Fascin Is a Key Regulator of Breast Cancer Invasion That Acts via the Modification of Metastasis-Associated Molecules

Monther Al-Alwan1*, Safiah Olabi1, Hazem Ghebeh1, Eman Barhoush1, Asma Tulbah2, Taher Al-Tweigeri3, Dahish Ajarim3, Chaker Adra1,4

1 Stem Cell Therapy Program, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia; 2 Department of Pathology and Laboratory Medicine, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia; 3 Department of Oncology, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia; 4 Transplantation Research Centre (TRC), Brigham and Women’s Hospital and Children’s Hospital Boston, Harvard Medical School, Boston, Massachusetts, United States of America

Abstract

The actin-bundling protein, fascin, is a member of the cytoskeletal protein family that has restricted expression in specialized normal cells. However, many studies have reported the induction of this protein in various transformed cells including breast cancer cells. While the role of fascin in the regulation of breast cancer cell migration has been previously shown, the underlying molecular mechanism remained poorly defined. We have used variety of immunological and functional assays to study whether fascin regulates breast cancer metastasis-associated molecules. In this report we found a direct relationship between fascin expression in breast cancer patients and, metastasis and shorter disease-free survival. Most importantly, in vitro interference with fascin expression by loss or gain of function demonstrates a central role for this protein in regulating the cell morphology, migration and invasion potential. Our results show that fascin regulation of invasion is mediated via modulating several metastasis-associated genes. We show for the first time that fascin down-regulates the expression and nuclear translocation of a key metastasis suppressor protein known as breast cancer metastasis suppressor-1 (BRMS1). In addition, fascin up-regulates NF-kappa B activity, which is essential for metastasis. Importantly, fascin up-regulates other proteins that are known to be critical for the execution of metastasis such as urokinase-type plasminogen activator (uPA) and the matrix metalloproteases (MMP)-2 and MMP-9. This study demonstrates that fascin expression in breast cancer cells establishes a gene expression profile consistent with metastatic tumors and offers a potential therapeutic intervention in metastatic breast cancer treatment through fascin targeting.

Citation: Al-Alwan M, Olabi S, Ghebeh H, Barhoush E, Tulbah A, et al. (2011) Fascin Is a Key Regulator of Breast Cancer Invasion That Acts via the Modification of Metastasis-Associated Molecules. PLoS ONE 6(11): e27339. doi:10.1371/journal.pone.0027339

Editor: Wanjin Hong, Institute of Molecular and Cell Biology, Singapore

Received May 25, 2011; Accepted October 13, 2011; Published November 4, 2011

Copyright: © 2011 Al-Alwan et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Research Advisory Council (proposal grant 2060 016), King Faisal Specialist Hospital and Research Centre. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: MAlwan@kfshrc.edu.sa

Introduction

Breast cancer is one of the leading causes of cancer mortality in women worldwide. In spite of significant advances in cancer treatment, mortality results from local invasion and/or distant metastasis and not from tumor in the primary site [1,2]. Therefore, there is a strong demand to understand the cellular and molecular mechanisms that regulate tumor invasion and metastasis in order to develop better treatment regimens. Metastasis is a multi-step process involving neovascularization, stromal invasion by cancer cells, and infiltration into vascular and lymphatic spaces, extravasation and growth at a secondary site [1–6]. Many cellular and molecular factors have been reported to regulate tumor metastasis [Reviewed in [7]]. However, a critical step of metastatic tumor cells is the ability to cross extracellular matrix (ECM) tissue boundaries [8,9], a process that can be accomplished by expression of active metalloproteases (MMPs) by cancer cells, which facilitate their migration. In addition, metastatic tumor cells can down-regulates metastatic suppressors such as the Breast Cancer Metastasis Suppressor 1 (BRMS1), which belongs to family of metastasis suppressors that suppress metastasis without blocking orthotopic tumor growth, BRMS1 has been reported to block lung and regional lymph node metastasis in experimental breast models [10] and decreased expression of this protein has been demonstrated to correlate with reduced disease-free survival in human breast cancer [11]. It was reported that BRMS1 suppression of tumor metastasis is mediated via inhibition of NF-kB and subsequent suppression of the urokinase-type plasminogen activator (uPA) [12], a serine protease that is known to activate the MMPs [13] leading to invasion and metastasis.

Manipulation of the actin cytoskeleton, leading to enhancement of cell motility, is one of the dominant cellular mechanisms that regulate metastasis [14]. Fascin is a member of the actin cytoskeletal proteins that bundles actin filaments into tertiary structures within dynamic cellular structures such as microspikes, stress fibers and membrane ruffles [15], consistent with its abundant expression at these sites. Interestingly, fascin expression is highly restricted to certain tissues such as brain and spleen and
abundant fascin expression was reported in normal specialized cells such as neurons, glia cells, endothelial cells and antigen presenting dendritic cells [16–19].

It is widely believed that de-regulation of normal tissue organization and homeostasis including cell adhesion, motility and cytoskeleton can contribute to many human diseases including cancers [19]. In fact, fascin over-expression induces membrane protrusion and has been reported to enhance cell motility in various systems [20]. In addition to its expression in specialized normal cells, many studies have reported induction of fascin expression in various transformed cells. In many human carcinomas including breast cancer, fascin expression correlates with clinically aggressive tumors and metastasis [21]. However, the exact function of fascin and its regulation of other genes in breast cancer are still poorly understood.

