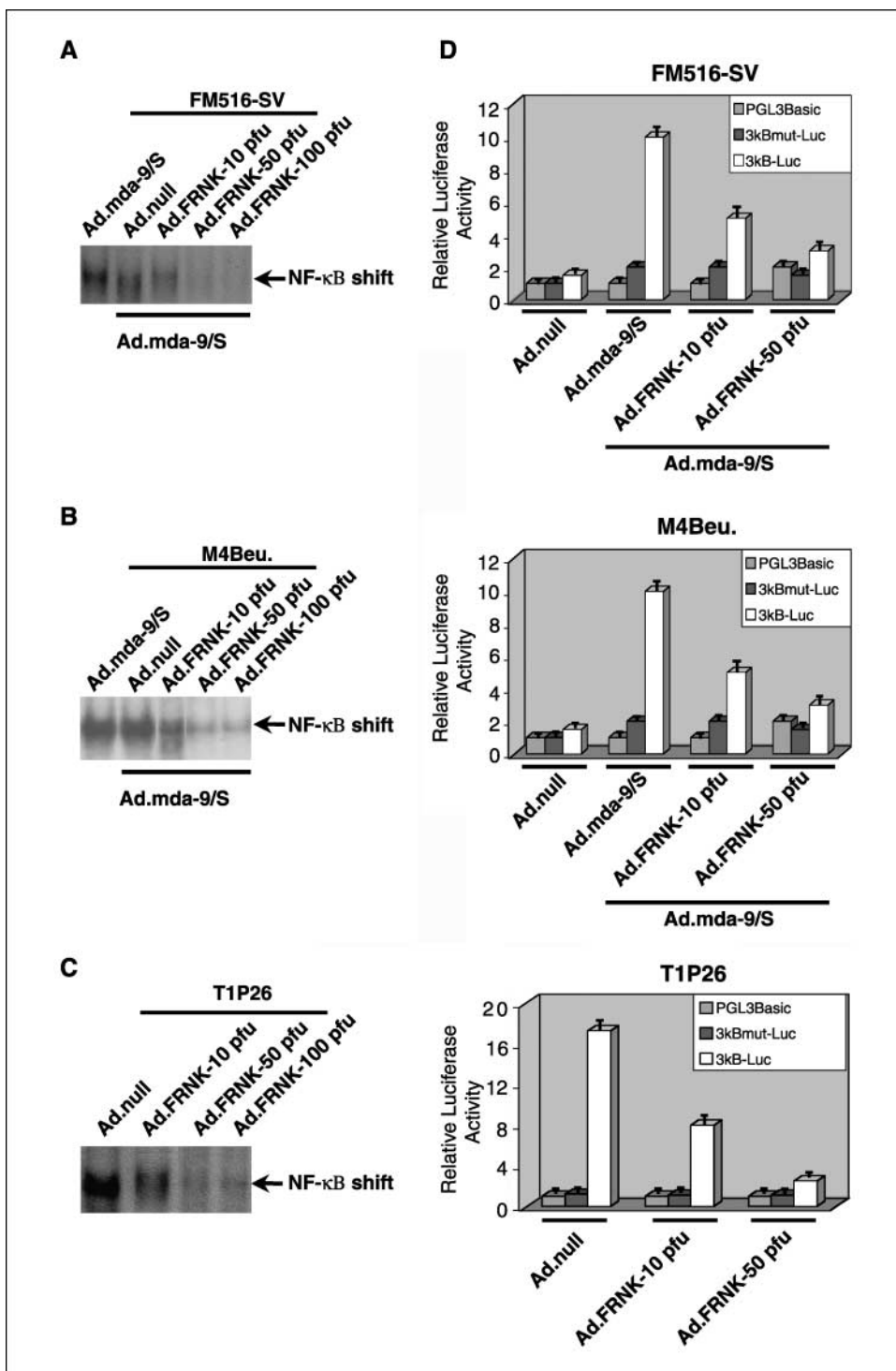


Figure 3. Ad.FRNK is a negative regulator of *mda-9*/syntenin-mediated NF-κB signaling. *A* and *B*, FM516-SV and M4Beu. cells were infected with Ad.null or Ad.mda-9/S, alone or in combination with Ad.FRNK, at the indicated pfu per cell. *C*, T1P26 cells were infected with Ad.null or Ad.FRNK at the indicated pfu per cell. Cells were then replated on fibronectin in serum-free medium for 2 to 4 h, and nuclear NF-κB binding activity was then assessed by EMSA. *D*, FM516-SV and M4Beu. cells were infected as above and then transfected 12 h later with the indicated reporter constructs. T1P26 cells infected with Ad.null or Ad.FRNK (100 pfu/cell) were transfected with the indicated plasmid. Cells were then seeded on fibronectin-coated plates in serum-free medium and luciferase activity was measured as described in Materials and Methods.

To further confirm the requirement for FAK in *mda-9*/syntenin-induced activation of NF-κB in FM516-SV and M4Beu. cells, a NF-κB luciferase reporter assay was done. As shown in Fig. 3D, coinfection of the dominant-negative Ad.FRNK with Ad.mda-9/S significantly blocked in a dose-dependent manner *mda-9*/syntenin-induced transcriptional activation of NF-κB. Infected cells that were transfected with either pGL3Basic or 3κBmut-Luc containing three mutated NF-κB sites showed only basal transcriptional

activation of NF-κB. Similarly, a dose-dependent decrease (10 and 50 pfu/cell) in NF-κB DNA binding activity (up to 80% reduction) and transcriptional activity of the NF-κB-responsive promoter (up to 8-fold) was observed in Ad.FRNK-infected T1P26 cells in comparison with Ad.null-infected cells (Fig. 3C and D). These results indicate that FAK activation is required for *mda-9*/syntenin-induced NF-κB activation and suggest that FAK might be the potential downstream signaling kinase that leads to the

increased transcriptional activity of NF-κB in response to *mda-9*/syntenin overexpression.

