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Original Article

ITGAV targeting as a therapeutic approach for treatment of metastatic breast cancer

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Abstract: During tumorigenesis and metastasis, integrins regulate localization and activity of proteolytic enzymes that remodel the extracellular matrix. Previous studies have demonstrated blocking of $\alpha_V\beta_3$ to effectively inhibit proliferation, angiogenesis, and the survival of various cancer cell types. However, little is known about the functional role of the integrin subunit alpha-V gene (*ITGAV*) in metastatic breast cancer. In this study, *ITGAV* knockdown was used to identify the molecular mechanism by which *ITGAV* promotes tumorigenesis, metastasis, proliferation, invasion, and cellular self-renewal. The effectiveness of an ITGAV antagonist, cilengitide, for breast cancer treatment was investigated *in vivo*. Analysis of publicly available data demonstrated that overexpression of *ITGAV* was associated with poor relapse free survival of breast cancer patients. Silencing of *ITGAV* inhibited cell proliferation, invasion, and self-renewal of breast cancer cell lines by altering expression of *BCL2* and *PXN*. The use of cilengitide significantly reduced lung metastasis in a metastatic breast cancer animal model. In conclusion, overexpression of *ITGAV* contributes to breast cancer metastasis through upregulation of *PXN*. Targeting *ITGAV* is a potential treatment for metastatic breast cancer as well as primary breast tumors with high *ITGAV* expression. *ITGAV* expression levels may be useful predictors of patient treatment and outcome responses.

Keywords: *ITGAV*, cilengitide, breast cancer, metastasis

Introduction

Worldwide, breast cancer is the most common cancer in women, affecting 2.09 million women with an age-standardized rate of 46.3 per 100,000 in 2018 [1]. There is a 25% increase in the incidence of breast cancer since 2012 [2]. Of note, loco-regional relapse and distant metastasis remain the major causes of breast cancer-related mortality. Up to 30% of breast cancer patients relapse with distant metastasis even when diagnosed at an early stage [3]. Therefore, there is an urgent need to identify genes associated with breast cancer progression and distant metastasis in order to develop targeted therapies that improve clinical outcomes.

During cancer development, epithelial cancer cells acquire a mesenchymal phenotype by epithelial to mesenchymal transition (EMT), a key process involved in carcinogenesis and metastasis. Cancer cells that have undergone EMT show increased cell motility, reduced intracellular adhesion, stem-cell like characteristics, and are capable of cell renewal and differentiation, conferring tumor heterogeneity [4, 5]. Heterogenetic cancer cells, with acquired phenotypes, have increased invasiveness that results in cancer progression and metastasis. Fundamental components of the extracellular matrix (ECM) are fibrous proteins, such as fibronectin and collagen, that regulate cell adhesion and cell attachment [6]. When cancer cells metastasize and invade, EMT-induced degradation of the cytoskeleton and ECM disrupts tight junctions and adherence between cells, which allow cancer cells to disseminate to distant organs [4, 5]. Degradation of the ECM can be achieved by dissociation or blocking of cellular receptors, such as integrins [7].

Integrins are heterodimeric cell surface receptors that regulate the localization and activity of proteolytic enzymes that promote cancer cell invasion and migration during ECM remodeling [7]. Formed by transmembrane α and β subunits, there are 24α and 9β human integrin subunits [8]. Expression of integrins varies in different cell types and tissues. For instance, αIIbβ3 is only expressed by platelets and $\alpha_6\beta_4$ expression is limited to keratinocytes, while $\alpha_V\beta_3$ is widely distributed in different cell types and tissues [9]. Further, several integrins are ligand-specific. Integrin $\alpha_5\beta_1$ is a fibronectin-specific receptor, while $\alpha_5\beta_3$ binds a wide range of fibrous proteins such as fibronectin, fibrinogen, and vitronectin [10]. During tumor progression and metastasis, integrin expression profiles are altered in a manner that enhances cell invasion and migration [11]. Moreover, integrins promote cancer invasion and metastasis by interacting with other cell types, such as endothelial cells and fibroblasts in the tumor microenvironment [10]. During tumor progression, growth factors such as vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF) released by tumor cells induce expression of integrins in angiogenic endothelial cells, thereby promoting the formation of new blood vessels [12].

