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The MDA-9/Syntenin/IGF-1R/STAT3 axis directs prostate cancer invasion

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Abstract

Although prostate cancer (PCa) is clinically manageable during several stages of progression, survival is severely compromised once cells invade and metastasize to distant organs. Comprehending the pathobiology of invasion is required for developing efficacious targeted therapies against metastasis. Based on bioinformatics data, we predicted an association of melanoma differentiation associated gene-9 (syntenin, or syndecan binding protein (SDCBP)) in PCa progression. Using tissue samples from various Gleason stage PCa patients with adjacent normal tissue, a series of normal prostate and PCa cell lines (with differing tumorigenic/metastatic properties), mda-9/syntenin manipulated variants (including loss-of-function and gain-of-function cell lines), and CRISPR/Cas9 stable MDA-9/syntenin knockout cells, we now confirm the relevance of and dependence on MDA-9/syntenin in PCa invasion. MDA-9/syntenin physically interacted with insulin-like growth factor-1 receptor (IGF-1R) following treatment with insulin-like growth factor binding protein-2 (IGFBP-2), regulating downstream signaling processes that enabled STAT3 phosphorylation. This activation enhanced expression of MMP-2 and MMP-9, two established enzymes that positively regulate invasion. Additionally, MDA-9/syntenin-mediated upregulation of pro-angiogenic factors including IGFBP-2, IL-6, IL-8, and VEGF-A also facilitated migration of PCa cells. Collectively, our results draw attention to MDA-9/syntenin as a positive regulator of PCa metastasis, and the potential application of targeting this molecule to inhibit invasion and metastasis in PCa and potentially other cancers.

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Competing Interests

P.B. Fisher is a co-founder and has ownership interest in CTS, Inc. Virginia Commonwealth University, Johns Hopkins University and Columbia University have ownership interest in CTS. P.B. Fisher is also a co-founder and has ownership in InVaMet Therapeutics, Inc. The remaining authors declare no competing financial interests.

Keywords

MDA-9/Syntenin; prostate cancer; invasion; IGF-1R; STAT3

Introduction

As for most solid malignancies, mortality from prostate cancer (PCa), the most commonly diagnosed non-cutaneous cancer, results from widespread metastases (1). In this context, even treatment of localized PCa is fraught with significant morbidity, however, once metastasis occurs, there are few available treatment options for patients that have failed to respond to androgen deprivation therapies. Patients diagnosed in this advanced stage usually die within 12 to 18 months (1), emphasizing the need for better definition of the signaling cascades that regulate metastasis, which embodies three main processes: invasion, intravasation and extravasation. Invasion initiates with loss of cell-cell adherence of metastatic competent cells, which become motile and breakdown the extracellular matrix allowing them to migrate and enter the circulation (2). Multiple intrinsic transcriptional and epigenetic molecular programs regulate epithelial-to-mesenchymal transition (EMT). This process results in loss of intracellular adhesion and epithelial polarization, cytoskeleton rearrangement facilitating mobility, and release of extracellular proteases including matrix metalloproteinases (MMPs) that degrade the extracellular matrix promoting tumor spread. Secretion of active MMPs release matrix bound growth factors and cytokines further enabling invasion (3).

Defining genes that control invasion has potential to identify new target(s) for intervening in metastasis, which would have profound therapeutic implications. To achieve this objective, we employed subtraction hybridization (4). *Melanoma differentiation associated gene-9* (*mda-9*), also known as *syntenin*, (*mda-9/syntenin*) was initially identified by subtraction hybridization as a novel gene displaying biphasic expression during terminal differentiation of human melanoma cells (5,6). *mda-9/syntenin*, a PDZ-domain protein, overexpressed in many types of human cancers, based on bioinformatics analyses http://cancergenome.nih.gov/, may function in tumor progression (7–9). Although both bioinformatics analyses and our observations (published and unpublished) indicate a positive correlation of *mda-9/syntenin* expression with aggressive phenotypes in multiple cancers, the precise roles of *mda-9/syntenin* in many of these neoplasms have not been ascertained. Suppression subtractive hybridization between a poorly invasive/non-metastatic and an invasive/metastatic breast cancer cell line identified *mda-9/syntenin* overexpression in metastatic cells (10). Overexpression of *mda-9/syntenin* is also evident in metastatic melanoma (11), breast (10), gastric (10) and bladder (12) cancer cells in comparison to their primary or poorly metastatic counterparts. Recently, we confirmed a role of this protein in glioma invasion (13). Notably, forced expression of *mda-9/syntenin* increased migration of non-metastatic cancer cells, which correlated with a more polarized distribution of F-actin and increased pseudopodia formation (11,14). Additionally, immunohistochemical analyses revealed a statistically significant continued increase in MDA-9/Syntenin expression from acquired melanocytic nevi to primary melanoma without or with progression to metastatic melanoma. These accumulated data strongly support the hypothesis that *mda-9/syntenin*

functions as a positive regulator of melanoma metastasis (11,15–17) as well as aggressiveness in breast and gastric cancers, and might contribute to invasion of multiple additional cancers.

The insulin-like growth factor (IGF) signaling axis plays a pivotal role in prostate cancer progression, confirmed both in preclinical and clinical studies (18). This signaling axis consists of extracellular ligands (IGF-1 and IGF-2 with their binding partners, *e.g.,* IGFBP-1 to -6) and receptors (IGF-1R and IGF-2R for IGF-1 and IGF-2, respectively). In 1998, Chan *et al.* first reported a positive correlation between IGF-1 levels and PCa risk (19), which was validated further in transgenic animals overexpressing IGF-1 in prostate epithelium that led to spontaneous neoplasia in the mouse prostate (20,21). Upon stimulation by specific ligands, IGF-1R is activated through auto phosphorylation resulting in phosphorylation of its' downstream targets insulin receptor substrate-1 (IRS-1) and Shc adaptor proteins, which play decisive roles in cellular proliferation/differentiation/cellular homeostasis through PI3K/AKT or Ras/Raf/Mek pathways (22). Additionally, activation of IGF-1R also associates with cellular invasiveness and metastatic phenotypes through activation of IRS-1 that directly influences the beta catenin pathway (23), or by regulating secretion of matrix metalloproteinases (24). As a receptor kinase, IGF-1R influences other growth factors or receptors such as VEGF and EGFR (Reviewed by Tao *et al.*(25)) and regulates angiogenesis and proliferation. Accordingly, IGF-1R activation or its' signaling cascade in PCa is fundamental, emphasizing clinical relevance and rationalizing the development of targeted therapeutics as reflected by the accelerated number of ongoing clinical trials employing inhibitors of the IFG-1R axis (reviewed by Heidegger *et al.*(22)).

The present study explores the relevance of MDA-9/Syntenin expression in PCa pathogenesis. We decipher a novel mechanistic signaling pathway for MDA-9/Syntenin in regulating PCa invasion. IGF-1R, through a physical interaction with MDA-9/Syntenin is central in this cascade. Overall, our findings show that MDA-9/Syntenin represents a novel molecular target for inhibiting invasion and metastasis, an ultimate cause of PCa-associated death.

Materials and Methods

Human cell lines

In the present study, the following cell lines were used, P69, RWPE-1, DU-145, PC-3, M12, M2182, ARCaP, ARCaP-E, and ARCaP-M (26). P69 (SV40 T antigen immortal normal human prostate epithelial cells), M2182 (progressed P69 cells displaying a tumorigenic phenotype) and M12 (progressed P69 cells displaying a tumorigenic and metastatic phenotype) were provided by Dr. Joy Ware, VCU School of Medicine, Richmond, VA. P69, M2182 and M12, provide a representative panel of human prostate cells (from normal immortal, tumorigenic, metastatic) and are described and cultured as indicated in our previous publication (26). Other cells, except ARCaP with its epithelial and metastatic variants, ARCaP-E and ARCaP-M, respectively, were obtained from ATCC (Manassas, VA) and maintained in culture as per ATCC recommendations. ARCaP, ARCaP-E and ARCaP-M cells were from Novicure Biotechnology (Birmingham, AL) and maintained in media recommended by the provider. HPV-immortal human prostate epithelial cells, RWPE-1,

were from ATCC. All cell lines were routinely checked for mycoplasma contamination using commercial kits. The majority of experiments used RWPE-1, DU-145 and ARCaP-M cells. All of these cell lines were purchased recently (within the last 3 years) and were strictly maintained as recommended by the manufacturer.

