MDA-9/Syntenin Is Essential for Factor VIIa-induced Signaling, Migration, and Metastasis in Melanoma Cells*

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Background: MDA-9/syntenin and tissue factor (TF) are overexpressed in most types of human cancer.

Results: Induction of MDA-9/syntenin in melanoma involves the binding of FVIIa and FX to TF on the cell surface, which initiates a signaling circuit essential for cell motility and metastasis of melanoma.

Conclusion: MDA-9/syntenin is an important TF-regulated gene.

Significance: Targeting TF-mediated MDA-9/syntenin may represent a novel therapeutic strategy for eliminating cancer.

Melanoma differentiation associated gene-9 (MDA-9), also known as syntenin, is a novel gene that positively regulates cancer cell motility, invasion, and metastasis through distinct biochemical and signaling pathways, but how MDA-9/syntenin is regulated in response to signals with the extracellular environment and promotes tumor progression is unclear. We now demonstrate that MDA-9/syntenin is dramatically up-regulated in a thrombin-independent signaling pathway and involves the Src family tyrosine kinases, cellular signaling, angiogenesis, tumor migration, and metastasis of melanoma. The present finding uncovers a novel role in signaling pathways by organizing networks of receptors and in targeting several cellular proteins to multiprotein complexes (2–4). MDA-9/syntenin is up-regulated in a large spectrum of human malignancies, including melanoma (4–7). We previously documented that overexpressed MDA-9/syntenin acts through the Src pathway and initiates a signaling cascade resulting in the activation of transcription factor NF-κB and matrix metalloproteinases (MMPs), which in turn promotes invasion and metastasis in vivo (8–10). However, the exact mechanisms by which MDA-9/syntenin gene regulation influences MDA-9/syntenin gene expression and the effects that these mechanisms have on tumor progression are still not well understood.

Melanoma differentiation associated gene-9 (MDA-9), also called syntenin, is a significant member of an expanding family of scaffolding PDZ domain-containing proteins, identified by a subtraction hybridization approach (1, 2). MDA-9/syntenin contains a tandem repeat of PDZ domains that plays a central role in signaling pathways by organizing networks of receptors and in targeting several cellular proteins to multiprotein complexes (2–4). MDA-9/syntenin is up-regulated in a large spectrum of human malignancies, including melanoma (4–7). We previously documented that overexpressed MDA-9/syntenin acts through the Src pathway and initiates a signaling cascade resulting in the activation of transcription factor NF-κB and matrix metalloproteinases (MMPs), which in turn promotes invasion and metastasis in vivo (8–10). However, the exact mechanisms by which MDA-9/syntenin gene regulation influences MDA-9/syntenin gene expression and the effects that these mechanisms have on tumor progression are still not well understood.

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2The abbreviations used are: MDA-9, melanoma differentiation associated gene-9; TF, tissue factor; PAR, protease-activated receptor; PAP, poly(A) polymerase.

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Tissue Factor FVIIa-FXa-induced MDA-9/Syntenin Expression

We presently uncover a novel role of MDA-9/syntenin as an important TF-FVIIa-Xa-regulated gene that can initiate through PAR-1 a signaling circuit essential for cell motility, invasion, and metastasis of melanoma cells. These intriguing observations suggest that induction of MDA-9/syntenin could represent a key molecular event linking hemostasis and tumor progression. In these contexts, inhibition of TF-FVIIa-Xa and its relevant downstream targets such as MDA-9/syntenin, may be useful for managing thrombogenic complications associated with malignancy but also for preventing tumor growth and dissemination.

MATERIALS AND METHODS

Reagents—Neutralizing anti-human tissue factor, anti-MDA-9, anti-HA tag antibody, anti-PAR-1 and anti-PAR-2, anti-Src, anti-p38, anti-MMP-2, anti-poly(A) polymerase (PAP) antibodies, and tissue factor, PAR-1, PAR-2, and PAP shRNAs lentiviral particles were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-anti-TF (H18528) serum (27) all of our studies were performed in cells grown in serum-free media to eliminate the unpredictable effect of factors present in the serum on the cellular responses of cell lines. Because FVII is equally present in plasma and in tissue, we used serum (27) all of our studies were performed in cells grown in serum-free media to eliminate the unpredictable effect of factors present in the serum on the cellular responses of cell lines.

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High Constitutive TF Expression Is Observed in Human Melanoma Cells Overexpressing MDA-9/Syntenin—An anti-TF, monoclonal antibody (TF9–10H10) showed that normal human melanocytes, a nonmetastatic radial growth phase primary melanoma cell line, WM35, and a weak metastatic human melanoma cell line, M4Beu, expressing low levels of MDA-9/syntenin, contained also TF at very low levels (Fig. 1A and Table 1). In contrast, the metastatic melanoma cells, T1P26, 7GP, and c8161 (expressing high levels of MDA-9/syntenin) showed a drastic increase in TF protein expression compared with normal melanocytes or poorly aggressive cell lines (Fig. 1C). In total, these findings suggest that TF expressed on these metastatic cells is functionally active and its expression correlates with the expression levels of MDA-9/syntenin and the metastatic potential of melanoma cells.

