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MDA-9/Syntenin and IGFBP-2 Promote Angiogenesis in Human Melanoma

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Abstract

Melanoma differentiation associated gene-9 (*mda-9/syntenin*) encodes an adapter scaffold protein whose expression correlates with and mediates melanoma progression and metastasis. Tumor angiogenesis represents an integral component of cancer metastasis prompting us to investigate a possible role of *mda-9/syntenin* in inducing angiogenesis. Genetic (gain-of-function and loss-of-function) and pharmacological approaches were employed to modify *mda-9/syntenin* expression in normal immortal melanocytes, early radial growth phase melanoma and metastatic melanoma cells. The consequence of modifying *mda-9/syntenin* expression on angiogenesis was evaluated using both *in vitro* and *in vivo* assays, including tube formation assays using human vascular endothelial cells, CAM assays and xenograft tumor animal models. Gain-of-function and loss-of-function experiments confirm that MDA-9/syntenin induces angiogenesis by augmenting expression of several pro-angiogenic factors/genes. Experimental evidence is provided for a model of angiogenesis induction by MDA-9/syntenin in which MDA-9/syntenin interacts with the ECM activating Src and FAK resulting in activation by phosphorylation of Akt, which induces HIF-1 α . The HIF-1 α activates transcription of Insulin Growth Factor Binding Protein-2 (IGFBP-2), which is secreted thereby promoting angiogenesis and further induces endothelial cells to produce and secrete VEGF-A augmenting tumor angiogenesis. Our studies delineate an unanticipated cell non-

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autonomous function of MDA-9/syntenin in the context of angiogenesis, which may directly contribute to its metastasis-promoting properties. As a result, targeting MDA-9/syntenin or its downstream-regulated molecules may provide a means of simultaneously impeding metastasis by both directly inhibiting tumor cell transformed properties (autonomous) and indirectly by blocking angiogenesis (non-autonomous).

Keywords

mda-9/syntenin; melanoma; angiogenesis; IGFBP-2; HuVECs; CAM assay

Introduction

Angiogenesis is a complex process involving formation of new blood vessels derived from pre-existing vessels. For survival and growth of solid tumors beyond 1-mm in diameter establishing an independent blood vessel system is mandatory (1–3). Vascularization of tumors promotes not only their survival and growth, but also facilitates metastases from primary to distant sites (4, 5). Consequently, angiogenesis may be an essential component of tumor metastasis and highly vascularized tumors metastasize at a significantly higher rate than less angiogenic tumors. Therefore, inhibiting tumor angiogenesis should in principle provide an effective strategy to obstruct cancer growth and metastasis. Although a number of angiogenesis inhibitors have shown promise in preclinical studies, very few have shown genuine therapeutic efficacy in clinical trials (6). Hence, understanding the molecular determinants controlling tumor angiogenesis is mandatory to develop clinically efficacious angiogenesis inhibitors for cancer therapy.

Melanoma differentiation associated gene-9 (mda-9), also known as syntenin, was cloned in our laboratory using subtraction hybridization as a gene displaying differential biphasic expression as a consequence of induction of irreversible growth arrest, terminal differentiation and loss of tumorigenic potential in HO-1 human metastatic melanoma cells following treatment with fibroblast interferon (IFN- β) and the protein kinase C activator mezerein (7). MDA-9/syntenin is a multifunctional scaffold protein that crosstalks with different classes of proteins and regulates diverse physiological and pathological processes, including tumor progression and metastasis, by activating defined cell signaling pathways (8–18). MDA-9/syntenin interacts with Src resulting in activation of Src/FAK complexes (10, 11). The signaling cascade, particularly the activation of Src, is implicated in various biological processes associated with cytoskeleton organization, including increased cell motility, invasiveness and survival. In the context of angiogenesis, this tyrosine kinase plays a role in regulating endothelial cell function and differentiation by augmenting multiple pro-angiogenic factors, e.g., VEGF-A and IL-8 (19–25). The observation that MDA-9/syntenin positively crosstalks with c-Src strongly supports the potential involvement of MDA-9/syntenin in angiogenesis.

The present studies elucidate a new role of *mda-9/syntenin* in regulating angiogenesis and identify IGFBP-2 as a major mediator of the pro-angiogenic functions of *mda-9/syntenin*. Using both genetic (gain and loss of function) and pharmacological approaches we clarify the biochemical pathways by which *mda-9/syntenin* modulates IGFBP-2 and how these molecules promote angiogenesis. These findings uncover a new mechanism by which the MDA-9/syntenin pro-metastatic gene contributes to progression in human melanoma and provide potential targets for intervening in the metastatic process.