Reagents and antibodies

Specific antibodies (MDA-9/Syntenin, Phospho-IGF-1R (Tyr-1135), IGF-1R, Phospho-Src (Tyr-416), Src, Phospho-FAK (Tyr-397), FAK, Phospho-STAT3 (Tyr-705), MMP-2, MMP-9, IGFBP-2, STAT3, Syntenin-2, β-actin and EF-1α) and reagents (IGFBP-2 and ELISA kits (IGFBP-2)) used in the present study are described in Supplementary Materials and Methods.

Tissue microarray

Two different PCa tissue microarrays, purchased from Novus Biologicals (Littleton, CO)) and USBiomax (Rockville, MD) were stained with MDA-9/Syntenin or IGFBP-2 antibody from Sigma-Aldrich (St. Louis). All clinical samples were received from a commercial source without any personal identifications, with the exception of sex (all are male), age and cancer stage. Images were captured by Vectra Polaris Quantitative Pathology Imaging System in VCU Cancer Mouse Models Developing Shared Resource Core. Staining intensity was quantified with inbuilt software supplied by PerkinElmer, Inc. (Houston, TX).

Real Time PCR

For qPCR, total RNA were extracted using miRNeasy kits (Qiagen, Valencia, CA) and cDNA was prepared as described (15). Quantitative qPCR was performed using an ABI ViiA7 fast real-time PCR system and Taqman gene expression assays according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

Constructs and stable cell clones

Vectors and stable cell clones used in this study, including *mda-9* and sh*mda-9* (15), and pRc.CMV.Stat3Y705F vectors and Pool 1 and Pool 2 *mda-9/syntenin* knockout, and *sgRNA control* clones are described in Supplementary Materials and Methods.

In vivo experiments

All *in vivo* experiments were performed using IACUC approved protocols. Metastasis assays using tail vein injection were performed by injecting 1×10^6 ARCaP-Luc cells, either infected with Ad.5/3-sh*con* or Ad.5/3-sh*mda-9,* and animals were monitored for lung fluorescence by bioluminescence (BLI) at 48 days and survival was monitored over 90 days. Details can be found in Supplemental Materials and Methods.

Co-immunoprecipitation

Co-Immunoprecipitation was performed as described previously (15,17) using a kit from Pierce (Pierce Biotechnology, Rockford, IL).

Invasion assays

Boyden chamber assays tested the invasive properties of cancer cells (15,17). Briefly, cells were infected with control shRNA or *mda-9/syntenin* shRNA expressing Ad.5/3 and plated on the Matrigel-coated upper chamber. After 18 hours, invasive cells in the lower chamber were photographed and analyzed. All data presented, mean ± S.D.

Statistical analysis

All data represent mean ± S.D. from three independent experiments. Statistical analysis was performed using either Student *t* test (Microsoft excel), Pearson Correlation (GraphPad prism software). $P < 0.05$ was considered significant.

Results

MDA-9/Syntenin expression is elevated in PCa

Computational analysis identified *mda-9/syntenin* mRNA overexpression in PCa compared to normal prostate (8). Immunohistochemistry (IHC) of MDA-9/Syntenin protein expression in two tissue microarray (TMA) slides containing 8 adjacent tumor tissue sections and 64 human prostate tumor samples (from individual patients) at different disease stage (Stage II (n = 17) and Stage III (n = 47)) supported the genomic profiling data (Fig. 1A). MDA-9/Syntenin expression was significantly upregulated in Stage II and Stage III compared with adjacent normal tissue. Due to the low number of cases, samples from “Stage I (n = 3)”, “metastatic stage (n = 2)”, “hyperplasia (n = 3)”, “small acinar carcinoma (n = 1)” and “carcinoid (n = 1)” were not included in the final analysis. In the two cases from metastatic stage higher expression of MDA-9/Syntenin was observed, however, the staining scores were not significantly different from Stage III adenocarcinoma. Next, prostate tumor sections from a cohort of Hi-*myc* mice, a well-studied spontaneous PCa model which develops PIN by 13-weeks and invasive adenocarcinoma by 26-weeks of age, were analyzed for MDA-9/Syntenin expression (27). In 2-month old and older Hi-*myc* mice, MDA-9/Syntenin expression was elevated in prostate adenocarcinomas (Fig. 1B).

mda-9/syntenin was elevated (Fig. 1C; Supplementary Fig. S1) in different human PCa cell lines in comparison with RWPE-1, a normal immortalized prostate epithelial cell line. Expression was significantly up-regulated in M12, a metastatic variant of P69 (non-tumorigenic prostate epithelial cells), suggesting an association of MDA-9/Syntenin with metastasis, which was supported further by *in vitro* invasion assays. MDA-9/Syntenin was also overexpressed in M2182 cells, which are tumorigenic but non-metastatic P69 variant cells, suggesting that in addition to metastasis, MDA-9/Syntenin might also contribute to tumorigenic phenotypes. In addition, higher expression of MDA-9/Syntenin was evident in mesenchymal ARCaP-M (metastatic variant) cells in comparison with epithelial variant ARCaP-E cells (Fig. 1C, **right panel**; Supplementary Fig. S1), providing additional confirmation of the potential relevance of MDA-9/Syntenin in PCa progression.

MDA-9/Syntenin expression regulates invasiveness of PCa cells

Invasion is an early event in metastasis (28). A role of MDA-9/Syntenin in invasion has been established in multiple cancer lineages (9). To investigate the potential role of this gene in

PCa invasion, we overexpressed *mda-9/syntenin* in normal immortal RWPE-1 cells, using an adenovirus expressing *mda-9/syntenin* (Ad.5/3-*mda-9*) (15,17). Adenovirus expressing empty vector was used as control. Overexpression of *mda-9/syntenin* (Supplementary Fig. S2A) caused a robust up regulation of invasion ability (~6-fold) in these minimally invasive cells (Fig. 2A). Additionally, we silenced MDA-9/Syntenin expression in three aggressive human PCa cell lines, PC-3, DU-145, and ARCaP-M, using an *mda-9/syntenin* targeted shRNA carrying adenovirus (Ad.5/3-sh*mda-9*) (15,17). A significant reduction (~30 to ~50%) in invasion was observed, which correlated with the down regulation of MDA-9/Syntenin expression (Fig. 2B; Supplementary Fig. S2B). Both PDZ domains affect MDA-9/Syntenin-mediated invasion, since ectopic expression of mutant constructs (Fig. 2C, **Left panel**: ΔPDZ1 and ΔPDZ2 (16,29)) containing only the PDZ1 (ΔPDZ2) or PDZ2 (ΔPDZ1) domain decreased invasion as compared with full length *mda-9/syntenin* (WT) (Fig. 2C, **right panel**). Deletion of the PDZ1 domain had greater impact on invasion than deleting the PDZ2 domain in PCa cells (Students *t* test, p=0.0002). A stable luciferase expressing ARCaP-M-sh*mda-9* cell line was engineered and introduced into athymic nude mice intravenously *via* tail vein injection. Mice were imaged to monitor lung metastasis progression. Tumor burden was reduced in the lungs of animals that received *mda-9/syntenin* manipulated (suppressed, Fig. 2D. **panel i**) ARCaP-M cells (Fig. 2D, **panel ii**). Mice receiving control cells had larger lungs (Fig. 2D, **panel iii**) and poorer survival in comparison with the *mda-9/syntenin* manipulated (suppressed) group (Fig. 2D, **panel iv**).