Inhibition of TF Signaling Prevents MDA-9/Syntenin Expression in Human Melanoma Cells—To test the hypothesis that TFVIIa binding to TF in melanoma cells may regulate the expression of endogenous MDA-9/syntenin, serum-starved metastatic cell lines, T1P26, 7GP, and c8161 were treated with rFVIIa for 60 min. As shown in Fig. 1D expression of MDA-9/syntenin was dose-dependently up-regulated by the addition of rFVIIa, which induces a very slight increase with 10 nM rFVIIa and a maximal response at 50 to 100 nM. In vivo, factor X zymogen binds to the TF-FVIIa complex (13), we determine whether rFVIIa was used at physiological plasma levels and combined with FX regulate MDA-9/syntenin expression. As anticipated, treatment of serum-starved metastatic cells with the combination of increasing concentrations of rFVIIa (0–10 nM) and 150 nM FX induces a significant dose-dependent increase of MDA-9/syntenin producing a signaling response comparable with that observed with 50 nM rFVIIa (Fig. 1E). Time course experiments showed that the peak of MDA-9/syntenin was already observed after initiation of the treatment. Furthermore, after initiation of the treatment, M4Beu melanoma cells expressing high levels of MDA-9/syntenin in these cells were infected with LV-TF/green fluorescent protein (GFP) to generated a stable M4Beu melanoma cell line expressing high levels of TF (M4Beu/TF). M4Beu/TF cells were serum starved, treated with a combination of rFVIIa (0–10 nM) and FX for 60 min. As shown in Fig. 1G, expression of MDA-9/syntenin was dose-dependently up-regulated by the addition of rFVIIa and FX. These results suggest that at levels of rFVIIa that approach physiological concentrations, the ternary complex, TF-FVIIa-Xa, is a more potent inducer of MDA-9/syntenin expression than the binary complex in our melanocyte/melanoma model.

Correlation Between TF Expression and Metastatic Potential of Melanoma Cells—Finally, we determined whether rFVIIai could compete with rFVIIa for MDA-9/syntenin. As shown in Fig. 2A, inhibition of rFVIIa/Xa-induced MDA-9/syntenin expression in serum-starved metastatic cell lines T1P26, 7GP, and c8161 was dose-dependent and reached a maximum level of inhibition (∼90–95% inhibition) with 100 nM rFVIIai compared with rFVIIa + FX-treated cells. The level of MDA-9/syntenin protein in rFVIIa + FX-stimulated melanoma cells was also inhibited by active site-directed factor Xa inhibitor (FXai), rivaroxaban (2 μM), but not by hirudin (100 nM), a specific thrombin inhibitor (Fig. 2B). Additionally, after treatment of melanoma cells with anti-TF neutralizing antibody, a significant inhibition of rFVIIa/Xa-induced MDA-9/syntenin expression was observed compared with control IgG-treated cells (Fig. 2C). Furthermore, when TF shRNA lentiviral particles (LV-TF)-infected melanoma cells were exposed to...
rFVIIa + FX, a substantial decrease of MDA-9/syntenin expression was evident at a multiplicity of infection of LV-TF as low as 1 and was maximal (~90–95% reduction) at doses of 20 m.o.i. when compared with a nonrelevant lentiviral control shRNA (LV-CTR) (Fig. 2D). In total, these findings employing rFVIIai, FXai, anti-TF antibody that block FX activation, and genetic approaches to block TF support the hypothesis that up-regulation of MDA-9/syntenin expression by tumor cells occurs through the TF-FVIIα-Xa pathway in malignant melanoma cells.

**Table 1**

| Anti-TF | ND*<br>NHEM | M4Beu | WM35 | 7GP | TIP26 | c8161 |
|--------|--------|--------|--------|--------|--------|--------|
| Positively staining cells | <2 | <2 | 85.2 ± 6.2 | 92.4 ± 5.8 | 98.2 ± 7.2 |

* ND, not detected.