Development of membrane protrusions by non-immune cells has been clearly demonstrated to enhance cell motility and to facilitate interactions with other cell types. Therefore, we proposed to investigate whether fascin expression in breast cancer cells is directly involved in promoting cell migration and invasion and to elucidate the molecular mechanism that regulate this process. Our results clearly demonstrated a key role for fascin in regulating both cell morphology and invasion. In addition, we have shown that fascin facilitates this process via modulation of metastasis-associated genes; specifically down-regulation of the metastasis suppressor BRMS1 and up-regulation of NF-kB activity as well as the induction of uPA, MMP-2, MMP-9 expression.

Materials and Methods

Cell lines

The human breast cancer cell line MDA-MB-231 (HTB-26) and T-47D (HTB-20) were purchased from ATCC and seeded in DMEM containing 10% FBS, 200 mM L-glutamine and antibiotics-antimycotic liquid (Invitrogen) at 37°C in a 5% CO2 humidified atmosphere.

Antibodies and reagents

The mouse anti-human fascin mAb and APC-conjugated secondary Ab were from Dako and Jackson Laboratories, respectively. The mouse mAb to detect MMP-2 (2C1-1D12) and MMP-9 (2C3) were from Invitrogen and Chemicon, respectively. The mouse mAb (ab57082), rabbit polyclonal Ab (ab65244) to detect BRMS1, mAb to detect uPA (PGM2005) and mAb to detect TATA binding protein (TBP) were all from Abcam. Also to detect p65 (C22B4), phospho-p65 (93H1), IκBζ (L35A3) and phospho-IκBζ (5A5), alpha-tubulin were all from Cell Signaling Technology. The GAPDH, β-actin, PCNA and p50 (C-19) were from Santa Cruz. The secondary anti-mouse HRP-conjugated and anti-goat HRP-conjugated were from Southern Biotech and ScIotec, respectively. The Rhodamine-phalloidin to detect F-actin was from Invitrogen. The remaining antibodies were from BD Bioscience. Fascin specific-, negative control-SiRNA and GAP-DH-SiRNA were purchased from Applied Biosystem. Fascin specific-ShRNA and control were purchased from Plasmid-12039) or control shRNA were obtained from Thermo Scientific. Recombinant lentivirus was produced in 293T cells as previous described [23] and mixed with MDA-MB-231 cells in the presence of polybrene (10 µg/ml) for 16 hour at 37°C, medium was replaced and cultured for additional 72 hours. Transduced cells were selected with puromycin followed by cloning and screening for the lack of fascin expressing using FACS. ShRNA were designed and used especially for the activation experiments where cells need to be seed in serum containing media before stimulation in serum-free media. This could not be done using the SiRNA approach as they were generated in a serum-free media.

Generation of eGFP-fascin expressing cell stable transfectants

Wild-type or mutant fascin-GFP fusion constructs were previously described [24] and kindly provided to us by Dr. Josephine C. Adam (Cleveland Clinic Foundation, Ohio, USA). Briefly, mutation in fascin was generated by substituting the major site that is phosphorylated by PKCα (Serine 39) with Aspartic Acid. After transfection using the Nucleofector reagent (Amaxa BioSystems), stable clones were isolated post selection with G418 for 2–3 weeks followed by cloning. Fascin expression levels and mean florescent intensity (MFI) were always higher in GFP positive cells as assessed by FACS and western blots, respectively.

Chemotaxis and invasion assay

The migration and metastatic potential of tumor cells were evaluated using the 24-well BD migration and BioCoat Matrigel Invasion Chamber, respectively, as per the manufacturer guideline (BD Bioscience). Cells (1–2 x10^5/well) were allowed to migrate for 18 hours and membranes were stained with a Diff Quick stain and mounted on slides. Cells that had migrated to the underside of the filter were counted using light microscope (Zeiss Axio Observer) in 5 randomly selected fields (magnification: 40x). Each assay was performed in triplicate and repeated at least 5 times. Due to variation in the number of migrated cells from different experiment, the results are normalized to control cells and the relative migration or invasion is expressed as mean ± SD of migrating cells relative to control cells.

Purification of total RNA and RT-PCR

Total RNA was extracted from cells using RNAasy Mini kit (QIAGEN). cDNA was synthesized using First Strand cDNA synthesis kit (Invitrogen). The relative mRNA copy number of a gene was determined by RT-PCR using molecular beacon method and detected by ABI PRISM 7900HT (Applied Biosystems) and expressed as previously described [25]. Sequences of forward, reverse and probe used in this experiment are shown in Table S1.
Western Blot

Cells were washed with cold PBS, total proteins were extracted using RIPA buffer containing protease inhibitors, and concentrations were determined using Bradford assay (Bio-Rad). Proteins (20 μg) were loaded onto an SDS-PAGE gel and transferred using semidry electrophotographing (Bio-Rad). Membranes were then incubated in 5% (w/v) skimmed milk before incubation with primary antibodies overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour. Chemiluminescence Super Signal System (Thermo Scientific) was used for subsequent detection of bound antibodies.

For activation, exponentially growing cells were stimulated with 10 ng/ml of TNF-α in serum-free media for the indicated time points in the figures. Nuclear and cytoplasmic fractionation was done as previously described [26]. Proteins (15 μg of cytoplasmic and 10 μg of nuclear extract) were loaded and assayed as above. Equal amounts of supernatants were loaded onto an SDS-PAGE gel and protein expression was detected by western blot as described above. Gelatin zymography was done as previously described [27].

