



β 1 Integrin is essential for fascin-mediated breast cancer stem cell function and disease progression

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Breast cancer remains the second cause of tumor-related mortality in women worldwide mainly due to chemoresistance and metastasis. The chemoresistance and metastasis are attributed to a rare subpopulation with enriched stem-like characteristics, thus called Cancer Stem Cells (CSCs). We have previously reported aberrant expression of the actin-bundling protein (fascin) in breast cancer cells, which enhances their chemoresistance, metastasis and enriches CSC population. The intracellular mechanisms that link fascin with its downstream effectors are not fully elucidated. Here, loss and gain of function approaches in two different breast cancer models were used to understand how fascin promotes disease progression. Importantly, findings were aligned with expression data from actual breast cancer patients. Expression profiling of a large breast cancer dataset (TCGA, 530 patients) showed statistically significant correlation between fascin expression and a key adherence molecule, β 1 integrin (ITGB1). *In vitro* manipulation of fascin expression in breast cancer cells exhibited its direct effect on ITGB1 expression. Fascin-mediated regulation of ITGB1 was critical for several breast cancer cell functions including adhesion to different extracellular matrix, self-renewability and chemoresistance. Importantly, there was a significant relationship between fascin and ITGB1 co-expression and short disease-free as well as overall survival in chemo-treated breast cancer patients. This novel role of fascin effect on ITGB1 expression and its outcome on cell self-renewability and chemoresistance strongly encourages for dual targeting of fascin-ITGB1 axis as a therapeutic approach to halt breast cancer progression and eradicate it from the root.

Introduction

Despite enormous efforts made toward increasing awareness, encouraging early screening and supporting the development of more effective cancer therapeutics, breast cancer-related mortality remains high. It accounts for almost a quarter of all types of female malignancies in Saudi Arabia.¹ In spite of

advancements in surgical, biological and chemotherapeutic treatment alternatives, a high mortality rate amongst breast cancer patients still persists. This has been mainly due to residual cancer cells that resist chemotherapy and remain in a quiescence state until triggered, where they have the potential to regrow and metastasize to distant organs.² This rare cell

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What's new?

Residual cancer stem cells (CSCs) have the ability to regrow tumors and to metastasize to distant organs, resulting in disease relapse and increased cancer mortality. In breast cancer, CSC populations are enriched by aberrant expression of the actin-bundling protein fascin, induction of which is also associated with chemoresistance and metastasis. In this study, fascin was found to upregulate $\beta 1$ integrin (ITGB1) expression, an effect that proved critical to breast cancer cell adhesion and self-renewal. Coexpression of fascin and ITGB1 was associated with decreased survival in chemotherapy-treated breast cancer patients. The findings identify the fascin-ITGB1 axis as a potential therapeutic target.

population is called cancer stem cells (CSCs), because they hijack the pathways and cellular mechanisms that normal stem cells (NSCs) utilize.³ Indeed, enriched CSCs were shown to be highly resistant to chemotherapy and more metastatic.^{4,5}

Increased cell motility that enhances the metastatic potential of cells was shown to be dependent on active actin cytoskeletal rearrangements.⁶ We have demonstrated that fascin, an actin-bundling protein that is induced in many neoplasms like breast cancer,⁷ can directly enhance the expression of metastasis-associated genes that augment metastasis and result in shorter survival.⁸ In a subsequent study, we have illustrated that fascin expression in breast cancer cells confers resistance to chemotherapy through activation of the PI3K/Akt signaling pathway.⁹ Both metastasis and chemoresistance are key features of CSCs. Indeed, we have demonstrated that fascin is a direct regulator of breast CSCs *via* the activation of the Notch self-renewal pathway.¹⁰ Therefore, a better understanding of the mechanism by which fascin regulates breast CSCs to confer chemoresistance and metastasis will encourage the development of more therapeutic targets and would impact the success rate of treatment.

One of the main factors that control cancer cell fate and response to therapy is their interaction and communication with adjacent cells or extracellular matrix (ECM). Integrins are a family of heterodimer (α and β) transmembrane adhesion proteins that promote cancer survival, proliferation and motility through regulation of adhesion molecule expression and signal transmission *via* their downstream targets. Through these signals, integrins provide support and trigger a cascade of events that regulate cell-cell and cell-ECM interaction *via* transcriptional modification and cytoskeletal rearrangements.¹¹ Integrins play an important role in maintaining the mammary stem cells in normal breast, where deletion of integrin such as $\beta 1$ impairs mammary gland development and function.¹² On the other hand, dysregulation of integrin signaling distorts cell-cell or cell-ECM interaction and promotes breast cancer progression *via* induction of chemoresistance and metastasis,¹³ two important functions of CSCs. Indeed, increased expression of integrins in cancer was reported on tumor-initiating cells and was used as markers for isolation of normal and neoplastic stem/progenitor cells (reviewed in¹⁴).

Here, we have demonstrated that fascin enhances the expression of $\beta 1$ integrin (ITGB1) in breast cancer cells and augments their adhesion to different ECM substrates. Furthermore, fascin-mediated ITGB1 expression promotes breast

self-renewability and chemoresistance. In breast cancer samples, fascin significantly correlated with ITGB1 expression and their co-expression was significantly associated with shorter disease-free and overall survival. This is the first study to demonstrate that fascin-mediated ITGB1 expression present an axis of evil that is critical for the maintenance of breast CSCs. Therefore, better understanding of how fascin/ITGB1 axis works may present excellent therapeutic targets for eradication of breast cancer from the root.

Materials and Methods**Cell culture**

The breast cancer cell lines MDA-MB-231 (HTB-26) and T-47D (HTB-133) were purchased from ATCC (Manassas, VA). All cells were cultured in DMEM media (Sigma) supplemented with 10% FBS and 1% Antibiotic Anti-mycotic (ABM) and 1% L-Glutamine (Gibco). Cells were incubated at 37°C, 5% CO₂ humidified incubator and single cells were harvested using the appropriate amount of 0.25% Trypsin EDTA (Gibco). Mycoplasma screening was routinely performed using a PCR-based kit (Intron, Korea) on each cell line to continuously ensure that the cells are mycoplasma-free.

Stable and transient transfection

Generation of stable fascin knockdown in the naturally fascin-positive MDA-MB-231 cells was previously described.⁸ Scrambled shRNA denotes fascin⁺, while fascin shRNA denotes fascin⁻ cells. Furthermore, fascin was ectopically expressed in the naturally fascin-negative T-47D cells or rescued in the fascin-knockdown (fascin⁻) MDA-MB-231 cells using lentiviral particles (Genecopoeia, Rockville, MD USA) expressing either fascin ORF (FORF; LP-D0369-Lv105-0205-S) or control ORF (NORF; LP-NEG-LV105-02000205). Scrambled shRNA (ShCon; sc-108,080) or ITGB1 shRNA (ShITGB1; sc-35,674-V) lentiviral particles were obtained from Santa Cruz Biotechnology, Inc (Dallas, TX). Stable fascin-expressing T-47D and stable ITGB1 knockdown MDA-MB-231 cells were generated as per manufacturers' transduction protocols. The efficiency of knockdown, expression or overexpression was confirmed using flow cytometry and western blot.