Materials and Methods

Cell lines and Reagents

Different melanoma cell lines were maintained in routine cell culture conditions as described previously (8, 10). A clone of normal immortal human melanocytes FM516-SV (referred to as FM-516, was initially provided by Dr. L. Diamond, Wistar Institute, Philadelphia, PA), radial growth phase melanoma WM35 (obtained from Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA). C8161.9 was a gift from Dr. Danny R. Welch, Kansas University Medical Center, KS. The metastatic melanoma cell lines MeWo and FO-1 were provided by Dr. Robert S. Kerbel, Sunnybrook Cancer Center, Toronto, Canada and Dr. Eliezer Huberman, Argonne National Laboratories, Argonne, IL, respectively. Vertical growth phase melanoma WM278 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). All the cells were routinely checked for morphology, *in vitro* phenotypes (such as invasive and anchorage independent growth) and mycoplasma contamination using a PCR-based mycoplasma detection kit (Applied Biosystems, Foster City, CA). Human Umbilical Vein Endothelial cells (HuVECs) were cultured according to the provider's protocol (Lonza Walkersville Inc., Walkersville, MD). Unless stated otherwise, all the experiments were performed in Cultrex® Basement Membrane Extract (Trevigen Inc., Gaithersburg, MD)-coated plates (diluted in PBS, 2.5 mg/mL). Human recombinant IGFBP-2 (rhIGFBP-2) and neutralizing antibody for both IL-8 and IGFBP-2 were purchased from R&D Biosystems, Minneapolis, MN.

Construction of Plasmids, Adenoviruses and Stable Cell Lines

Small hairpin RNA for *mda-9*/syntenin (sh*mda-9*/syntenin) has been constructed with pSilencer™ hygro Expression vectors according to the manufacturer's protocol (Ambion Inc. TX). Specific hairpin siRNA oligonucleotides (sense 5'-GATCCGCGGATGGCACCAAGCATTTTCAAGAGAAATGCTTGGTGCCATCCGCTTTTTTGAAA-3' and antisense 5'-AGCTTTTCCAAAAAAGCGGATGGCACCAAGCATTTCTCTTGAAAATGCTTGGTGCCATCCGCG-3') were annealed and ligated to pSilencer vector by T4 DNA ligase. The genomic sequence of *mda-9*/syntenin was amplified by PCR using genomic DNA as template and primers, sense: 5'-CTGCAAAAATGTCTCTCTATCC-3' and anti-sense: 5'-GGTGCCGTGAATTTTAAACCTCAG-3'. The PCR product was cloned into pREP4 expression vectors from where it was digested and released with *Xho* and *Bam*HI and subcloned into the pcDNA3.1 (+hygro) (Invitrogen, Carlsbad, CA). *mda-9*/syntenin expression plasmid was constructed using genomic DNA as template and stable clones were established in immortal primary human melanocyte FM-516-SV40 cells. sh*IGFBP-2* expression plasmid was purchased from OriGene, Rockville, MD.

The DNA fragment (702 bp) containing the U6 promoter followed by oligonucleotide encoding shRNA designed for the *mda-9*/syntenin gene was isolated from *Eae*I-digested pSilencer-sh*mda-9* plasmid and then cloned into *Not*I site of pShuttle (pSh) plasmid generating pSh*mda-9* shuttle vector. To construct shuttle vector pShCMV-*mda-9*, *Bam*HI and *Eco*RV DNA fragment (990 bp) containing the *mda-9*/syntenin gene was isolated from plasmid p0tg-CMV-*mda-9* and cloned between *Bgl*II and *Eco*RV sites downstream of the CMV promoter in plasmid pShuttle-CMV. The shuttle plasmids were recombined with genomic DNA of Ad.5/3.*Luc*1 vector as we previously described (26) to derive plasmids pAd.5/3-sh*mda-9* or pAd.5/3-*mda-9*. The resultant plasmids were cleaved with *Pac*I to release the recombinant Ad. genomes and then transfected to HEK-293 cells to rescue the corresponding Ad.5/3-based vectors. The rescued viruses were upscaled using HEK-293 cells and purified by cesium chloride double ultracentrifugation using standard protocol (27)

and the titers of infectious viral particles are determined by plaque assay using HEK-293 cells as described (28).

Co-culture of HuVECs and tumor cells

In the co-culture system, both cell types were maintained in complete EGM-2 medium (Walkersville, MD). The tumor cells express green fluorescent protein (GFP) to discriminate them from HuVECs. For growth curves, cells were cultured in six-well plates in triplicate on the BME-coated plates. To dissociate cells from the gel, dispase in PBS without calcium, magnesium, and EDTA, was used at a concentration of 1 unit per mL. Cells were counted using a haemocytometer on a fluorescence microscope to discriminate between the colorless HuVECs and the green tumor cells.

Western blotting analyses

Western blotting analyses were performed as described (10). The primary antibodies used were anti-MDA-9 (1:1000; mouse polyclonal, Abnova, Walnut, CA), anti-c-Src, (1:200, Santa Cruz Biotechnology), anti-FAK (1:1000, Transduction Laboratories), anti-pAKT (1:2,000; rabbit polyclonal; Cell Signaling Technology), anti-AKT (1:2,000; rabbit polyclonal; Cell Signaling Technology), anti HIF-1 α (1:1000; mouse monoclonal, Abcam) and anti α V β 3 (1:500; Mouse monoclonal antibody; Novus Biologicals, Littleton, CO). Blots were stripped and normalized by re-probing with anti-EF-1 α antibody (1:1000; mouse monoclonal, Upstate Biotechnology, Waltham, MA). Digitized images of immunoblots were quantified using a Kodak Station 440.