Anchorage-independent growth and cell motility of melanoma cells depend on *mda-9*/syntenin induction of NF-κB. The ability of cancer cells to grow autonomously in an anchorage-independent manner and the processes of migration/invasion are two prominent attributes of the metastatic phenotype of tumor cells. Using a soft agar assay that distinguishes between tumorigenic cells with the capacity to metastasize *in vivo* versus tumorigenic cells that lack this ability and a cell invasion/migration assay (27), we addressed the question of whether these two biological phenomena were dependent on NF-κB activation induced by *mda-9*/syntenin. For this analysis, we used an adenovirus expressing the mt32IκBα superrepressor (IκBα-mt32), Ad.IκBα-mt32, which inhibits IκBα degradation and subsequent NF-κB nuclear translocation (32). As

shown in Fig. 4A, Ad.IκBα-mt32 (50 pfu/cell) was as effective as Ad.*mda-9*/AS (50 pfu/cell) in inhibiting the number (approximately 70–75%) and the size of T1P26 colonies in agar when compared with mock- or Ad.*null*-infected cells. Given the apparent equal efficiencies of Ad.IκBα-mt32 and Ad.*mda-9*/AS in inhibiting anchorage-independent growth, we tested the ability of this mutant, Ad.IκBα-mt32, to compete with endogenous NF-κB for DNA binding, thereby resulting in suppression of colony formation in agar. As shown in Fig. 4A, the number and the size of the colonies of FM516-SV and M4Beu. cells were significantly enhanced on infection with Ad.*mda-9*/S (50 pfu/cell), with a more dramatic enhancement observed in M4Beu. cells than in FM516-SV cells (15). In contrast, this enhancement was significantly inhibited by more than 75% to 85% when cells were coinfectd with Ad.IκBα-mt32 and Ad.*mda-9*/S (50 pfu/cell of each virus; Fig. 4A).