Associations between integrin expression and cancer progression have been reported. The $\alpha_5\beta_1$, $\alpha_5\beta_3$, and $\alpha_5\beta_5$ integrins all contain the integrin subunit alpha-V (*ITGAV*). Each regulates angiogenesis through a distinct pathway [11]. Overexpression of $\alpha_5\beta_3$ has been associated with breast cancer metastasis and tumor progressiveness [10, 13]. Overexpression of *ITGAV* has been reported in several solid tumors including bladder, colorectal, prostate, and breast [14-17]. Changes in integrin expression activate downstream proteins through phosphorylation of the mitogen-activated protein kinase (MAPK) cascade. The paxillin (*PXN*) gene is a 68-kDa tyrosine-containing protein that acts as an adaptor protein that integrates integrin, adhesion molecule, and growth factor signals [18]. PXN interacts with integrins during matrix organization and tissue remodeling by destabilizing focal adhesions, such as the linkage between PXN and actin filaments [19]. PXN is overexpressed in many cancers with that overexpression resulting in activation of ERK and Src signaling pathways [18]. Overexpression of *PXN* has also been reported to increase chemo-resistance in lung cancer [20].

Cilengitide is an ITGAV antagonist, which targets $\alpha_V\beta_3$ and $\alpha_V\beta_5$ and reduces angiogenesis. Cilengitide was first developed as a novel therapy for glioblastoma [21]. Several registered, U.S. National Institutes of Health clinical trials have used cilengitide as a single agent or in combination with other agents for treatment of metastatic or unresectable cancers including breast, lung, and prostate [22]. *In vivo* preliminary results have shown cilengitide to reduce breast cancer bone metastasis [23]. However, the mechanistic basis for this result is unresolved. An understanding of the molecular mechanism(s) of *ITGAV*-mediated breast cancer tumorigenesis and metastasis would facilitate development of potential drug targets.

Hence, the aims of this study were to delineate *ITGAV*-mediated regulatory pathway in breast cancer and to explore the potential use of cilengitide for the treatment of metastatic breast cancer. Integrin-mediated signaling cascades are important contributors to cancer development. Treatment decisions that include individual gene expression profiles would allow for stratification of patient subsets with similar gene expression characteristics. Inclusion of *ITGAV* in those expression profiles may further improve treatment efficacy.

Methods

Ethics approval and consent to participate

Ethics approval of animal experiments was obtained from the Committee on the Use of Live Animals in Teaching and Research (CULATR) of the University of Hong Kong. The collection and storage of tissue samples from breast cancer patients were approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority West Cluster. Written informed consent was obtained from all participants.

Cell lines and transfection

Five human breast cancer cell lines, MDA-MB-231 (MB231), MDA-MB-468 (MB468), SKBR3, MCF7, and T-47D, were used in this study. MB231, MB468, and T-47D were cultured in RPMI-1640 medium. MCF7 was cultured in DMEM/F12 medium and SKBR3 was cultured in DMEM medium. Cells were cultured in the respective medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Life tech-

nologies, Carlsbad, CA, USA) and 1% antibiotic-antimycotic (anti-anti) (Gibco) and kept at 37°C in a humidified atmosphere containing 5% carbon dioxide. All transfection experiments were performed with Lipofectamine 3000 for 3 days (Invitrogen, Life technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA from cells and tumor tissues was extracted using a Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). Extracted RNAs were reverse-transcribed into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Target gene expression was determined with a LightCycler 480 Real-time PCR system (Roche, Basel, Switzerland) by the comparative threshold cycle (Ct) method. Expression levels were calculated with the formula $2^{-\Delta\Delta Ct}$. Beta-actin (β-actin) was used as an internal control to calculate relative fold change.