Preparation of conditioned media (CM)

CM were harvested from different cultures and filtered with 0.2 μ M filters and further concentrated 8-fold on a Centricon-100 (Millipore).

In vitro cell invasion assays

Cell invasion was performed in a modified Boyden chamber (BD Bioscience, Bedford, MA, USA) according to the manufacturer's instructions.

Capillary-like tube formation assays

Tube formation assays were performed as described previously using an In Vitro Angiogenesis Assay Kit (Chemicon). Based on visual patterns, a numerical value was assigned according to manufacturer's recommendation and results are expressed as relative units for degree of angiogenesis progression.

Human angiogenesis arrays

Equal amounts of protein (500 μ g) in 100 μ L samples were assayed using Human Angiogenesis Antibody Arrays (R&D Biosystems, Minneapolis, MN) and processed according to the manufacturer's instructions.

Enzyme linked immunosorbant assay (ELISA) for IGFBP-2, PTX3 and VEGF-A

Protein levels in conditioned media (CM) were measured using corresponding ELISA Kits, purchased from R&D Biosystems, Minneapolis, MN and processed according to the manufacturer's instructions. For CM, 200 μ L were collected from triplicate samples, analyzed for the target (IGFBP-2, PTX3 or VEGF-A) protein levels, and normalized for total protein using the Bradford method.

Chorioallantoic membrane (CAM) assay

To detect *in vivo* angiogenesis, we performed CAM assays as described previously (29). Either cells or CM in a collagen sponge were implanted onto the CAM at day 8 of fertilization. At day 12, CAMs were fixed with 10% formalin; the neovasculature was examined and photographed. Angiogenesis was quantified by counting the blood vessel branch points under a stereomicroscope.

Immunohistochemistry

Formalin-fixed tumors were embedded in paraffin, sectioned, and mounted on glass slides. Immunohistochemical staining was performed with anti-mouse MDA-9/syntenin and anti mouse CD31 (Glostrup, Denmark) antibodies as described previously (8).

Xenograft studies in athymic nude mice

Subcutaneous xenografts were established in the flanks of athymic nude mice (4–5 week old male mice, purchased from Charles River, Wilmington, MA) using 1×10^6 cells and followed for four weeks. Tumor volume was measured twice weekly with a caliper and calculated using the following formula: $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$. In a separate experiment, C8161.9 (1×10^6) cells were subcutaneously xenotransplanted in the flanks of nude mice and after establishment of visible tumors of $\sim 75 \text{ mm}^3$, intratumoral injections of different adenoviruses were given at a dose of 1×10^8 plaque-forming units in 100 μL of PBS. The injections were given 3X a week for the first week and then 2X a week for two more weeks for a total of seven injections and followed for 3 weeks. All experiments were performed with at least 5 mice in each group, and all of the experiments were repeated three times.

Statistical analysis

The data are reported as the mean $\pm$ S.D. of the values from three independent determinations and statistical analysis was performed using the Student's t test in comparison with corresponding controls. Probability values < 0.05 were considered statistically significant. One-way analysis of variance (ANNOVA) was used to test the difference between the means of several subgroups of a variable (Prism Statistical Software).

Results

mda-9/syntenin promotes tumor progression by augmenting angiogenesis

To examine the effect of persistent downregulation/overexpression of *mda-9/syntenin* we established several *shmda-9/syntenin* and *mda-9/syntenin* overexpressing stable clones in highly metastatic (C8161.9) melanoma and primary immortal human melanocytes (FM-516 SV, indicated as FM-516), respectively (Fig. S1A and S1B). These clones were evaluated for growth and biological traits characteristic of the metastatic phenotype, e.g., invasion (Fig. S1C and S1D) and anchorage independent growth (Fig. S1E and S1F). Manipulating *mda-9/syntenin* expression did not affect cellular proliferation, whereas it profoundly affected expression of the transformed/metastatic phenotype. After confirming a direct relationship between *mda-9/syntenin* expression and the *in vitro* transformed/invasive phenotype, we assessed C8161.9-con-sh, C8161.9-*shmda-9* Cl.4 and C8161.9-*shmda-9* Cl. 13 clones for *in vivo* tumorigenesis by establishing subcutaneous xenografts in athymic nude mice. Knockdown of *mda-9/syntenin* intensely inhibited the tumorigenic capacity of C8161.9 cells (Fig. 1A), which directly correlated with a marked inhibition of angiogenesis as revealed by CD31 staining that is indicative of microvessel density (Fig. 1B and Fig. S2A). To authenticate these findings, we established subcutaneous xenografts of C8161.9