MDA-9/Syntenin regulates PCa invasion through STAT3 activation

Many tumor-derived cell lines as well as human tumors, including PCa, express a constitutively active STAT3 protein (30–32). Consistent with previous observations, STAT3 activity (measured by phosphorylated (Tyr705) STAT3) was significantly higher in three aggressive PCa cells and showed a positive correlation with MDA-9/Syntenin levels (Fig. 3A **vs.** Fig. 1C for MDA-9/Syntenin). Next, we assessed the expression of phospho-STAT3 by Western blotting in *mda-9/syntenin* over-expressing RWPE-1 cells or in different PCa cells where *mda-9/syntenin* was silenced. As seen in Fig. 3B, forced expression of *mda-9/syntenin* upregulated STAT3 activation in RWPE-1 and ARCaP-E cells (Supplementary Fig. S3). Conversely, downregulation of *mda-9/syntenin* in different PCa cells (DU-145, ARCaP and ARCaP-M) negatively regulated STAT3 activation supporting a correlation between MDA-9/Syntenin expression and STAT3 activity (Supplementary Fig. S3). To provide additional confirmatory evidence, an additional experiment was conducted using STAT3 reporter constructs from SA Bioscience (Valencia, CA), which encode a firefly luciferase reporter gene under the control of a minimal CMV promoter and tandem repeats of the SIE transcriptional response element. These constructs can monitor both increases and decreases in the transcriptional activity of STAT3-containing dimers, and hence the activity of the STAT3 signaling pathway. Evidence for *mda-9/syntenin*-mediated STAT3 activation was verified by co-transfection of a STAT3 reporter with *mda*-9/*syntenin* or sh*mda-9* in RWPE-1 and DU-145 cells, respectively (Fig. 3C). Finally, the anti-invasive activity of *mda-9/syntenin* silencing was rescued by overexpressing a constitutively active STAT3 in transiently *mda-9/syntenin* knockdown PCa cells (Fig. 3D). Blocking secretion, using Brefeldin A, significantly reduced STAT3 activity in DU-145 and ARCaP-M cells indicating a role of secretion in activation (Fig. 4A). To further characterize the secretory molecules

involved in MDA-9/Syntenin-mediated STAT3 regulation, we evaluated the role of IGFBP-2, a downstream secretory target of MDA-9/Syntenin (15), which was expressed at elevated levels in aggressive PCa cells (33,34). A TMA slide (Novus Biologicals) was immunostained for IGFBP-2 and compared with MDA-9/Syntenin stained sections. In 46 samples, including both normal (n = 8) and adenocarcinomas (8 and 30 cases from Stage II and Stage III, respectively), both Stage II and III cases showed significantly higher staining intensity compared with adjacent normal tissue (Fig. 4B). The values for MDA-9/Syntenin and IGFBP-2 from individual cases were analyzed for Pearson correlation using statistical software (Prism). A positive correlation ($r^2 = 0.4813$, $p<0.0001$) between these two proteins was observed when comparing the 46 samples (Fig. 4C) or only Stage II (n = 8, $r^2 = 0.8451$, $p<0.0083$) and Stage III (n = 30, $r^2 = 0.5965$, $p<0.0005$) samples. Additionally, when comparing ARCaP series for both *mda-9/syntenin* and *IGFBP-2* mRNA expression, maximum fold changes (relative to RWPE-1) for both genes was evident in ARCaP followed by ARCaP-M and ARCaP-E (Supplementary Fig. S4A). Similar patterns were also apparent in DU-145 and PC-3ML cells (metastatic PCa cells isolated from a bone metastatic lesion) (Supplementary Fig. S4A). Finally, using a loss-of-function experiment, we confirmed that *mda-9/syntenin* expression directly regulates *igfbp-2* at both mRNA and protein level (Supplementary Fig. S4B and S4C). In contrast, manipulation of MDA-9/syntenin did not alter the expression of Syntenin-2 (Supplementary Fig. S4C and S4D). Downregulation of secretory IGFBP-2 was evident by ELISA in the media collected from *mda-9/syntenin* manipulated cell lines (Supplementary Fig. S4E). Co-transfection studies with various combinations of genetic manipulation defined the effects of IGFBP-2 on STAT3 activation and the potential role of MDA-9/Syntenin in this pathway. RWPE-1 cells, which have very low levels of MDA-9/Syntenin expression, displayed higher STAT3 activity following elevated *mda-9/syntenin* expression. This activity was significantly suppressed in the presence of sh*igfbp-2*, indicating the importance of IGFBP-2 (Fig. 4D; Supplementary Fig. S5A) in MDA-9/Syntenin-mediated STAT3 activation. Similarly, transiently silencing *mda-9/syntenin* impacted STAT3 activation in DU-145 and ARCaP-M cells and the effect was more robust when both genes (*mda-9/syntenin* and *igfbp-2*) were knocked down) (Fig. 4E; Supplementary Fig. S5B). Loss-of-function studies in ARCaP-M and DU-145 cells confirmed that exogenous hIGFBP-2 (recombinant protein)-mediated STAT3 activation depended on endogenous MDA-9/Syntenin expression levels (the effects of hIGFBP-2 was not evident in *mda-9/syntenin*-knockdown cells, Fig. 4F; Supplementary Fig. S5C).

MDA-9/Syntenin physically interacts with IGF-1R and activates STAT3

IGF-1R is a tyrosine kinase that phosphorylates STAT3 (35). IGF-1R, which belongs to the IGF receptor family, is a transmembrane receptor tyrosine kinase (RTK). In response to insulin-like growth factor 1 ligand binding, IGF-1R is activated via auto phosphorylation (Tyr 980), leading to activation of various signaling cascades including STAT3. Because IGF-1R can also potently activate the STAT3 pathway, one could envision a model in which IGFBP-2 (through binding with IGF-1) might activate STAT3 in an IGF-1R-dependent manner. To explore this possibility, we treated RWPE-1 with recombinant IGFBP-2 (hIGFBP-2) in the absence of serum and Western blotting was performed to determine auto phosphorylation, representing the activation state of IGF-1R. The presence of hIGFBP-2 in the absence of exogenous IGF-1 (not ruling out the possibility of endogenous- or cell-

derived IGF-1 in the media) activated IGF-1R, which only occurred in the presence of MDA-9/Syntenin (Fig. 5A; Supplementary Fig. S6A). Enhanced STAT3 activation was also observed in these samples. These findings support the importance of MDA-9/Syntenin in IGF-1R-mediated STAT3 activation. Further experiments revealed that MDA-9 physically interacts and colocalizes with IGF-1R (Fig. 5B and C), which might play an essential role in transmitting activation signals to STAT3. To obtain more insight into potential binding site(s) and the consequences of this interaction, we overexpressed different deletion mutants (ΔPDZ1 and ΔPDZ2) of *mda-9/syntenin* in RWPE-1 cells and examined IGF-1R activation (Fig. 5D; Supplementary Fig. S6B). Expression of a PDZ1-deleted fragment (ΔPDZ1) failed to activate IGF-1R (following hIGFBP-2 treatment) indicating that the potential binding site of IGF-1R resides in the PDZ1 domain of MDA-9/Syntenin. STAT3 activity also correlated with IGF-1R activation further confirming that MDA-9/Syntenin-mediated STAT3 activation is a downstream consequence of MDA-9/IGF-1R interactions. Two PCa cells, with genetically modified *mda-9/syntenin* expression (using Ad.5/3-sh*mda-9*), were exposed to exogenous hIGFBP-2 to investigate IGF-1R activation. As anticipated, lack of *mda-9/syntenin* expression robustly impacted IGFBP-2 activity (Fig. 5E; Supplementary Fig. S6C).

Based on prior studies with MDA-9/Syntenin (11,15,16) and current experimental evidences we hypothesized that MDA-9/Syntenin and IGF-1R physically interact, enhancing stability and activating STAT3 through phosphorylation at tyrosine 705. Phospho-STAT3 forms a dimer and translocates into the nucleus to induce various genes that actively participate in PCa progression. To test this hypothesis, we determined the expression of MMP-2 and MMP-9, the most common, invasion-related downstream targets of STAT3 in *mda-9/syntenin*-knockdown cell variants (transient or stable). Knocking down *mda-9/syntenin* downregulated basal expression of MMP-2 and MMP-9 (Fig. 6A; Supplementary Fig. S7A and S7B). Exogenous stimulation (using recombinant hIGFBP-2) upregulated both proteins in wild type cells, however, this did not occur in *mda-9/syntenin* downregulated cells. This data and that provided in Fig. 4F (hIGFBP-2-mediated STAT3 activation) demonstrate that endogenous MDA-9/Syntenin expression is critical for IGFBP-2-mediated MMP-2 and MMP-9 up regulation through STAT3 activation. *mda-9/syntenin*-mediated downregulation of MMP-2 and MMP-9 was rescued when over expressing a constitutively active STAT3 (Fig. 6B and C; Supplementary Fig. S8A and S8B). To confirm these findings, we developed two stable *mda-9/syntenin* knockout variant ARCaP-M cells using the CRISPR/Cas9 approach, one with minimal (Pool 1) and the other with a complete knockout (Pool-2) (Fig. 7A) of expression. These knockout pools showed a significant downregulation of *igfbp-2* (Fig. 7B), *MMP-2* (Fig. 7C) and *MMP-9* (Fig. 7C) mRNA. In addition, qPCR data from the complete knockout MDA-9/Syntenin cells (Pool 2) demonstrated a significant downregulation of STAT3 downstream targets, *e.g., Interleukin-6 (IL-6), IL-8 (IL-8), and VEGF-A* mRNA, further supporting the critical role of MDA-9/Syntenin in regulating STAT3 activity in PCa (Fig. 7D). These clones significantly lose their invasive potential *in vitro* in Boyden chamber assays and this phenotype is rescued significantly by a constitutive active STAT3 (Fig. 7E). A hypothetical model for MDA-9/Syntenin in regulating PCa progression via IGF-1R/STAT3 regulation is shown in Fig. 7F.