Cell viability assay

Cell viability of transfected cells or cells treated with cilengitide was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium reduction assay. Briefly, 5,000 cells in 100 µl of culture medium were seeded into 96-well flat-bottom culture plates in triplicate and were incubated for 1 day before transfection or drug treatment. After 3 days, culture medium was removed and replaced with 100 µl of culture medium with 3% MTT. Medium containing 3% MTT was removed after 2 hours incubation. Dimethylsulfoxide (DMSO) was added to each well, followed by a 30 minute incubation at room temperature. Absorbance at 570 nm was detected with a microplate photometer (Thermo Scientific, USA).

Tumorsphere assay

Cells were seeded into 24-well ultra-low attachment plates in serum free medium supplemented with 20 ng/ml human recombinant EGF (Gibco), 20 ng/ml FGFb (Merck Millipore), 1X N2 supplement (Gibco), and 1X anti-anti. After 7 days, the number of tumorspheres was determined with a microscope.

Soft agar colony formation assay

Culture medium containing 10% FBS, 1X anti-anti, and 0.5% noble agar were loaded into each well of a 6-well plate as the bottom layer and allowed to solidify for 30 minutes. Untreated cells and transfected cells resuspended in culture medium containing 10% FBS, 1X anti-anti and 0.3% noble agar at a density of 5,000 cells/well were loaded over the bottom layer and allowed to solidify for 30 minutes. Cells were cultured for 17 days to allow formation of colonies. Colonies were stained with crystal violet for quantification.

Cell invasion assay

Invasion potential of transfected cells was assessed in 24-well BioCoat Matrigel invasion chambers (BD Biosciences) according to the manufacturer's protocol. Briefly, trypsinized cells were resuspended in serum free medium at a density of 2×10^4 cells/ml. Cells were incubated for 24 hours and then washed with PBS to remove non-invasive cells. Invaded cells were stained with crystal violet for 1 hour and chambers were then washed with PBS before quantification by microscope.

Immunofluorescence staining

Cells were plated into 12-well plates containing coverslips in each well. After overnight incubation, cells were transfected with siRNA for 3 days. Cells were fixed with 4% paraformaldehyde for 15 minutes and then permeabilized with 0.1% Triton X-100 in PBS for 1 hour. Permeabilized cells were incubated with primary antibodies diluted in blocking solution overnight at 4°C. After washing, cells were incubated with Alexa fluor-conjugated secondary antibodies and DAPI (4',6-diamidino-2-phenylindole), used as nuclear counterstain. Cells were visualized with a Nikon Eclipse E600 microscope.

Western immunoblot

Protein expression of integrin α_V was examined by western blot analysis. Cell pellets were lysed with ice-cold buffer containing proteinase and phosphatase inhibitors. Protein concentrations in lysed cells were quantified using the Bio-Rad DC™ protein assay. Forty µg of protein was mixed with loading buffer and resolved by SDS-polyacrylamide gel electrophoresis. Proteins

were transferred to a Polyvinylidene difluoride (PVDF) membrane followed by 1 hour of membrane blocking. Membranes were immunoblotted with anti-ITGAV (Abcam, 1:1000) and anti-β-actin (CST, 1:5000) primary antibodies overnight at 4°C, followed by incubation with peroxidase-conjugated secondary antibodies for 1 hour. Membranes were developed with enhanced chemiluminescence (ECL) and exposed to X-ray film (FUJI photo Film, Tokyo, Japan) for visualization. Protein intensity was quantified using ImageJ.

Immunohistochemistry (IHC)

Anti-Ki67 (CST) and anti-ITGAV (Abcam) were used to examine protein levels in formalin-fixed, paraffin-embedded xenograft tumors. Protein expression of ITGAV (Abcam) was examined in formalin-fixed, paraffin-embedded paired tumor and adjacent normal tissues from breast cancer patients. In brief, tissue sections (6 μm) were dewaxed and rehydrated with ethanol. Tissue sections were incubated with 10 mM citrate buffer solution and 3% hydrogen peroxide, followed by 1 hour incubation with blocking solution at room temperature. After blocking, sections were incubated with primary antibodies overnight at 4°C. Tissue sections were incubated with secondary antibodies for 30 minutes and visualized using Nikon Eclipse E600 microscope using ultraView Universal DAB detection kit (Ventana).