For transient integrin knockdown, siRNA for ITGB1 (siITGB1) was purchased from Ambion-Thermo Scientific Fisher (Waltham, MA, USA) and cells were transfected as per the manufacture recommendation. In brief, the transfection complex was prepared by mixing siRNA in siPORT transfection agent in

serum-free Opti-MEM media. The recommended cell numbers, which were previously prepared in serum-free Opti-MEM media, were overlaid on the transfection complex and mixed by tilting the plate. The silencing efficiency was assessed by flow cytometry and was determined at 48–72 h of incubation.

Reagents

Monoclonal mouse anti-human fascin primary antibody was purchased from Dako. The secondary goat anti-mouse IgG1 antibody (APC) was purchased from Jackson ImmunoResearch Labs. The purified anti-ITGB1 antibodies for immunohistochemistry (clone EPR1040Y), western blot (clone P5D2) or the APC-labeled anti-ITGB1 antibody for FACS were purchased from GeneTex (Irvine, CA), Abcam (Cambridge, MA) and BD Biosciences (San Jose, CA), respectively.

The chemotherapy drug used in the assays (doxorubicin) and focal adhesion kinase (FAK) inhibitor were obtained from Sigma (St Louis, MO) and the stock solutions were diluted to desired concentrations in culture medium.

Flow cytometry and cell sorting

Staining of all cell surface and intracellular (fascin) molecules was performed as previously described.¹⁰ Spontaneous or chemotherapy-mediated cell apoptosis was analyzed using Annexin V staining kit (Life Technologies) as previously described.⁹ A minimum of 10^4 live cells per sample was collected and analyzed using flow cytometry (LSR II; Becton Dickinson, Mountain View, CA).

Cell sorting approach was used to select for ITGB1^{high} and ITGB1^{low} expressing cells from the fascin⁺ and fascin⁻ live cells (DAPI-negative). Cells were gated and sorted based on the expression levels (MFI) of ITGB1, where the top 5–10% and the bottom 5–10% were considered ITGB1^{high} and ITGB1^{low} population, respectively. FACSaria[®] (Becton Dickinson, Mountain View, CA) was used for cell sorting and the number of cells sorted depended on the requirement of the assay.

Stable ITGB1 knockdown (ShITGB1) and control (ShCon) were generated in the MDA-MB-231 cells that were previously transfected with ShFascin (fascin⁻) or ShCon (fascin⁺). ITGB1 knockdown cells were selected by multiple sorting for at least six consecutive rounds. The efficiency of the ITGB1 knockdown was routinely confirmed by flow cytometry and western blot.

Western blot

Cells lysates were prepared from the different cell pellets using RIPA buffer containing 100x protease and phosphatase inhibitor as previously described.⁸ Briefly, proteins were quantified using Bradford method (Bio-Rad, Hercules, CA) and an optimized concentration of protein samples was loaded on a 10% SDS-PAGE gel followed by transfer using iBlot pre-made PVDF membrane and dry transfer system (Bay Cities Tool & Supply Inc., Newark, CA, USA). Membranes were blocked, washed and incubated with the desired primary antibody for overnight before incubation with an HRP-conjugated

secondary antibody for 45 min. The bounded antibodies were detected using chemiluminescence Super Signal System (Thermo Scientific) and were captured on ImageQuant LAS4010 Biomolecular Imager (GEHealthcare, Pittsburgh, PA). Imager bands were quantified and analyzed using the QuantityOne Software.

Patients' selection

Breast cancer specimens (137 patients) were obtained from archived paraffin-embedded tumors of patients with local breast cancer at presentation who received adjuvant chemotherapy and undergo a tumor excision surgery. Treatment protocol was previously described.⁹ A waiver of consent were obtained from the King Faisal Specialist Hospital and Research Centre institutional board under RAC# 2160 011.

Immunohistochemistry

Formalin-fixed, paraffin-embedded breast cancer sections of 137 patients were deparaffinized and rehydrated as previously described.⁹ Antigen retrieval was done in citrate solution pH 6 (Dako) for 10 min using pressure cooker. Sections were incubated with 3% hydrogen peroxide (Sigma) in methanol to block endogenous peroxidase followed by 60 min incubation with 10% goat serum (Sigma). Sections were then incubated with anti-ITGB1 antibody for overnight at 4°C. The slides were washed before incubation with labeled Polymer (EnVision +) HRP detection kit (Dako) followed by DAB substrate (Novocastra). The sections were counterstained with instant hematoxylin (Shandon) and the intensity of staining and the percentages of ITGB1 positive cells were quantified at 5 to 10 increments by an anatomical pathologist who had no prior knowledge of patient details. Assessment of fascin expression on these patients has been previously described.⁹

Tumorsphere and colony forming assays

The tumorsphere assay was performed using a special medium, which was previously described by Dontu *et al.*,¹⁵ and seeding 500 cells per well in 96-well ultra-low attachment plates (Corning) as previously described.¹⁰ At day 7–10, primary tumorspheres were collected after counting using EVOS digital inverted microscope and dissociated to generate single cells for secondary tumorspheres.

The organoid assay was performed using GrowDex[®] hydrogel (UPM Biochemicals, Helsinki, Finland). About 0.5% of GrowDex hydrogel was freshly prepared using 1x PBS in a glass test tube and the solution was mixed by pipetting until it is homogenous. Cell suspension that was prepared in the special media as above were then added to the diluted GrowDex and gently mixed until evenly dispersed. A 100 μ L containing 2000 cells and GrowDex mix was added per well in 96-well ultra-low attachment plates and incubated at 37°C, 5% CO₂ humidified incubator for 5–8 days. Wells were carefully hydrated with 50 μ L of the special media every second day. Tumorspheres were counted using EVOS digital inverted microscope if they were ≥ 50 μ m in size.

The colony forming assay was performed in 6-well plates (Corning). Fascin-positive or -negative cells that express or lack ITGB1 were seeded at 100 cell/well in 3 ml of complete DMEM media for 10–12 days at 37°C, 5% CO₂ humidified incubator. Media was then removed followed by washing, fixation and crystal violet staining to visualize and count colonies (blue dots) as previously described.¹⁰

Adhesion assay

A plate (96-well) was coated overnight at 4°C with different concentration of ECM substrates including; Matrigel (Corning), 40 µg of Collagen IV (Millipore), 5 µg of Fibronectin (Millipore) and 60 µg of Laminin (Millipore) as previously described.¹⁶ Non-specific binding was blocked by 4% Bovine Serum Albumin (BSA) before serum-starved cells were seeded and incubated at 37°C, 5% CO₂ for 45 min. At the end of the incubation, adherent cells were fixed using 1% Glutaraldehyde for 25 min followed by staining with 0.1% Crystal Violet for 1 h in the dark. After washing and solubilization with 1% SDS, plate was read on an ELISA reader at OD570nm absorbance.¹⁷

Expression analysis of human breast tumor microarray datasets

The Cancer Genome Atlas (TCGA) dataset consisting of 530 mRNA expression profiles of breast invasive carcinoma samples was previously described¹⁸ and the data were downloaded in accordance with TCGA Data Access Policies (<https://tcgadata.nci.nih.gov/tcga/>). Calculation of the stemness score was previously described.¹⁹ Pearson's correlation coefficient (r), which reflects the correlations between continuous data, and all related statistical analyses were performed as previously described.¹⁸

Statistical analysis

The analysis was performed using two-tailed paired student t-test between control and treated samples. Data was presented in mean or replicates ± SD. Any value of $p < 0.05$ was considered statistically significant.

