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Vasodilator-Stimulated Phosphoprotein (VASP) depletion from breast cancer MDA-MB-231 cells inhibits tumor spheroid invasion through downregulation of Migfilin, β -catenin and urokinase-plasminogen activator (uPA)

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Abstract

A hallmark of cancer cells is their ability to invade surrounding tissues and form metastases. Cell-extracellular matrix (ECM)-adhesion proteins are crucial in metastasis, connecting tumor ECM with actin cytoskeleton thus enabling cells to respond to mechanical cues. Vasodilator-stimulated phosphoprotein (VASP) is an actin-polymerization regulator which interacts with cell-ECM adhesion protein Migfilin, and regulates cell migration.

We compared VASP expression in MCF-7 and MDA-MB-231 breast cancer (BC) cells and found that more invasive MDA-MB-231 cells overexpress VASP. We then utilized a 3-dimensional (3D) approach to study metastasis in MDA-MB-231 cells using a system that considers mechanical forces exerted by the ECM. We prepared 3D collagen I gels of increasing concentration, imaged them by atomic force microscopy, and used them to either embed cells or tumor spheroids, in the presence or absence of VASP. We show, for the first time, that VASP silencing downregulated Migfilin, β -catenin and urokinase plasminogen activator both in 2D and 3D, suggesting a matrix-independent mechanism. Tumor spheroids lacking VASP demonstrated impaired invasion, indicating VASP's involvement in metastasis, which was corroborated by Kaplan-Meier plotter showing high VASP expression to be associated with poor remission-free survival in lymph node-positive BC patients. Hence, VASP may be a novel BC metastasis biomarker.

Keywords

Vasodilator Stimulated Phosphoprotein; extracellular matrix; tumor spheroid invasion; urokinase plasminogen activator; Migfilin

Introduction

A characteristic hallmark of malignant tumors that, in fact, differentiates them from benign is the ability of cancer cells comprising the tumor, to dissociate from the original tumor

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mass, migrate and invade through surrounding tissues, forming metastasis.^{1,2} Indeed, the primary tumor is almost never the cause of death for cancer patients, as the majority of them die from cancer cell metastasis and related complications.³ However, despite worldwide research efforts on cancer, there is no available metastasis biomarker or effective pharmacological intervention, to date.

As cancer cells do not act in isolation, but rather interact with other cells within the tumor as well as with the tumor extracellular matrix (ECM), which is an important component of tumor microenvironment, understanding this communication is fundamental for the elucidation of metastatic mechanisms and thus, their inhibition.

Evidently, cell-ECM adhesion proteins are key molecules in metastasis. Indeed these proteins, known to form multi-protein complexes at cell-ECM adhesion sites with direct or indirect connections to the actin cytoskeleton, are greatly disrupted in cancer, thereby allowing tumor cells to detach from the original tumor^{2,4–6}, adhere to blood and lymphatic vessels, and invade through them.⁷

Vasodilator-stimulated phosphoprotein (VASP), a potent regulator of actin polymerization, is a member of the Ena-VASP protein family originally purified from human platelets, that is localized to cell-ECM adhesions⁸, where it has been found to interact with Filamin Binding protein/Migfilin.⁹ Moreover, VASP enhances actin barbed-end elongation, protrusion and propulsion speed and is generally targeted to the leading edge of migratory cells where lamellipodia are formed, playing an important role in cell migration.^{10–12} VASP's involvement in cell migration and invasion has been supported in many studies.^{11,38–42} However, the type of regulation (positive or negative) differs depending on the cell system studied and the exact mechanism is vague, with Rac activation,^{13,14} VASP phosphorylation,¹⁵ and actin polymerization,¹¹ being among the potential mechanisms proposed.

Interestingly, this connection of cell-ECM adhesion proteins to the actin cytoskeleton enables cells to respond to mechanical cues by modulating their shape and migration patterns, which is important for cancer progression and metastasis.^{16,17} In fact, cancer tissues normally exert larger mechanical compressive forces on cancer cells promoting metastasis, as they are typically stiffer than normal tissues, containing large amounts of ECM proteins and other molecules.^{7,16–24}

Although traditional two-dimensional (2D) monolayer cultures cannot take into account the ECM stiffness of the tumor microenvironment or the cell-ECM interactions taking place²⁵, the majority of *in vitro* studies using cancer cell lines are performed in 2D. Three-dimensional (3D) *in vitro* models however, are more physiologically relevant and are, thus, receiving more attention with several models being developed to also consider the mechanical forces exerted by the tumor microenvironment on cancer cells during cancer progression and metastasis.^{25,26}

Hence, in the present study, we focused on VASP, as a potent regulator of actin cytoskeleton, which is fundamental in cell migration. We investigated its role in the highly invasive MDA-MB-231 breast cancer (BC) cells in 2D monolayer and in 3D collagen I cultures of increasing concentration²⁵, as well as in tumor spheroid invasion in 3D collagen gels.