Dual-luciferase reporter assay

NF-κB luciferase activity in cell lysates was measured using dual-luciferase reporter assay system (Promega) previously described [28]. pXPG-hIL-8p luciferase reporter plasmid was a generous gift from Dr. Abbas Haowari, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia. Each experiment group was done in triplicate and repeated at least 5 times. Firefly luciferase was divided by the Renilla luciferase activity to normalize for transfection efficiency and the relative values are presented as fold change over non-stimulated control.

Immunohistochemistry

Formalin-fixed, paraffin-embedded breast cancer sections of 71 patients deparaffinized and rehydrated. Antigen retrievals were done by microwaving for 15 minutes in a special citrate solution pH 6 (Dako). Endogenous peroxidase were blocked for 15 minutes with 3% hydrogen peroxide (Sigma) in methanol. Sections were then blocked with 10% goat serum (Sigma) for 60 minutes, followed by addition of a primary mouse anti-human fascin (1/200) or rabbit anti-BRMS1 (1/1000) antibodies for overnight incubation at 4°C. After washing, sections were incubated with labeled Polymer (EnVision+) HRP detection kit (Dako) for 30 minutes at room temperature. The HRP was detected using DAB substrate (Novocastra) for 4 minutes and the sections were counterstained for 1 minute with Instant hematoxylin (Shandon). The intensity of staining and the percentages of fascin and BRMS1 positive cells were quantified at 5 to 10 increments by an anatomical pathologist (AT) who had no prior knowledge of patient details. Type of breast cancer was confirmed at the time of reading. Histological grades of breast cancer sections were evaluated according to Scarff-Bloom-Richardson (SBR) classification [29].

For immunoflorescence staining of BRMS1, the above method of fixation and primary staining was used followed by Alexa-555 anti-rabbit secondary antibody. F-actin staining was done as previously described [30]. For cellular localization of BRMS1 or F-actin detection, 100 cells were assessed using attovision software on Pathway 855 (BD, Mountain View, CA).

Statistical Analysis

The significance (0.05) of relationship between fascin expression and patient’s clinicopathological parameters was assessed using Fisher exact test. The software package SAS 9.1 (SAS Institute, Cary, NC) was used for these analyses.

Results

Fascin expression in breast cancer is associated with poor prognosis, metastasis and reduced disease-free survival

Fascin expression in breast cancer correlates with poor prognosis of the disease and shorter disease-free and overall survival [21]. Here we have used immunohistochemistry to re-examine the relationship between the expression of fascin and metastasis in 71 breast cancer patients, which were diagnosed with invasive ductal carcinoma. Fascin was negative in normal breast luminal cells, but weak to moderate expression was seen in the myoepithelial and endothelial cells (data not shown), consistent with previous study [21]. In breast cancer samples however, fascin was expressed in the tumor cells of 40.84% of breast cancer patients. There were a strong correlation between fascin expression and basal-like phenotype (<0.001), hormone receptor-negative (ER−, P<0.001), (PR−, P = 0.020), larger tumor size (P = 0.034), high histological grade tumors (P = 0.091), known poor prognostic markers (Table 1). Interestingly, fascin also significantly correlated with increased expression level of B7-H1 (P = 0.008), a T cell inhibitory molecule that is associated with bad prognostic markers in breast cancer [31]. Importantly, our data showed significant (P = 0.017) correlation between fascin expression and local as well as systemic metastasis (Table 1). In addition, there was a highly significant (P<0.001) association between fascin expression and decreased disease-free survival (Figure 1A), but the association with the overall survival (Figure 1B) was borderline significant (P = 0.058). Our in vivo findings demonstrate a strong correlation between fascin induction in breast cancer cells and; poor prognostic markers, increased tumor metastasis and reduced disease-free survival.

Fascin regulates the morphology and migration of breast cancer cells

To address the relationship between fascin expression and metastasis we measured parameters important for this process using MDA-MB-231 breast cancer cell line as a model. This cell line is known to be ER/PR-negative, a feature of breast cancer cells with more invasive and motile phenotype [32–34]. FACS analysis (Figure 2A) and western blot (Figure 2A) demonstrate that these cells are fascin positive. To study fascin function in MDA-MB-231 cells, we transiently down-regulated its expression using fascin-specific siRNA (SiFascin). FACS histogram (Figure 2A) shows that 60–70% of the SiFascin treated cells became fascin-negative compared with the control-siRNA (SiCon) treated group. The mean florescence intensity (MFI) of SiFascin-treated cells was also reduced by greater than 60%. Similar to the FACS data, western blot indicated 70% inhibition of fascin in the knockdown cells (Figure 2A). Inhibition of the house keeping gene (GAPDH) using specific siRNA was used as positive control in all of the assays (data not shown).

We examined whether fascin expression in human breast cancer cells induces morphological changes, as we have previously observed in mouse immune cells [22]. In culture, fascin-knockdown cells lost their typical/normal spindle shape that is seen in the control cells as shown by phase contrast inverted microscope (Figure 2B). Furthermore, immunohistochemistry staining demonstrated that while the control cells expressed fascin and have a spindle shape with fine dendrites, fascin-knockdown cells expressed lower fascin levels and exhibited more rounded morphology (Figure 2B Top). Immunoflorescent staining of
SiFascin cells demonstrated these alterations in cell morphology and showed changes in the distribution of F-actin (Figure 2B Bottom). Fascin expression had no effect on MDA-MB-231 cell proliferation as assessed using the cell proliferation reagent WST1 and CSFE dye (data not shown).