**Figure 1. Induction of MDA-9/syntenin by rFVIIa and FX in human melanoma cells.** A, surface expression of TF by flow cytometry analysis. Normal human epidermal melanocytes (NHEM) and human melanoma cell lines derived from tumors at different stages of progression were incubated with anti-TF monoclonal antibody, anti-MDA-9 monoclonal antibody, and anti-EF1α antibody. The bar graphs represent densitometric results (mean ± S.E.) from three or four independent experiments. C, measurement of TF activity. 2 × 10⁶ cells were seeded in each well of a 96-well plate and cultured for 16 h. FXa generation was revealed by addition of its chromogenic substrate PNAPEP25 as described under "Materials and Methods." The data are presented as mean ± S.D. (n = 3). * different from normal human epidermal melanocyte (p < 0.01) according to Student's t test analysis. D and E, serum-starved melanoma cell lines TIP26, c8161, and 7GP were treated for 60 min either with varying concentrations of rFVIIa (10, 50, 100, and 150 nM) (D) or with a combination of increasing concentrations of rFVIIa (1, 5, and 10 nM) and FX (150 nM) (E), and then subjected to SDS-PAGE and Western blot analysis with anti-MDA-9/syntenin antibodies. The bar graphs represent densitometric results (mean ± S.E.) from three or four independent experiments. F, time course study of MDA-9/syntenin expression following treatment of serum-starved melanoma cells with rFVIIa (10 nM) and FX (150 nM). Lysates were analyzed by Western blotting with anti-MDA-9/syntenin antibody and anti-EF1α antibodies. Control refers to basal (time 0). The bar graphs represent densitometric results (mean ± S.E.) from three or four independent experiments. G, serum-starved stable M4Beu melanoma cells overexpressing TF (M4Beu/Tf) or vector-transfected cells were treated with a combination of increasing concentrations of rFVIIa and FX (150 nM) and then subjected to SDS-PAGE and Western blot analysis with anti-MDA-9 and anti-EF1α antibodies. The bar graphs represent densitometric results (mean ± S.E.) from three or four independent experiments.

**Figure 2. FVIIa-Xa-induced MDA-9/Syntenin Expression Signals through PAR-1 in Human Melanoma Cells.—** Because the ternary complex TF-FVIIa efficiently activates both PAR-1 and PAR-2, as a Fxa-dependent manner (23, 24), we first measured using flow cytometry expression levels of PAR-1 and PAR-2 in cells. All metastatic cell lines, TIP26, 7GP, and high levels of PAR-1 and moderate levels of PAR-2 surface (Fig. 2E and Table 2). However, only PAR-1 and PAR-2 expression was evident at a multiplicity of infection of LV-AS-infected melanoma cells (50 pfu/cell) or vector-transfected cells (Fig. 3A). Additionally, when serum-starved melanoma cells were treated with rFVIIa + FX, the level of expression of PAR (M6000, ~90,000) increased significantly compared with untreated cells (Fig. 2G), whereas MDA-9/syntenin protein levels significantly decreased (~90–95% reduction) upon infection of these cells with LV-PAP compared with ShRNA-infected cells (Fig. 2H). In total, these results suggest that TF-Xa-mediated up-regulation of MDA-9/syntenin in melanoma cells is regulated by a TF-FVIIα-Xa pathway at a post-transcriptional level, which includes MDA-9/syntenin mRNA stability. In total, these findings employing genetic approaches to block PARs mediated expression of MDA-9/syntenin induced activation of c-Src and JNK in malignant melanoma cells.

**Figure 3. PAP Augments FVIIa-Xa-Mediated MDA-9/Syntenin Expression in Human Melanoma Cells—** TF-Xa-induced MDA-9/syntenin expression was significantly decreased on infection of serum-starved melanoma cells with PAR-1 shRNA lentiviral particles (LV-PAR-1) (Fig. 2D). In total, these findings employing rFVIIai, FXai, anti-TF antibody that block FX activation, and genetic approaches to block TF support the hypothesis that up-regulation of MDA-9/syntenin expression by tumor cells occurs through the TF-FVIIα-Xa pathway in malignant melanoma cells.

**Table 2**

| Control | 1 nM | 5 nM | 10 nM | 150 nM |
|--------|------|------|-------|-------|
| M4Beu | 85.2 | 92.4 | 98.2 | 98.2 |
| WM35 | 85.2 | 92.4 | 98.2 | 98.2 |
| 7GP | 85.2 | 92.4 | 98.2 | 98.2 |