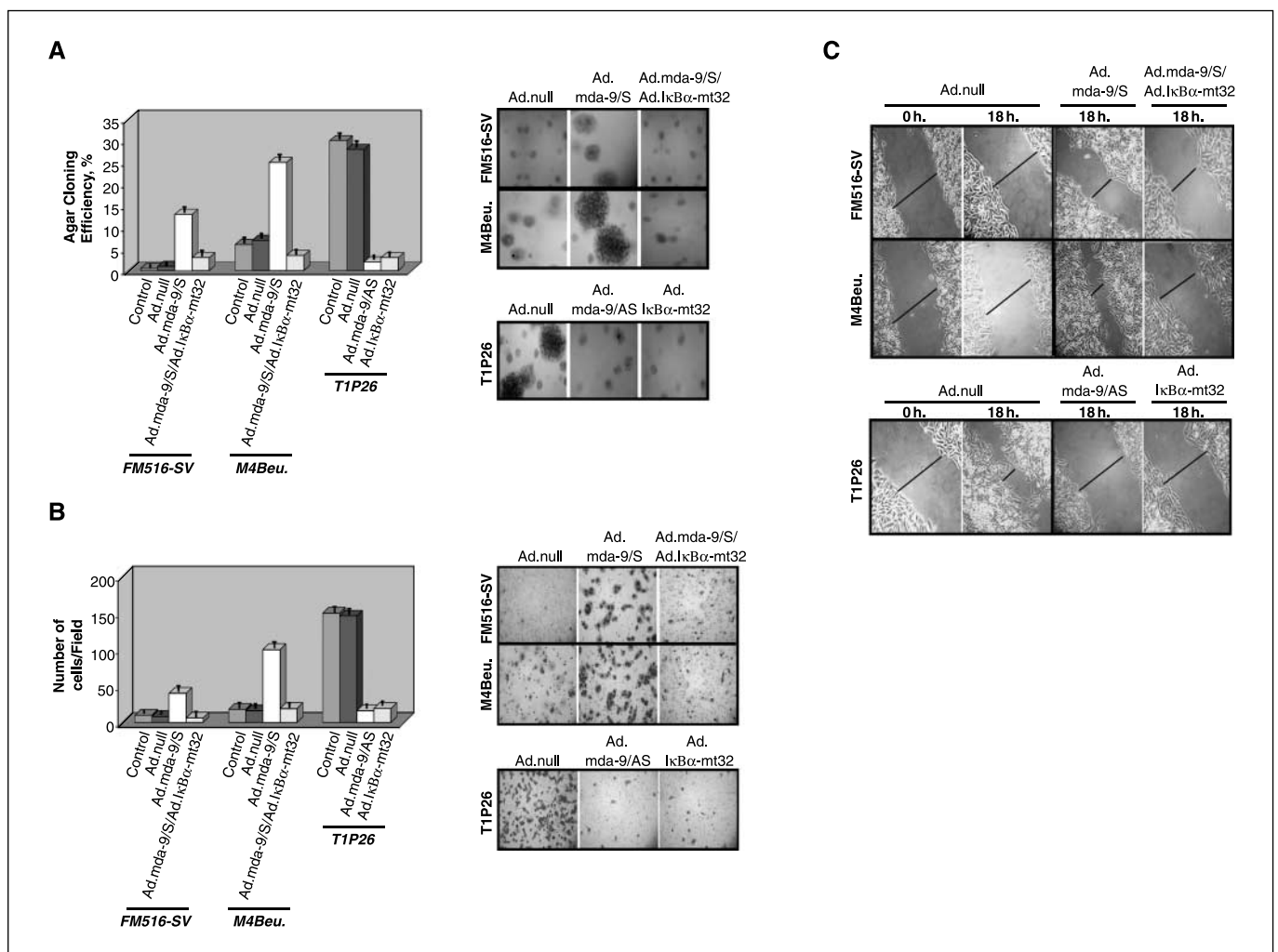


Figure 4. A dominant-negative mutant of p65/RelA, Ad.IκBα-mt32, blocks *mda-9*/syntenin-mediated anchorage-independent growth, invasion, and migration of melanoma cells. **A**, anchorage-independent growth of FM516-SV and M4Beu. melanoma cells either uninfected or infected with Ad.*null*, Ad.*mda-9*/S, or Ad.*mda-9*/S + Ad.IκBα-mt32 (50 pfu/cell of each virus). T1P26 cells were either uninfected or infected with Ad.*null*, Ad.*mda-9*/AS, or Ad.IκBα-mt32 (100 pfu/cell). Forty-eight hours later, 1×10^5 cells were replated in complete culture medium containing 0.6% agar on top of a 0.6% agar base layer. Macroscopic colonies were scored after 2 wks. Columns, mean of triplicate colony counts from three independent experiments; bars, SD (left). Representative photomicrographs of colonies 2 wks after seeding in agar (right). Magnification, $\times 20$. **B**, migration/invasion of FM516-SV, M4Beu., and T1P26 melanoma cells either uninfected or infected with the indicated adenovirus as described in (A). Assays were done as described in Materials and Methods. Ten fields per cell line were counted. Columns, mean of triplicate sample from three independent experiments; bars, SD (left). Representative photomicrographs of cell migration/invasion taken 48 h after seeding in Matrigel (right). **C**, representative photomicrographs of cell migration/invasion taken 48 h after seeding in Matrigel using FM516-SV and melanoma cells infected with the indicated adenovirus as described in (A). Phase-contrast images were taken 18 h after scrape wounding.

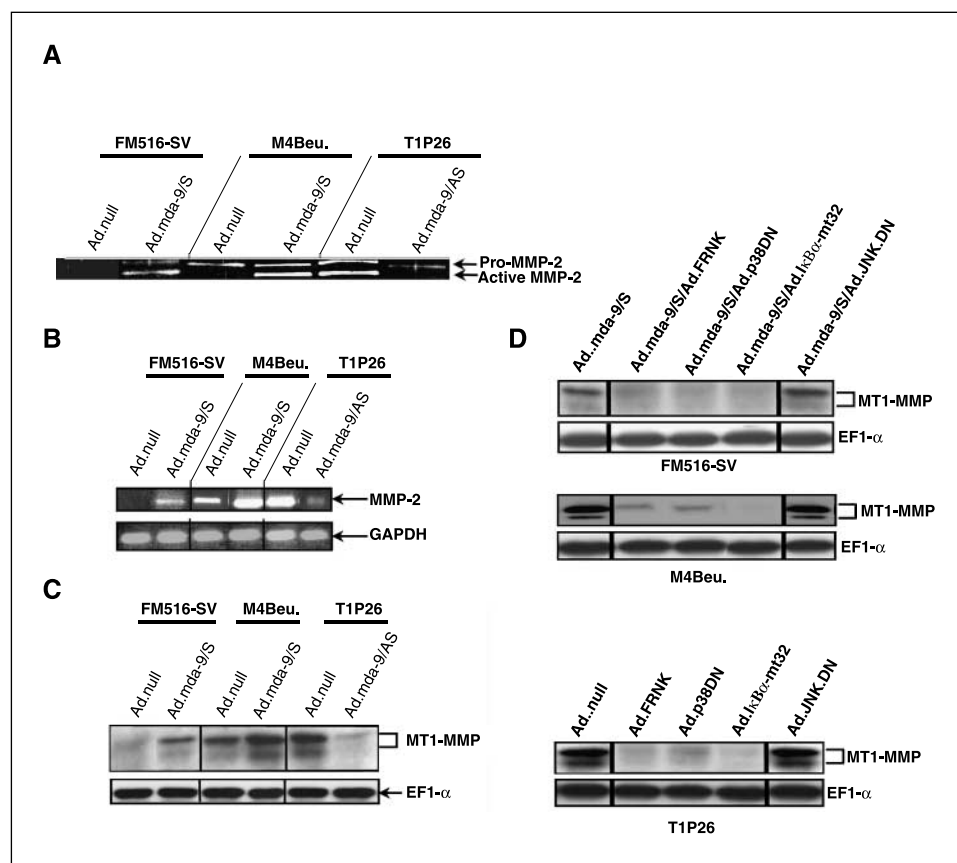


Figure 5. Inhibition of *mda-9*/syntenin-mediated MMP-2 activation through induction of MT1-MMP by dominant-negative mutants of FRNK and p38 MAPK and Ad.I κ B α -mt32. **A**, Ad.*mda-9/S* stimulates activation of pro-MMP-2 in immortal melanocytes and melanoma cells. FM516-SV, M4Beu., and T1P26 cells were infected with the indicated adenovirus at a MOI of 100 pfu/cell. Forty-eight hours later, cells were replated on fibronectin in serum free medium. The conditioned medium was collected and processed by gelatin zymography as described in Materials and Methods. **B**, total RNA was isolated from FM516-SV and melanoma cells that were infected with the indicated virus as described in (A). RT-PCR was done with primer pairs to amplify MMP-2 or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). **C**, Ad.*mda-9/S* stimulates activation of MT1-MMP in immortal melanocytes and melanoma cells. FM516-SV, M4Beu., and T1P26 cells were infected with the indicated virus at a MOI of 100 pfu/cell and replated on fibronectin in serum free-medium. Forty-eight hours later, cell lysates were analyzed by Western immunoblotting for MT1-MMP protein expression. **D**, *mda-9*/syntenin induces MT1-MMP expression by activating p38 MAPK and NF- κ B pathways. FM516-SV and M4Beu. cells were infected with Ad.*null*, Ad.*mda-9/S*, or Ad.*mda-9/S* + the different Ad.dominant-negative or Ad.I κ B α -mt32 viruses (50 pfu/cell of each virus) and then replated on fibronectin in serum-free medium. T1P26 cells were infected with Ad.*null*, the Ad.dominant-negative, or Ad.I κ B α -mt32 viruses as above. Cell lysates were analyzed for MT1-MMP expression by Western blotting.