We examined if fascin-mediated morphological changes affect breast cancer cell motility. Migration of the fascin-knockdown cells was significantly (P<0.001) inhibited by more than 50% compared with control cells (Figure 2C). Furthermore, SiFascin cells were also less migratory after 72 hours using the traditional wound healing assay (Figure S2). Difference in wound closure was not a reflection of cell division as similar number of cells was seeded and became confluent at the same time before the wound was made.

To specifically link fascin expression to enhanced migration, we have generated MDA-MB-231 cells stably over-expressing wild-type (WT) or mutant fascin. Contrary to fascin-knockdown data, over-expression of WT (Figure S3) and not mutant fascin significantly (P = 0.01) enhanced MDA-MB-231 cell migration (Figure 2D). Collectively, our data shows that fascin expression in breast cancer cells regulates their morphology and migratory potential.

Fascin Regulates Breast Cancer Invasion

Fascin involvement in mediating breast cancer metastasis was then tested using a well-established invasion assay. Global actin polymerization inhibitor (Cytochalasin D), at a dose that did not effect cell viability (data not shown), altered MDA-MB-231 cell invasion at 72 hours using the traditional wound healing assay (Figure S2). Difference in wound closure was not a reflection of cell division as similar number of cells was seeded and became confluent at the same time before the wound was made.

To specifically link fascin expression to enhanced migration, we have generated MDA-MB-231 cells stably over-expressing wild-type (WT) or mutant fascin. Contrary to fascin-knockdown data, over-expression of WT (Figure S3) and not mutant fascin significantly (P = 0.01) enhanced MDA-MB-231 cell migration (Figure 2D). Collectively, our data shows that fascin expression in breast cancer cells regulates their morphology and migratory potential.

Table 1. *Correlation between fascin expression in tumor cells and the clinico-pathological parameters of 71 breast cancer patients.

| Parameters          | Fascin         | χ²     | P    |
|---------------------|---------------|--------|------|
| **Age**             |               |        |      |
| <40 years           | 16 *(76)      | 5 (24) | 0.575|
| ≥40 years           | 33 (66)       | 17 (34) |      |
| **Tumor Size**      |               |        |      |
| <4 CM               | 31 (79)       | 8 (21) | 0.035|
| ≥4 CM               | 18 (56)       | 14 (44) |     |
| **Histological Grade (SBR)** | |        |      |
| I                   | 1 (30)        | 1 (50) | 0.091|
| II                  | 27 (82)       | 6 (18) |      |
| III                 | 21 (58)       | 15 (42) |     |
| **Her2/neu status** |               |        |      |
| 0                   | 23 (68)       | 11 (32) | 0.072|
| 1                   | 0 (0)         | 2 (100) |     |
| 2                   | 10 (31)       | 8 (33) |      |
| 3                   | 16 (67)       | 8 (33) |      |
| **ER status**       |               |        |      |
| Negative            | 9 (39)        | 14 (61) | <0.001|
| Positive            | 70 (83)       | 5 (17) |      |
| **PR status**       |               |        |      |
| Negative            | 20 (56)       | 16 (44) | 0.020|
| Positive            | 29 (83)       | 6 (17) |      |
| **B7H1**            |               |        |      |
| Negative            | 49 (72)       | 19 (28) | 0.008|
| Positive            | 0 (0)         | 3 (100) |     |
| **Basel**           |               |        |      |
| Negative            | 46 (77)       | 14 (23) | <0.001|
| Positive            |               |        |      |

*When interpreting data and correlating it with clinico-pathological parameters, the 5% expression of cells was the cut-off point below which were considered as negative and above as positive.

*S*Numbers between brackets are the percentages of patients,

*1* Patients were reported as no show or dead,

*P* values in bold represent a significant data.

doi:10.1371/journal.pone.0027339.t001

Figure 1. Fascin is associated with disease-free survival in human breast cancer samples. Survival with or without the disease was reported by oncologist and blotted in relation to fascin expression. Survival curves showing decreased disease-free (A) or overall (B) survival in patients that have fascin positive tumor.

doi:10.1371/journal.pone.0027339.g001
altered morphology compared with control cells (Figure 3B). On
the contrary, over-expression of WT and not mutant fascin
in MDA-MB-231 cells significantly (P = 0.003) enhanced their
invasive capability (Figure 3C). Interference with fascin expression
in MDA-MB-231 had no effect on cell proliferation or survival
(data not shown). Collectively, these data demonstrate that fascin
expression in breast cancer cells is strongly associated with
enhanced cell motility and invasiveness.

Fascin inhibits nuclear expression of BRMS1 in breast
cancer cells

BRMS1 is a known metastasis suppressor in many cancer types
including breast cancer [35]. We examined whether fascin-
mediated breast cancer cell invasion has an effect on the
expression levels and cellular localization of BRMS1. SiFascin
breast cancer cells showed significantly enhanced BRMS1 RNA
(Figure 4A) and protein (Figure 4B left) expression compared with
SiCon, consistent with the role of fascin in enhancing breast
cancer metastatic potential. Conversely, over-expression of WT
fascin in MDA-MB-231 dramatically suppresses BRMS1 expres-
sion (Figure 4B right), further confirming the inverse relationship
between these two proteins. BRMS1 expression was found to be
predominantly in the nucleus and fascin-knockdown clearly leaded
to enhanced expression of nuclear BRMS1 (Figure 4C). Exam-
ining the relationship between BRMS1 and fascin in our breast
cancer patient samples demonstrated variations in the intensity
and subcellular distribution of BRMS1 (Figure 4D). Most
importantly, 36 patients that were scored as having high levels
of nuclear BRMS1 (≥50% of tumor cells express high levels
(intensity of +3) of nuclear BRMS1) showed reduced levels of
fascin (Figure 4E), demonstrating an inverse relationship between
fascin and nuclear BRMS1 that is statistically significant
(P<0.001). This data demonstrates that fascin can directly or
indirectly regulate the tumor suppressor BRMS1 nuclear expres-
sion.