* ND, not detected.
Because c-Src induces actin remodeling through activation of Cdc42 and Rac1 proteins (34), and both can activate JNKs (35), we investigated whether TF/FVIIa-Xa-induced MDA-9/syntenin expression is involved in the activation of these two GTP-binding proteins. As apparent in Fig. 3B, the activity of Rho-like GTPases Cdc42 and Rac1 were significantly increased in rFVIIa + FX-treated Ad.null-infected melanoma cell lines, T1P26 and c8161, compared with untreated cells (Fig. 3C). In contrast, infection of melanoma cells with either Ad.MDA-9/AS or Ad.Src.DN (50 pfu/cell) significantly blocked FVIIa +
FX-induced Rac1 and Cdc42 activity, when compared with untreated or rFVIIa + FX-treated Ad.null-infected cells, respectively (Fig. 3B). In total, these results suggest a signaling pathway consisting of c-Src-Cdc42-Rac1-JNK mediates TFFVIIa-Xa-induced MDA-9/syntenin expression in malignant melanoma cells. Phosphorylation of paxillin, a focal adhesion-associated protein, was recently identified as a novel JNK substrate and can impact cell migration (36). As expected, infection of metastatic cells, T1P26 and c8161, with either Ad.MDA-9/AS, Ad.Src.DN, or Ad.JNK.DN (50 pfu/cell) or Ad.Rac1.DN + Ad.Cdc42.DN (25 pfu/cell of each virus), induce a robust decrease in paxillin phosphorylation of Ser178 compared with rFVIIa + FX-stimulated Ad.null-infected cells (Fig. 3C). Given that JNK directly induce phosphorylation of paxillin on Ser178, we next considered whether rFVIIa or anti-FXa-mediated MDA-9/syntenin expression was involved in the association of paxillin with FAK. Serum-starved melanoma cell lines treated for 60 min with rFVIIa (10 nM) and FX (150 nM), in the presence of neutralizing anti-TF antibody or control IgG antibody (Fig. 4B), showed significant (up to 7-fold) decrease of levels of phospho-Ser-119, 9251/J/9260 compared with Ad.NK.DN, but not with Ad./NK.DN compared with rFVIIa + FX-treated Ad.null-infected cells (Fig. 4A). We also determined whether TFFVIIa-Xa-mediated expression of MDA-9/syntenin requires phosphorylation of the macromolecular Ikb kinase (IKK) complex, IkBα and RelA/p65. As shown in Fig. 4B, rFVIIa + FX increased the levels of phospho-Ser-119, 9251/J/9260 and phospho-Ser-IkBα in both c8161 and T1P26 melanoma cell lines. In sharp contrast, overexpression of MDA-9/AS or dominant-negative forms of Src or Rac1 and Cdc42 by means of adenoviral vectors (50, 50, and 25 pfu/cell of each virus, respectively) suppressed rFVIIa-Xa-induced phosphorylation of phospho-Ser-119, 9251/J/9260 and phospho-Ser-IkBα in melanoma cells, whereas adenovirus-mediated dominant-negative JNK (Ad.mt32IbH9260) has no effect (Fig. 4B). To confirm the role of NF-κB activation by TFFVIIa, in JNK kinase expression, MPP-2, a NF-κB responsive promoter compared with untreated cells with either Ad.MDA-9/AS or an adenovirus expressing the mt32IbH9260 superrepressor (Ad.mt32IbH9260), which prevents p65 nuclear translocation when compared with rFVIIa + FX-stimulated Ad.null-infected cells (Fig. 5, A and B). As a corollary, treatment of rFVIIa + FX-stimulated metastatic cells with rivaroxaban, or LV-PAR-1 shRNAs significantly (~85–95%) decreased activity of c-Src, Rac1, Cdc42, JNK, and paxillin in comparison with rFVIIa + FX-treated LV-control shRNAs-infected cells (Fig. 5C). Similarly, NF-κB transcriptional activity and active MMP-2 was significantly decreased following infection of rFVIIa + FX-stimulated melanoma cells with Ad.MDA-9/AS, LV-PAR-1 shRNAs, or treatment with rivaroxaban, compared with rFVIIa + FX-stimulated LV-control shRNAs-infected cells or rFVIIa + FX-stimulated Ad.null-infected cells (Fig. 5, D and E).