Materials and Methods

Antibodies and reagents

Anti-VASP antibody was purchased from Santa Cruz Biotechnology (sc-46668), and anti- β -actin antibody from Sigma (A2228). Alexa-488 donkey anti-mouse secondary antibody was purchased from Life Technologies (A21202) and 4',6-diamidino-2-phenylindole (DAPI) was obtained from Roche (Cat.#10236276001). Gelatin was from Sigma (Cat.#48723), Collagen I high concentration solution was purchased from Corning (Cat.# 354249), while collagenase D (11088858001) and human insulin solution (I9278) were obtained from Sigma.

Breast cancer cell lines

BC cell lines MCF-7, and MDA-MB-231 were purchased from ATCC. All cells were cultured in Dulbecco's Modified Eagle Medium (Gibco 41965-039) supplemented with 10% Fetal Bovine Serum (Sigma F4135), 1% L-Glutamine (Sigma G7513) and 1% Penicillin/Streptomycin (Sigma P4333), and incubated in a CO₂-incubator at 37°C.

Transfection with siRNAs

MDA-MB-231 cells were transfected with 100 nM non-specific control siRNA or siRNA against VASP using the HiPerfect reagent (Qiagen Cat.#301705). The siRNA used for VASP silencing was purchased from Santa Cruz Biotechnology (sc-29516) whereas the Control siRNA-A (sc-37007) was used as Non Specific Control (NSC) siRNA. Cells were harvested 48h post-transfection and silencing efficiency was verified by western blot and/or real time PCR as specified in each experiment.

RNA isolation and Real Time PCR

Total RNA was extracted from BC cell lines using Trizol (Thermo-Fisher Scientific Cat.#15596026), purified using RNeasy mini kit (Qiagen Cat.# 74104) and transcribed to cDNA using Superscript III Reverse Transcriptase (Thermo-Fisher Scientific Cat.#18080093). Quantification of gene expression was performed by real-time PCR using CFX96 Real Time PCR (BioRad). B-actin was used as a housekeeping gene. Reactions were done in triplicate and at least 3 independent experiments were performed. All primers used are shown in Table 1. Quantification of relative gene expression was performed using the

Ct method. Cells grown in 0.5mg/ml collagen I gels and cells transfected with NSC siRNA were used as calibrators, as specified in each experiment.

Protein extraction and Western blot analysis

For protein expression analysis, cells embedded in collagen I gels were harvested by scraping and treated with 1mg/ml collagenase D in phosphate buffered saline (PBS) for 30min at 37°C. Cell suspensions were then centrifuged at 300g for 5min to remove collagen and cell pellets were kept for gene expression analysis. Total cell lysates were obtained from cell pellets taken from 2D culture or collagenase D treatment using 1% sodium dodecyl sulfate in RIPA buffer (20mMTris/Cl pH7.5, 150 mM NaCl, 0.5% NP-40, 1% TX-100, 0.25% sodium deoxycholate, 0.6-2 μ g/ml aprotinin, 10 μ M leupeptin, 1 μ M pepstatin).

Protein concentrations in the samples were determined by the BCA protein assay kit (Pierce Cat.#23227). Cell lysates were run on a 10-12% acrylamide gel and transferred to a PVDF membrane (Millipore Cat.# IPVH00010) using the BioRad Semi-dry transfer system (BioRad). Membrane was blocked in 5% non-fat milk in Tris-Buffered saline and Tween 20 (TBST) buffer for 1h and was then incubated with appropriate antibodies overnight in 5% milk. Standard western blot procedure steps were followed thereafter and signal was analyzed using chemiluminescent substrate from Pierce and Kodak Biomax light films. Films were subsequently scanned using an HP Scanjet G4010 scanner and scanned images were analyzed by Adobe Photoshop software by being converted to greyscale after discarding color. No other image manipulation was involved.

Quantification of protein expression in western blots

Whenever quantification of protein expression in western blots was deemed necessary, it was performed using the National Institute of Health (NIH) ImageJ software. The mean intensity from relative protein bands from at least three different western blots corresponding to three independent experiments was used for the quantification, as specified in each figure. A *p* value of <0.05 was considered as statistically significant.