Results

Strong association between fascin, ITGB1 and stem-associated gene expression in breast cancer patients

We have previously shown that fascin regulates metastasis,⁸ chemoresistance⁹ and self-renewal, which are three main features of CSCs.¹⁰ How fascin can directly interact and regulate these processes is not fully elucidated. Using the best candidate approach method, we have identified β1 integrin (ITGB1) as a key adhesion molecule previously reported to promote metastasis, chemoresistance and self-renewal of CSCs.^{20–22} More specifically, we have previously demonstrated that fascin confers breast cancer resistance to chemotherapy *via* enhancing the phosphorylation of focal adhesion kinase (FAK),⁹ which is known to bind ITGB1²³ and fascin²⁴ in breast cancer cells.

Nonetheless, whether fascin interact with ITGB1 in breast cancer cells is not yet being investigated. Here, we have used the publically available breast cancer patients' gene expression dataset from The Cancer Genome Atlas (TCGA, $n = 530$ patients) to examine the relationship between fascin and ITGB1 expression. The TCGA dataset showed significant correlation ($r = 0.27$, $p = 2.00 \times 10^{-9}$) between fascin and ITGB1 expression in breast cancer samples (Fig. 1a). Consistent with our previous results,¹⁰ analysis of the 530 samples in the TCGA dataset showed significant correlation ($r = 0.42$, $p = 2.97 \times 10^{-21}$) between fascin expression and stemness score (Fig. 1b). Indeed, the significant correlation with the stemness score ($r = 0.4$, $p = 0.008$) was maintained in the samples that co-expressed fascin and ITGB1 (Fig. 1c), but it was lost ($r = -0.09$, $p = 0.6$) in the samples that lacked fascin and ITGB1 expression (Fig. 1d), suggesting a role for this correlation in the progression of breast cancer.

Fascin up-regulates ITGB1 expression and enhances adhesion of breast cancer cells

The highly significant correlation between fascin and ITGB1 expression in breast cancer patients further encouraged us to address the impact of fascin on ITGB1 expression. To this end, we used our previously generated fascin knocked down in the naturally fascin⁺ breast cancer cell line (MDA-MB-231) to examine its ITGB1 expression. Western blot analysis showed significant ($p < 0.05$) inhibition of ITGB1 in fascin knocked down (fascin⁻) cells as compared to their fascin⁺ counterparts (Fig. 2a). Similarly, FACS analysis showed significantly reduced mean fluorescent intensity (MFI) of ITGB1 on fascin⁻ MDA-MB-231 cells as compared to their fascin⁺ counterparts (Supporting Information Fig. S1 and Figure 2b). To validate the dependence of ITGB1 on fascin expression, we restored fascin expression in fascin⁻ cells by transfection with fascin ORF (FORF) and compared them with scrambled negative control (NORF). Indeed, rescuing fascin expression (FORF) in fascin⁻ MDA-MB-231 cells was able to restore the expression levels of ITGB1 as compared to their negative (NORF) counterparts (Supporting Information Fig. S2). This gain and loss of function data strongly support a significant role for fascin in regulating ITGB1 expression in the MDA-MB-231 breast cancer cells. To further confirm that the observed ITGB1 up-regulation was a direct effect of fascin expression, fascin ORF (FORF) was used to induce fascin expression in another breast cancer cell line (T-47D) that are naturally fascin-negative and their ITGB1 level was tested. Western blot and FACS (MFI) analysis showed significant induction of ITGB1 in fascin-expressing T-47D cells (FORF) as compared to their fascin negative counterparts, which were transfected with negative ORF (NORF) (Supporting Information Fig. S3). Altogether, fascin loss of function/restoration in MDA-MB-231 and gain of function in T-47D confirmed its critical role for ITGB1 up-regulation in breast cancer cells.

We next tested whether fascin-mediated ITGB1 expression in breast cancer cells influences their function. ITGB1 as a key

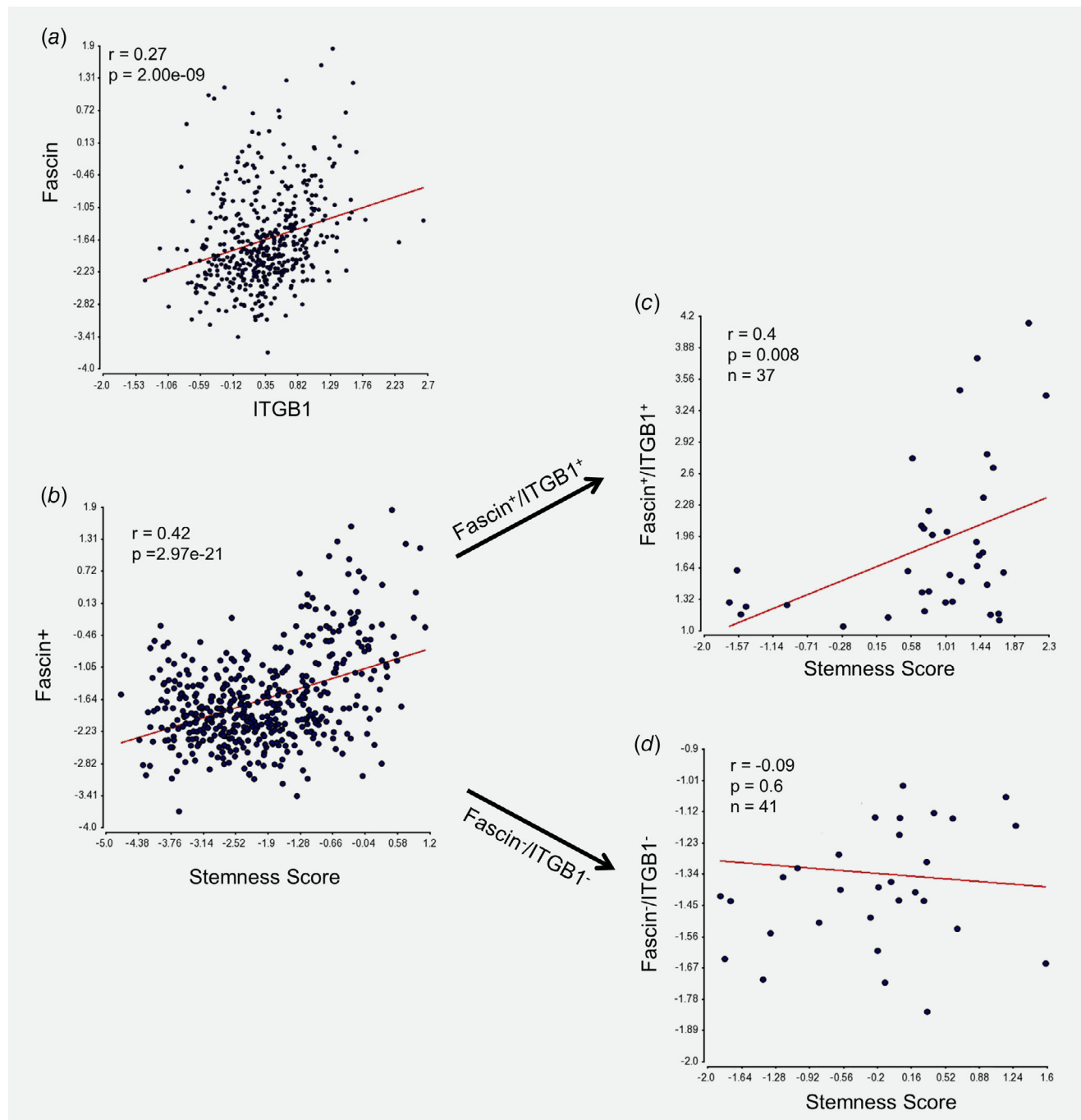


Figure 1. *a–d*: Significant association between fascin and ITGB1 expression in breast cancer samples. (a) Scatter plot showing mRNA expression levels of fascin against ITGB1, which were obtained from the publicly available breast cancer patients' gene expression dataset from The Cancer Genome Atlas (TCGA, $n = 530$). (b) Scatter plot showing mRNA expression levels of fascin against stemness score, which consist of 100 stem-associated genes and calculated from the TCGA gene expression dataset ($n = 530$) as previously described.¹⁸ Scatter plots showing the mRNA expression levels of samples that are (c) fascin⁺/ITGB1⁺ ($n = 37$) or (d) fascin⁻/ITGB1⁻ ($n = 41$) against stemness score. Pearson correlation coefficients (r) and associated p values (p) for the correlation test is shown. [Color figure can be viewed at wileyonlinelibrary.com]