Discussion

Using computational-based analyses to interrogate online public data bases, confirmation was provided for the potential clinical significance of *mda-9/syntenin* with cancer prognosis (8). The bioinformatics data established and supported previous pre-clinical observations indicating a correlation of MDA-9/Syntenin with GBM (13) and melanoma (11) pathogenesis in clinical settings. Scrutiny of the public data bases also predicted a relationship between MDA-9/Syntenin expression and PCa prognosis. Moreover, it has been suggested that MDA-9/Syntenin is present in secreted exosomes (36), where its function requires clarification. Our present study confirms a direct relationship between expression of *mda-9/syntenin* and PCa progression and provides direct molecular mechanistic insights into precisely how this gene regulates PCa invasion (Fig. 7).

MDA-9/Syntenin is an adaptor scaffold protein that elicits its diverse functions by physically interacting with subsets of unique proteins in different regions of the cell (7,9). In melanoma, MDA-9/Syntenin physically interacts with Src (16) activating the transcription factor NFκB resulting in upregulation of invasion/angiogenesis associated genes (e.g., MMP-9, IGFBP-2). EGFR and MDA-9/Syntenin interaction was observed in urothelial cancer (12) and in radiation-treated GBM (37) cells. Menezes *et al.* demonstrated a physical interaction of MDA-9/Syntenin with TGFβ that induced EMT in breast cancer cells (29). Interestingly, MDA-9/Syntenin was also reported as a partner of the cytoplasmic domain of TGFβ receptor in the context of breast cancer (38). In head and neck cancer, small proline-rich protein 1B interacts with the PDZ domain of MDA-9/Syntenin regulating angiogenesis by upregulating VEGF-R (39). Additionally, a number of interacting partners and the consequences of these interactions have been documented in non-cancerous conditions. MDA-9/Syntenin interacts with the cytoplasmic domain of CD4 in T-helper cells allowing successful HIV entry (40). MDA-9/Syntenin and ALIX interactions are essential for exosome biogenesis (41). T-cell migration towards a chemoattractant is regulated by an association of MDA-9/Syntenin and myosin phosphatase Rho interacting protein (42). Thus, the diversity of interactions and the corresponding signaling changes in different cancerous or non-cancerous disorders suggests that specific interactions might be context dependent and influenced by both intrinsic (e.g., affinity, availability) and extrinsic (ECM, growth factors) factors. Defining the precise role of MDA-9/Syntenin in diverse pathological conditions is of value in clarifying its complete repertoire of functions. Our present study identified IGF-1R as a new MDA-9/Syntenin binding partner. Ligensa *et al.* identified a type I PDZ domain binding site ((S/T)XV) at the C-terminal of IGF-1R that could serve as the docking site for MDA-9/Syntenin (43). Further studies are necessary to define the critical genetic site(s) involved in defining IGF-1R-MDA-9/Syntenin interactions and what additional partner proteins may facilitate or antagonize these interactions.

Following IGF-1R binding to MDA-9/Syntenin STAT3 is activated. STAT3 is one of the downstream targets of IGF-1R. For example, Zong *et al* demonstrated activation of STAT3 by phosphorylation at tyrosine 705 position by IGF-1R upon IGF-1 stimulation (35). In pancreatic cancer, an IGF-1R-targeted small molecule inhibitor NVP-AEW541 elicited a direct effect on cell proliferation and abrogated cellular migration through inhibition of STAT3 activation (44). NT157, another IGF-1R targeting molecule, similarly caused

significant down regulation of IGF-1R in a pre-clinical model of metastatic melanoma progression (45). A role of IGF-1R-mediated STAT3 activation (31) also occurs in PCa and its clinical relevance has been described (32). In summary, these studies and our previous collaborative study demonstrating that IL-6 activates STAT3 through IGF-1R in PCa (31) establishes a link between IGF-1R and STAT3 activation. These observations raise intriguing questions as to how STAT3 is recruited by IGF-1R. One study demonstrated that RACK1, an adaptor protein, served as a mediator and facilitated the recruitment of STAT3 with IGF-1R for phosphorylation (46). Theoretically, being an adaptor protein MDA-9/Syntenin may enable the assembly of a large complex containing both STAT3 and IGF-1R proteins, which requires experimental confirmation. Apart from inflammation, active STAT3 transcriptionally regulates various genes involved in survival, proliferation, invasion and angiogenesis (47). Since MDA-9/Syntenin did not show any direct impact on cell survival or proliferation in PCa cells based on *in vitro* experiments, we predict that MDA-9/Syntenin-mediated up regulation of STAT3 activity may be more relevant in regulating cell invasion rather than cell survival, which is experimentally supported in our present study.

Expression of IGFBP-2 is upregulated in PCa patients. Recently, a meta-analysis using 17 prospective and two cross-sectional data, which included more than ten thousand patients and healthy volunteers, demonstrated a positive correlation of IGFBP-2 with PCa risk. Along with IGFBP-2, this study also revealed that levels of IGF-1, IGF-2, IGFBP-3 positively correlated with disease progression (48). IGFBP-2 modulates IGF-1 function through IGF-1R. However, IGFBP-2 may also function in an IGF-1-independent manner via association with cytoplasmic or nuclear binding partners. Using cell fractionation approaches, IGFBP-2 was shown to translocate into the nucleus through importin-α, via a classical nuclear import mechanism, and activated VEGF-A transcription in multiple types of cancer including PCa (49). IGFBP-2 mediates EGFR-dependent STAT3 activation in glioblastoma cells. Although the activation of IGF-1R was not explored, IGFBP-2 and EGFR co-localized and accumulated in the nucleus, transactivated STAT3 and initiated the transcription of several STAT3-regulated genes including *c-Myc, Bcl-xL, Cyclin D* (50). Our experimental evidences suggest a potential role of IGFBP-2 in activation of STAT3 through IGF-1R in PCa, where MDA-9/Syntenin plays a decisive role.

Support is now provided for a signaling cascade in which MDA-9/Syntenin physically interacts with IGF-1R upon IGFBP-2 stimulation, resulting in STAT3 phosphorylation, thereby facilitating the expression of invasion/angiogenesis-related genes particularly *MMP-2, MMP-9* and *VEGF-A* (Fig. 7). MDA-9/Syntenin-mediated MMP-9 and VEGF-A expression occurs in multiple cancers (11,12). The present study, firmly establishes MDA-9/Syntenin mechanistic functions in PCa pathogenesis, principally through interactions with partner protein(s) that are influenced by IGFBP-2, a protein upregulated and found in PCa patients' body fluids (48). Although, both PDZ domains are important for MDA-9/Syntenin function (16), recent studies confirm the importance of the PDZ1 domain in forming relevant interactions independent of the PDZ2 domain. As discussed, the PDZ1 domain of MDA-9/Syntenin is the interacting domain with TGFβ1 (29), TGFβ1 receptor (38), small proline rich protein B (39) and now IGF-1R. We demonstrate that disrupting the MDA-9/Syntenin/IGF-1R/STAT3 loop profoundly affects PCa pathogenesis suggesting that targeting MDA-9/Syntenin could provide a strategy for inhibiting PCA progression and metastasis.