Cell Immunostaining

Cells were plated on gelatin-coated glass coverslips 24h post-siRNA-transfection. The following day, and 48h post-transfection they were fixed in 4% paraformaldehyde (Sigma P6148), permeabilized in a buffer containing PBS, 2mg/ml Bovine Serum Albumin (Sigma Cat.#A2153), and 0.1% Triton X-100 (Sigma X100), and stained with anti-VASP antibody, following a previously described protocol.²⁷ Alexa 488-donkey anti-mouse antibody was used as secondary antibody and nuclei were stained using DAPI. Pictures were taken under an Olympus BX53 fluorescent microscope and attention was paid to always have equal exposure time in all photos taken.

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was performed in lysates from MCF-7 and MDA-MB-231 treated with NSC or VASP siRNA for 48h using the Quantikine ELISA human u-plasminogen activator/urokinase immunoassay (Cat.#DUPA00 from R& D systems) following the company's guidelines. The same kit was used for lysates from MCF-7 and MDA-MB-231 cells that were treated with NSC or VASP siRNA for 24h and then embedded in 3D collagen gels (0.5, 1.0 or 3.0 mg/ml collagen) for an additional 24h. Collagen gels containing cells were subsequently treated with collagenase D (1mg/ml collagenase D in PBS for 30min at 37°C) and cell suspensions were centrifuged at 300g for 5min to remove collagen. Cells were finally lysed using the recommended lysis buffer from R&D systems (Cat#890713) and the ELISA assay was performed according to the provided protocol. Two independent experiments were performed.

Atomic Force Microscopy

Atomic Force Microscopy (AFM) was used to characterize 3D collagen I gels. More specifically, for high resolution imaging of collagen I gel fibers AFM images of the collagen

films were obtained in air using a PicoPlus AFM system from Molecular Imaging/Agilent Technologies. Briefly, part of the collagen solution (90µl) was flushed on 13mm circular glass cover glasses (AGL46R13, Agar Scientific) and the samples were incubated in a cell culture incubator for 30 min. Samples were then mounted on 15mm specimen AFM metal discs (AGF7003, Agar Scientific). All images were obtained at room temperature in contact mode with Sicon (Applied Nanostructures) probes. The surface images were acquired at a fixed resolution (512×512 data points) with scan rate between 0.5 and 1 Hz.

The AFM image processing was performed by using the freeware scanning probe microscopy image analysis software WSxM 5.0 dev.2.1.28

Culture of cells in 3D collagen I gels

To create a 3D collagen I gel of desired concentration, we utilized the high concentration collagen I solution (Corning 354249) and adjusted the pH using a modification of previously published protocols.²⁹ More specifically, we added the desired amount of collagen I so as to get a final collagen concentration of 0.5, 1.0 or 3.0 mg/ml, in a solution containing 10% 10x Minimal Essential Medium (Gibco 21430-020), 1% human insulin solution (Sigma I9278), and distilled water. The pH was adjusted to 7.4 by adding 1N NaOH. Cancer cells were added to the collagen solution before it solidified at a concentration of 2.5×10^5 cells/ml²⁹ and normal complete culture medium was added on top of the collagen gel containing cells 4h after its solidification. Cells were cultured in 3D collagen I gels of 0.5, 1.0 or 3.0 mg/ml for 3 days and were then subjected to gene expression analysis at the mRNA level as specified. In experiments involving VASP silencing, siRNA transfection was performed in traditional 2D culture 1 day prior to embedding cells in the collagen gels. Cells were left to grow in the gels for at least one more day before being harvested and analyzed for gene expression.

Cancer cell spheroid formation and spheroid invasion assay

MDA-MB-231 cell spheroids were formed using the “hanging drop” technique, as described previously.^{30–32} Briefly, cells were trypsinized, counted and put in suspension at a concentration of 2.5×10^4 cells/ml. Hanging drops containing 500 cells each were placed on the inside of the cover of a culture dish. Drops were left for at least 24h to allow for spheroid formation. Subsequently, formed spheroids were transferred into wells of a 96-well plate containing 0.5, 1.0 or 3.0 mg/ml collagen I gel using a glass Pasteur pipette. Pictures were taken immediately (time zero) using a Nikon Eclipse optical microscope equipped with a digital camera and spheroids were then incubated at 37°C for 2–24h to select the optimum time point to observe cell invasion. Cell invasion in surrounding collagen was measured using the NIH ImageJ software and spheroids’ size (average of the major and minor axis length) at the designated time was compared to the initial size at time zero.²² In experiments where cells were subjected to VASP siRNA-mediated silencing, siRNA transfection was performed 24h prior to formation of hanging drops, hanging drops were left for an additional 24h for the spheroids to be formed and they were then placed in collagen gels. Pictures were taken at time zero (48h post-silencing) and at 8h post implantation in collagen gels (56h post-silencing). At least 8 spheroids were analyzed per condition and at least three independent experiments were performed.