Flow cytometry

Apoptotic rate and stem-like phenotypes of breast cancer cells were investigated using flow cytometry. All experiments were performed using FACSCalibur flow cytometry (Becton Dickinson) and were analyzed by FlowJo. For apoptosis assessment, cells were washed and resuspended in 50 μl of binding buffer containing 2.5 μl of annexin V-FITC and 2.5 μl of propidium iodide (PI). Resuspended cells were incubated for 15 minutes and 300 μl of buffer added to the resuspended cells before quantification.

The stem-like phenotype of breast cancer cells was characterized by cell surface markers $CD44^{high}/CD24^{low}$ and aldehyde dehydrogenase (ALDH) enzymatic activity. For cell surface marker expression, cells were incubated with fluorescently-conjugated anti-CD24 and anti-CD44 in the dark for 20 minutes at room temperature before quantification. For ALDH enzymatic activity, cells were incubated at 37°C with ALDEFLUOR assay buffer containing ALDH substrate for 40 minutes. Cell aliquots stained with diethylaminobenzaldehyde (DEAB) were used as negative controls.

Tumorigencity

The expression of *ITGAV* was investigated in a human xenograft breast cancer model and a metastatic breast cancer model in NOD/SCID mice. For the human xenograft breast cancer model, 1×10^6 cells/ml (100 μl) were injected into the mammary fat pad of the mice. For the metastatic breast cancer model, breast cancer cells were trypsinized and resuspended in PBS at a concentration of 5×10^6 cells/ml (200 μl) for intravenous injection. Mice were sacrificed after 6 weeks and tumors extracted for histological examination.

Clinical data analysis

Relapse-free survival (RFS) analysis of overall breast cancer, luminal A, luminal B, HER2, and TNBC was conducted with the Kaplan Meier (KM) plotter for breast cancer based on the 2017 version of the database with probe ID ITGAV (202351_at) [24].

Statistical analysis

Statistical analysis was performed using ANOVA or Student's t test with SPSS, release 20 or PRISM 5. All *p*-values are two-sided with a $P<0.05$ considered statistically significant.

Results

ITGAV is highly expressed in the tumor tissue of breast cancer patients

To investigate the expression levels of *ITGAV* in breast cancer, IHC was performed in paired tumor and adjacent normal tissue of breast cancer patients. Results demonstrated *ITGAV* to be highly expressed in tumor tissue when compared to adjacent normal tissue (**Figure 1**).

ITGAV expression is selectively linked to RFS of breast cancer patients

To assess the prognostic value of *ITGAV* in different intrinsic subtypes of breast cancer, the association between *ITGAV* expression and RFS was analyzed using publicly available mi-