integrin functions by promoting the adhesion of cells in the tissues.²⁵ To this end, adhesion of the un-manipulated fascin-positive (MDA-MB-231) and -negative (T-47D) breast cells were tested on different ECM including matrigel, laminin, collagen IV

and fibronectin, which are important integrin ligands in the breast tumor microenvironment. Adhesion of MDA-MB-231 cells to the different ECM was significantly superior to that of the T-47D cells (Supporting Information Fig. S4A), demonstrating

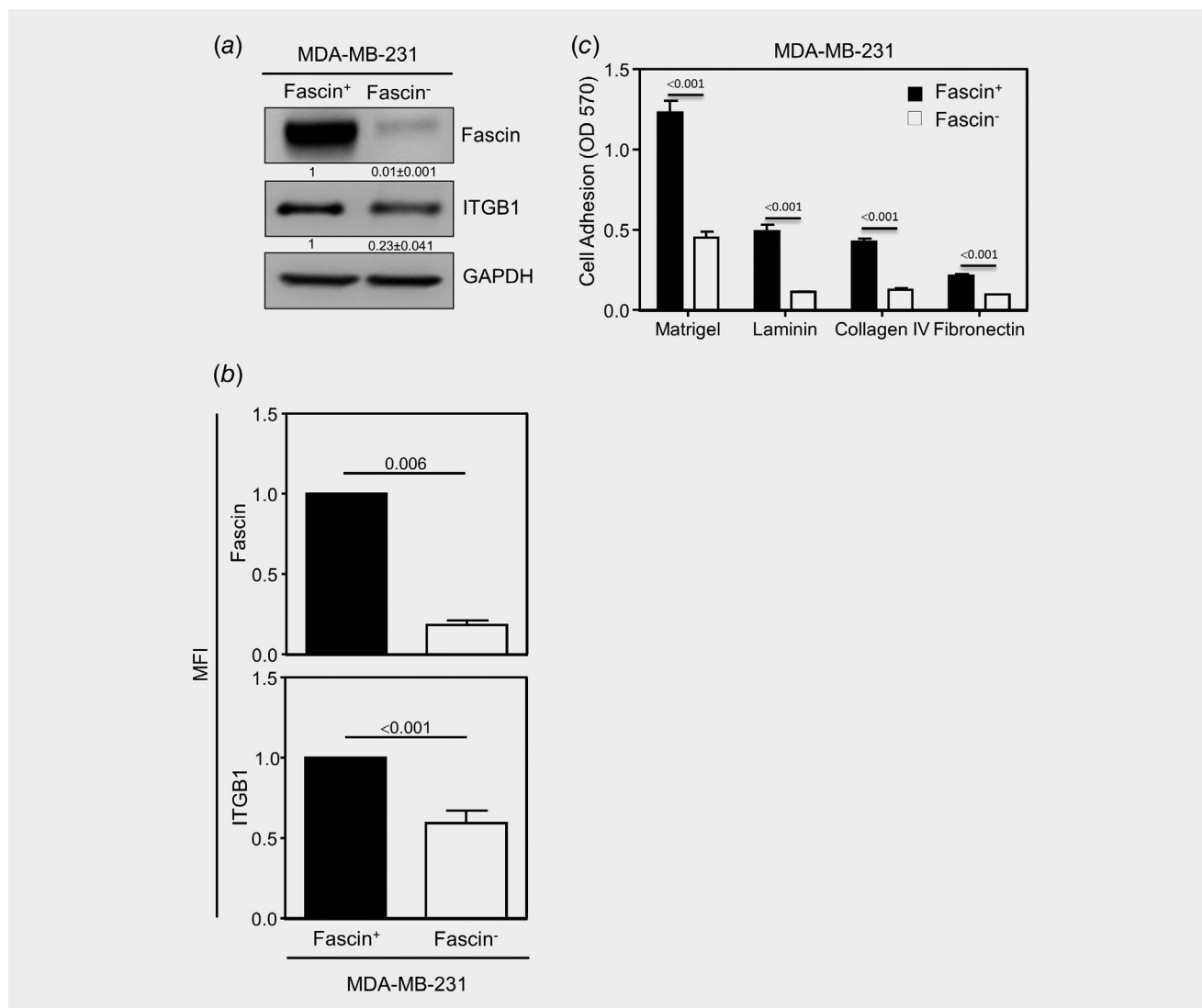


Figure 2. *a–c:* Fascin enhances ITGB1 expression and adhesion of MDA-MB-231 breast cancer cells. *(a)* Western blots showing the level of fascin and ITGB1 expression in fascin⁻ and fascin⁺ cells. ITGB1 and fascin were normalized on GAPDH and the numbers shown below the images indicate the mean fold changes (of 3 independent experiments ± SD) in fascin⁻ in reference to fascin⁺ group. *(b)* Bar graph obtained from flow cytometry showing mean fluorescent intensity (MFI) of fascin (top) and ITGB1 (bottom) in fascin⁺ and fascin⁻ cells. *(c)* Bar graph showing adhesion of the fascin⁺ and fascin⁻ MDA-MB-231 cells to different ECM. Absorbance (570 nm) was measured as detailed in the methods and used as an indication of cells' adhesion to different ECM. The results are mean of 4 independent experiments ± SD.

a correlation between fascin expression and enhanced adhesion. To confirm that the enhanced adhesion of MDA-MB-231 cells was due to fascin expression, the effect of fascin knockdown on cell adhesion was tested. Fascin⁺ MDA-MB-231 cells exhibited significantly superior adhesion ability to the different ECM as compared to their fascin⁻ counterparts (Fig. 2c). Moreover, expression of fascin in T-47D breast cancer cells (FORF) increased their adhesion potential to the different ECM as compared to their fascin-negative counterparts (NORF) (Supporting Information Fig. S4B). These results demonstrated a critical role for fascin in up-regulating ITGB1 expression, which was consistent with enhanced breast cancer cell adhesion ability.