Kaplan-Meier plotter analysis

Kaplan-Meier plotter, an *in silico* online tool was used to predict survival of BC patients depending on the level of VASP expression. The Kaplan-Meier plotter uses Affymetrix microarray gene expression data from multiple BC studies and integrates them simultaneously with clinical data including relapse free and overall survival information. 33,34 Information on a total of 4,142 BC patients was available in the database and we searched for VASP expression in all patients, as well as in lymph node (LN) positive and negative patients (945 and 1813 patients respectively). Kaplan Meier plotter can be found at: <http://kmplot.com/analysis/index.php?p=service&cancer=breast>

Statistical analysis

Comparison of means using Statgraphics software was used for the statistical analysis. Student's *t*-test was performed and a *p*-value <0.05 was considered statistically significant.

Results

2D culture system study

VASP is upregulated in more aggressive MDA-MB-231 cells compared to MCF-7 cells—We first set out to test VASP expression in the highly invasive MDA-MB-231 BC cells and the less invasive MCF-7 BC cells in 2D cell culture monolayers. We found VASP to be significantly upregulated in the aggressive MDA-MB-231 cells compared to the less aggressive MCF-7 cells both at the protein (Figure 1A, B, and D) and mRNA (Figure 1C) level, as shown by immunoblotting (Figure 1 A, B), immunocytochemistry (Figure 1D) and real time PCR analysis (Figure 1C), respectively. This, along with the fact that VASP plays a central role in cell migration, indicates that it might be important for cancer cell invasion in BC cells. Thus, to further understand its role and mechanism of action in cancer cell invasion, we focused on the invasive MDA-MB-231 cell line for the rest of the study.

VASP is efficiently silenced in MDA-MB-231 cells cultured in 2D cell monolayers—In order to investigate the role of VASP in the aggressive BC cells and the molecular mechanism of its action, we performed siRNA-mediated gene silencing in MDA-MB-231 cells. As shown in Figure 2, VASP was significantly and effectively silenced both at the protein (Figure 2A and B) and mRNA (Figure 2C) level compared to cells being transfected with non-specific control (NSC) siRNA.

VASP silencing in 2D cultures reduces Migfilin, β -catenin and uPA mRNA expression but does not affect Integrin-Linked Kinase (ILK) expression—As VASP is localized to cell-ECM adhesion sites⁸, we first wondered whether its elimination affects important cell-ECM adhesion proteins, such as Integrin-Linked Kinase (ILK). As shown in Figure 2D, VASP silencing has no effect on ILK mRNA expression. Interestingly, however, its silencing results in dramatic downregulation of Migfilin (Figure 2E), which is a cell-ECM adhesion protein that has been reported to be a VASP binding partner at the cell-ECM adhesion sites.⁹ Importantly, Migfilin has been found to be concurrently present at the cell-ECM and cell-cell adhesions of the cell. In fact, at cell-cell adhesions, it is concentrated

at discrete clusters bridging neighboring cells being closely (within 100 nm) associated with β -catenin.²⁷ Taking this into account, we tested the effect of VASP silencing on β -catenin mRNA level. Our results show that β -catenin was also downregulated following VASP silencing in MDA-MB-231 cells (Figure 2F). To further test whether VASP silencing affects key molecules involved in matrix degradation, we then tested the mRNA expression of urokinase Plasminogen Activator (uPA), a known protease involved in cancer progression and metastasis. Figure 2G shows that uPA was also significantly downregulated following VASP silencing compared to NSC-treatment in MDA-MB-231 cells. Finally, to further corroborate our real time PCR results, we performed an enzyme-linked immunosorbent assay (ELISA) to assess changes in uPA secretion following VASP silencing both in MCF-7 and MDA-MB-231 cells. As shown in Figure 2H, and consistent with the gene expression analysis at the mRNA level (Figure 2G) uPA secretion was significantly downregulated in VASP-depleted MDA-MB-231 cells. Of note though is the fact that, as expected, non-invasive MCF-7 cells exhibited no uPA secretion.

3D collagen culture system and spheroids' analysis

Defining the 3D culture system of Collagen I gels—To better understand the role of VASP in cancer cell invasion, we needed to also consider the interactions of cells with surrounding ECM, which is more physiologically relevant, and cannot be studied in a 2D culture system. In that regard, we employed a culture system that would permit cancer cells to grow in 3D while allowing us to modulate the stiffness of the surrounding matrix, mimicking the stiffer microenvironment present in most solid tumors.^{6, 14–18} This would also provide us with the opportunity to study the effect of ECM-originating mechanical cues on cancer cells with regard to tumor growth and metastasis.