Fascin enhances metastasis-associated genes

We tested whether fascin enhances metastasis via counteracting
BRMS1 effect on key downstream mediators that are involved in
this process such as uPA [12]. Consistent with enhanced invasion,
cells that over-expressed WT and not mutant fascin showed higher
levels of uPA expression (Figure 5A). To directly link invasion to
well established mediators of metastasis, we blocked activity of
MMP-2 and MMP-9, which are activated by uPA [9] and are
among the most well established proteases known to degrade
ECM and facilitate invasion and metastasis [36–38]. MMP-2 and
MMP-9 selective inhibitors significantly (P = 0.013) reduced the
invasion of control cells to comparable level with the fascin-
knockdown cells (Figure 5B), implicating a role for these proteases

Figure 2. Fascin knockdown alters cell morphology and
reduces cell migration. MDA-MB-231 cells were treated with control
(SiCon) or Fascin (SiFascin) SiRNA for 72 hours. A) Cells were then
collected and stained for fascin before detection by flow cytometry. B)
Top: Immunohistochemistry showing cell morphology and fascin
expression after treatment with SiCon or SiFascin in 8-well chamber.
Bottom: Immunofluorescent staining showing the altered morphology
and distribution of F-actin (red) in cells after treatment with SiFascin in
8-well chamber. Blue color indicates nuclear stain (Dabi). C) Bar graph
showing reduced migration in fascin knockdown MDA-MB-231 cells.
Data was normalized to SiCon cells and the relative migration is
expressed as mean ± SD of triplicate experiments. D) Bar graph
showing enhanced cell migration in MDA-MB-231 cells that over-
express wild type (WT) fascin compared with mutant (M) over-
expressing cells. Data was normalized to parental MDA-MB-231 and
the relative migration is expressed as mean ± SD of triplicate
experiments.
doi:10.1371/journal.pone.0027339.g002
In fascin-mediated invasion, Cytochalasin D, which significantly diminished the cell invasion capability (Figure 3A), also inhibited the expression of MMP-2 and MMP-9 (Figure S4).

Since TNF-α was shown to trigger uPA expression in MDA-MB-231 cells [12], we activated our cells with TNF-α to further test the link between fascin and uPA secretion in the supernatants. Maximum induction of uPA RNA was seen at 6 hours post TNF-α stimulation in fascin-positive breast cancer cells, while fascin-knockdown cells failed to induce significant up-regulation compared with the control cells (Figure 5C). uPA protein secretion in control cells was detected in the supernatant 6 hours post TNF-α stimulation and notable reduction was observed in fascin-knockdown cells 12 hours post activation (Figure 5D). In parallel, MMP9 was also detected in control cells 6 hours post TNF-α stimulation and only detected at reduced levels 12 hours post TNF-α stimulation of fascin-knock down cells. Furthermore, reduced MMP-2 protein expression in fascin-knockdown cells that was observed above is in line with decreased TNF-α-mediated enzymatic activity in gelatin zymograms (Figure 5E).

**Fascin enhanced NF-κB activity**

Since uPA promoter was reported to contain an NF-κB binding site [39], we asked if fascin enhanced uPA secretion after TNF-α activation is mediated via enhancement of NF-κB activity. Inhibition of the NF-κB pathway significantly reduced cell invasion in a dose-dependent manner (Figure 6A), implicating the importance of NF-κB in this process. Importantly, fascin-knockdown cells showed significantly (P = 0.040) impaired NF-κB luciferase activity upon TNF-α activation when compared with control cells (Figure 6B). In contrast, cells that over-expressed WT fascin exhibited significantly (P = 0.002) enhanced NF-κB luciferase activity upon TNF-α activation (Figure 6C), indicating that fascin positively regulates NF-κB transcriptional activity. No NF-κB transcriptional activities was observed upon TNF-α activation, when mutant NF-κB-luciferase reporter was used (data not shown). Collectively, our data strongly demonstrated that fascin suppressed BRMS1 and enhanced NF-κB activity.

Phosphorylation of the inhibitory subunit (IκBα) leads to liberation and translocation of NF-κB to the nucleus [40,41]. We thus examine whether the observed fascin mediated NF-κB transcriptional activity is related to enhanced NF-κB nuclear translocation. The cytoplasmic levels of total IκBα were reduced in control and fascin knockdown cells in response to activation (Figure 7A). However, the magnitude of IκBα inhibition was more pronounced in the control cells especially at earlier time points (Figure 7B). There were a time dependent increase in IκBα phosphorylation in control cells after activation, which was severely reduced in the fascin-knockdown cells (Figure 7 A and C). The effect of fascin on IκBα phosphorylation and degradation have also been observed in another breast cancer cell line (T47-D). Expression of wild type fascin in T47-D cells, which are fascin negative (Figure S3A), reduced BRMS1 expression (Figure S3B). Interestingly, fascin expressing T47-D cells demonstrated reduced total levels of IκBα and enhanced magnitude of IκBα phosphorylation in response to activation especially at later time points (Figure 8A).