cells in nude mice and intratumorally injected a tropism-modified serotype 5/3 adenovirus expressing shRNA-targeting *mda-9*/syntenin (Ad.5/3-sh*mda-9*). These experiments documented a significant reduction in tumor volume and tumor weight (Fig. 1C) as well as tumor angiogenesis detected by CD31 staining (Fig. 1D and Fig. S2B) upon injection of Ad. 5/3-sh*mda-9* versus Ad. *vec*, the control empty adenovirus. Further validation was obtained when C8161.9-con-sh, C8161.9-sh*mda-9* Cl.4, FM-516 and FM-516-*mda-9* Cl.14 clones were implanted onto the chicken chorioallantoic membrane (CAM). After 8-days of incubation, the underside of the tumors was photographed to view neo-vascularization. Tumor size was significantly larger (~5 times) and extensive vascularization was detected in C8161.9-con-sh cells as compared to C8161.9-sh*mda-9* Cl.4 cells (Fig. 1E). Similarly, overexpression (gain-of-function) of *mda-9*/syntenin in FM-516 cells resulted in larger tumors with significant vascularization when compared to the control FM-516 cells (Fig. 1E). These findings support the hypothesis that augmentation of angiogenesis plays a decisive role in mediating *mda-9*/syntenin-induced tumor progression and metastasis.

***mda-9*/syntenin promotes angiogenesis in HuVEC cultures**

Tumor cells mediate tumor angiogenesis by direct cellular interactions with endothelial cells as well as by secreting soluble factors that enhance endothelial cell proliferation, migration and tube formation (30). In order to explore a potential role for intercellular interactions, we co-cultured C8161.9 or its sh*mda-9* expressing clones *in vitro* with human umbilical vein endothelial cells (HuVECs). A significant temporal increase in HuVEC cell number was evident in cell growth assays (spanning 5 days) when co-cultured in the presence of C8161.9-con-sh cells as compared with C8161.9-sh*mda-9* cells (Fig. 2A) demonstrating that interactions between HuVECs and C8161.9 cells in co-culture promote HuVEC proliferation. Next, we examined tube formation of HuVECs, when seeded on Matrigel-coated plates with tumor cells. Culturing HuVECs in a complex matrix like Matrigel itself resulted in significant tube formation, which was further augmented upon co-culture with C8161.9-con-sh cells (Fig. 2B). However, co-culturing HuVECs with C8161.9-sh*mda-9* clones resulted in a marked inhibition of HuVEC tube formation (Fig. 2B) indicating that *mda-9*/syntenin stimulates angiogenesis in HuVECs. We also determined the effects of C8161.9-con-sh and C8161.9-sh*mda-9* clones on HuVEC motility by plating tumor cells onto the lower chambers of TransWellR cell cultures (Fig. 2C, top panel). Serum-starved HuVECs were plated on the inserts, cultured for 18 hours and the number of cells crossing the Matrigel membrane was scored. Significantly higher numbers (~55%) of HuVECs crossed the Matrigel layer towards the C8161.9-con-sh cells when compared with C8161.9-sh*mda-9* cells (Fig. 2C, bottom panel) indicating that soluble factors produced by tumor cells expressing *mda-9*/syntenin promoted HuVEC motility.

To define directly the involvement of *mda-9*/syntenin-regulated soluble factor(s) released from tumor cells in mediating angiogenesis, we determined HuVEC proliferation, migration and tube formation in the presence of conditioned media (CM) collected from both *mda-9*/syntenin overexpressing and knockdown clones as well as corresponding parental cells. HuVEC proliferation (Fig. 2D), invasion (Fig. 2E) and tube formation (Fig. 2F) in CM directly correlated with the *mda-9*/syntenin status of the producing cells, i.e., *mda-9*/syntenin overexpression promoted, while *mda-9*/syntenin knockdown inhibited these *in vitro* phenotypes. Additionally, *in vivo* CAM assays also revealed that CM from C8161.9-con-sh and FM-516-*mda-9* Cl.14 cells profoundly induced angiogenesis compared to CM from C8161.9-sh*mda-9* Cl.4 and FM-516 cells, respectively (Fig. 2G).

IGFBP-2 is a *mda-9*/syntenin-induced angiogenic factor

Angiogenesis is induced and controlled by the relative balance of pro- and anti-angiogenic factors present in the tumor microenvironment. Accordingly, an angiogenesis array using

CM from C8161.9-con-sh and C8161.9-sh*mda-9* Cl.4 cells was used to identify potential *mda-9*/syntenin-regulated angiogenesis-associated factors (Fig. 3A and Fig. S3). The expressions of interleukin-8 (IL-8), Insulin Growth Factor Binding Protein-2 (IGFBP-2) and Pentraxin 3 (PTX3) were markedly downregulated and that of VEGF-A, IGFBP-1 and -3, and EGF were modestly downregulated in C8161.9-sh*mda-9* Cl.4 cells as compared to the parental C8161.9-con-sh cells. Moreover, overexpression of IL-8, IGFBP-2 and PTX3 was consistently found in *mda-9*/syntenin overexpressing FM-516 clones compared to the parental FM-516 cells (Fig. S4). As the range of expression changes was variable, the identified proteins might contribute to variable extents and at different threshold levels to the overall angiogenic process induced by *mda-9*/syntenin.