Type I collagen was selected for the generation of the 3D matrix environment as the most abundant protein found in tissues's ECM,³⁵ making the system more physiologically relevant. Hence, we generated gels containing 0.5, 1.0 or 3.0 mg/ml collagen I. To characterize the generated gels in terms of structure, we used Atomic Force Microscopy (AFM), which is a unique tool for imaging collagen without destroying its fibrillar structure.^{36,37} As shown in Figure 3A-C by AFM nanoscale topography images taken in contact mode (in air), collagen gels consisted of fibers with random orientation, confirming that the formed gels mimic collagen-rich tissues. Furthermore, the density of the fibers in the formed gels was increased with collagen concentration showing a correlation with collagen stiffness, as it has been previously shown in studies using collagen gels of increasing concentration^{38,39}. More importantly, using higher resolution imaging, we demonstrated that the formed collagen fibers possessed the characteristic D-band periodicity (Figure 3D). This is of great significance, as transverse D-banding periodic pattern occurs only in naturally-formed collagen fibers, it has been correlated with fibers' mechanical strength and it has been proposed to be recognized by cells⁴⁰.

No significant effect of 3D culture on cell morphology and VASP expression was observed—MCF-7 and MDA-MB-231 BC cells were then embedded in the collagen gels, according to previously published protocols,^{29,41} and were allowed to grow for 3 days. As shown in Figure 3, MCF-7 (Figure 3 F-H) and MDA-MB-231 cells (Figure 3 J-L)

taking advantage of the presence of matrix around them, grew at different levels in all three dimensions within the 3D collagen matrix, although no significant change in their morphology was observed in different concentrations.

We next sought to find out whether VASP expression in MDA-MB-231 cells is affected by their growth in 3D collagen gels of increasing concentration or not. As shown in Figure 4, VASP protein (Figure 4A and B) and mRNA expression (Figure 4C) was not significantly altered in response to increased collagen I concentration.

VASP is efficiently silenced in MDA-MB-231 cells grown in 3D Collagen I gels of increasing concentration leading to reduced Migfilin, β -catenin and uPA mRNA expression

—As VASP expression did not seem to be affected by growth in 3D, we wondered what the effect of its silencing would be. More specifically, we performed siRNA-mediated VASP silencing in MDA-MB-231 cells and the next day we embedded them in collagen gels of increasing concentration where they were allowed to grow for at least another 24h. Since VASP was efficiently silenced in all three collagen concentration conditions (Figure 5A), we studied further the effect of its depletion. Consistent with the effect seen in 2D following VASP depletion (Figure 2), we observed a dramatic reduction in the mRNA expression of Migfilin (Figure 5B), β -catenin (Figure 5C) and uPA (Figure 5D) after VASP silencing in collagen gels of all concentration conditions. Moreover, uPA secretion was also evaluated. As shown in Figure 5E, MCF-7 cells do not secrete uPA while MDA-MB-231 cells secrete high levels of uPA which are significantly reduced following VASP silencing in collagen 1.0 and 3.0 mg/ml gels.

VASP silencing in MDA-MB-231 cell spheroids embedded in 3D collagen gels inhibits invasion

—To study the functional effect of VASP silencing and uPA downregulation, we wanted to test the invasive potential of VASP-depleted cells using the spheroid invasion assay in an attempt to better control invasion routes of cells within the surrounding matrix and better recapitulate a breast tumor *in vitro*. Thus, we first generated MDA-MB-231 cell spheroids using the hanging drop method,^{22,30,31} and embedded them in culture wells containing 0.5, 1.0 or 3.0 mg/ml collagen gels, considering the time of implantation as time zero. We then performed a time-course experiment to select the optimum time to observe spheroid cell invasion. The size of the cancer cell spheroids was measured using ImageJ software and taking the mean of major and minor axis length, while invasion was assessed as the percent difference in spheroids' size at a certain time point compared to the size at time zero. Based on our observations, we found that significant differences in invasion are seen as early as 6h post embedding with 8h being the optimum time point, which was selected for all subsequent experiments.

MDA-MB-231 cells were then subjected to VASP siRNA transfection and one day later were used to generate cancer cell spheroids that were placed inside collagen gels of increasing concentration 24h later (48h post-siRNA transfection). Pictures were taken at the time of embedding (time zero) and at 8h post-embedding. As shown in Figure 6, VASP silencing in MDA-MB-231 cells resulted in significant inhibition of cell spheroid invasion in 0.5 and 1.0 mg/ml collagen matrices but had no effect on spheroids embedded in 3.0 mg/ml collagen gels.