We recently developed a novel MDA-9/Syntenin PDZ1-targeted small molecule pharmacological agent (*PDZ1i*) using a combination of fragment-based drug discovery techniques guided by *in silico* docking and NMR-based design (37). This small molecule was efficacious in interrupting the interaction of MDA-9/Syntenin with EGFR and inhibited downstream signaling involved in the invasion gain in radiation-surviving GBM cells. *PDZ1i* also displayed a strong synergistic effect when used in combination with radiation in terms of survival in animals containing a primary human GBM (37). Further studies, not in the scope of this paper, are clearly warranted to define the impact of *PDZ1i* on MDA-9/IGF-1R interactions and downstream signaling events in PCa progression and metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance

This study provides new mechanistic insight into the pro-invasive role of MDA-9/Syntenin-1 in prostate cancer and has potential for therapeutic application to prevent prostate cancer metastasis.

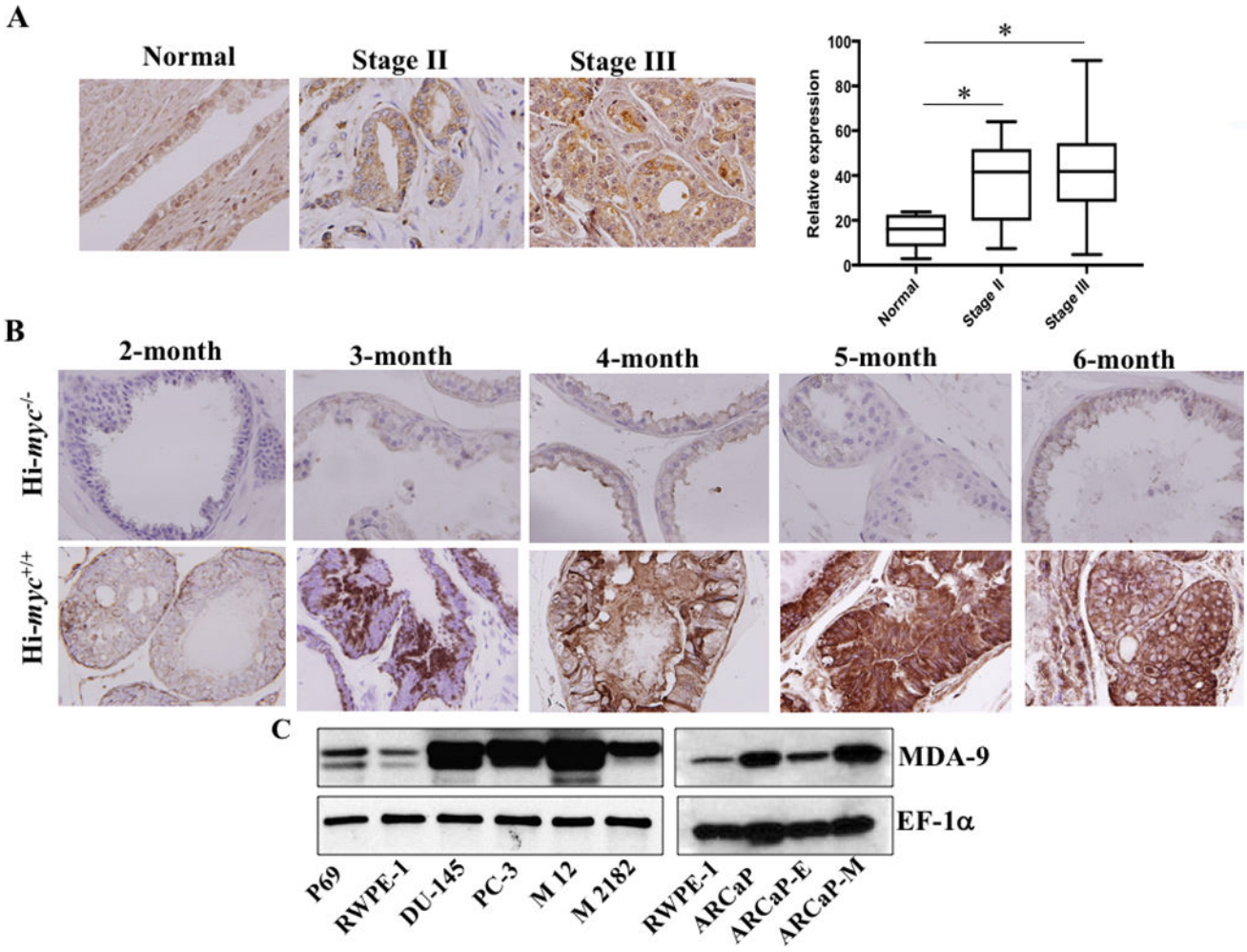

Figure 1. MDA-9/Syntenin is upregulated in advanced PCa

A) Representative photomicrographs of MDA-9/Syntenin expression in various stages of prostate adenocarcinoma. Staining intensity was measured using imaging software and the average value with S.D. for Stage II and III disease is presented. **B)** Prostates from Hi-*Myc* positive or negative male mice at different ages (n = 3) were collected, paraffin embedded and sectioned. Representative photomicrographs of MDA-9/Syntenin immunostained slides. C) Expression of MDA-9/Syntenin in P69, RWPE-1 and different human PCa cells.