In addition to soft agar assays, coinfection of FM516-SV or M4Beu. cells with Ad.I κ B α -mt32 and Ad.*mda-9/S* (50 pfu/cell of each virus) induced a significant decrease in cell migration/invasion (approximately 85–90%) compared with Ad.*mda-9/S*-infected cells (Fig. 4B). Similarly, Ad.I κ B α -mt32-infected as well as Ad.*mda-9/AS*-infected T1P26 cells displayed decreased migration/invasion by approximately 85% to 90% (Fig. 4B). In addition to Transwell assays, we also compared the cell motility of infected cells using a wound healing/scratch assay (15). Eighteen hours after generating the wound in a monolayer of cells grown on fibronectin, the closure was almost complete in Ad.*mda-9/S*-infected FM516-SV or Ad.*mda-9/S*-infected M4Beu. cells compared with Ad.*null*-infected cells, whereas healing (cell migration) was inhibited by ~70% when cells were coinfecting with Ad.I κ B α -mt32 and Ad.*mda-9/S* (50 pfu/cell of each virus; Fig. 4C). Similarly, healing was inhibited by ~85% in Ad.*mda-9/AS*-infected or Ad.I κ B α -mt32-infected T1P26 cells compared with Ad.*null*-infected cells (Fig. 4C).

***mda-9*/syntenin stimulates activation of pro-MMP-2 in melanoma.** Because the NF- κ B pathway modulates the expression of essential genes involved in cancer cell migration/invasion

and metastasis (23), we hypothesized that *mda-9*/syntenin-induced NF- κ B activation might affect the activity and/or expression of MMPs, a group of ECM-degrading enzymes. A key MMP specifically implicated with progressive invasiveness of melanoma is MMP-2 (33). As shown by gelatin zymography, Ad.*mda-9/S*-infected FM516-SV or M4Beu. cells displayed increased expression of both the latent (72-kDa protein) and the active (64-kDa protein) forms of MMP-2 gelatinase in conditioned medium versus mock- or Ad.*null*-infected cells (Fig. 5A). This enzymatic activity was further confirmed to result from the MMPs because incubation of the zymographic gel in the presence of 10 mmol/L EDTA completely extinguished the gelatinase activity *in vitro* (data not shown). In contrast, an increase in the levels of active MMP-2 and pro-MMP-2 expression (gelatinolytic activity) was prevented (up to a 90–95% reduction) when T1P26 cells were infected with Ad.*mda-9/AS* compared with Ad.*null*-infected cells (Fig. 5A). Consistent with this finding, we detected by reverse transcription-PCR (RT-PCR) a significant increase in the expression of MMP-2 mRNA in Ad.*mda-9/S*-infected FM516-SV or M4Beu. cells,

whereas a significant reduction of MMP-2 mRNA expression was observed in Ad.*mda-9/AS*-infected T1P26 cells (Fig. 5B).

***mda-9/syntenin* induces MT1-MMP expression by activating FAK, p38, and NF- κ B.** Because activation of pro-MMP-2 is controlled by MT1-MMP in participation with the tissue inhibitor metalloproteinase-2 (TIMP-2; ref. 34), we examined whether *mda-9/syntenin*-induced MMP-2 activation occurs through up-regulation of MT1-MMP. As shown in Fig. 5C, the expression levels of both pro-MT1-MMP expression (65-kDa protein) and the active cleaved form of MT1-MMP (60-kDa protein) significantly increased in Ad.*mda-9/S*-infected FM516-SV or M4Beu. cells compared with Ad.*null*-infected cells. Conversely, the protein levels of both forms of MT1-MMP decreased significantly in Ad.*mda-9/AS*-infected T1P26 cells compared with Ad.*null*-infected cells (Fig. 5C).

mda-9/Syntenin promotes tumor cell invasion/migration through activation of FAK and specific signal transduction pathways, including JNK and p38 MAPK (15). Based on this consideration, the involvement of these pathways in the activation of the NF- κ B signaling pathway leading to induction of MT1-MMP and subsequent activation of pro-MMP-2 was further studied using dominant-negative mutants of either FAK, p38, JNK, or NF- κ B. As shown in Fig. 5D, expression of both forms of MT1-MMP was significantly inhibited by greater than 75% to 80% when FM516-SV or M4Beu. cells were coinfecting with Ad.*mda-9/S* and Ad.FRNK, Ad.p38DN, or Ad.I κ B α -mt32 (50 pfu/cell of Ad.*mda-9/S* + 50 pfu/cell of the Ad.dominant-negative) compared with Ad.*null*-infected cells. In contrast, MT1-MMP proteins were essentially unchanged in Ad.*mda-9/S* + Ad.JNK.DN-infected or Ad.*mda-9/S*-infected FM516-SV or M4Beu. cells. Similarly, Ad.FRNK, Ad.p38DN, or Ad.I κ B α -mt32 infection of T1P26 cells significantly decreased expression of MT1-MMP, whereas Ad.JNK.DN again had no effect (Fig. 5D). These results strongly suggest that *mda-9/syntenin* signaling through FAK and p38 MAPK activates the NF- κ B pathway, leading to induction of MT1-MMP and subsequent activation of pro-MMP-2 (Fig. 6).

Discussion

Cell motility and invasion are two critical determinants of cancer progression and metastasis. Previous studies confirmed that *mda-9/syntenin*, identified by subtraction hybridization (1, 2), is a positive regulator of cancer development (15). When expressed by means of a replication-incompetent adenovirus in melanoma, Ad.*mda-9/S* regulates cytoskeletal organization, promotes cell motility *in vitro*, and regulates tumor cell dissemination *in vivo* by enhancing the activity of critical components of the signaling pathways involved in establishing a motile phenotype, including FAK and MAPK pathways, such as phospho-p38 and phospho-JNK (15).