Kaplan Meier Plotter analysis

High VASP mRNA expression is associated with poor prognosis for remission-free survival in BC patients and in lymph node (LN) positive patients but not in lymph node negative patients—As VASP silencing led to inhibition of cancer cell spheroid invasion through collagen gels, we wanted to further validate our findings in human patients. Thus, we utilized the Kaplan Meier plotter, an *in silico* online tool which performs meta-analysis of Affymetrix microarray gene expression data from multiple studies involving BC patients^{33,34} to predict survival depending on VASP expression. We first analyzed the microarray data using all the available information on 4,142 BC patients and found that high VASP mRNA expression had no effect on overall survival (Figure 7A) but correlated with poor prognosis for remission-free survival (Figure 7B). Moreover, when we analyzed the data based on LN metastasis status, high VASP mRNA expression had no effect on LN negative patients (Figure 7C) but was associated with worse prognosis for remission-free survival in 945 LN positive patients (Figure 7D), indicating an important role of VASP in tumor progression and metastasis in BC patients.

Discussion

In the current work, we employed a dual approach to study the role of VASP in BC cell invasion *in vitro*. First, we cultured MCF-7 and MDA-MB-231 BC cells in 2D monolayer and also in 3D collagen gels of increasing concentration that were characterized by AFM, and second, we generated cancer cell spheroids in invasive MDA-MB-231 cells and placed them in the collagen gels monitoring their invasiveness in relation to matrix concentration.

Our results demonstrate that changes in the invasive capacity of MDA-MB-231 cell spheroids are more obvious at the 0.5 and 1.0 mg/ml collagen condition showing a minimum invasion at the 3.0mg/ml collagen gel concentration (Figure 6B). This indicates that cancer cell spheroid invasion was severely impaired in the stiffer matrix condition suggesting that the denser and stiffer 3D matrix presents obstacles to the formation of cell protrusions and proper invasion⁴⁴.

All in all, it is no news that VASP is involved in cell migration regulation, and invasion in cancer cells as multiple studies have supported this involvement.^{11,38–42} However, the type of regulation (positive or negative) differs depending on the cell system studied and the exact mechanism of its action is unclear with multiple mechanisms proposed to date. For instance, Rac activation,^{13,14} VASP phosphorylation,¹⁵ and actin polymerization,¹¹ are among the mechanisms reported.

In the present study, we show for the first time that VASP silencing downregulates Migfilin (Figure 2E and 5B), which is a known binding partner of VASP.⁹ Regarding Migfilin's role in migration and invasion, though, studies are perplexing. Migfilin has been shown to promote migration and invasion in glioma cells,⁴⁵ and hepatocellular carcinoma cells,⁴⁶ but it has also been shown to inhibit cell migration and invasion in esophageal cancer cells.⁴⁷ Interestingly, one of the first studies on Migfilin showed that it regulates cell migration in a biphasic manner, as both its depletion and its overexpression impair cancer cell migration,⁹ which might explain the seemingly controversial findings. Taking all the above into

consideration, we could postulate that Migfilin acts in concert with VASP to regulate BC cell migration and invasion, being downregulated following VASP silencing. Hence, it would be expected that its overexpression in VASP-depleted MDA-MB-231 cells could reverse the inhibition in invasiveness induced by VASP silencing. Nevertheless, the exact interplay and interaction strength between VASP and Migfilin would be interesting to be investigated further to better understand their molecular interdependence and function in BC as well as other cancer cells.

Furthermore, we show herein that VASP depletion also reduces the mRNA expression of β -catenin (Figure 2F and 5C), which is a fundamental component of cell-cell adhesions proven to be closely associated with Migfilin (within a distance of 100 nm as shown by electron microscopy).²⁷ Thus, it is not surprising that downregulation of Migfilin is also accompanied by β -catenin downregulation. It should be noted though that several reports exist indicating that Migfilin is a negative regulator of β -catenin^{46–48} leading us to assume that an alternative mechanism might be in place in MDA-MB-231 cells or that VASP inhibits β -catenin through a different pathway that does not include Migfilin.

Finally, VASP depletion both in 2D and 3D cultures also reduces the mRNA expression and secretion of uPA (Figure 2G&H and 5D), a critical protease implicated in metastasis.⁴⁹ This is a completely novel association. To the best of our knowledge, it has not been reported, to date, that VASP or Migfilin affect uPA expression in any way. Interestingly though, it has been shown that β -catenin regulates uPA in differential ways depending on cell type.^{50,51} More importantly, several studies have demonstrated that uPA is induced upon cytoskeletal reorganization,^{52,53} suggesting that disruption of cell-ECM adhesions by VASP silencing may disorganize the cytoskeleton and lead to the observed inhibition of uPA.