We initially focused our attention on IL-8, PTX-3 and IGFBP-2, the three factors modulated maximally by *mda-9*/syntenin. We confirmed the role of IL-8, an established angiogenic factor (25), by examining the effects of C8161.9-con-sh CM treated with neutralizing antibody to IL-8 on HuVECs. Neutralization of IL-8 blocked HuVEC proliferation (Fig. S5A), migration (~20%) (Fig. S5B) and tube formation (~34%) (Fig. S5C) as compared to control IgG. Previous studies indicated that PTX-3 functions as an anti-angiogenic factor by binding to bFGF (31). Knocking down PTX-3 with siRNA in C8161.9 cells did not alter HuVEC phenotypes suggesting that PTX-3 may not play a direct role in melanoma angiogenesis. Moreover, the expression of bFGF in C8161.9 cells did not change after *mda-9*/syntenin knockdown, further inferring that PTX-3 expression might not contribute to angiogenesis in melanoma.

We next investigated the effect of recombinant human IGFBP-2 (rhIGFBP-2) on HuVECs. Proliferation (Fig. 3B), invasion (Fig. 3C) and tube formation (Fig. 3D) in HuVECs were significantly stimulated by rhIGFBP-2 and neutralizing antibody to IGFBP-2 prevented these effects. Similarly, HuVECs treated with rhIGFBP-2 produced ~6-fold more vascularization compared with basal media in CAM that was reduced by IGFBP-2 neutralizing antibody (Fig. 3E and Fig. S6A). CM from C8161.9 cells undergoing transient knockdown of *IGFBP-2* by siRNA inhibited HuVEC tube formation (upper panel in Fig. 3F, and Fig. S6B,) and neovascularization in CAM (lower panel in Fig. 3F, and Fig. S6C) when compared to control siRNA-treated HuVECs. Similar results were obtained in FM-516 clones overexpressing *mda-9*/syntenin treated with IGFBP-2 siRNA. Pooled clones of C8161.9 cells with stable knockdown of *IGFBP-2* by shRNA were significantly less aggressive in tumor formation in athymic mice compared with pooled clones expressing control shRNA (Fig. 3G). This effect was associated with a reduction in CD31 positive cells establishing IGFBP-2 as a potential pro-angiogenic factor (Fig. 3H and Fig. S6D).

***mda-9*/syntenin-mediated HIF-1 α activation induces IGFBP-2 expression**

The molecular mechanism of enhanced IGFBP-2 expression by *mda-9*/syntenin was studied. Compared to FM-516 and WM-35 radial growth phase melanoma cells, cell-derived CMs from the metastatic melanomas contained significantly higher levels of IGFBP-2 that positively correlated with the levels of *mda-9*/syntenin expression (Fig. 4A and Fig. S7A). Overexpression of *mda-9*/syntenin in FM-516 cells by adenovirus (Ad.5/3-*mda-9*) transduction resulted in a dose- and time-dependent induction of IGFBP-2 expression in both mRNA and protein levels (Fig. 4B). Similar results were also obtained in WM-35 cells.

MDA-9/syntenin is a scaffold protein and following interaction with the ECM it might crosstalk with different protein(s), thereby activating multiple signaling pathways. FM-516 cells were infected with different concentrations of Ad.5/3-*mda-9* and then plated on a thin basement membrane extract (BME) that mimics ECM resulting in a dose-dependent increase in the phosphorylation of AKT at serine 473 (Fig. 4C, left panel and Fig. S7B, upper panel) at 30 min post-seeding. Conversely, knocking down *mda-9*/syntenin by Ad.5/3-sh*mda-9* in

C8161.9 cells and plating cells on BME significantly reduced AKT activation (Fig. 4C, middle panel and Fig. 7B, lower panel). Stable clones of FM-516 cells overexpressing *mda-9/syntenin* and C8161.9 cells expressing *mda-9/syntenin* shRNA also showed similar trends in AKT activation or deactivation, respectively (Fig. 4C, right panel and Fig. S7C). *mda-9/syntenin*-induced AKT activation facilitated production of hypoxia inducible factor 1- α (HIF-1 α), a transcription factor that regulates the transcription of IGFBP-2 in breast cancer (32). Inhibition of the PI3K/AKT pathway by the chemical inhibitor LY294002 significantly abrogated *mda-9/syntenin*-induced augmentation of HIF-1 α and IGFBP-2 expression in FM-516 cells indicating that induction of IGFBP-2 by *mda-9/syntenin* is mediated through AKT and HIF-1 α (Fig. 4D and Fig. S7D).

We previously demonstrated that when plated on fibronectin, MDA-9/syntenin physically interacts with c-Src resulting in sequential activation of FAK, p38 MAPK and NF- κ B thereby promoting metastasis (8–11). When plated on BME, siRNA-mediated transient knockdown of c-Src and FAK inhibited *mda-9/syntenin*-mediated AKT activation, HIF-1 α induction and IGFBP-2 expression in FM-516 cells (Fig. 4E and Fig. S7E). A dose-dependent decrease in *mda-9/syntenin*-induced IGFBP-2 expression was observed with the selective c-Src inhibitor PP-2 (1–5 μ M), but not by the inactive congener PP-3 (Fig. 4F). Moreover, CM from C8161.9 cells with transient knockdown of c-Src induced less tube formation by HuVECs and were less angiogenic (~2-fold) in CAM compared to control siRNA-treated C8161.9 cells confirming the role of c-Src in *mda-9/syntenin*-mediated IGFBP-2 expression and angiogenesis (Fig. 4G and Fig. S7F).