We now provide evidence for a mechanism that underlies induction of motility and tumor cell growth based on the functional activation of the transcription factor NF- κ B and its cooperation with FAK. (a) When *mda-9/syntenin* expression is elevated by a replication-incompetent adenovirus Ad.*mda-9/S*, immortalized human melanocytes and poorly metastatic melanoma cells plated on fibronectin exhibited significant activation of NF- κ B compared with unmodified or Ad.*null*-infected cells, as documented by EMSA and luciferase reporter assays. Conversely, down-regulation of endogenous *mda-9/syntenin* expression using Ad.*mda-9/AS* decreased NF- κ B activity in metastatic melanoma

cells. (b) Blocking FAK activation by infecting immortalized human melanocytes and poorly metastatic melanoma cells plated on fibronectin with Ad.*mda-9/S* and an adenovirus expressing the COOH-terminal noncatalytic domain of FAK, FRNK (Ad-FRNK), inhibited *mda-9/syntenin*-induced transcriptional activation of NF- κ B in a dose-dependent manner. (c) Interaction and subsequent migration/invasion of immortalized human melanocytes and poorly metastatic melanoma cells was significantly inhibited following coinfection with an Ad.I κ B α -mt32, which inhibits I κ B α degradation and subsequent NF- κ B nuclear translocation, or Ad.*mda-9/S* compared with unmodified or Ad.*null*-infected cells. Similarly, down-regulation of endogenous transcriptional activation of NF- κ B resulted in a significant inhibition of the haptotactic motility of highly metastatic cells. (d) When Ad.*mda-9/S* and Ad.I κ B α -mt32 were expressed together, immortalized human melanocytes displayed a less transformed phenotype and poorly metastatic melanoma cells exhibited a less aggressive transformed phenotype than unmodified or Ad.*null*-infected parental cells, as documented by induced/enhanced restrictive anchorage-independent growth, respectively. Similarly, down-regulation of endogenous transcriptional activation of NF- κ B by Ad.I κ B α -mt32 rendered highly metastatic cells poorly aggressive. Collectively, these data (i.e., the

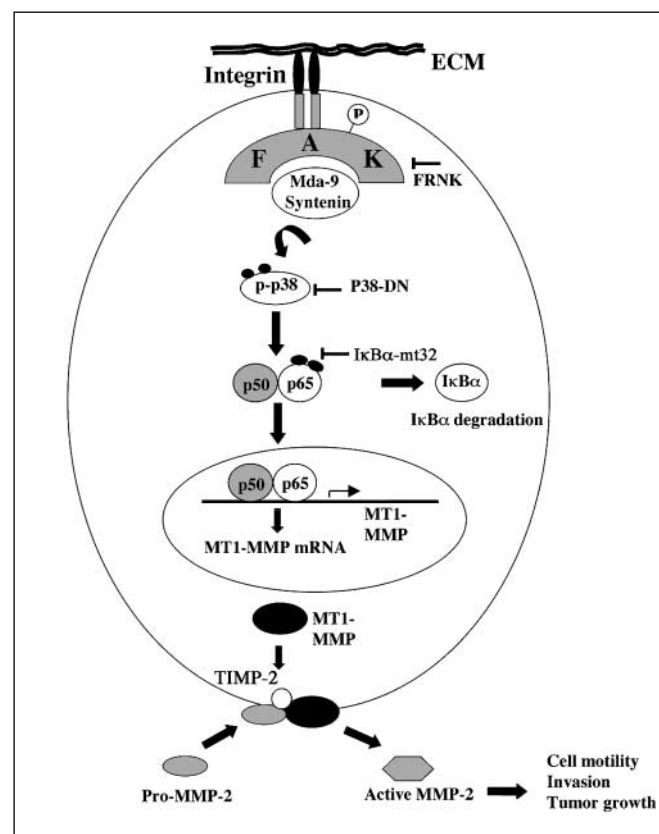


Figure 6. Hypothetical model of *mda-9/syntenin* activation of defined signaling pathways leading to enhanced cell motility, invasion, and cancer cell growth. On interacting with the ECM (fibronectin), FAK is phosphorylated by *mda-9/syntenin*, which results in phosphorylation and activation of p38 MAPK. This results in degradation of I κ B α 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.

gain or loss of function with sense or antisense gene delivery strategies, respectively) provide the first mechanistic evidence for *mda-9/syntenin* as a mediator of NF- κ B activation in melanoma cancer progression.

Our studies also suggest a causal relationship between *mda-9/syntenin*-induced FAK activation and subsequent NF- κ B activation, leading to an increase in tumor cell motility and anchorage-independent growth in soft agar. Cancer cell migration is a highly dynamic process, governed predominantly by cell adhesion molecules and requiring organization of a complex structure, termed focal adhesion, on the cytoplasmic side of the plasma membrane (35, 36). The tyrosine kinase FAK plays a central role in promoting cell migration in response to ECM proteins (36). Experiments using adenoviral gene delivery of FRNK, a dominant-negative inhibitor of FAK, argue that *mda-9/syntenin* acting through FAK is a critical determinant of enhanced NF- κ B activation. Of importance, overexpression of FAK in malignant cells, such as human leukemia, HL-60, also leads to enhanced activation of the transcription factor NF- κ B, lending further support of a direct link between *mda-9/syntenin*, the activation of FAK, and the stimulation of the NF- κ B pathway (Fig. 6; ref. 37). The link established in this study between *mda-9/syntenin*, FAK, and NF- κ B has significant implications for the regulation of cell migration events associated with tumor metastases because *mda-9/syntenin* is colocalized with FAK and stress fibers and promotes migration of human melanoma, breast, and gastric cancer cells *in vitro* and melanoma metastasis *in vivo* (15, 17). These data, together with previous work showing high levels of expression of both FAK (36, 31) and NF- κ B (18–20, 23, 38) in several different tumors, including cancers of the breast, head and neck, ovary, and melanoma, strongly support a role of *mda-9/syntenin*-induced FAK activation in enhancing cell motility and metastatic potential of melanoma through activation of the NF- κ B pathway.