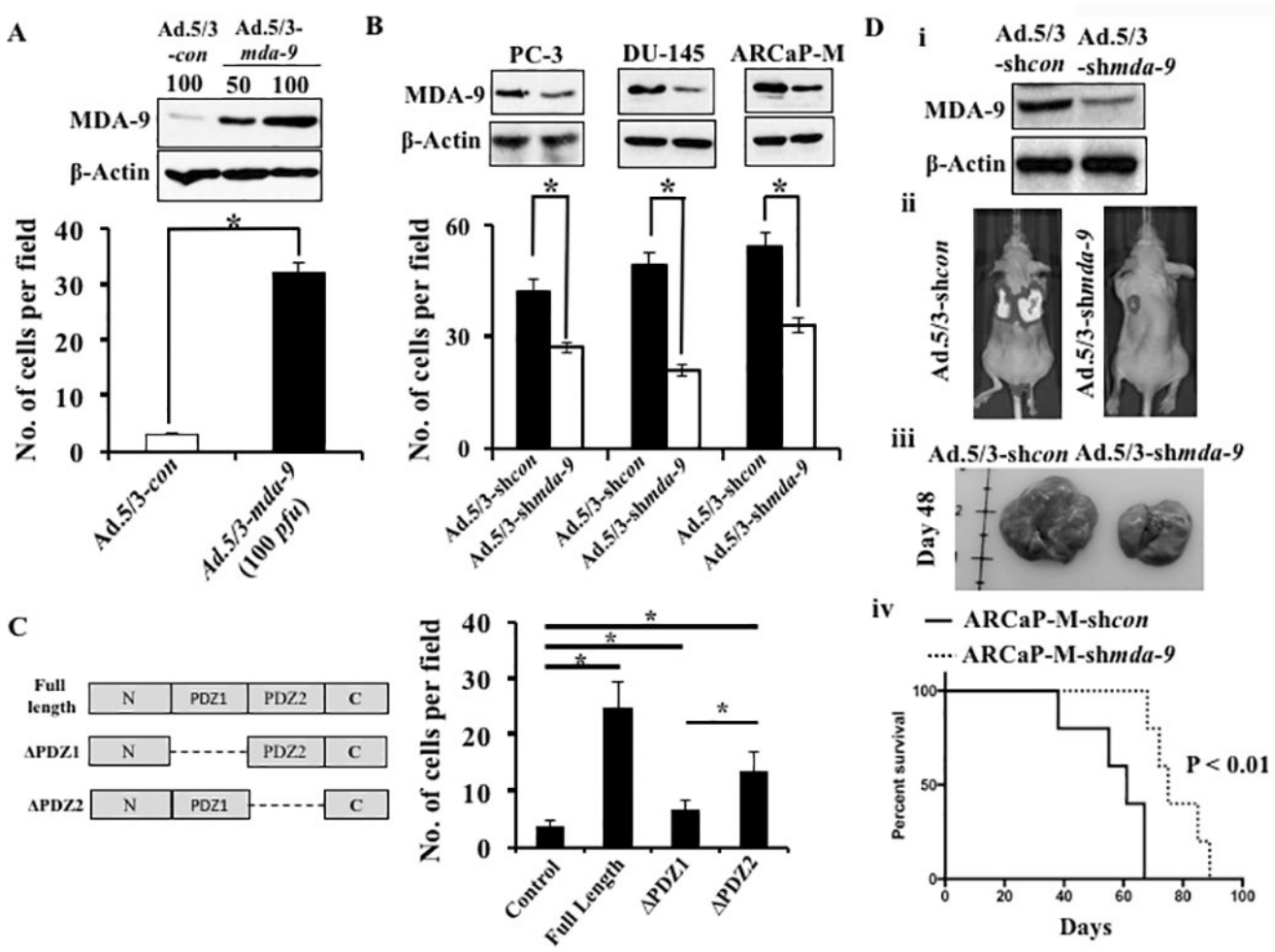

Figure 2. MDA-9/Syntenin regulates PCa progression

A) RWPE-1 cells infected with various adenoviruses at the indicated M.O.I. After 48 hours, infected cells were subjected to Matrigel invasion assays as described in Materials and methods. Expression of MDA-9/Syntenin was confirmed by Western blotting. **B)** MDA-9/Syntenin levels and invasiveness of different PCa cells after infection with Ad.5/3-sh*con* or Ad.5/3-sh*mda-9*. **C)** Left panel: Schematic diagram of mutant *mda-9/syntenin* constructs, ΔPDZ1 and ΔPDZ2. Right panel: RWPE-1 cells transfected with full length and mutants of *mda-9/syntenin* and 48 hours after transfection invasion was evaluated. Number of invaded cells presented as a bar graph. Average with S.D. from three independent experiments. "*" represents statistical significance ($p<0.05$) between indicated groups. **D)** Downregulation of MDA-9/Syntenin was confirmed in Ad.sh*mda-9* infected cells (i). Luciferase expressing ARCaP-M cells carrying sh*con* or sh*mda-9* were inoculated I.V. into athymic nude mice by tail vein. Representative photographs of BLI (ii) and gross morphology of lungs (iii) 48 days after inoculation. Kaplan-Meier survival graph using Graph Pad software (iv).

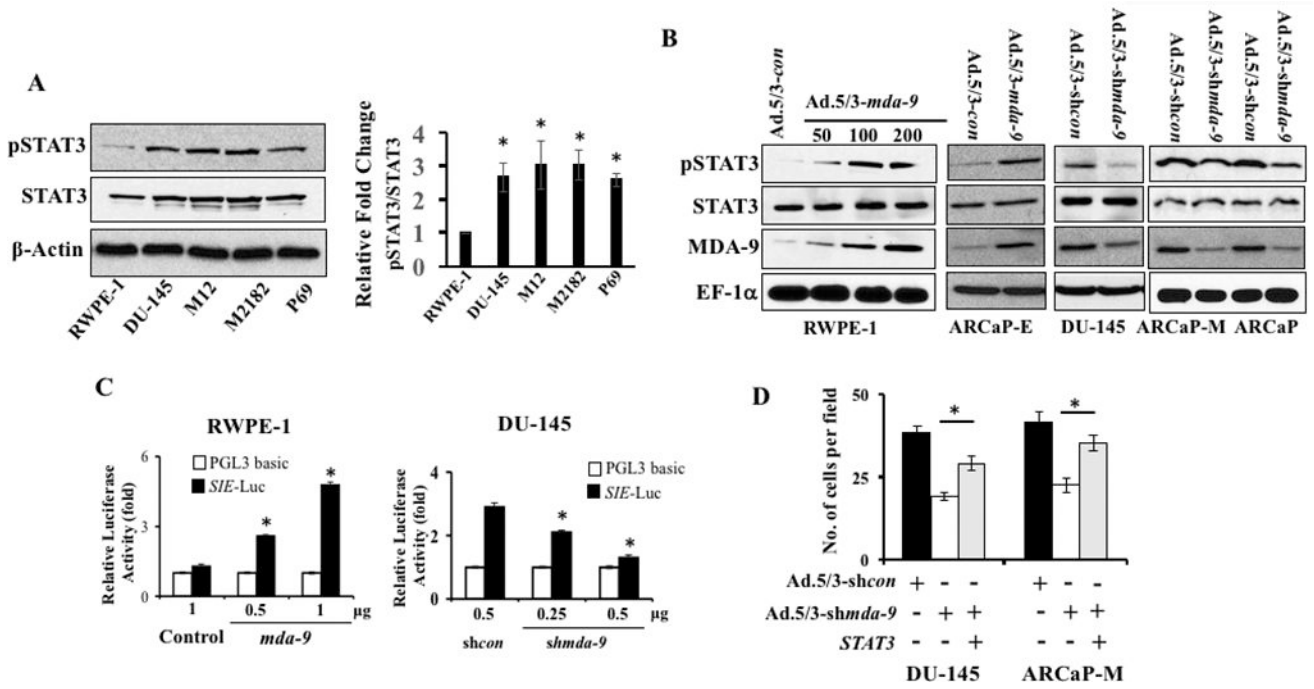

Figure 3. MDA-9/Syntenin regulates STAT3 activity in PCa cells

A) Western blotting analysis of phospho-STAT3 (Tyr705) and total STAT3 (left panel). Band intensity was quantified and relative-fold change in different cancer cells vs. RWPE-1 is presented (right panel). Average with S.D. from three independent experiments. "*" represents statistical significance ($p<0.05$) between indicated groups. **B)** Cells were either infected with adenoviruses for 48 hours and reseeded on fibronectin-coated plates. Total cell lysates prepared 1 hour after plating and Western blotting using indicated antibodies. **C)** Cells were co-transfected with a reporter gene and empty vector, *mda-9/syntenin* or *mda-9/syntenin* shRNA and after 48 hours, luciferase activity was measured. Data presented as fold-change vs. the control group (empty vector). **D)** Cells were co-transfected with different expression plasmids and 48 hours later, cells were trypsinized and invasion assayed. Cells counted using bright field microscopy.

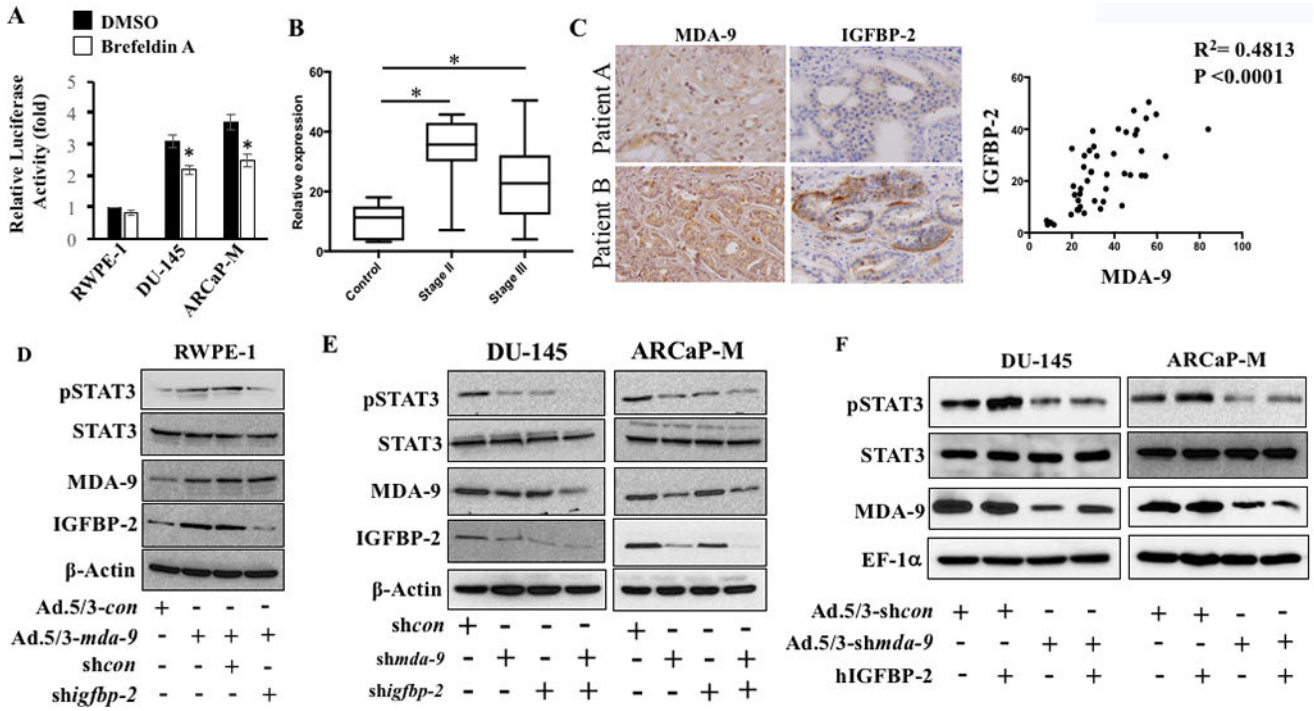

Figure 4. MDA-9/Syntenin plays a decisive role in IGFBP-2-induced STAT3 activation