To verify fascin-dependent ITGB1 effect on cell function, we have generated ITGB1 stable knockdown in our fascin⁺ and fascin⁻ MDA-MB-231 cells. Both western blot (Fig. 3a) and FACS (Supporting Information Fig. S5A and B) results confirmed ITGB1 knockdown in ShITGB1 compared to their ShCon counterpart in both fascin⁺ and fascin⁻ MDA-MB-231 cells. Consistent with our previous study,⁹ fascin expression enhances FAK activation (phosphorylation), but this enhancement was suppressed in the absence of ITGB1 (Fig. 3a), strongly suggesting that FAK activation is dependent on fascin-mediated ITGB1 expression. Interestingly, there was no effect of ITGB1 knockdown (ShITGB1) on the expression of fascin and consistent with our previous results,¹⁰ fascin loss promotes cells to gain

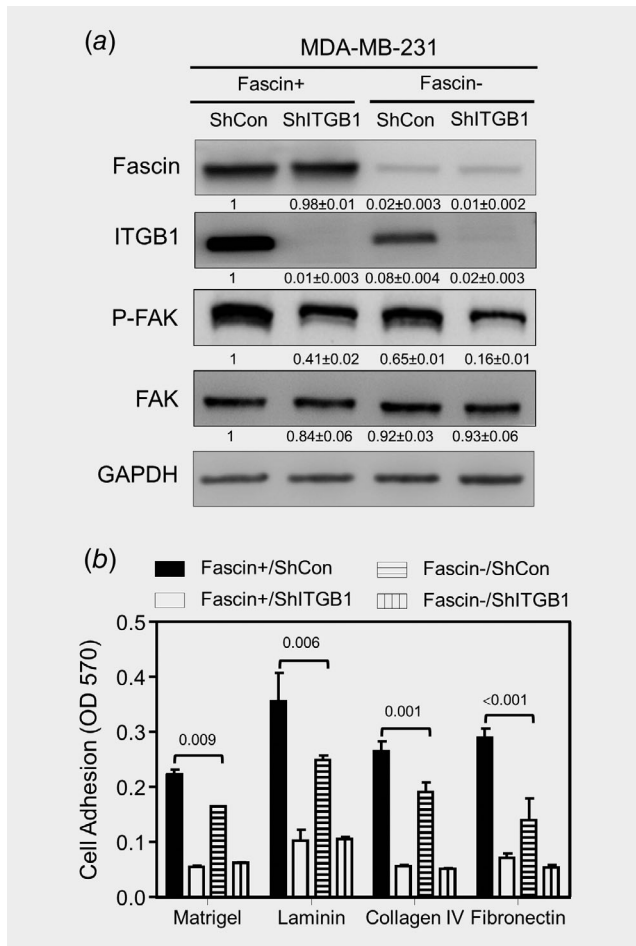


Figure 3. *a* and *b*: ITGB1 expression in MDA-MB-231 breast cancer cells is critical for fascin-mediated adhesion. (*a*) Western blot showing fascin, ITGB1 and FAK expression following stable knockdown of ITGB1 using specific ShRNA (ShITGB1) or scrambled control (ShCon) in either fascin⁺ or fascin⁻ MDA-MB-231 cells. The numbers shown below the images indicate the mean fold changes (of 3 independent experiments ± SD) in reference to fascin⁺ ShCon group. (*b*) Bar graph showing adhesion of ShITGB1 and ShCon cells in fascin⁺ and fascin⁻ group. Absorbance (570 nm) was measured as in the methods and used as an indication of cells' adhesion to different ECM. The results are mean of 3 independent experiments ± SD.

epithelial-like morphology (Supporting Information Fig. S5C). Similarly, ShITGB1 fascin⁺ cells acquire epithelial-like morphology resemble fascin⁻ cells. On the other hand, fascin⁻ cells did not show further noticeable morphological changes when ITGB1 was knocked down. Functionally, fascin-mediated increased adhesion to the different ECM was significantly impaired when ITGB1 was knocked down (Fig. 3*b*). Upon ITGB1 knockdown, adhesions were not significantly different between fascin⁺ and fascin⁻ cells. ITGB1 was also transiently knocked down in fascin expressing T-47D (FORF) (Supporting Information Fig. S4C) and their adhesion ability was tested on the different ECM (Supporting Information Fig. S4D). When ITGB1 was transiently knocked down, FORF cells lost their adhesion advantage to

the different ECM. In fact, adhesion of FORF/SiITGB1 was not significantly different than NORF cells that express ITGB1 (NORF/SiCon). Collectively, the data from T-47D further supported that of the MDA-MB-231 and confirmed the complete dependence of fascin-mediated adhesion promotion on ITGB1 expression in breast cancer cells.

Fascin-mediated ITGB1 expression is critical for enhancement of breast cancer cell self-renewability

The results above demonstrated that fascin up-regulates ITGB1 expression and enhances adhesion function. We have previously demonstrated the critical role of fascin as a regulator of breast CSCs.¹⁰ In addition, other studies showed higher expression of ITGB1 on breast CSCs that is important in regulating their self-renewability.²⁶ To investigate the impact of fascin-mediated ITGB1 up-regulation on breast cancer cell self-renewability, we used tumorsphere assays on our established ITGB1 stable knockdown in our fascin⁺ and fascin⁻ MDA-MB-231 cells. Our data demonstrated that the increase number of tumorsphere forming cells in the fascin⁺ cells was preferentially mediated through ITGB1. In a classical tumorsphere assay, ITGB1 stable knockdown in fascin⁺ cells demonstrated reduced primary and secondary tumorsphere forming capability (Fig. 4*a* top and bottom). Interestingly, fascin⁺ cells lost their tumorsphere forming advantage when ITGB1 was knocked down as demonstrated by the comparable number of tumorsphere formed by fascin⁺ cells that lack ITGB1 and fascin⁻ cells that express ITGB1. When using the organoid assay, which adds another degree of complexity that mimics more the *in vivo* environment, similar trends of reduced primary and secondary tumorspheres were observed upon ITGB1 loss in fascin⁺ cells (Fig. 4*b* top and bottom). In fact, the organoid forming potential was abolished when both fascin and ITGB1 were knocked down as compared to a milder effect in the classical tumorsphere assay. Altogether, these results strongly suggested a direct role for fascin-mediated ITGB1 expression in regulating the breast cancer tumorsphere formation potential.

To exclude the effect of clonal selection when the stable knockdown of ITGB1 was generated, a limitation of ShRNA technology, we have transiently silenced ITGB1 (SiITGB1) in fascin⁺ MDA-MB-231 cells using specific SiRNA. Silencing of ITGB1 significantly suppress their tumorsphere forming ability to a level near that of the fascin⁻ counterparts in a similar fashion to that of the stable ShRNA clones (Supporting Information Fig. S6A). Indeed upon ITGB1 silencing, fascin⁺ cells had no tumorsphere forming advantage than their fascin⁻ counterparts, supporting ITGB1 essential role in fascin-mediated tumorsphere enhancement. Furthermore, provided that ITGB1 is a surface molecule we have sorted high and low ITGB1 from fascin⁺ as well as fascin⁻ MDA-MB-231 cells without manipulation and tested their tumorsphere formation ability (Supporting Information Fig. S6B and C). ITGB1^{high} from fascin⁺ cells were able to form tumorspheres more potently than their counterparts that express ITGB1^{low} (Supporting Information Fig. S6B, left).