**FIGURE 2.** Blockage of TF, Xa activity, PAR-1, or PAR PAP prevents MDA-9/syntenin expression in human melanoma cells. Serum-starved normal human epidermal melanocytes (NHEM) or melanoma cell lines TIP26, 7GP, and c8161: A, either untreated (UNR) or treated for 60 min with rFVIIa (10 nM), FX (150 nM), and varying concentrations of active site-inactivated FVIIa (rFVIIai); B, either untreated (control) or treated for 60 min with rFVIIa (10 nM) and FX (150 nM) in the presence of neutralizing anti-TF antibody or control IgG antibody (Cir IgG); D, either uninfected (UNR) or infected either with control shRNA lentiviral particles (LV-CTR) or TF shRNA lentiviral particles (LV-TF), at m.o.i. of 1 and 20 as described under “Materials and Methods” and treated 96 h post-infection with rFVIIa (10 nM) and FX (150 nM) for 60 min. Lysates were analyzed by Western blotting with anti-MDA-9 and anti-anti-EF1α antibodies; E, incubated with anti-PAR-1, anti-PAR-2 monoclonal antibodies (gray shaded histograms), or a nonimmune mouse serum (open histograms) and surface expression of PAR-1 and PAR-2 was analyzed by flow cytometry. Rabbit anti-mouse IgG/F(ab')2 antibody conjugated to FITC was added to the cells. Results are representative of one experiment of three; F, treated with hirudin (100 nM) or either uninfected (UNR) or infected with PAR-1 (LV/PAR1, 20 m.o.i.), PAR-2 (LV/PAR2, 20 m.o.i.), shRNA lentiviral particles, or control shRNA lentiviral particles (LV-CTR), 96 hours later, cells were treated with rFVIIa (10 nM) and FX (150 nM) for 60 min. Lysates were analyzed by Western blotting with anti-MDA-9 and anti-EF1α antibodies; G, and H, either untreated or (control) treated with rFVIIa (10 nM) and FX (150 nM) for 1 h, G, or, infected with control shRNA lentiviral particles (LV-CTR) or PAP shRNA lentiviral particles (LV/PAP), at m.o.i. of 10 and 20, then treated 96 h later with rFVIIa (10 nM) and FX (150 nM) for 1 h, H, whole cell lysates were analyzed by Western blotting with anti-PAP, anti-MDA-9, or anti-EF1α antibodies. The bar graphs represent densitometric results (mean ± S.E.) from three or four independent experiments.
FIGURE 3. Effect of TF-FVIIa-FXa-mediated MDA-9/syntenin expression on activation of the mitogen-activated protein kinase (MAPK) signaling pathway. Serum-starved stable melanoma cell lines, T1P26 and c8161, either untreated (UNR) or infected with Ad.null, Ad.MDA-9/AS, Ad.Src.DN, Ad.MEK1.DN, Ad.p38.a.DN, Ad.JNK.DN (50 pfu/cell), or Ad.Rac1.DN + Ad.Cdc42.DN (25 pfu/cell of each virus) and/or transfected with HA-FAK were treated for 1 h with rFVIIa (10 nM) and FX (150 nM). Following treatment, cell lysates were subjected (A) to SDS-PAGE and Western blot analysis with specific antibodies against phosphorylated c-Src, ERK, p38, and JNK MAPKs. Membranes were reprobed with specific antibodies directed against total enzyme. + or — refer to the presence or absence of adenovirus (Ad). Note that overexpression of the dominant-negative form of c-Src, MEK1, p38, or JNK markedly increased total c-Src and MAPK ERK1/2, p38, and JNK in comparison with Ad.null vector; B, pulldown analysis of activated (GTP-bound) Rac1 and Cdc42. GTP-bound Rac1 and Cdc42 were affinity precipitated with a recombinant Pak1-CRIB and immunoblotted with anti-Rac1 and Cdc42 antibodies as described under “Materials and Methods.” Total Rac1 and Cdc42 was detected using anti-Rac1 and Cdc42 antibodies. Lysates from melanoma cells infected with the indicated adenovirus were blotted with anti-c-Src antibody. Note that overexpression of the dominant-negative form of c-Src (Ad.Src.DN) markedly increased total c-Src in comparison with Ad.null vector or Ad.MDA-9/AS; C, SDS-PAGE and Western blot analysis with anti-phosphorylated paxillin. Total paxillin was detected using an anti-paxillin antibody; D, immunoprecipitation with anti-HA antibody followed by Western blotting with anti-phospho-Ser178, Tyr118, or Tyr118 paxillin and anti-HA antibodies. The bar graphs represent densitometric results (mean ± S.E.) from three or four independent experiments.
TF-FVIIa-Xa Promotes the Interaction of MDA-9/Syntenin with c-Src in Human Melanoma Cells—When overexpressed by means of an adenoviral expression system, MDA-9/syntenin associates with c-Src tyrosine kinase and initiates a signaling cascade that culminates in enhanced cell migration and invasion of melanoma cells (8). rFVIIa + FX induce a robust increase in MDA-9/syntenin protein expression and c-Src activity in melanoma cells. These findings prompted us to investigate whether interactions between MDA-9/syntenin and c-Src in melanoma cells are influenced by rFVIIa + FX. Immunoprecipitation with anti-MDA-9 antibody pulls down a significant level of p-Src interacting with MDA-9/syntenin in rFVIIa + FX-treated serum-starved metastatic cells, T1P26 and c8161, in comparison with control IgG antibody or untreated cells (Fig. 6A, left panel). In contrast, this interaction was prevented in rFVIIa + FX-treated cells that were infected with Ad.MDA-9/AS (50 pfu/cell), compared with control IgG or Ad.null-infected cells (Fig. 6A, right panel). Double immunofluorescence also documented co-localization of c-Src and MDA-9 in rFVIIa + FX-treated T1P26 and c8161 cells (Fig. 6B and data not shown).

GST pulldown assay further verified the interaction between MDA-9/syntenin and p-Src in rFVIIa + FX-stimulated tumor cells. Physical interaction of MDA-9/syntenin and p-Src was significantly increased following incubation of rFVIIa + FX-treated serum-starved lysates from T1P26 or c8161 melanoma cells in comparison with untreated cells (Fig. 6, C and D). Additionally, GST-MDA-9/syntenin fusion proteins lacking both PDZ-1 and -2 domains (MDA-9/PDZ1-2) failed to interact with endogenous p-Src compared with full-length GST-MDA-9/syntenin in rFVIIa + FX-stimulated tumor cells (Fig. 6, C and D), providing further documentation of the in vivo interaction of MDA-9/syntenin with p-Src that requires exogenous rFVIIa and FX.

Cell Migration, Invasion, and Metastasis of Human Melanoma Cell Depend on TF-FVIIa-Xa-mediated MDA-9/syntenin Expression—To assess further the involvement of TF-FVIIa-FXa-induced MDA-9/syntenin expression in tumor cell migra-
Tissue Factor FVIIa-FXa-induced MDA-9/Syntenin Expression

As shown in Fig. 7, A and B, rFVIIa and 150 nM FX induce a significant dose-dependent increase of migration and invasion (~4- to ~6-fold) of 7GP, T1P26, and c8161 cells, compared to untreated Ad.null-infected T1P26 and c8161 cells, respectively. This effect was observed in both the rFVIIa+ and FX+ conditions. The increase in migration and invasion was also accompanied by an increase in MMP-2 secretion from melanoma cells, as determined by ELISA (Fig. 7, E).