IGFBP-2 induces angiogenesis via interaction with α V β 3 integrin and activation of PI3K/AKT in HuVECs

In glioblastoma, IGFBP-2 and VEGF are co-expressed and expression positively correlates with angiogenic phenotypes (33). VEGF-A is a well-known pro-angiogenic factor involved in the induction of angiogenic phenotypes in HuVECs (34). Based on these considerations, we analyzed VEGF-A expression in HuVECs after stimulating with rhIGFBP-2. VEGF-A expression, both RNA and protein, and VEGF-A promoter activity were dose-dependently upregulated by rhIGFBP-2 indicating that IGFBP-2 regulates VEGF-A expression at a transcriptional level (Fig. 5A and B). Interestingly, rhIGFBP-2 treatment of HuVECs stimulated AKT activation within 15 min (Fig. 5C and Fig. S8A) and blocking activation by LY294002 significantly inhibited VEGF-A expression (Fig. 5D) indicating that IGFBP-2-mediated PI3K/AKT activation results in VEGF-A production. It has been reported that integrin α V β 3 is highly expressed in HuVECs (35) and interacts with IGFBP-2 (36). Treatment of HuVECs with anti α V β 3 antibody blocked rhIGFBP-2-induced AKT activation and elevated VEGF-A expression (Fig. 5E and Fig. S8B) as well as tube formation in Matrigel (Fig. 5F and Fig. S8C) and neovascularization in CAM (Fig. 5G and Fig. S8D) indicating that interaction of IGFBP-2 with α V β 3 integrin initiates a cascade of events resulting in augmentation of angiogenesis. Additionally, consistent with previous findings by Russo and colleagues (37), we also observed that IGF-1R (Insulin growth factor-1 receptor) critically regulates IGFBP-2-mediated angiogenesis, since transiently knocking down the receptor in HuVECs significantly prevented tumor-derived condition media-induced angiogenic phenotypes in *in vitro* assays (data not shown).

Discussion

In the present study, we describe a new mechanism of melanoma progression involving angiogenesis induction through expression of *mda-9/syntenin* and IGFBP-2 (Fig. 6). *mda-9* is an adaptor protein that facilitates tumor progression and metastasis of melanoma cells (8–13). Conclusive evidence is now provided that *mda-9/syntenin* can function as a potent inducer of angiogenesis, which is an essential non-autonomous component of the tumor-

promoting functions of this cancer-promoting gene. Important components of angiogenesis include endothelial cell proliferation, migration, interaction with the ECM, morphological differentiation, cell adherence and tube formation (30). Although the 'crosstalk' between cell types might be bidirectional, in this study, we focused our investigations on melanoma-induced changes in endothelial cells. We demonstrate using an *in vitro* co-culture system that *mda-9/syntenin* can stimulate endothelial cell proliferation, migration and differentiation through direct (contact-mediated) and indirect (contact-independent) interactions between human melanoma cells and endothelial cells. The observation that physically separated melanoma cells induce HuVEC migration and conditioned media (CM) from melanoma cells modifies endothelial cell phenotypes suggests that metastatic melanoma cells produce pro-angiogenic factors that can directly modify endothelial cell behavior in a *mda-9/syntenin*-dependent manner. Additionally, vasculogenesis induced by parental melanoma cells vs. *mda-9/syntenin* downregulated clones established the involvement of *mda-9/syntenin* in promoting angiogenesis.

Through human angiogenesis antibody arrays and both gain-of-function and loss-of-function experiments, we identified IGFBP-2 as a key contributor to angiogenesis in melanoma. High serum IGFBP-2 levels have been detected in individuals with diverse types of cancer, including cancer of the central nervous system (CNS) (38), lung (39), lymphoid organs (40), colon (41), adrenal gland (42) and prostate (43), and expression positively correlates with the aggressive behavior of prostate cancer and melanoma cells (44). In melanoma (45), IGFBP-2 is overexpressed in dysplastic nevi and primary melanomas when compared to benign nevi and the expression of IGFBP-2 increases in melanocytic lesions with tumor progression. Although high IGFBP-2 expression has been identified in different malignancies, the role of IGFBP-2 in tumor progression is poorly understood. In a limited number of studies, IGFBP-2 has been shown to regulate tumor cell phenotypes, including cell proliferation and adhesion, through interaction with different signaling pathways (46–48). With respect to angiogenesis, the enhancing role of IGFBP-2 has only been suggested in glioma based on observations that IGFBP-2 is co-expressed with VEGF in pseudopalisade cells surrounding necrotic areas in tumors (33). During the preparation and submission of our manuscript two research groups independently demonstrated that IGFBP-2 secreted by metastatic tumor cells can recruit endothelial cells (49) and transcriptionally regulate VEGF-A secretion (50) that ultimately culminates in angiogenesis, providing additional support for our observations. Our study is the first to provide decisive proof of pro-angiogenic functions of IGFBP-2 in the context of human melanoma, where the pro-metastatic gene MDA-9 is documented to play a pivotal role (8–11).