To test whether reduced IκBα phosphorylation in fascin knockdown cells would affect the phosphorylation of p65 and translocation of p65/p50 to the nucleus, cytoplasmic and nuclear fractions were extracted from cells after activation and the level of these proteins and the status of phosphorylation were evaluated. TNF-α activation induced time-dependent p65/p50 translocation into the nucleus in control and fascin knockdown cells (Figure 7 D, E and F). However, the levels of p65/p50 translocation into the nucleus in fascin knockdown cells were notably lower especially at the earlier time points, indicating less sustainable p65 nuclear translocation in fascin knockdown cells. There were also time-
dependent increases of p65 phosphorylation in control and fascin knockdown cells. However, the magnitude of induction of p65 phosphorylation in fascin knockdown cells was notably lowered (Figure 7 D and G). In T47-D cells, TNF-α activation triggered early p65 translocation into the nucleus which declined after 30 minutes (Figure 8B). Nuclear translocation of p65 in response to TNF-α activation was more pronounced in the fascin expressing T47-D cells. Most importantly, the nuclear translocation of p65 after 30 minutes was more sustainable in fascin expressing T47-D cells. Furthermore, there were also time-dependent increases of p65 phosphorylation in fascin expressing T47-D cells, which was not seen in T47-D parental cells till 24 hours. Collectively, our data demonstrated that fascin positively regulate NF-κB nuclear translocation and transcriptional activity.

Discussion

Metastasis and not the primary tumors remained the main causes of cancer mortalities [1,2], stressing the need to understand the cellular and molecular mechanisms that regulate this process. It is a complex process where cytoskeletal proteins were reported to regulate multiple cellular processes including morphological changes and motility, which are critical steps for metastasis (Reviewed in [42]). In this study, we have shown an association between the actin-bundling protein (fascin) and expression of bad prognosis, metastasis and reduced disease-free survival. Our in vitro data demonstrated fascin involvement in regulation of breast cancer cell invasiveness and identifies some of the underlying molecular mechanisms.

Breast cancers mainly arise from the luminal compartment of the breast [43], but a small minority of tumors arise from multi-potent progenitor cells, which can differentiate into luminal or myoepithelial lineages [44,45]. The aggressive breast cancer with a basal-like phenotype is triple negative (ER, PR and HER2) and expresses both luminal and myoepithelial markers [46]. Our breast cancer patients showed an association between fascin expression and basal-like phenotype and high histological grade tumors, a type of breast cancers that are associated with metastasis [46,47]. Furthermore, our patients showed an association between fascin expression, increase metastasis and shorter disease-free survival. Our in vitro data demonstrated fascin involvement in regulation of breast cancer cell invasiveness and identifies some of the underlying molecular mechanisms.

Breast cancers mainly arise from the luminal compartment of the breast [43], but a small minority of tumors arise from multi-potent progenitor cells, which can differentiate into luminal or myoepithelial lineages [44,45]. The aggressive breast cancer with a basal-like phenotype is triple negative (ER, PR and HER2) and expresses both luminal and myoepithelial markers [46]. Our breast cancer patients showed an association between fascin expression and basal-like phenotype and high histological grade tumors, a type of breast cancers that are associated with metastasis [46,47]. Furthermore, our patients showed an association between fascin expression, increase metastasis and shorter disease-free survival. Our in vitro data demonstrated fascin involvement in regulation of breast cancer cell invasiveness and identifies some of the underlying molecular mechanisms.

Discussion

Metastasis and not the primary tumors remained the main causes of cancer mortalities [1,2], stressing the need to understand the cellular and molecular mechanisms that regulate this process. It is a complex process where cytoskeletal proteins were reported to regulate multiple cellular processes including morphological changes and motility, which are critical steps for metastasis (Reviewed in [42]). In this study, we have shown an association between the actin-bundling protein (fascin) and expression of bad prognosis, metastasis and reduced disease-free survival. Our in vitro data demonstrated fascin involvement in regulation of breast cancer cell invasiveness and identifies some of the underlying molecular mechanisms.

NF-κB pathway is constitutively activated in many cancers, which leads to enhancement of metastasis (reviewed in [48]) and positively regulates the expression of uPA [39], which in turn can activate the MMPs to facilitate invasion by degrading the ECM [13]. BRMS1 negatively regulates uPA expression through inhibition of the NF-κB activity in breast cancer and melanoma cells [12]. Consistent with those findings, our data showed that fascin expression in breast cancer cells, which enhances invasion,
suppresses BRMS1 and counteract its effect on downstream targets by increasing NF-κB activity, uPA secretion and MMP enzymatic activity. Although we could not conclude whether fascin suppression of BRMS1 is directly responsible for the inhibition of BRMS1 downstream targets, we demonstrated that fascin regulates MMPs expression and activation, key molecules that facilitate invasion and metastasis. Furthermore, TNF-α stimulation was shown to induce over-expression of fascin that in turn up-regulate MMP-9 expression in cholangiocarcinoma [49].