In conclusion, our results provide evidence for a novel mechanism by which TF-FVIIa-FXa signaling is involved in tumor malignancy, with potential implications for the development of new therapeutic strategies.
Tissue Factor FVIIa-FXa-induced MDA-9/Syntenin Expression

FIGURE 6. rFVIIa and FX enhance association of MDA-9/syntenin with c-Src. A, serum-starved melanoma cells T1P26, or treated for 60 min with rFVIIa (10 nM) and FX (150 nM) were either untreated (UNT), or infected with Ad.DN-mt32, Ad.mda-9/AS, or Ad.null (25 pfu/cell), or infected with Ad.mda-9/AS (50 pfu/cell), and then exposed to rFVIIa and FX (right panel). Cell lysates were immunoprecipitated with c-src antibody (Fig. 7, A and B). This increased migration and invasion induced by rFVIIa + FX was also strongly inhibited (~85–90%) on infection of rFVIIa + FX-stimulated melanoma cells with Ad.SrcDN, Ad.JNKDN, Ad.mt32/mt32 (50 pfu/cell), or Ad.DN_Rac1 + CDC42 (25 pfu/cell) compared with rFVIIa + FX-stimulated Ad.null-infected cells (Fig. 8, A and B). Despite the differences in the migratory and invasive potential of melanoma cell lines, cells treated with rFVIIa + X in the presence or absence of adenoviral infection with Ad.MDA-9/AS (50 pfu/cell) did not show marked differences in their proliferation (Fig. 9, A–C). Because rFVIIa + X induced MDA-9/syntenin expression in M4Beu/TFT cells, we predicted that rFVIIa + X would induce migration and invasion of M4Beu/TFT cells. In agreement, increasing concentrations of rFVIIa (0.1, 1, and 10 nM) and 150 nM FX induce a significant dose-dependent increase of migration and invasion of melanoma cells overexpressing TF compared with rFVIIa alone (Fig. 7C). In contrast, rFVIIai, rivaroxaban, anti-

with rFVIIa alone. In contrast, the P2Y12 receptor antagonist ticagrelor (2 μM), neutralized the synergistic effect of both rFVIIa and FX, markedly inhibited cell migration and invasion of melanoma cells, compared with rFVIIa alone, and increased the specificity of this effect deduced by the use of a PE-purified c-src antibody (Fig. 7, A and B). This increase in migration and invasion of rFVIIa + FX-stimulated melanoma cells with shRNAs LV-PAR-1 (20 m.o.i.) or Ad.MDA-9/AS (50 pfu/cell) but not LV-PAR-2 also dramatically decreased (~85–95%) cell migration and invasion, in comparison with rFVIIa + FX-stimulated LV-control shRNA-infected cells, or rFVIIa + FX-stimulated Ad.null-infected cells, respectively (Fig. 7, A and B). This increased migration and invasion induced by rFVIIa + FX was also strongly inhibited (~85–90%) on infection of rFVIIa + FX-stimulated melanoma cells with Ad.SrcDN, Ad.JNKDN, Ad.mt32/mt32 (50 pfu/cell), or Ad.DN_Rac1 + CDC42 (25 pfu/cell) compared with rFVIIa + FX-stimulated Ad.null-infected cells (Fig. 8, A and B). Despite the differences in the migratory and invasive potential of melanoma cell lines, cells treated with rFVIIa + X in the presence or absence of adenoviral infection with Ad.MDA-9/AS (50 pfu/cell) did not show marked differences in their proliferation (Fig. 9, A–C). Because rFVIIa + X induced MDA-9/syntenin expression in M4Beu/TFT cells, we predicted that rFVIIa + X would induce migration and invasion of M4Beu/TFT cells. In agreement, increasing concentrations of rFVIIa (0.1, 1, and 10 nM) and 150 nM FX induce a significant dose-dependent increase of migration and invasion of melanoma cells overexpressing TF compared with rFVIIa alone (Fig. 7C). In contrast, rFVIIai, rivaroxaban, anti-

A direct involvement of FVIIa-TF-induced expression of MDA-9/syntenin in tumor cell metastasis was further evaluated in vivo. As demonstrated in Fig. 10A, the average number of metastatic lung lesions in untreated (146 ± 8 and 150 ± 10 lung nodules) or LV-control shRNA-infected cells, T1P26 and c8161 (152 ± 10 and 148 ± 8 lung nodules), was significantly decreased on infection of T1P26 and c8161 metastatic cells with shRNAs LV-TF (8 ± 2 and 12 ± 2 lung nodules). Similarly, the average number of lung metastatic foci was significantly increased in the MDA-9/syntenin group (86 ± 12 lung nodules) and the TF group (98 ± 10 lung nodules) compared with control M4Beu cells (12 ± 2 lung nodules). In total, these in vitro and in vivo studies confirm a cause and effect relationship between TF-mediated MDA-9/syntenin expression and metastatic competence in human melanoma cells.
DISCUSSION