A) Cells transfected with a STAT3 reporter gene and after 36 hours were treated with Brefeldin A (5 μg/ml) for 30 min. Media was removed and cells were cultured an additional 3 hours in serum-free media and luciferase activity measured. **B)** Immunostaining for IGFBP-2 was performed in tissue microarray and staining intensity was quantified with Polaris Image Analysis software. The average value with S.D. from different stages is plotted. "*" represents statistical significance between groups. **C)** The expression of MDA-9/Syntenin and IGFBP-2 correlates in sections from the same patient (upper patient A (low expression) and patient B (high expression) bottom). Pearson correlation determined using 46 samples and presented with R^2 and *p* value. **D)** RWPE-1 cells expressing MDA-9/Syntenin in presence of control shRNA or *igfbp-2* shRNA for 48 hours. Cells were trypsinized and reseeded onto fibronectin-coated plates. Samples collected after 1 hour and Western blotting performed. **E)** Endogenous MDA-9/Syntenin and IGFBP-2 expression in PCa cells treated with sh*mda-9* and sh*igfbp-2* either alone or in combination. Cells were reseeded on fibronectin-coated plates for 1 hour. Western blotting with indicated antibodies. **F)** PCa cells infected with Ad.5/3-sh*con* or Ad.5/3-sh*mda-9* (100 pfu/cell) for 48 hours. Cells re-seeded on fibronectin-coated plates and treated with recombinant hIGFBP-2 (100 ng/ml) for 1 hour. Western blotting for indicated proteins.

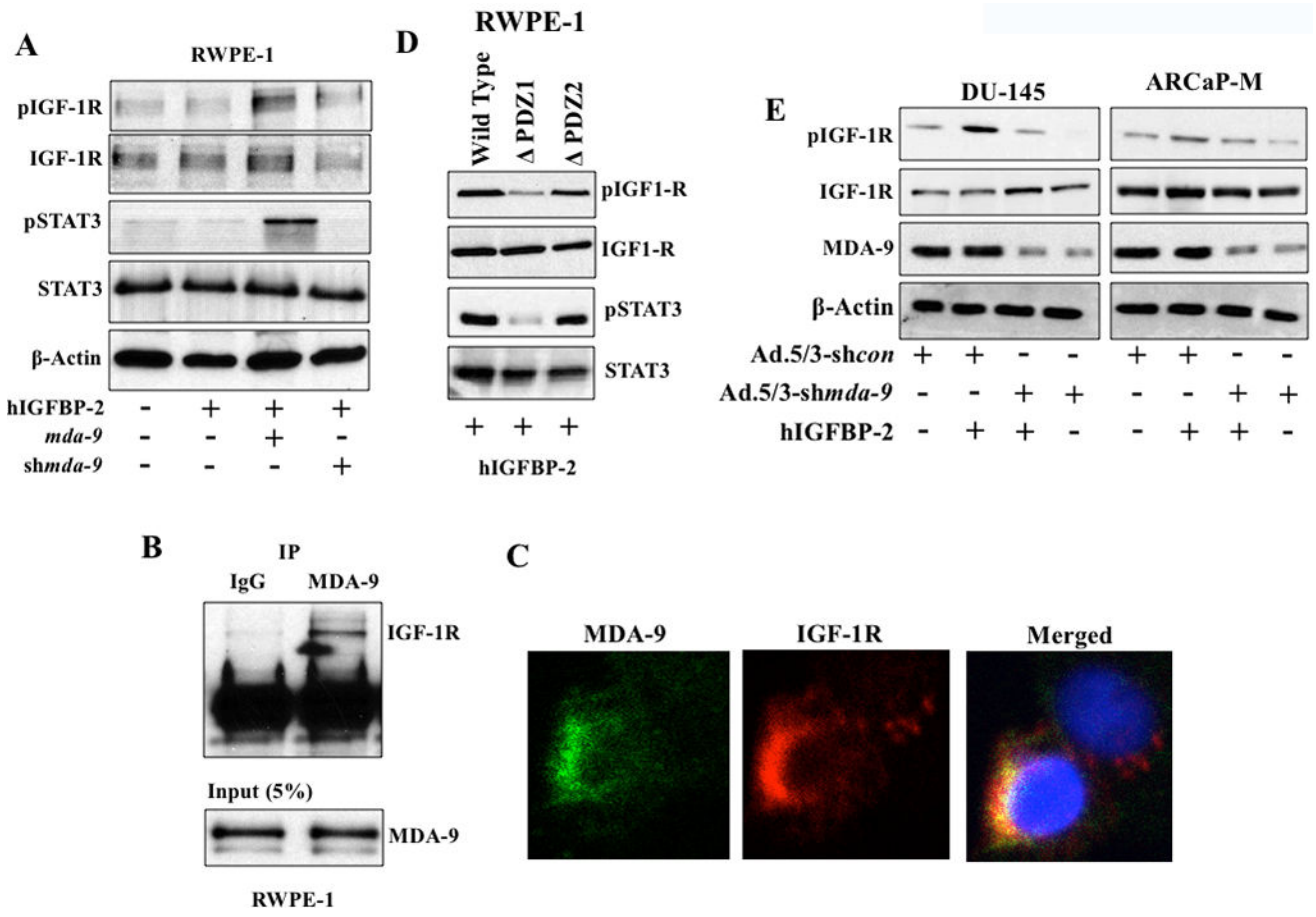

Figure 5. IGF-1R and MDA-9/Syntenin physically interact and regulate STAT3 activity

A) RWPE-1 cells treated with hIGFBP-2 under different conditions and phospho-IGF-1R expression determined by Western blotting. **B)** 200 μg of total protein from ARCaP-M cells incubated with MDA-9/Syntenin overnight for immunoprecipitation and Western blotting performed with anti-IGF-1R antibody. **C**) Immunofluorescence assay to determine co-localization of proteins. Confocal microscopy images of MDA-9/Syntenin and IGF-1R as "separate" or "merged" images. **D**) RWPE-1 cells transfected with wild type or mutant *mda-9* vectors. 48 hours later, cells replated on fibronectin-coated plates for 30 mins and cell lysates analyzed using the indicated antibodies. **E)** PCa cell lines infected with Ad.5/3-sh*con* or Ad.5/3-sh*mda-9* for 48 hours. Cells reseeded on fibronectin-coated plates and treated with recombinant hIGFBP-2 (100 ng/ml) for 1 hour. Western blotting analysis to determine auto-phosphorylation.