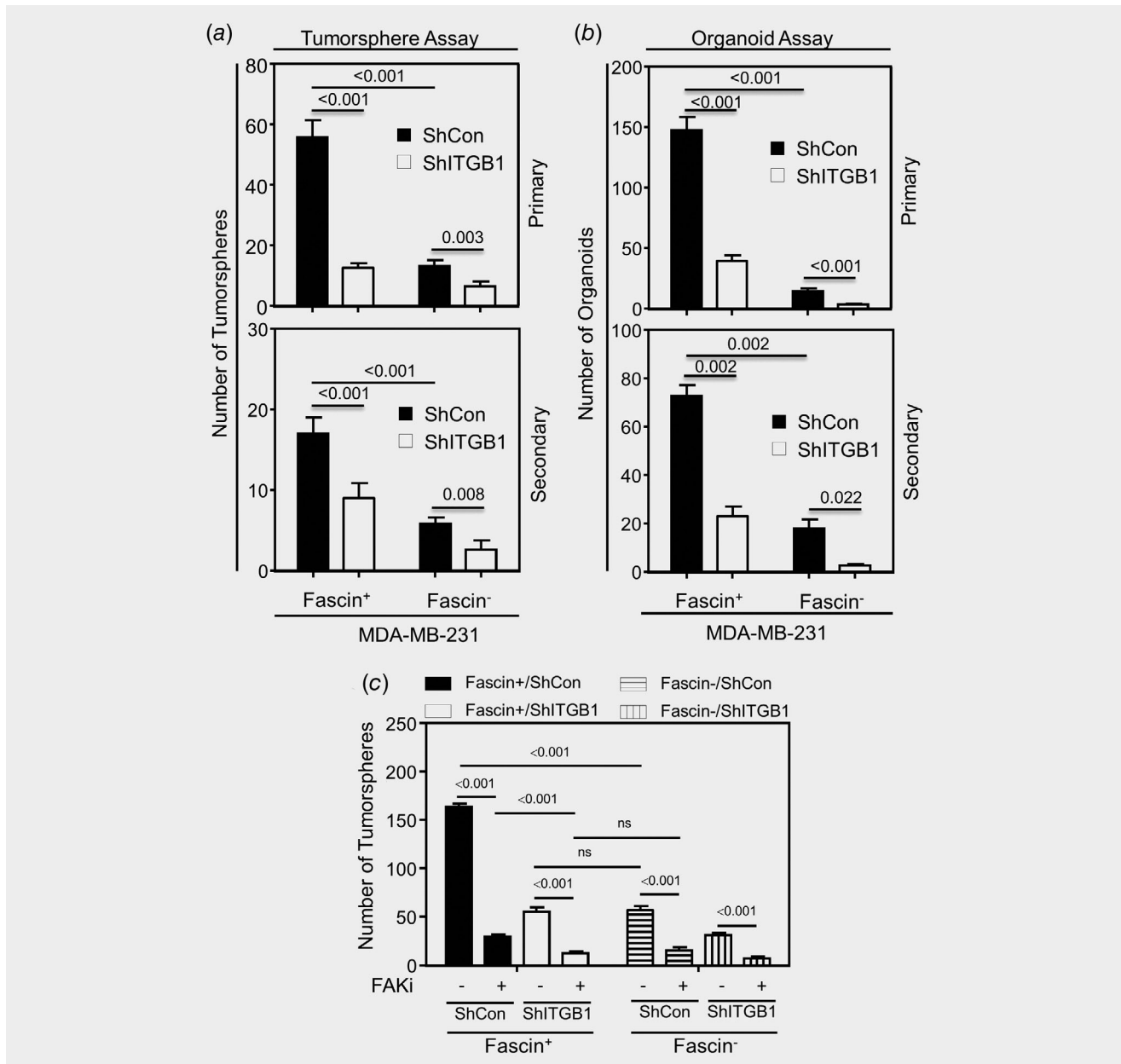


Figure 4. *a-c*: ITGB1 expression in MDA-MB-231 breast cancer cells is vital for fascin-mediated enhanced self-renewability. Bar graph showing the number of tumorspheres (500 seeded) and organoids (2000 seeded) formed by fascin⁺ and fascin⁻ MDA-MB-231 cells in the presence (ShCon) or absence (ShITGB1) of ITGB1. Primary (top) and secondary (bottom) tumorspheres (*a*) or organoids (*b*) are mean of 3 independent experiments \pm SD. (*c*) Number of tumorspheres formed by fascin⁺ and fascin⁻ MDA-MB-231 cells in the presence (ShCon) or absence (ShITGB1) of ITGB1 \pm 2 μ M of FAK inhibitor (FAKi). The results are mean of 2 independent experiments \pm SD.

Interestingly, there was no significant difference between cell expressing different levels of ITGB1 in fascin⁻ cells (Supporting Information Fig. S6B, right). In consistent with the MDA-MB-231 data, sorted ITGB1^{high} from fascin-expressing T-47D breast cancer cells (FORF) formed more tumorsphere as compared to their fascin-negative (NORF) counterparts (Supporting Information Fig. S6D and E). In this later experiment, fascin expression in T-47D cells had no tumorsphere formation advantage in the presence of low levels of ITGB1. Collectively, these data

demonstrated that expression of ITGB1 is crucial for fascin-mediated enhancement of breast cancer cell self-renewability.

We have demonstrated that fascin-mediated ITGB1 expression is critical for FAK activation (Fig. 3*a*). Several studies have reported that FAK is essential for breast cancer self-renewability.^{27,28} Therefore FAK was inhibited in the presence or absence of fascin and/or ITGB1 to delineate whether fascin/ITGB1 enhancement of self-renewability is FAK mediated (Fig. 4*c*). Fascin⁺ITGB1⁺ breast cancer cells lost their tumorsphere formation