We presently document, for the first time, that rFVIIa binding to tumor cell TF initiates a signaling cascade that culminates in the induction of MDA-9/syntenin leading to cell migration, invasion, and metastatic spread. Consistent with previous reports (23, 24, 38), we found that rFVIIa, at a near plasma concentration (10 nM), is insufficient to induce MDA-9/syntenin expression in melanoma, and that FXa in the ternary TF/rFVIIa/Xa complex efficiently induces expression of this gene in melanoma. We also document that a poly(A) polymerase, a TF-regulated gene that is up-regulated in multiple cancers in response to exposure to FVIIa (33) promotes MDA-9/syntenin mRNA stability and translatability, thereby leading to an increase in melanoma cell motility and invasion. Importantly, we show that specifically blocking the functionality or expression of the pertinent signaling molecules can induce a signaling axis consisting of PAR-1/c-Src/Cdc42/Rac1/JNK/paxillin and mediates TF/rFVIIa/Xa-induced MDA-9/syntenin expression.

FIGURE 7. MDA-9/syntenin mediates cell migration and invasion of melanoma cells induced by rFVIIa and FX. A–C, serum-starved melanoma cell lines, 7GP, T1P26, and c8161 or M4Beu/TF were added to the upper well and rFVIIa ranging from 0.1 to 10 nM + FX (150 nM) or rFVIIa (10 nM) + FX (150 nM) were added to the lower well in the presence or absence of different agonists, including rFVIIa (100 nM), rivaroxaban (RVX, 5 μM), or anti-TF antibody cH36 (100 μg/ml) or a nonimmune mouse serum, in serum-free medium (migration). A and C, for invasion, FVIIa + FX and other agonists were added to the lower wells that contained NIH3T3 cell-conditioned media (invasion by Matrigel invasion assay). B and C, in all cases, cells were infected with control shRNA, PAR-1, or PAR-2 shRNA lentiviral particles (LV-CTR, LV/PAR-1, and LV/PAR-2, 20 m.o.i, Ad.null or Ad-MDA-9/AS (50 pfu/cell), and analyzed for migration and invasion. Recombinant hirudin (100 μM) was added during invasion and migration assays to eliminate any effects of thrombin. The number of cells that migrated to the underside of the membrane after 18–20 h incubation at 37 °C or migrated across the Matrigel matrix to the underside of the membrane after a 48-h incubation at 37 °C was determined. Columns, mean ± S.D. (n = 3); *, different from rFVIIa + X-treated cells (p < 0.01); +, different from rFVIIa + X-treated control IgG cells (p < 0.01); double +, different from rFVIIa + X-treated LV-control-infected cells (p < 0.01); **, different from rFVIIa + X-treated Ad.null-infected cells (p < 0.01); according to Student’s t test analysis.
that leads to NF-κB activation and subsequent expression of MMP-2, thereby promoting migration/invasion and metastatic potential of human melanoma cells.

Our findings provide strong evidence that PAR-1 is the central signaling receptor responsible for TF-FVIIa-FXa-induced MDA-9/syntenin in melanoma, as a reduction in PAR-1 pro-
Tissue Factor FVIIa-FXa-induced MDA-9/Syntenin Expression

FIGURE 9. Effect of rFVIIa-Xa on proliferation of melanomas. A, human melanoma cells (A375, H9260, and H18528) were seeded in 96-well tissue culture plates (1.5 × 10^3 cells/well) and treated with FVIIa (10 nM) and FX (150 nM) for 48 h. At indicated time points, the medium was removed, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well as indicated under "Materials and Methods." The optical density from the plates was read on a THERMO max microplate reader at 540 nm. All experiments were performed at least three times using quadruplicate sample. Data are presented as mean ± S.E.

question is how coagulation factors FVIIa and FX can orchestrate MDA-9/syntenin-induced tumor cell migration/invasion in extravascular space? Extravascular blood coagulation is observed in multiple cancers, including melanoma (42). In preliminary studies, we were unable to detect FX mRNA expression in melanoma cells by RT-PCR. However, FX is frequently detected in several cancer specimens, including melanoma (18), and may be locally delivered to the tumor microenvironment by macrophages/inflammatory cells that bind FX through Mac-1 (43). These observations together with the fact that hypoxia in multiple cancers also enhances expression of FVII (44), lend further support for a critical role of MDA-9/syntenin in the TF-FVIIa-Xa/PAR-1 signaling axis that regulates cell motility and tumor progression.