In addition to promoting migration of melanoma cells through NF- κ B activation, *mda-9/syntenin* also induced an increase in the production of the MMP-2 as well as activation of its proteolytic activity. Such activation of MMP-2 is correlated with increased levels of MT1-MMP expression and cleavage by cell membrane-bound MT1-MMP (34). Because TIMP-2 and MT1-MMP form a “receptor” complex that binds pro-MMP-2 resulting in the proteolysis of bound MMP-2 by an adjacent free MT1-MMP (39), we have also detected an increased level of TIMP-2 expression in Ad.*mda-9*-infected melanoma cells (data not shown). These results suggest that *mda-9/syntenin* facilitates a shift in balance toward increasing proteolytic activity of MMP-2. These data are consistent with a previously described pathway for progression of melanocytic lesions that is dependent on the expression of MT1-MMP and activation of MMP-2 by a NF- κ B-dependent pathway (40, 41).

How might *mda-9/syntenin* mediate MT1-MMP that leads to MMP-2 activation? Experiments using Ad.FRNK or Ad.I κ B α -mt32 support the view that *mda-9/syntenin*-stimulated pro-MMP-2 activation occurs through FAK- and NF- κ B-mediated induction of MT1-MMP. Such regulation of activation of MMP-2 by *mda-9/syntenin*-induced NF- κ B pathway is likely to be important in the development of melanoma metastases. This possibility is supported by our previous observations showing that poorly metastatic cells overexpressing *mda-9/syntenin* grew faster in a restrictive anchorage-independent growth assay and acquired a highly metastatic phenotype *in vivo* compared with highly

metastatic variants, which display a reduced metastatic phenotype when infected with Ad.*mda-9/AS* (15). Accordingly, it is conceivable that an important scaffolding protein, such as *mda-9/syntenin*, acting through FAK, induces enhanced activation of the transcription factor NF- κ B in our melanoma model, which in turn allows tumor cells to produce active MMP-2 and acquire a highly migratory phenotype that facilitates their ability to metastasize (Fig. 6; ref. 42). These observations together with the fact that blockade of NF- κ B in several human-derived cell lines, including human prostate cancer cells, breast, murine lung alveolar carcinoma cells, and melanoma, resulted in suppression of invasion and metastasis (19, 43) lend further support for a critical role of *mda-9/syntenin*-mediated NF- κ B activation in promoting cellular invasion and motility during melanoma progression.

This raises the obvious question about the mechanism by which *mda-9/syntenin*-induced NF- κ B activation, acting through FAK, affects the expression of tumor-promoting genes, such as *MT1-MMP*, and subsequent MMP-2 activation, thereby promoting tumor migration/invasion? Previous data from our laboratory indicated that members of the MAPKs, JNK and p38, are important signaling components for *mda-9/syntenin*-dependent cytoskeletal reorganization, cell migration, and invasion of melanoma cells (15). Our findings that specific pharmacologic inhibitors of p38 MAPK (data not shown) or a p38 α DN-expressing adenoviral construct significantly inhibited NF- κ B activity are consistent with the interpretation that p38 MAPK is involved in the regulation of MT1-MMP in our melanoma model, whereas JNK kinase does not seem to be essential (Fig. 6). This difference may reflect the distinct signaling pathways involved in the regulation of MMP production in melanoma cells (44). The functional importance of p38 MAPK in MMP-2 expression in melanoma cells was also suggested by a recent report showing that inhibition of p38 MAPK by the specific inhibitor SB203580 down-regulated MMP-2 expression resulting in reduced invasive potential of malignant melanoma cells (45). Moreover, in different cell types, the p38 MAPK-dependent pathway interferes with the transactivation properties of NF- κ B (46–48). Although the mechanisms by which p38 MAPK activates NF- κ B are controversial, with evidence supporting regulation of NF- κ B-dependent gene transcription via modulation of activation of the transcription factor I κ B and/or post-translational modifications, including phosphorylation and acetylation, of the RelA/p65 subunit, our data strongly suggest that *mda-9/syntenin* stimulates MMP-2 expression by activating MT1-MMP through the p38 MAPK and NF- κ B pathways leading to melanoma cell migration and invasion.

Because NF- κ B is already constitutively activated in immortalized human melanocytes and the poorly metastatic M4Beu melanoma cells as shown in this study and previous research (21), the present findings raise the intriguing possibility that *mda-9/syntenin*, acting through FAK and p38 MAPK, may function as an “amplifier” that dramatically potentiates the transcriptional activity of NF- κ B and subsequently accelerates tumor cell motility and invasion. In support of this possibility, a molecular profile of genes modulated by NF- κ B in malignant progression of a squamous cell carcinoma revealed that this transcription factor regulated a diverse repertoire of genes, including integrins and plasminogen activator, potentially involved in cell migration and invasion (23). In this context, loss of *mda-9/syntenin* would result in a complete disarray of the signaling

proteins in cells, and loss of their ability to interact efficiently to promote appropriate signaling. How *mda-9*/syntenin holds multiprotein signaling complexes together is currently under investigation.