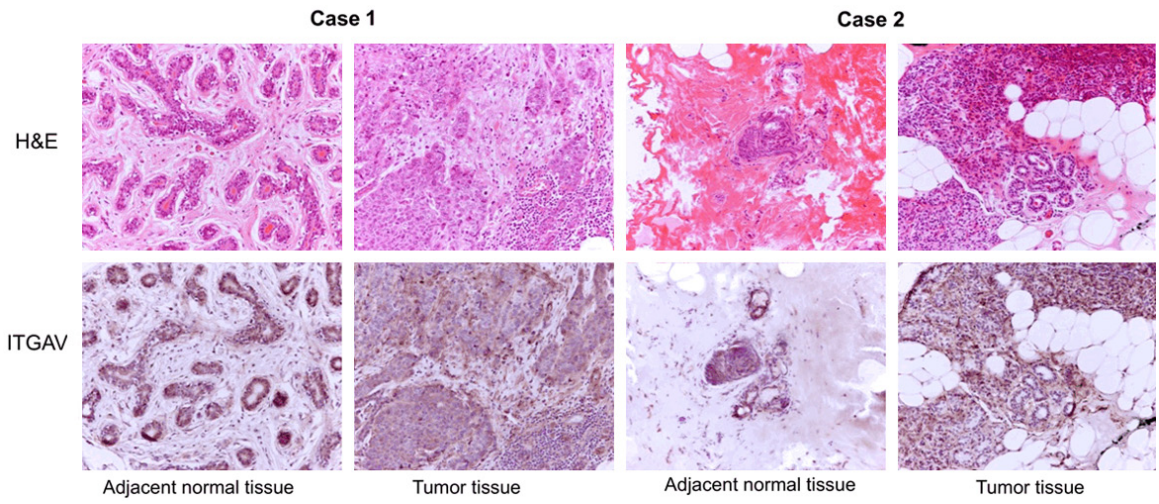


Figure 1. ITGAV expression level in breast cancer patients. Representative H&E and IHC images (20×) showing expression levels of ITGAV in paired tumor and adjacent normal tissues in breast cancer patients.

croarray data. Overexpression of *ITGAV* in primary tumors was strongly associated with poor RFS in luminal B, HER2, and triple-negative breast cancer patients (**Figure 2A-E**).

Silencing of ITGAV inhibits cell proliferation, invasion and migration of breast cancer cells

To investigate whether there is differential *ITGAV* expression in different breast cancer molecular subtypes, baseline gene expression levels of five breast cancer cell lines were evaluated. There was no significant difference in *ITGAV* baseline expression level for any of the cell lines (**Figure 3A**). Cell proliferation, invasion, and migration potential for different breast cancer subtypes were assessed *in vitro* by knockdown of *ITGAV* in MB231 and T47D cells. *ITGAV*-silencing was confirmed by qPCR, western blot (**Figure 3B**), and by immunofluorescence staining of MB231 cells (**Figure 3C**). Cell proliferation of MB231 and T47D was assessed after silencing of *ITGAV* with siRNA or the antagonist, cilengitide. Silencing of *ITGAV* with siRNA significantly inhibited cell proliferation of MB231 and T47D (**Figure 3D**). As well, treatment with cilengitide significantly inhibited cell proliferation of MB231 and T47D (**Figure 3E**). Further, silencing *ITGAV* with siRNA and cilengitide also significantly inhibited cell proliferation of MCF7, MB468, and SKBR3 with varying degrees of effect (Supplementary Figure 1). Colony formation by *ITGAV*-siRNA transfected cells was significantly inhibited in soft agar, anchorage-free cultures (**Figure 3F**). These results demonstrate breast cancer invasiveness to be compromised by *ITGAV* silencing (**Figure 3G**).

Effect of ITGAV upregulation on breast cancer cell tumorigenesis and metastasis

To investigate the expression levels of integrins during cancer progression, gene expression of several integrins was examined in MB231-induced tumorspheres. Stem cell markers *COX2*, *NOTCH2*, *SNAI2*, as well as *ITGAV,* were significantly upregulated in tumorspheres (**Figure 4A**). Furthermore, the $CD44^{high}/CD24^{low}$ subpopulation was increased in subsequent passages of the tumorspheres, suggesting an increase in stem-cell like features (**Figure 4B**). Expression of *ITGAV* and Ki67 was higher in tumorsphere-induced tumors than in MB-231-induced tumors (**Figure 4C**). Further, silencing *ITGAV* with siRNA significantly reduced tumorsphere formation (**Figure 4D**). High levels of ALDH activity have been associated with stem-cell like properties. Analysis of ALDH by flow cytometry demonstrated silencing of *ITGAV* to significantly reduce the number of ALDH-positive cells (**Figure 4E**). These data suggest that *ITGAV* confers stem cell-like properties on breast cancer cells.