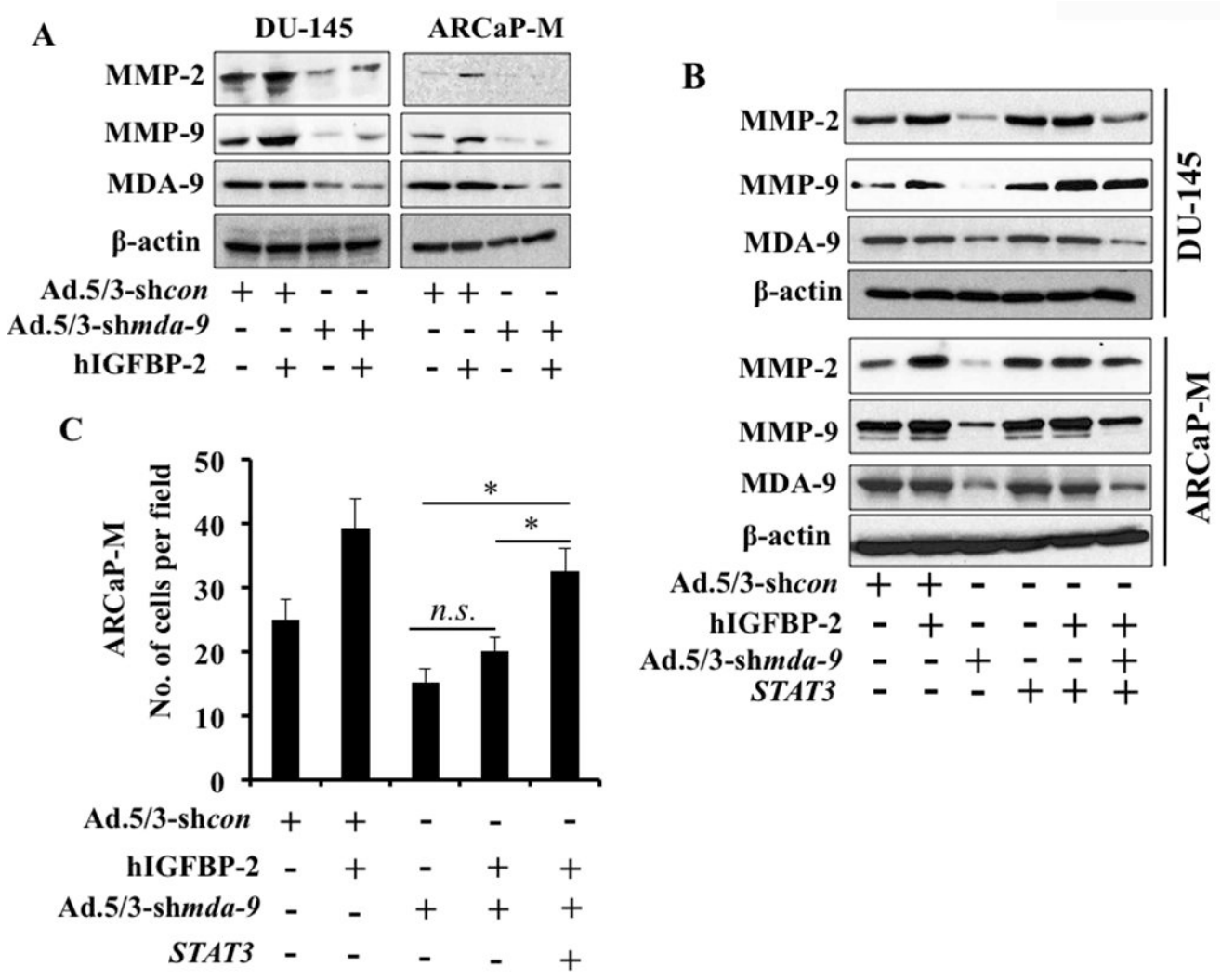

Figure 6. MMP-2 and MMP-9 are key regulators of MDA-9/Syntenin-mediated PCa invasion

A) PCa cell lines infected with Ad.5/3-sh*con* or Ad.5/3-sh*mda-9* for 48 hours at 100 pfu/cell. Cells reseeded on fibronectin-coated plates and treated with recombinant hIGFBP-2 (100 ng/ml) for 1 hour. Western blotting to determine MMP-2 and MMP-9 expression. **B)** Cells were co-transfected/infected with different adenovirus/plasmids, followed by 48 hours incubation for transgene expression. Western blotting performed using anti-MMP-2 and anti-MMP-9. **C)** Cells were co-transfected with different expression plasmids. 48 hours later, cells were trypsinized and invasion determined. Average with S.D. from three independent experiments. "*" represents statistical significance ($p<0.05$) between indicated groups.

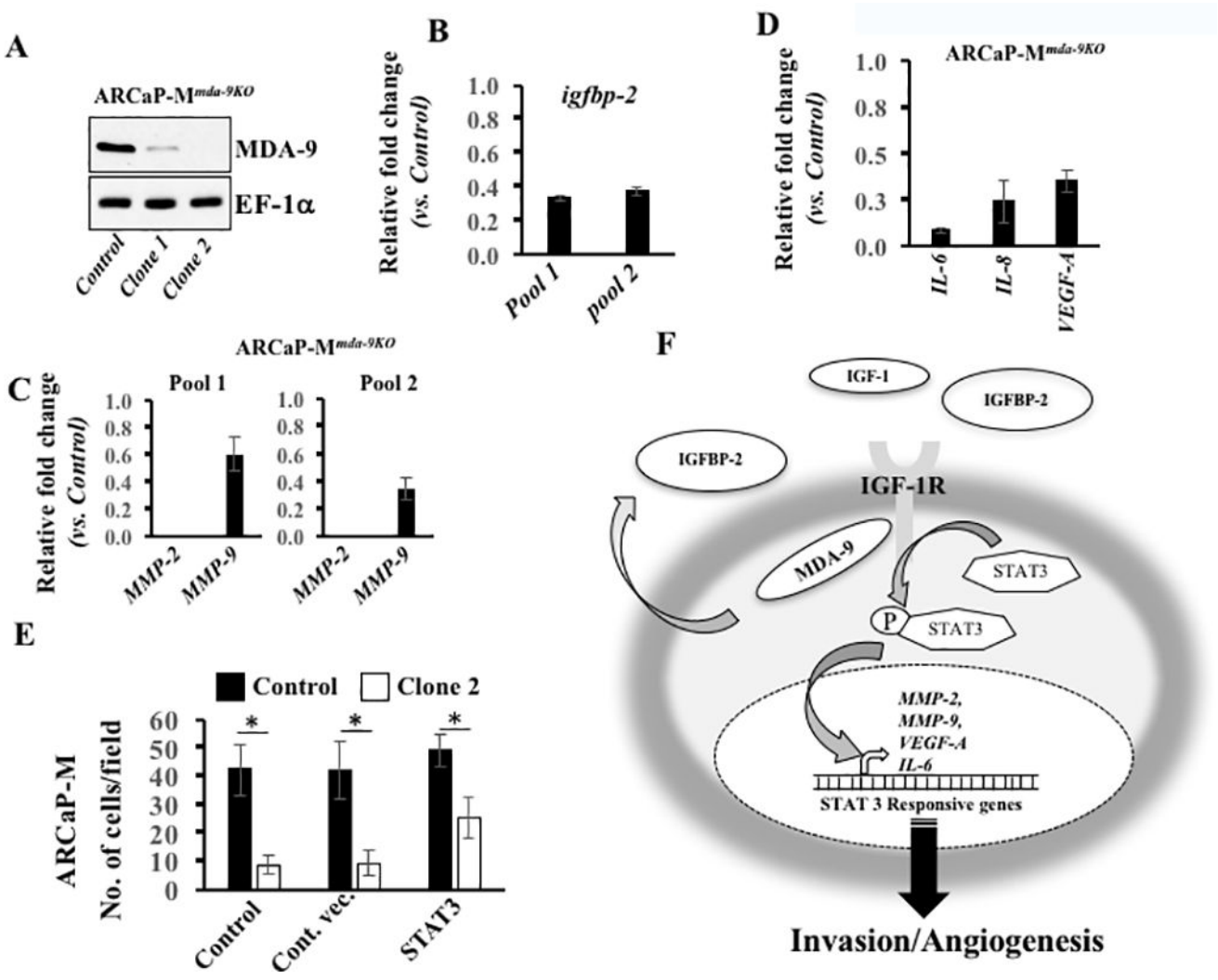

Figure 7. Knockout of MDA-9/Syntenin in PCa inhibits invasion

A) MDA-9/Syntenin expression in two clones from CRISPR/Cas9-*mda-9/syntenin* transfected cells. **B & C)** Expression of *igfbp-2* (**B**) or *MMP-2* and *MMP-9* mRNA (**C**) determined in Pool-1 and Pool-2, presented as fold-change vs. control CRISPR/Cas9 transfected cells. **D)** Expression of indicated mRNA determined in Pool-2. Data presented as fold-change vs. control. **E)** Cells co-transfected with different expression plasmids. 48 hours later, cells trypsinized and invasion determined. Cells counted using bright field microscopy. Average with S.D. from three independent experiments. "*" represents statistical significance ($p<0.05$) between indicated groups. **F)** Hypothetical model of MDA-9/ Syntenin-mediated PCa progression.