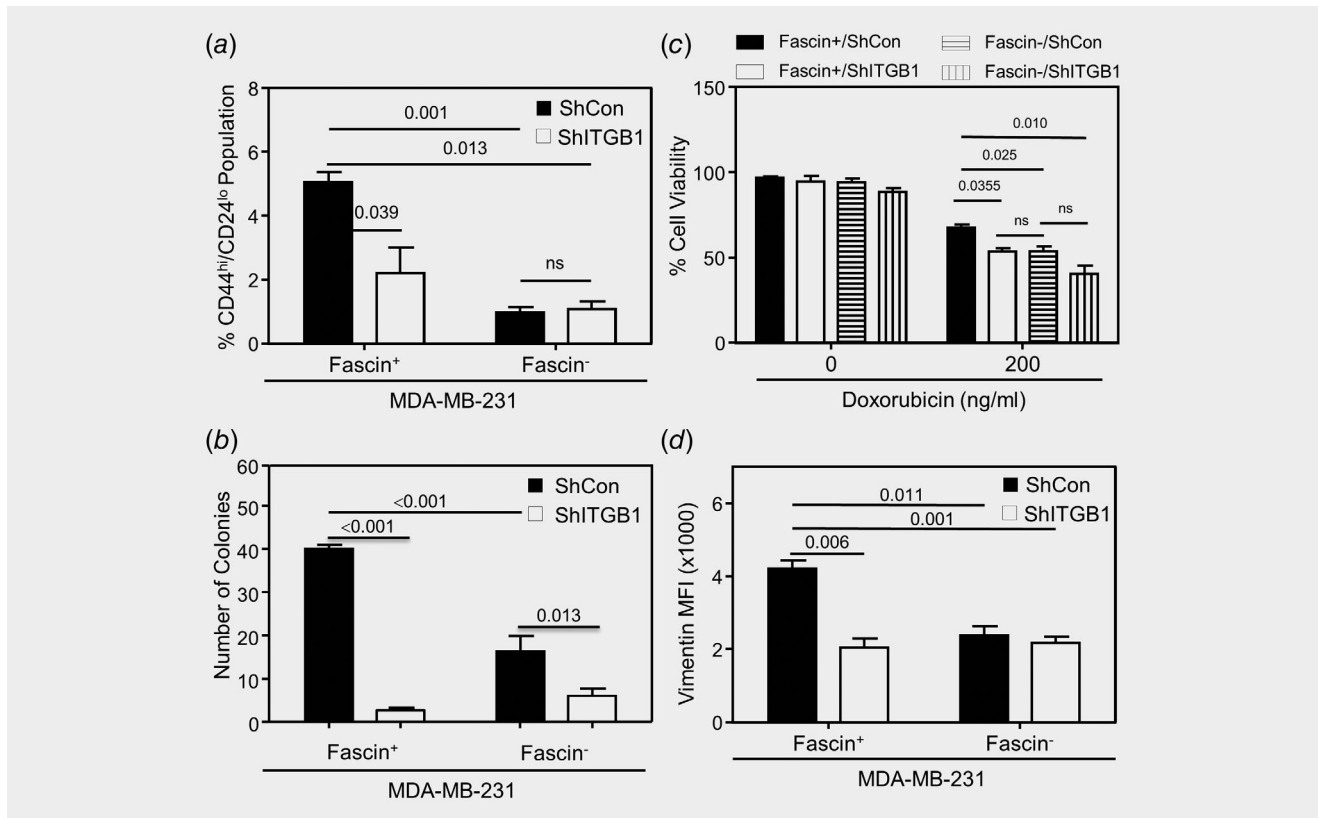


Figure 5. *a–d*: Fascin enrichment of CSCs in MDA-MB-231 breast cancer cells is ITGB1-dependent. CSC features were assessed in fascin⁺ and fascin⁻ MDA-MB-231 cells in the presence (ShCon) or absence (ShITGB1) of ITGB1. Bar graphs showing the (a) percentage of CD44^{hi}/CD24^{lo} positive population, (b) number of colony formed, (c) percentage of cell survival post treatment with doxorubicin and (d) MFI of vimentin. The results are mean of at least 3 independent experiments \pm SD.

advantage when FAK was inhibited. In addition, fascin⁺ITGB1⁻ tumorsphere formation potential was inhibited compared to their fascin⁺ITGB1⁺ counterparts despite the presence of FAK. This data demonstrated that fascin-mediated tumorsphere formation is significantly impaired in the absence of either ITGB1 or FAK. Similarly, tumorsphere formation by fascin⁻ breast cancer cells was impaired even in the presence of FAK and ITGB1. Together, the fact that majority of the tumorsphere formation potential by fascin⁺ITGB1⁺ cells is lost when FAK was inhibited, strongly supports that fascin collaborates with ITGB1 to promote self-renewability, at least partially, through FAK.

Fascin-mediated ITGB1 expression enriches for breast CSCs

We then tested the effect of fascin-ITGB1 interaction on other CSC features. The number of cells with CSC phenotype (CD44^{hi}/CD24^{lo}), colony forming potential and chemotherapy resistance were assessed in the presence or absence of fascin and/or ITGB1. Fascin⁺ITGB1⁺ cells have greater number of CD44^{hi}/CD24^{lo} breast CSC population as compared to their fascin⁺ITGB1⁻ counterparts, while there was no advantage for ITGB1⁺ over ITGB1⁻ cells in the absence of fascin (Supporting Information Fig. S7 and Fig. 5a). Similarly, the colony forming potential of fascin⁺ cells was also significantly impaired when

ITGB1 was knocked down (Fig. 5b). In fact, the number of colonies formed by ShITGB1 fascin⁺ cells became like their ShITGB1 fascin⁻ counterparts. Consistent with our previous results,⁹ fascin expression provides breast cancer cells with chemoresistance advantage over their fascin⁻ counterpart (Fig. 5c). Importantly, this chemoresistance advantage was lost when ITGB1 was knocked down as demonstrated by the comparable level of cell viability between fascin⁺ that lack ITGB1 (fascin⁺/ShITGB1) and fascin⁻ cells that express ITGB1 (fascin⁻/ShCon). We have further checked the EMT status upon ITGB1 as a feature of CSCs. There was reduced protein expression of vimentin in fascin⁺ITGB1⁻ cells to levels comparable to that of the fascin⁻ITGB1⁺ cells (Fig. 5d) in line with the observed morphological changes (Supporting Information Fig. S5C). Collectively, the above data clearly demonstrated a critical role for fascin-mediated ITGB1 expression in enrichment of breast CSCs.

Fascin-dependent ITGB1 protein expression is associated with worse survival of breast cancer patients

The above mRNA data from TCGA analysis showed significant correlation between fascin and ITGB1 expression in breast cancer cells. We have further showed a direct dependence of

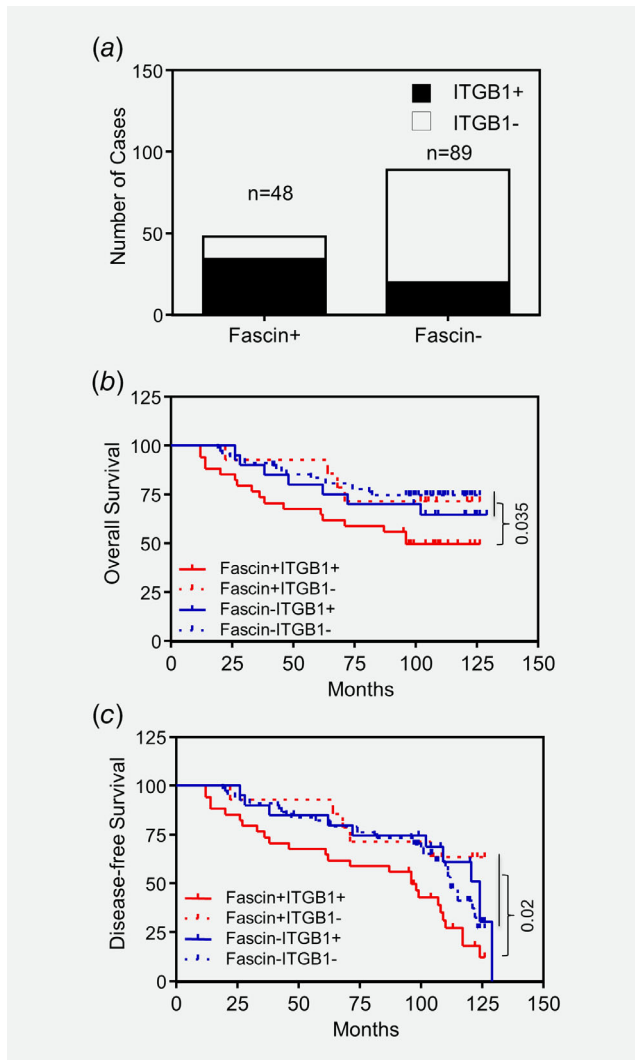


Figure 6. a–c: Significant correlation between fascin and ITGB1 expression in neoadjuvant treated breast cancer patients is associated with shorter survival. (a) Bar graph showing the distribution of fascin and ITGB1 expression in our invasive breast cancer samples (137 cases). Kaplan–Meier survival curves showing overall (b) and disease-free (c) survival of patients blotted in relation to the distribution of fascin and ITGB1 expression.

fascin function related to enrichment of CSC pool on ITGB1. To understand the relevance these findings to breast cancer patients, we have analyzed fascin and ITGB1 expression at the protein level in 137 patients, who were diagnosed with invasive ductal carcinoma and received neoadjuvant therapy, and traced their disease progression and survival data. Our breast cancer samples showed highly significant ($p < 0.001$) correlation between fascin and ITGB1 expression as majority (70.8%) of the fascin-positive samples expressed ITGB1, while majority (77.52%) of the fascin-negative samples were ITGB1-negative (Fig. 6a). This result is in line with the demonstrated *in vitro* relationship between fascin and ITGB1. Clinically, fascin⁺/ITGB1⁺ patients who received chemotherapy had significantly

shorter overall and disease-free survival compared to their fascin⁻/ITGB1⁺, fascin⁺/ITGB1⁻ or fascin⁻/ITGB1⁻ counterparts (Figs. 6b and 6c). Collectively, these data provides strong evidence that co-expression of fascin and ITGB1 in breast cancer patients promotes resistance to chemotherapy.