![Figure 5. Fascin enhanced uPA, MMP-2 and MMP-9 expression.](image)

A) Western blot showing increased expression of uPA in MDA-MB-231 cells over-expressing WT fascin compared with the mutant over-expressing cells. B) Bar graph showing reduced invasion of SiCon-treated cells when MMP-2/MMP-9 inhibitor II (20 μM) was used. Data was normalized to untreated SiCon cells and the relative invasion is expressed as mean ± SD of triplicate experiments. C) Bar graph showing reduced RNA expression of uPA in fascin knockdown cells as assessed by real time PCR. D) Western blot showing reduced uPA (top) or MMP-9 (bottom) secretion in supernatants of fascin knockdown cells. E) MMP-2 gelatinolytic activity showing reduced enzymatic activity of secreted MMP-2 in the supernatants of fascin knockdown cells.

doi:10.1371/journal.pone.0027339.g005

![Figure 6. Fascin enhanced NF-κB-dependent transcriptional activity.](image)

A) Bar graph showing inhibition of MDA-MB-231 cell invasion that were treated with NF-kB inhibitor. Data was normalized to untreated cells and the relative invasion is expressed as mean ± SD of triplicate experiments. B) ShCon or ShFascin MDA-MB-231 cells were co-transfected with the NF-kB luciferase promoter and Renilla promoter as in methods. Cells were stimulated with or without (20 ng/ml) TNF-α and luciferase activity was assessed as in methods. C) MDA-MB-231 parental or cells that over-express WT or mutant fascin were co-transfected with the NF-kB luciferase promoter and Renilla promoter as in methods. Cells were stimulated with or without (20 ng/ml) TNF-α and luciferase activity was assessed as in methods.

doi:10.1371/journal.pone.0027339.g006
While our data demonstrated a suppression of BRMS1 by fascin, the exact mechanism by which this mediated has not been elucidated. Interestingly, previous study reported down-regulation of fascin when they expressed BRMS1 in a human ovarian carcinoma, but the supporting data was not presented [50]. Both Zhang et al and our findings support the existence of a direct or indirect interaction between fascin and BRMS1. Fascin is mainly a cytoplasmic protein with higher expression at the submembrane and around the nuclear membrane as shown in our work and reviewed by Kureishy N et al [51]. Although BRMS1 is predominantly nuclear, it is expressed in the cytoplasm as shown by our data and by Frolova N et al [52]. It is therefore possible that there is an active shuttling of this molecule between the cytoplasm and the nucleus, where its translocation is controlled by direct or indirect interaction with fascin molecules.

Our study showed that fascin acts at several cellular fronts to facilitate cell invasion. It may act by negatively regulating the expression of BRMS1, thereby enhancing NF-κB activity and subsequent augmentation of uPA and MMPs expression. It is also possible that fascin and BRMS1 have a feedback loop where BRMS1 may also down-regulate fascin to inhibit cancer cells from metastasis. This assumption is supported by a study where BRMS1 was demonstrated to down-regulate fascin expression in human ovarian carcinoma cell line without showing data [50], and concluded that this could be a potential mechanism underlying BRMS1 suppression of metastasis. Alternatively, fascin may act via yet unknown pathway to positively regulate NF-κB transcriptional activity, which in turn enhances the expression of uPA and MMPs.

This study demonstrates a clear role for fascin in regulating breast cancer invasion partially through modifying the expression

Figure 7. Fascin enhanced NF-κB nuclear translocation in MDA-MB-231. A) Western blot showing reduced phosphorylation of IKBα in response to TNF-α stimulation in fascin knockdown cells. B and C) Bar graph showing quantitation of total and phosphorylated IKBα in ShCon and ShFascin cells after stimulation with TNF-α for the indicated time. Results showed the mean of triplicate experiments after normalization to actin and each time point is normalized to 0 time. D) Western blot showing reduced nuclear translocation and phosphorylation of p65 in response to TNF-α stimulation in Fascin knockdown cells. E-G) Bar graph showing quantitation of nuclear P50 and P65 and phosphorylated P65 in ShCon and ShFascin cells after stimulation with TNF-α for the indicated time. Results showed the mean of triplicate experiments after normalization to PCNA and each time point is normalized to 0 time.

doi:10.1371/journal.pone.0027339.g007
of metastasis-associated genes, making fascin a good target for therapeutic intervention in metastatic breast cancer cells. The relevance of our finding becomes more significant after the recent finding of a synthetic compound (migrastatin) that specifically targets fascin and inhibits its metastasis-mediated activity [53]. Identifying the specific molecular mechanism of fascin regulation of breast cancer invasion and metastasis will have a great impact on designing a better therapy for breast cancer metastasis.

Supporting Information

Figure S1  A) Insert is the western blot showing fascin (top) or GAPDH (bottom) in MDA-MB-231 cells after treatment with SiCon and SiFascin and bar graph showing the level of expression of fascin after normalization to GAPDH as assessed by densitometry. B) Morphology of MDA-MB-231 cells in culture after treatment with SiCon and SiFascin.

Figure S2  A) Wound healing assay showing wound healing impairment in SiFascin treated cells compared with the SiCon cells. B) Bar graph showing measurements of the wound size in ShCon and ShFascin MDA-MB-231 cells at the indicated time points. Measurements were collected from 3 different sites and normalized to the size at 0 time.

Figure S3  Flow cytometry histogram showing increase MFI of fascin in MDA-MB-231 over-expressing wild type (WT; gray line) compared with the parental cells (MDA-MB-231; black line).

Figure S4  Western blot showing reduced expression levels of MMP-2 and MMP-9 in MDA-MB-231 cells after treatment with Cytochalasin D (20 μM) for 30 minutes. Cells were washed and cultured for over night prior to harvesting for western blot.

Figure S5  A) FACS dot plot showing fascin expression in T47-D cells after transfection with wild type GFP-fascin. Cells were then stained with APC-labeled anti-fascin antibody to confirm fascin expression in GFP-positive cells. B) Western blot showing reduced expression of BRMS1 in fascin expressing T47-D cells.

Table S1  Table showing the sequences of forward and reverse primers as well as the probes of the different genes that were used in this experiment.

Acknowledgments

The authors are grateful to the administration of Research Centre and Research Affair Council for their support. The authors are grateful to Dr.
References

1. Bashyam MD (2002) Understanding cancer metastasis: an urgent need for using differential gene expression analysis. Cancer 94: 1021–1029.