Interaction of *mda-9/syntenin* with the ECM in melanoma cells results in c-Src and FAK activation that subsequently activates the PI3K/AKT pathway resulting in HIF-1- α -mediated induction of IGFBP-2 (Fig. 6). In endothelial cells, secreted IGFBP-2, via its interaction with $\alpha V\beta 3$ integrin, activates the PI3K/AKT pathway leading to the generation of the pro-angiogenic factor VEGF-A. It is well established that the PI3K/AKT pathway plays an important role both in the generation of VEGF-A in different cancer cells as well as in its subsequent function in endothelial cells. IGFBP-2 expression inversely correlates with PTEN expression, a known tumor suppressor and negative regulator of the PI3K/AKT pathway. Additionally, the expression of PTEN itself is down regulated by IGFBP-2 (47) indicating that PTEN-dependent activation of PI3K/AKT might also be important in upstream and downstream events regulating IGFBP-2 expression. However, in melanoma cells we did not observe changes in PTEN expression by *mda-9/syntenin* indicating that multiple and distinct pathways may regulate IGFBP-2 expression in different target cells.

In summary, our present study reveals the functional roles of MDA-9/syntenin in regulating angiogenesis and identifies the signaling events and downstream effectors important in

regulating this central component of the tumorigenic and metastatic process. Additionally, we identify IGFBP-2 as a direct downstream target of *mda-9/syntenin* that regulates endothelial cell proliferation, migration and invasion. Our findings expand the diverse cell autonomous and non-autonomous tumor-promoting functions of *mda-9/syntenin* and establish the rationale for developing cancer therapies based on the targeted disruption of *mda-9/syntenin* or its regulated pathways, including IGFBP-2 through novel small molecular inhibitors using high throughput screening approaches, including combinatorial chemistry and proteomics/bioinformatics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

In vivo assessment of tumor formation in mice and growth in the chicken embryo chorioallantoic membrane (CAM) assay after modulation of *mda-9*/syntenin expression. A) Subcutaneous xenografts were established in athymic nude mice (n=15) and tumor volume was measured twice a week and tumor weight at the end of the study at 4 weeks. Data represents mean $\pm$ S.D. B) Serial sections of formalin-fixed, paraffin-embedded tumor tissues were immunostained for MDA-9/syntenin and the endothelial cell marker CD31 and counterstained with hematoxylin. C) Subcutaneous xenografts from C8161.9-con-sh cells were established in the flanks of athymic nude mice (n = 15) and injected with the indicated Ad.5/3 at different m.o.i. Tumors were excised and photographed and tumor weight was measured at the end of the experiment (4 weeks). D) Serial sections of formalin-fixed, paraffin-embedded tumor tissues (as described in C) were immunostained for MDA-9/syntenin and CD31 and counterstained with hematoxylin. E) Different clones were implanted onto the CAM. Representative photomicrographs of tumors underneath the CAM are shown. Asterisks indicate statistically significant differences ($p < 0.05$) from corresponding controls.

Figure 2.

Effect of *mda-9*/syntenin on the angiogenic phenotype of human vascular endothelial cells (HuVECs). A) Time course analysis of growth of HuVECs in co-culture with either C8161.9-con-sh or C8161.9-sh*mda-9* clones. B) Analysis of tube formation by HuVECs co-cultured with C8161.9-con-sh or C8161.9-sh*mda-9* clones on Matrigel-coated plates grown in serum-starved media conditions. Left panel, photomicrograph. Right panel, graphical representation of quantification of tube formation. C) HuVEC migration towards melanoma cells. C8161.9-con-sh or C8161.9-sh*mda-9* clones were cultured in the lower chamber and HuVECs were cultured on the inserts, in TransWellIR cell culture plates as depicted in the upper panel. HuVEC migration was quantified as described in Materials and Methods and graphical representation is provided in the lower panel. D) Time course analysis for growth of HuVECs cultured in conditioned media (CM) from the indicated cell type. E) HuVEC migration through Matrigel in the presence of CM from the indicated cells. The assay was scored after 18 h. Left panel, photomicrograph. Right panel, graphical representation of quantification of migration. F) Analysis of tube formation by HuVECs in the presence of CM from the indicated cells. Left panel, photomicrograph. Right panel, graphical representation of quantification of tube formation. G) CM from the indicated cells was implanted onto the CAM (n = 10) and after 4 days photographs were taken for visualization (left panel) and quantification (right panel) of neovascularization. Asterisk indicates statistically significant difference (p<0.05) from corresponding controls. In panel A), and D), different italicized letters indicate significant differences between groups assessed by Student's t-test (p<0.05) at a corresponding time point. Data represents Mean $\pm$ S.D.

Figure 3.