Expression of PXN is correlated with ITGAV expression

To delineate genes involved in *ITGAV*-mediated regulatory pathways, gene expression in MB231 cells after silencing of *ITGAV* was com-

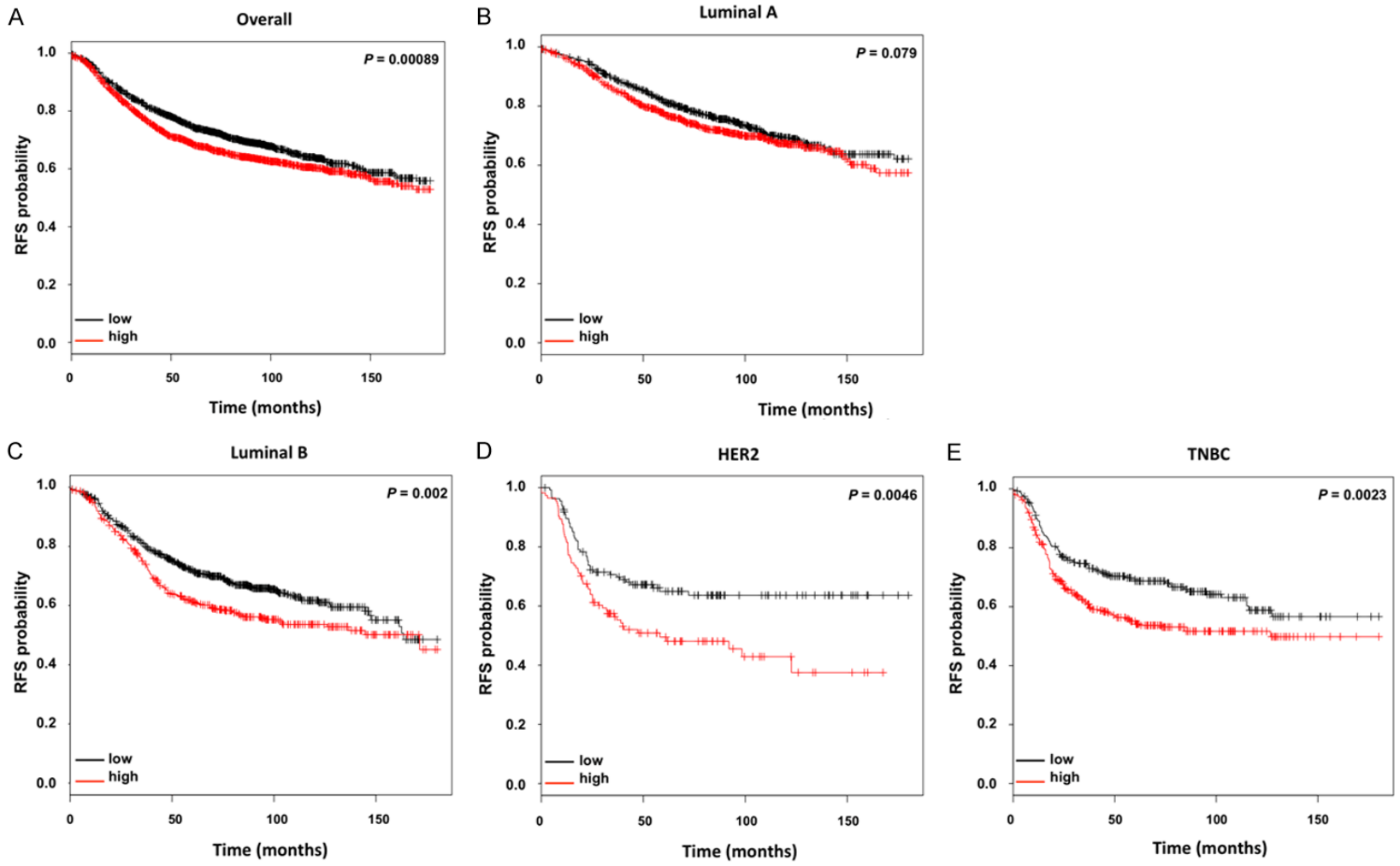


Figure 2. Kaplan-Meier plot of relapse-free survival (RFS) of breast cancer patients, stratified by expression of *ITGAV* in their primary tumors. A. Kaplan-Meier plot of breast cancer patients. B. Kaplan-Meier plot of breast cancer patients with luminal A subtype. C. Kaplan-Meier plot of breast cancer patients with luminal B subtype. D. Kaplan-Meier plot of breast cancer patients with HER2 subtype. E. Kaplan-Meier plot of breast cancer patients with triple-negative subtype. *P* values were calculated using log-rank test.