2. Shambars AF, Namvar GN, Varghese HJ, Naidakuri KV, MacDonald IC, et al. (2001) Critical steps in hematogenous cancer metastasis: an overview. Surg Oncol Clin N Am 10: 243–255, vii.

3. Fidler IJ (2001) Seed and soil revisited: contribution of the organ microenvironment to cancer metastasis. Surg Oncol Clin N Am 10: 197–208.

4. Nicolson GL (1988) Origin specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. Cancer Metastasis Rev 7: 135–188.

5. Saaristo A, Karpunen T, Alitalo K (2000) Mechanisms of angiogenesis and their use in the inhibition of tumor growth and metastasis. Oncogene 19: 6122–6129.

6. Woodhouse EC, Chuanqi RF, Liotta LA (1997) General mechanisms of tumor metastasis. Cancer 80: 1529–1537.

7. Smith SC, Theodorescu D (2009) Learning therapeutic lessons from metastasis suppressor proteins. Nat Rev Cancer 9: 253–264.

8. Kim J, Yu W, Kovaski K, Ossowski L (1998) Requirement for specific proteases in invasion of prostate cancer cells. J Biol Chem 273: 27440–27445.

9. Mignatti P, Rifkin DB (1993) Biology and biochemistry of proteinases in tumor invasion. Physiol Rev 73: 161–193.

10. Seraj MJ, Samant RS, Verderame MF, Welch DR (2000) Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13. Cancer Res 60: 2764–2769.

11. Hicks DG, Yoder BJ, Short S, Tarr S, Prescott N, et al. (2006) Loss of breast cancer metastasis suppressor 1 protein expression predicts reduced disease-free survival in subsets of breast cancer patients. Clin Cancer Res 12: 6702–6708.

12. Ciciek M, Fujiyama R, Welch DR, Sizemore N, Casey G (2005) Breast cancer metastasis suppressor 1 inhibits gene expression by targeting nuclear factor-kappaB activity. Cancer Res 65: 3536–3539.

13. Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Nielsen LS, et al. (1985) Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 44: 139–286.

14. Jiang P, Endo M, Takahashi M (2000) Cell biology of the movement of breast cancer cells: Intracellular signalling and the actin cytoskeleton. Cancer Lett 149: 123–133.

15. Edwards RA, Bryan J (1995) Fascins, a family of actin bundling proteins. Cell 80: 667–678.

16. Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Nielsen LS, et al. (1985) Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 44: 139–286.

17. Mosialos G, Birkenbach M, Ayeunie S, Matsumura F, Pinkus GS, et al. (1996) Circulating human dendritic cells differentially express high levels of a 53-kd actin-binding protein. Am J Pathol 148: 503–600.

18. Mosialos G, Yamashiro S, Baughman RW, Matsudaira P, Varia L, et al. (1994) Epstein-Barr virus infection induces expression in B lymphocytes of a novel gene encoding an evolutionarily conserved 53-kilodalton actin-binding protein. J Virol 68: 7320–7328.

19. Pinkus GS, Pinkus JL, Langhoff E, Matsumura F, Yamashiro S, et al. (1997) Fascin, a sensitive new marker for Reed-Sternberg cells of Hodgkin's disease. Evidence for a dendritic or B cell derivation? Am J Pathol 150: 343–352.

20. Yamashiro S, Yamakita Y, Otsu S, Matsumura F (1998) Fascin, an actin-binding protein, increases membrane protrusions and increases cell motility of normal human keratinocytes. Mol Cell Biol 9: 993–1006.

21. Yoder BJ, Too T, Slack CM, Petit J, Tarr S, et al. (2005) The expression of fascin, an actin-binding protein, correlates with hormone receptor-negative breast cancer and a more aggressive clinical course. Clin Cancer Res 11: 186–192.

22. Al-Ahwan MM, Rowden G, Lee TD, West KA (2001) Fascin is involved in the antigen presentation activity of mature dendritic cells. J Immunol 166: 333–345.

23. Tran J, Kung SK (2007) Lentinular vectors mediate stable and efficient gene delivery into primary and primary marine natural killer cells. Mol Ther 15: 1331–1339.

24. Adam MJ, Schwartz MA (2000) Stimulation of fascin spines by thrombospondi-1 is mediated by the GTPases Rac and Cdc42. J Cell Biol 150: 807–822.

25. Liviak KJ, Schmittin TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 492–498.

26. Wang W, Furneaux H, Cheng H, Caldwell MC, Hutter D, et al. (2000) HuR regulates p21 mRNA stabilization by UV light. Mol Cell Biol 20: 760–769.

Author Contributions

Conceived and designed the experiments: MAA. Performed the experiments: SO HG EB. Analyzed the data: AT TAT DA. Wrote the paper: MAA. Obtained fund for SO and provided administrative and materials support: CA.
51. Kureishy N, Sapountzi V, Prag S, Anilkumar N, Adams JC (2002) Fascins, and their roles in cell structure and function. Bioessays 24: 350–361.
52. Frolova N, Edmonds MD, Bodenstine TM, Seitz R, Johnson MR, et al. (2009) A shift from nuclear to cytoplasmic breast cancer metastasis suppressor 1 expression is associated with highly proliferative estrogen receptor-negative breast cancers. Tumour Biol 30: 148–159.
53. Chen L, Yang S, Jakoncic J, Zhang JJ, Huang XY (2010) Migastatin analogues target fascin to block tumor metastasis. Nature 464: 1062–1066.