Taking all the above into account, the present work provides an indication of the molecular mechanism underlying the inhibition of spheroid invasion seen in MDA-MB-231 cells after VASP silencing. More importantly, these changes in gene expression of Migfilin, β -catenin and uPA are observed following VASP silencing both in 2D and 3D culture conditions regardless of the concentration of the collagen gels, suggesting that the molecular mechanism involved is independent of matrix stiffness. It should be noted though, that VASP silencing inhibited spheroid invasion through collagen gels of 0.5 and 1.0mg/ml while it had no effect on the spheroids embedded in 3.0mg/ml collagen gels (Figure 6B), most likely due to the fact that cell invasion in the stiffest matrix is already low, and thus VASP silencing cannot inhibit it any further.

Lastly, analysis of the Kaplan Meier survival plots using the online Kaplan Meier plotter tool³³ further supported our findings showing that high expression of VASP does not seem to affect overall survival or LN negative patients (Figure 7A and C), but is associated with poor remission free survival in all BC patients as well as in LN positive patients (Figure 7B and D) indicating that VASP is crucial for BC cell metastasis.

In conclusion, this is the first study to show that VASP silencing severely impairs MDA-MB-231 tumor spheroid invasion in collagen gels of increasing concentration through downregulation of Migfilin, β -catenin, and uPA, although the exact sequence of events in the

molecular pathway needs still to be elucidated. As uPA is considered one of the most prominent biomarkers for cancer recurrence, the discovery of novel ways to inhibit its expression and secretion is always necessary, and VASP is worth being investigated as such a target.

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Abbreviations

AFM	Atomic Force Microscopy
DAPI	4',6-diamidino-2-phenylindole
ECM	extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ILK	integrin-linked kinase
NSC	non-specific control
LN	lymph node
PBS	phosphate buffered saline
TBST	Tris-Buffered saline and Tween 20
uPA	Urokinase plasminogen activator
VASP	Vasodilator Stimulated Phosphoprotein
2D	two-dimensions
3D	three-dimensions

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Figure 1. VASP protein and mRNA expression is elevated in more aggressive BC cells compared to less aggressive

A) Representative western blot showing VASP protein expression in MCF-7, and MDA-MB-231 cells. B-actin was utilized as loading control. B) Relative VASP protein expression normalized to β-actin using the NIH ImageJ software. Seven (7) different immunoblots were analyzed by imageJ to generate the respective graph. C) Relative VASP mRNA expression in MCF-7, and MDA-MB-231 cells cultured in traditional 2D culture conditions. Three (3) independent Real Time PCR experiments were performed, and data were analyzed using the Ct method and having MCF-7 cell sample as calibrator. A p value of less than 0.05 was

considered as statistically significant. D) Immunofluorescent staining of MCF-7 and MDA-MB-231 cells with anti-VASP antibody (green) and DAPI for visualization of the nuclei. Pictures were taken under 40x or 100x objective as indicated using an Olympus BX53 fluorescent microscope and attention was paid to always have equal exposure time in all photos taken. Asterisk (*) represents a statistically significant difference (p value <0.05).

Figure 2. VASP is effectively silenced in 2D conditions in MDA-MB-231 cells and its silencing downregulates Migfilin, β -catenin and uPA but does not affect ILK

A) Representative western blot showing VASP protein expression in MDA-MB-231 treated with NSC or VASP siRNA for 48h. β -actin was utilized as loading control. B) Relative VASP protein expression normalized to β -actin using the NIH ImageJ software. Three (3) different immunoblots from three independent experiments were analyzed by imageJ to generate the respective graph. C-G) Relative mRNA expression of VASP (C), ILK (D), Migfilin (E), β -catenin (F) and uPA (G) in MDA-MB-231 cells cultured in traditional 2D culture conditions and treated with NSC or VASP siRNA for at least 48h. At least three (3)

independent Real Time PCR experiments were performed, and data were analyzed using the

Ct method and having NSC-treated cells as calibrators. A p value of less than 0.05 was considered as statistically significant. H) Human uPA protein (pg/ml) secreted by MCF-7 and MDA-MB-231 cells following VASP silencing using ELISA. Two independent experiments were performed. Asterisk (*) represents a statistically significant difference (p value <0.05).