Multiple studies now indicate that TF-FVIIa-Xa complexes initiate signal transduction, resulting in the activation of a number of pathways that shape the microenvironment of the tumor (25, 26). Interestingly, our results demonstrated that the PDZ scaffold protein MDA-9/syntenin forms a signaling complex with Src that only promotes cleavage of PAR1 and PAR2 by the tethered ligand thrombin responses in endothelial cells (41), may allow tumor cells to acquire a highly aggressive phenotype. An intriguing protein by siRNA significantly decreases melanoma cell migration/invasion. These phenotypic changes did not involve thrombin formation suggesting that the promigratory and proinvasive roles of MDA-9/syntenin in melanoma resulted from the predominant activation of PAR-1 by FXa. It has been reported that PAR-1 and PAR-2 are complexed together and that cleaved PAR-1 transactivates PAR-2 to induce chemotaxis in multiple cancer cells (39, 40). Interestingly, thrombin, but not FXa, activated the PAR-1/PAR-2 complex (39, 41). Accordingly, it is conceivable that in our melanoma model increased expression of an important scaffolding protein, such as MDA-9/syntenin through a TF-FVIIa/PAR-1-dependent mechanism facilitates melanoma invasion and migration early in the metastatic process and subsequent activation of PAR-2 by the tethered ligand of cleaved PAR1, which has been described to contribute to thrombin responses in endothelial cells (41), may allow tumor cells to acquire a highly aggressive phenotype. An intriguing observation that further supports a role for MDA-9/syntenin is that knocking down MDA-9/syntenin interactions with c-Src blocked TF-FVIIa-Xa-induced Rac-1/Cdc42 and JNK signaling and that inhibiting JNK prevented TF-FVIIa-Xa-induced paxillin Ser178, and Tyr 31/Tyr118 phosphorylation (Fig. 4A, C), consistent with the observation that Rac-1 and Cdc42 efficiently activate NF-κB (50), or more importantly inhibition of the Src/paxillin signaling complex induced by a TF-FVIIa-Xa pathway most likely facilitates tumor cells to leave their original tumor site and migrate to the lungs.

Several additional lines of evidence suggest that the MDA-9/syntenin-Src signaling complex is upstream of Rac-1 and Cdc42 and that these two signaling molecules functionally cooperate with the TF-FVIIa-Xa-induced NF-κB/MMP-2 pathway to promote melanoma cell migration and invasion. Dominant-negative Rac-1 and Cdc42 mutants, but not JNK mutant, blocked NF-κB activation induced by TF-FVIIa-Xa (Fig. 4A), consistent with the observation that human Rac-1 and Cdc42 efficiently activate NF-κB and JNK (46, 47). Overall, these findings and our present study suggest a series of coordinated signaling transduction events involving MDA-9/syntenin that ultimately leads to the acquisition of a motile phenotype by melanoma cells. Indeed, blocking MDA-9/syntenin in response to rFVIIa and FX, or interfering with TF (48), Rho proteins (49), NF-κB-regulated genes such as MMP-2 (50), or more importantly inhibition of the Src/paxillin signaling pathway (45, 51) has been shown to inhibit tumor growth and metastasis in preclinical studies and clinical trials.

In summary, we presently uncover a critical and hitherto unappreciated role of MDA-9/syntenin as an important TF-FVIIa-Xa/PAR-1-regulated gene that initiates a novel signaling circuit essential for cell motility, ECM invasion, and metastasis. Our findings support a hypothetical model whereby the ternary TF-FVIIa-Xa signaling complex acting through PAR-1 promotes expression and interaction of MDA-9/syn-
Tissue Factor FVIIa-FXa-induced MDA-9/Syntenin Expression

FIGURE 10. Effect of fFVIIa-Xa signaling on MDA-9/syntenin-induced melanoma metastasis in vivo. A, lung metastasis with M4Beu cells either untreated (UNR) or overexpressing stable TF (M4Beu/TF) or stable MDA-9 (M4Beu/MDA-9) and TIP26 melanoma cells either untreated (UNR) or expressing stable TF silencing by shRNA lentivirus, or control shRNA lentiviral particles (LV-CTR) (bottom panel). The mean ± S.D. (n = 8) of metastatic nodules were determined after 4–5 weeks using a dissecting microscope. *, different from control shRNA lentiviral particles (LV-CTR, p < 0.01); **, different from control shRNA lentiviral particles (LV-CTR, p < 0.001); +, different from untreated melanoma cells (M4Beu) (p < 0.01), according to Student’s t test analysis (top panel). Representative photographs of lungs metastasis in mice (bottom panel). B, hypothetical model whereby the TF-FVIIa-Xa ternary complex acting through MDA-9/syntenin promotes tumor progression. The TF-FVIIa-H18528 complex signals for the regulation of motility events associated with TF-dependent mechanism, with the ability to recruit and organize the TF signaling complexes may significantly affect melanoma progression and potentially progression of other malignant tumors. Therapeutics that prevent TF-FVIIa-Xa complex formation and its regulated genes may be useful for managing thrombotic complications associated with malignancy but also for preventing tumor growth and dissemination.

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Cell Biology: MDA-9/Syntenin Is Essential for Factor VIIa-induced Signaling, Migration, and Metastasis in Melanoma Cells

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