In summary, we presently uncover a novel role of *mda-9*/syntenin as an important scaffolding protein that can initiate a signaling cascade resulting in the induction of NF- κ B in human melanoma cells, which by itself is a critical prerequisite for tumor growth and metastasis. Our results support a model in which a specific increase in FAK tyrosine phosphorylation and activation of the p38 MAPK pathway induced by *mda-9*/syntenin in melanoma cells may represent an essential mechanism that subsequently leads to MMP-2 activation through NF- κ B-mediated induction of MT1-MMP (Fig. 6). Given that melanoma is a disorder that manifests diverse sets of clinical presentations and aggressiveness, it is possible that increased expression of *mda-9*/syntenin during melanoma progression may provide a survival advantage for cancer cells through activation of the NF- κ B signaling pathway, which may then accelerate malignant progression by regulating several coordinated genes involved in invasion, metastasis, and angiogen-

esis. In this context, up-regulation of an important PDZ scaffolding signaling protein, such as *mda-9*/syntenin, with the ability to recruit and organize the formation of multimeric protein complexes that influence cytoskeleton dynamics leading to activation of NF- κ B pathways may significantly affect melanoma progression and potentially progression of other malignant tumors. Of potential therapeutic relevance, targeting of the *mda-9*/syntenin pathway using antisense or small interfering RNA approaches or with small-molecule inhibitors could provide a means for developing effective antimetastatic cancer therapies.

Acknowledgments

Received 10/19/2006; accepted 12/5/2006.

Grant support: NIH grant R01 CA35675 and P30 AR044535 (D. Sarkar), the Samuel Waxman Cancer Research Foundation (SWCRF), the Chernow Endowment (P.B. Fisher), and Philippe Foundation supplemental salary support (H. Boukerche). P.B. Fisher is the Michael and Stella Chernow Urological Cancer Research Scientist and a SWCRF Investigator.

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

References

- Lin JJ, Jiang H, Fisher PB. Characterization of a novel melanoma differentiation-associated gene, *mda-9*, that is down regulated during terminal differentiation. *Mol Cell Differ* 1996;4:317-33.
- Lin JJ, Jiang H, Fisher PB. Melanoma differentiation associated gene-9, *mda-9*, is a human γ interferon responsive gene. *Gene* 1998;207:105-10.
- Sarkar D, Boukerche H, Su ZZ, Fisher PB. *mda-9*/syntenin: recent insights into a novel cell signaling and metastasis-associated gene. *Pharmacol Ther* 2004;104:101-15.
- Grootjans JJ, Zimmermann P, Reekmans G, et al. Syntenin, a PDZ protein that binds syndecan cytoplasmic domains. *Proc Natl Acad Sci U S A* 1997;94:13683-8.
- Gimferrer I, Ibanez A, Farnos M, et al. The lymphocyte receptor CD6 interacts with syntenin-1, a scaffolding protein containing PDZ domains. *J Immunol* 2005;175:1406-14.
- Latysheva N, Muratov G, Rajesh S, et al. Syntenin-1 is a new component of tetraspanin-enriched microdomains: mechanisms and consequences of the interaction of syntenin-1 with CD63. *Mol Cell Biol* 2006;26:7707-18.
- Ko J, Yoon C, Piccoli G, et al. Organization of the presynaptic active zone by ERC2/CAST1-dependent clustering of the tandem PDZ protein syntenin-1. *J Neurosci* 2006;26:963-70.
- Fialka I, Steinlein P, Ahorn H, et al. Identification of syntenin as a protein of the apical early endocytic compartment in Madin-Darby canine kidney cells. *J Biol Chem* 1999;274:26233-9.
- Fernandez-Larrea J, Merlos-Suarez A, Urena JM, Basella J, Arribas J. A role for a PDZ protein in the early secretory pathway for the targeting of proTGF- α to the cell surface. *Mol Cell* 1999;3:423-33.
- Geijsen N, Uings IJ, Pals C, et al. Cytokine-specific transcriptional regulation through an IL-5R α interacting protein. *Science* 2001;293:1136-8.
- Grootjans JJ, Reekmans G, Ceulemans H, David G. Syntenin-syndecan binding requires syndecan-syntenin and the co-operation of both PDZ domains of syntenin. *J Biol Chem* 2000;275:19933-4.
- Mortier E, Wuytens G, Leenaerts I, et al. Nuclear speckles and nucleoli targeting by PIP2-PDZ domain interactions. *EMBO J* 2005;24:2556-65.
- Baril P, Nejjar M, Scoazek JY, Boukerche H. Blocking a novel 55-kDa melanoma-associated cell surface antigen inhibits the development of spontaneous metastases and interactions with frozen lung section. *Int J Cancer* 2002;99:315-22.
- Boukerche H, Baril P, Tabone E, et al. A new Mr 55,000 surface protein implicated in melanoma progression: association with a metastatic phenotype. *Cancer Res* 2000;60:5848-56.
- Boukerche H, Su ZZ, Emdad L, et al. *mda-9*/syntenin: a positive regulator of melanoma metastasis. *Cancer Res* 2005;65:10901-11.
- Helmke BM, Polychronidis M, Benner A, et al. Melanoma metastasis is associated with enhanced expression of the syntenin gene. *Oncol Rep* 2004;12:221-8.
- Koo TH, Lee JJ, Kim EM, et al. Syntenin is overexpressed and promotes cell migration in metastatic human breast and gastric cancer cell lines. *Oncogene* 2002;21:4080-8.
- Vermeulen L, Vanden Berghe W, Haegeman G. Regulation of NF- κ B transcriptional activity. *Cancer Treat Res* 2006;130:89-102.
- Baldwin AS, Jr. Control of oncogenesis and cancer therapy resistance by the transcription factor NF- κ B. *J Clin Invest* 2001;107:241-6.
- McNulty SE, del Rosario R, Cen D, Meyskens FL, Jr., Yang S. Comparative expression of NF- κ B proteins in melanocytes of normal skin vs. benign intradermal naevus and human metastatic melanoma biopsies. *Pigment Cell Res* 2004;17:173-80.
- Shattuck-Brandt RL, Richmond A. Enhanced degradation of I- κ B contributes to endogenous activation of NF- κ B in Hs294T melanoma cells. *Cancer Res* 1997;57:3032-9.
- Ghosh S, Karin M. Missing pieces in the NF- κ B puzzle. *Cell* 2002;109 Suppl:S81-96.
- Loercher A, Lee TL, Ricker JL, et al. Nuclear factor- κ B is an important modulator of the altered gene expression profile and malignant phenotype in squamous cell carcinoma. *Cancer Res* 2004;64:6511-23. Erratum in: *Cancer Res* 2004;64:8130-2.
- Boukerche H, Su ZZ, Kang DC, Fisher PB. Identification and cloning of genes displaying elevated expression as a consequence of metastatic progression in human melanoma cells by rapid subtraction hybridization. *Gene* 2004;343:191-201.
- Heidkamp MC, Bayer AL, Kalina JA, Eble DM, Samarel AM. GFP-FRNL disrupts focal adhesions and induces anoikis in neonatal rat ventricular myocytes. *Circ Res* 2002;90:1282-9.
- Emdad L, Sarkar D, Su ZZ, et al. Activation of the NF- κ B pathway by astrocyte elevated gene-1: implications for tumor progression and metastasis. *Cancer Res* 2006;66:1509-16.
- Fidler IJ, Li LM, Ananthaswamy HN, et al. Correlation of growth capacity of cells in hard agarose with successful transfection by the activated c-Ha-ras oncogene and *in vivo* proliferative capacity at metastatic sites. *Anticancer Res* 1991;11:17-24.
- Kuphal S, Poser I, Jobin C, Hellerbrand C, Bosserhoff AK. Loss of E-cadherin leads to upregulation of NF- κ B activity in malignant melanoma. *Oncogene* 2004;23:8509-19.
- Benoliel AM, Kahn-Perles B, Imbert J, Verrando P. Insulin stimulates haptotactic migration of human epidermal keratinocytes through activation of NF- κ B transcription factor. *J Cell Sci* 1997;110:2089-97.
- Dhawan P, Richmond A. A novel NF- κ B-inducing kinase-MAPK signaling pathway up-regulates NF- κ B activity in melanoma cells. *J Biol Chem* 2002;277:7920-8.
- Owens LV, Xu L, Craven RJ, et al. Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. *Cancer Res* 1995;55:2752-5.
- Brown K, Gerstberger S, Carlson L, Franzoso G, Siebenlist U. Control of I- κ B- α proteolysis by site-specific, signal-induced phosphorylation. *Science* 1995;267:1485-8.
- Vaisanen A, Tuominen H, Kallioinen M, Turpeenniemi-Hujanen T. Matrix metalloproteinase-2 (72 kD type IV collagenase) expression occurs in the early stage of human melanocytic tumour progression and may have prognostic value. *J Pathol* 1996;180:283-9.
- Malemud CJ. Matrix metalloproteinases (MMPs) in health and disease: an overview. *Front Biosci* 2006;11:1696-701.
- Harlozinska A. Progress in molecular mechanisms of tumor metastasis and angiogenesis. *Anticancer Res* 2005;25:3327-33.
- McLean GW, Carragher NO, Avizienyte E, et al. The role of focal-adhesion kinase in cancer—a new therapeutic opportunity. *Nat Rev Cancer* 2005;5:505-15.
- Sonoda Y, Matsumoto Y, Funakoshi M, et al. Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. *J Biol Chem* 2000;275:16309-15.
- Rayet B, Gelinas C. Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* 1999;18:6938-47.
- Zucker S, Drews M, Conner C, et al. Tissue inhibitor of metalloproteinase-2 (TIMP-2) binds to the catalytic domain of the cell surface receptor, membrane type 1-matrix metalloproteinase 1 (MT1-MMP). *J Biol Chem* 1998;273:1216-22.
- Amiri KI, Richmond A. Role of nuclear factor- κ B in