Discussion

ITGB1 expression is known to be associated with enhanced features of CSCs including EMT, metastasis and resistance to chemotherapy.^{20–22} On the other hand, we have also demonstrated in previous study the critical role of fascin expression on the maintenance of CSC pool.¹⁰ Here, we have shown for the first time the direct effect of fascin on ITGB1 expression in breast cancer cells and the dependence of fascin on ITGB1 in promoting cellular functions including adhesion and self-renewability. More importantly, the data extends to breast cancer patients response to chemotherapy and overall survival.

Fascin is an actin bundling protein that package actin filaments into structures to facilitate several dynamic functions in the cell.²⁹ Similarly, microtubules in the cell regulate many processes like focal adhesion structures by directing the delivery of key signaling molecules to stabilize these structures.^{30,31} In fact, actin and microtubules share common signaling cascades and are dynamically regulated by tuning the turnover of the focal adhesion structures to control cell migration. Interestingly, one study reported that fascin loss reduces focal adhesion dynamics in the filopodia³² and subsequent study demonstrated a direct interaction between fascin and microtubules and that the disruption of this binding reduces the focal adhesion turnover and cell migration.²⁴ Thus, in response to extracellular signals, fascin may act as scaffold to promote localization and/or interaction of key signaling molecules with ITGB1 or may simply facilitate the transport of key signaling molecule-containing vesicles along microtubules to amplify ITGB1-mediated signaling cascade. Moreover, upon binding to ECM, ITGB1 becomes activated and acts as scaffold for multiple proteins and signaling molecules to trigger downstream actin cytoskeletal rearrangements, which in turn regulate cell adhesion and spreading.³³ The demonstrated loss of mesenchymal-like and acquisition of epithelial-like morphology in our fascin⁺ cells when ITGB1 was knocked down is consistent with reported role of integrins in governing cell morphology, migration and invasion.³⁴ Specifically, our results are consistent with that of Parvani JG *et al.* where they reported acquisition of epithelial-like morphology when ITGB1-deficient MDA-MB-231 cells were used.³⁵

The abundant expression of fascin within dynamic cellular structures in the submembranous and dendritic processes²⁹ of the cell makes it a good candidate to interact and/or facilitate its functions through key transmembrane proteins such as ITGB1. Our finding that fascin positively regulates ITGB1 expression in breast cancer cells, which in turn enhances cells' adhesion and self-renewability, indicates that ITGB1 is a requirement of fascin role in promoting worse outcome. The data in this report is consistent with the fact that integrins regulate functions other

than adhesion, which are related to increase cell survival, tumor cell propagation or stemness.¹³ On the other hand, β integrins like ITGB1 possess long cytoplasmic tail, but they lack enzymatic activity.³⁶ Therefore, ITGB1 interaction with specific downstream targets occurs *via* recruitment of adaptors that link the ECM to the actin cytoskeleton to facilitate the signal transmission inside the cells. Through recruitment of adaptors, integrins either directly transmit intercellular signals or synergize with other growth factors to maximize the response.

Interaction between integrins and ECM recruits many adaptors and triggers many downstream signaling pathway including Ras, PI3K and small GTPases.^{37,38} Integrin-linked kinases (ILK) is an example of adaptor proteins that was shown to be recruited and directly binds to the cytoplasmic domain of β 1 and β 3 integrins.³⁹ It was reported that ILK play a key role in actin rearrangements, survival, proliferation and migration⁴⁰ and its depletion suppressed the survival and invasion potential of many cancer cells.⁴¹ Moreover, another study reported impaired Notch1 activation when ILK was inhibited in breast cancer cells.⁴² Given that we have demonstrated that fascin regulate breast CSCs *via* activation of the Notch self-renewal pathway,¹⁰ it is possible that our fascin-mediated ITGB1 expression enhanced self-renewability and conferred resistance to chemotherapy probably through activating ILK and its downstream signaling targets. FAK is another integrin adaptor that directly binds to the cytoplasmic tail of β 1 integrin and activates intercellular signaling pathways that induces survival, migration and invasion of breast cancer cells.^{11,43,44} Furthermore, FAK expression is frequently up-regulated in breast cancer.⁴⁵ Importantly, we have previously demonstrated that fascin can confer breast cancer chemoresistance by regulating FAK/Akt activation.⁹ Another study was able to demonstrate that fascin can regulate the proliferation and migration of human hepatic stellate cells through FAK-PI3K-AKT signaling pathway.⁴⁴ Villari *et al.* group was able to identify interaction between fascin and FAK that in turn controls focal adhesion stability and motility.²⁴ Our findings that fascin-mediated ITGB1 expression is critical for FAK activation and given that FAK binds fascin as well as ITGB1²³ strongly support a critical role for FAK in facilitating how fascin-mediate ITGB1 expression enhances breast cancer self-renewability. It is possible to speculate that fascin influences β 1 integrin expression and its downstream-related function *via* targeting selected miRNAs, which have gained much attention in recent years due to their functions in RNA silencing and post-transcriptional regulation of gene expression. Indeed, miR-7 was reported to inhibit EMT and metastasis of breast

cancer⁴⁶ and invasion of glioblastoma⁴⁷ *via* targeting FAK expression. Furthermore, miR-199a has been demonstrated to suppress ITGB1 and inhibit the invasion of the MDA-MB-231 breast cancer cells by targeting Ets-1 *via* FAK/Src/Akt/mTOR signaling axis.⁴⁸

Elevated expression of fascin and ITGB1 were independently reported in various cancers including breast and were found to associate with enhanced self-renewability, which present serious challenge to effective treatment of cancer. The patient results described in this study were consistent with our previous data where we have reported a significant correlation between increased fascin expression in breast cancer patients and shorter survival.⁹ Furthermore, our patient data is in agreement with the reported role of increased ITGB1 and decreased survival of invasive breast cancer.⁴⁹ Importantly, the survival of breast cancer patients worse in fascin⁺ITGB1⁺ tumors compared to ITGB1⁻ tumor (irrespective of fascin expression) or ITGB1⁺ fascin⁻ tumors. The outcome of patients based on fascin and ITGB1 expression was consistent with our *in vitro* findings where fascin effect on CSCs were dependent on ITGB1 expression, while ITGB1 has minimal effect in the absence of fascin. Findings presented in this study of fascin and ITGB1 cooperative effect on FAK activation and its significance on the CSC function may present a novel therapeutic window of targeting the fascin-ITGB1-FAK axis for effective eradication of cancer from the root.

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Authors' contributions

R.B. and S.A. performed the experiments and analyzed some of the data; D.C. supervised and performed all bioinformatics analyses, interpretation. A.T. and T.A. performed the pathological and patient survival analysis. M.F. obtained fund for S.A. and provided materials support. D.M. performed authentication of cell lines. H.G. performed some data interpretation and edited the manuscript. M.A. conceived and designed the experiments, analyzed the data and wrote the paper.

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