Pro-angiogenic activity of IGFBP-2. A) Top panel, an antibody array comparing the expression levels of regulators of angiogenesis in CM from C8161.9-con-sh and C8161.9-shmda-9 clones was performed. Bottom panel, Graphical representation of the band intensity quantified by densitometry. B) HuVECs were cultured in the presence of recombinant human IGFBP-2 (rhIGFBP-2) protein alone or with neutralizing antibody (NA) and growth kinetics were determined by trypan blue dye exclusion assay. C, D and E) HuVECs were treated with rhIGFBP-2 with or without NA and migration (C), tube formation (D) and vascularization in CAM (E) were analyzed. F) C8161.9 cells were transfected with either scrambled RNA (si-con) or si-IGFBP-2 and CM were analyzed for tube formation on Matrigel (upper panel) and vascularization in CAM (lower panel). G) Pooled clones of C8161.9 cells stably expressing shIGFBP-2 were established and evaluated for tumor generation ability in athymic nude mice (n = 15). Pooled clones of C8161.9 cells stably expressing control scrambled shRNA (con-sh) and C8161.9-shmda-9 Cl.4 were used as controls. H) Serial sections of formalin-fixed, paraffin-embedded tumor tissues were immunostained for the endothelial cell marker CD31 and counterstained with hematoxylin. In panel B), C), D) and G), italicized different letters indicate significant differences between groups assessed as defined by the Student's t-test ($p < 0.05$). Data represents Mean $\pm$ S.D.

Figure 4.

mda-9/syntenin enhances IGFBP-2 expression through c-Src- and AKT-dependent pathways. A) Expression levels of IGFBP-2 in the indicated cell-derived CM were determined by ELISA and the levels of MDA-9/syntenin protein in cell lysates were determined by Western blotting. EF-1α expression was used as a loading control. B) FM-516 cells were infected with either Ad.5/3-vec or Ad.5/3-*mda-9* at different m.o.i. as indicated. At different time points, ELISA analyzed CM for IGFBP-2 expression. C) FM-516 cells were infected with Ad.5/3-*mda-9* and C8161.9 cells were infected with Ad.5/3-*shmda-9* at the indicated m.o.i. and expression of the indicated protein was determined by Western blotting. D) FM-516 cells were infected with either Ad.5/3-vec or Ad.5/3-*mda-9* and then treated or untreated with LY294002 (2.5 μM) for 12 or 24 h. Expression of HIF-1α and EF-1α were analyzed by Western blotting using cell lysates (top panel) and expression of IGFBP-2 was analyzed by ELISA in CM (bottom panel). All experiments were performed at least three times. E) Western blot analysis of the indicated proteins (left panel) and ELISA of IGFBP-2 in CM (right panel) after transient knockdown of c-Src and FAK in FM-516 cells infected with Ad.5/3-vec or Ad.5/3-*mda-9*. F) FM-516 cells were infected with Ad.5/3-*mda-9* and then treated with the pharmacological inhibitor of c-Src PP2 or its inactive analogue PP3 and IGFBP-2 expression was determined by ELISA. G) C8161.9 cells treated with either control siRNA or *c-Src* siRNA, and cell lysates and CM were collected. Left panel, Western blot analysis of the indicated proteins in cell lysates. Middle panel, HUVECs were treated with CM and tube formation was analyzed; Right panel, CM was implanted in CAM and neovascularization was photomicrographed. Data represents Mean ± S.D.

Figure 5.

IGFBP-2 upregulates the expression of vascular endothelial growth factor (VEGF-A) through the AKT pathway in HuVECs. A) The expression of VEGF-A mRNA (top panel) and protein (bottom panel) were measured by real-time PCR and ELISA, respectively, after treating HuVECs with the indicated doses of rhIGFBP-2. B) HuVECs were transfected with VEGF-A promoter luciferase reporter plasmid and treated with the indicated concentrations of rhIGFBP-2. 48 h after transfection, cells were harvested for luciferase assays as described in Materials and Methods. C) HuVECs (1×10^6 cells) were treated or untreated with the specified doses of rhIGFBP-2 for the indicated times and expression of pAKT and AKT was analyzed by Western blotting. D) HuVECs were pre-treated with LY294002 (30 min) and then treated with rhIGFBP-2. Analysis of expression of VEGF-A by ELISA in the CM. E) HuVECs were treated with rhIGFBP-2 together with anti- $\alpha V\beta_3$ integrin antibody or anti-mouse IgG. Top panel, analysis of expression of pAKT and AKT by Western blotting. Bottom panel, analysis of expression of VEGF-A by ELISA in the CM. F & G) HuVECs were treated as in E and CM was used to analyze tube formation (F) and neovascularization in CAM (G). All experiments were performed at least three times. In panel A) and B), italicized different letters indicate significant differences between groups assessed by Student's t-test ($p < 0.05$).

Fig. 6. Hypothetical model of MDA-9/syntenin induction of angiogenesis. MDA-9/syntenin upon interaction with c-Src, activates HIF-1α in an AKT-dependent pathway and induces IGFBP-2 expression. IGFBP-2 acts as a chemo-attractant for endothelial cells and induces VEGF-A secretion resulting in angiogenic phenotypes.