3D collagen I gels **MCF-7 cells** **MDA-MB-231 cells**

Figure 3. Visualization of collagen fibers by AFM and morphology of MCF-7 and MDA-MB-231 cells grown in 3D collagen gels in conditions of increasing matrix concentration

A-C) AFM Topography images of the nanosurface of 0.5, 1.0 and 3.0 mg/ml collagen gels (contact mode in air, with PicoPlus AFM system). The topographic AFM images are presented in a color scale which represents the Z axis. D) High resolution 3D AFM topography image of collagen fibers with the characteristic D-band periodicity in collagen gel. E-H) Morphology of MCF-7 cells grown in 2D culture (0mg/ml collagen) (E), or embedded in collagen gels of 0.5mg/ml (F), 1.0mg/ml (G) and 3.0mg/ml (H) concentration. I-L) Morphology of MDA-MB-231 cells grown in 2D culture (0mg/ml collagen) (I), or

embedded in collagen gels of 0.5mg/ml (J), 1.0mg/ml (K) and 3.0mg/ml (L) concentration. Pictures were taken using a Nikon Eclipse TS100 optical microscope equipped with digital camera.

Figure 4. VASP expression is not affected by culture in conditions of increased collagen concentration

A) Representative western blot showing VASP protein expression in all concentration conditions in MDA-MB-231 cells. B-actin was utilized as loading control. B) Relative VASP protein expression in MDA-MB-231 cells cultured in collagen gels of 0.5, 1.0 and 3.0mg/ml concentration normalized to actin using the ImageJ software (NIH). The results shown represent the mean value from three different western blots. C) Relative VASP mRNA expression in MDA-MB-231 cells cultured in collagen gels of 0.5, 1.0 and 3.0mg/ml concentration. Eight (8) independent Real Time PCR experiments were performed, and data were analyzed using the $\Delta\Delta C_t$ method and having 0.5mg/ml collagen I gel as a calibrator. A p value of less than 0.05 was considered as statistically significant.

Figure 5. VASP silencing leads to downregulation of Migfilin, β -catenin and uPA in MDA-MB-231 cells cultured in 3D

Relative mRNA expression of VASP(A), Migfilin(B), β -catenin(C) and uPA (D) in MDA-MB-231 cells cultured in 3D collagen gels of increased concentration and treated with NSC or VASP siRNA for at least 48h. Three (3) independent Real Time PCR experiments were performed, and data were analyzed using the Ct method and having NSC-treated cells as calibrators for each condition of collagen. E) Human uPA protein (pg/ml) secreted by MCF-7 and MDA-MB-231 cells following VASP silencing in cells cultured in 0.5, 1.0 or 3.0

mg/ml collagen gels using ELISA assay. Two independent experiments were performed. Asterisk (*) represents a statistically significant difference (p value <0.05).

Figure 6. VASP silencing in MDA-MB-231 spheroids embedded in 3D collagen gels leads to reduced cell invasion in a stiffness-independent manner

A) MDA-MB-231 cells treated with NSC or VASP siRNA for 24h were used to generate cell spheroids. Twenty four hours later (48h post-gene silencing) cell spheroids were embedded in collagen gels of 0.5, 1.0 or 3.0mg/ml concentration at time zero and left to grow for 8h post embedding. B) Percentage (%) change in MDA-MB-231 spheroids' size (average of major and minor axis) within the specified time post embedding. At least 8 spheroids were analyzed per condition and 3 independent experiments were performed. A *p* value of less

than 0.05 was considered as statistically significant compared to the NSC and was denoted by an asterisk (*).

A Overall survival **B Remission-free survival** **C Remission-free survival in LN- patients** **D Remission-free survival in LN+ patients**

Figure 7. High VASP mRNA expression is correlated with reduced remission-free survival in all BC patients as well as in LN positive patients

Kaplan-Meier plotter was used to predict survival of BC patients depending on the level of VASP expression. A-B) Overall survival (A) and remission-free survival (B) was estimated using the available information on a total of 4,142 patients in the database. C-D) Remission-free survival was assessed in LN negative (C) and LN positive (D) patients (1813 and 945 patients respectively).

Table 1
Nucleotide sequence of the primers used for mRNA expression analysis

Primer name	Sequence
β-actin	Forward: 5'-CGAGCACAGAGCCTCGCCTTTGCC-3' Reverse: 5'-TGTCGACGACGAGCGCGGCGATAT-3'
β-catenin	Forward: 5'-ACAAACTGTTTTGAAAATCCA-3' Reverse: 5'-CGAGTCATTGCATACTGTCC-3'
ILK	Forward 5' GAC ATG ACT GCC CGA ATT AG 3' Reverse 5' CTG AGC GTC TGT TTG TGT CT 3'
Migfilin	Forward: 5'-CGAATGCATGGGAAGAAACT-3' Reverse: 5'-GCAGGTTAGGAAGGGAAACC-3'
uPA	Forward: 5'-GCTGCTGACCCACAGTGGAA-3' Reverse: 5'-AAAGTCATGCGGCCTTGGAG-3'
VASP	Forward: 5'-GAA AAC CCC CAA GGA TGA AT-3' Reverse: 5'-GGA AGT GGT CAC CGA AGA AG-3'