- melanoma progression. *Cancer Metastasis Rev* 2005;24:301–13.
41. Hofmann UB, Westphal JR, Zendman AJ, et al. Expression and activation of matrix metalloproteinase-2 (MMP-2) and its co-localization with membrane-type 1 matrix metalloproteinase (MT1-MMP) correlate with melanoma progression. *J Pathol* 2000;191:245–56.
42. Jiang WG, Davies G, Martin TA, et al. Expression of membrane type-1 matrix metalloproteinase, MT1-MMP in human breast cancer and its impact on invasiveness of breast cancer cells. *Int J Mol Med* 2006;17:583–90.
43. Orłowski RZ, Baldwin AS, Jr. NF- κ B as a therapeutic target in cancer. *Trends Mol Med* 2002;8:385–9.
44. Munshi HG, Wu YI, Mukhopadhyay S, et al. Differential regulation of membrane type 1-matrix metalloproteinase activity by ERK 1/2- and p38 MAPK-modulated tissue inhibitor of metalloproteinases 2 expression controls transforming growth factor- β 1-induced pericellular collagenolysis. *J Biol Chem* 2004;279:39042–50.
45. Denkert C, Siegert A, Leclere A, Turzynski A, Hauptmann S. An inhibitor of stress-activated MAP-kinases reduces invasion and MMP-2 expression of malignant melanoma cells. *Clin Exp Metastasis* 2002;19:79–85.
46. Maulik N, Sato M, Price BD, Das DK. An essential role of NF- κ B in tyrosine kinase signaling of p38 MAP kinase regulation of myocardial adaptation to ischemia. *FEBS Lett* 1998;429:365–9.
47. Carpentier I, Declercq W, Malinin NL, et al. TRAF2 plays a dual role in NF- κ B-dependent gene activation by mediating the TNF-induced activation of p38 MAPK and I κ B kinase pathways. *FEBS Lett* 1998;425:195–8.
48. Madrid LV, Mayo MW, Reuther JY, Baldwin AS, Jr. Akt stimulates the transactivation potential of the RelA/p65 subunit of NF- κ B through utilization of the I κ B kinase and activation of the mitogen-activated protein kinase p38. *J Biol Chem* 2001;276:18934–40.