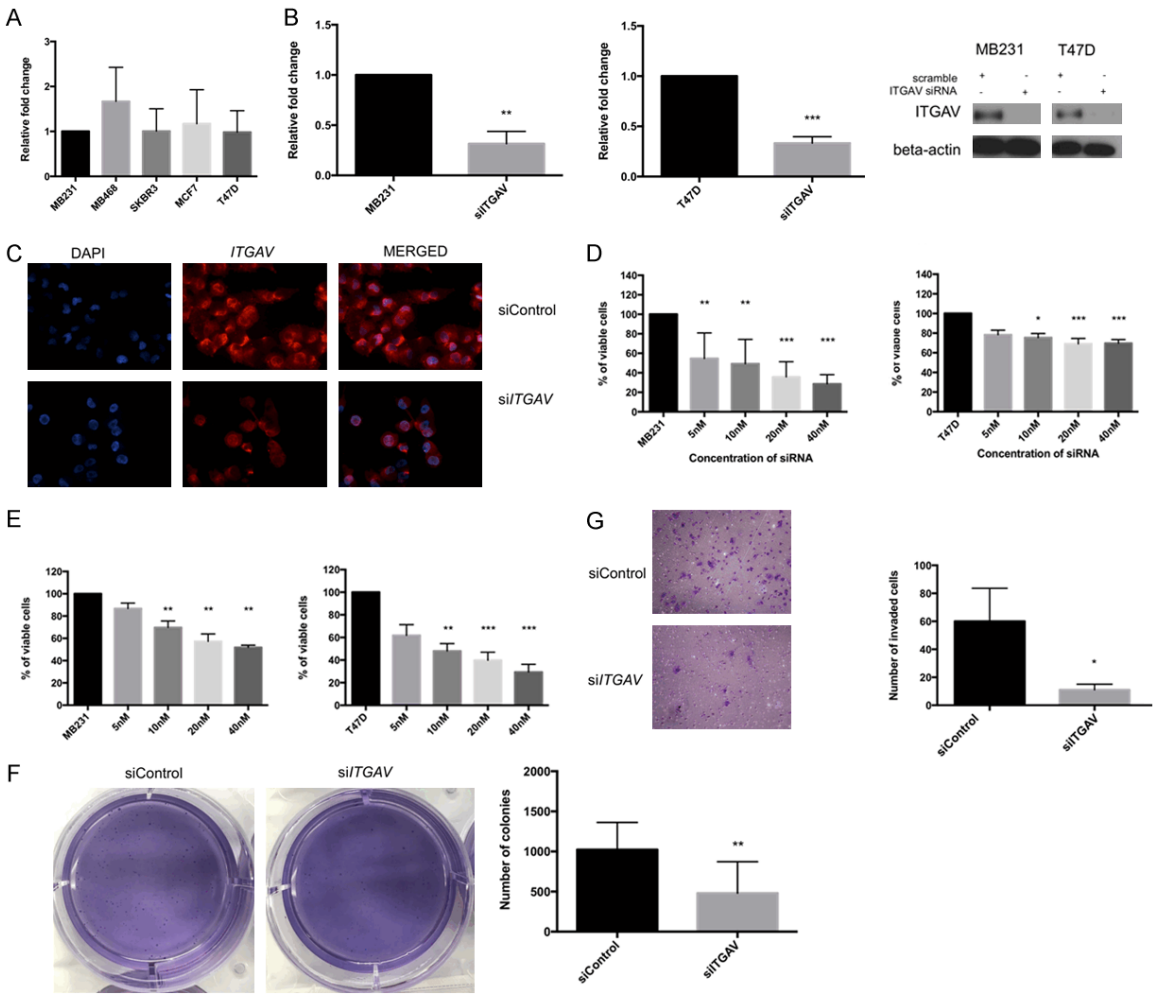
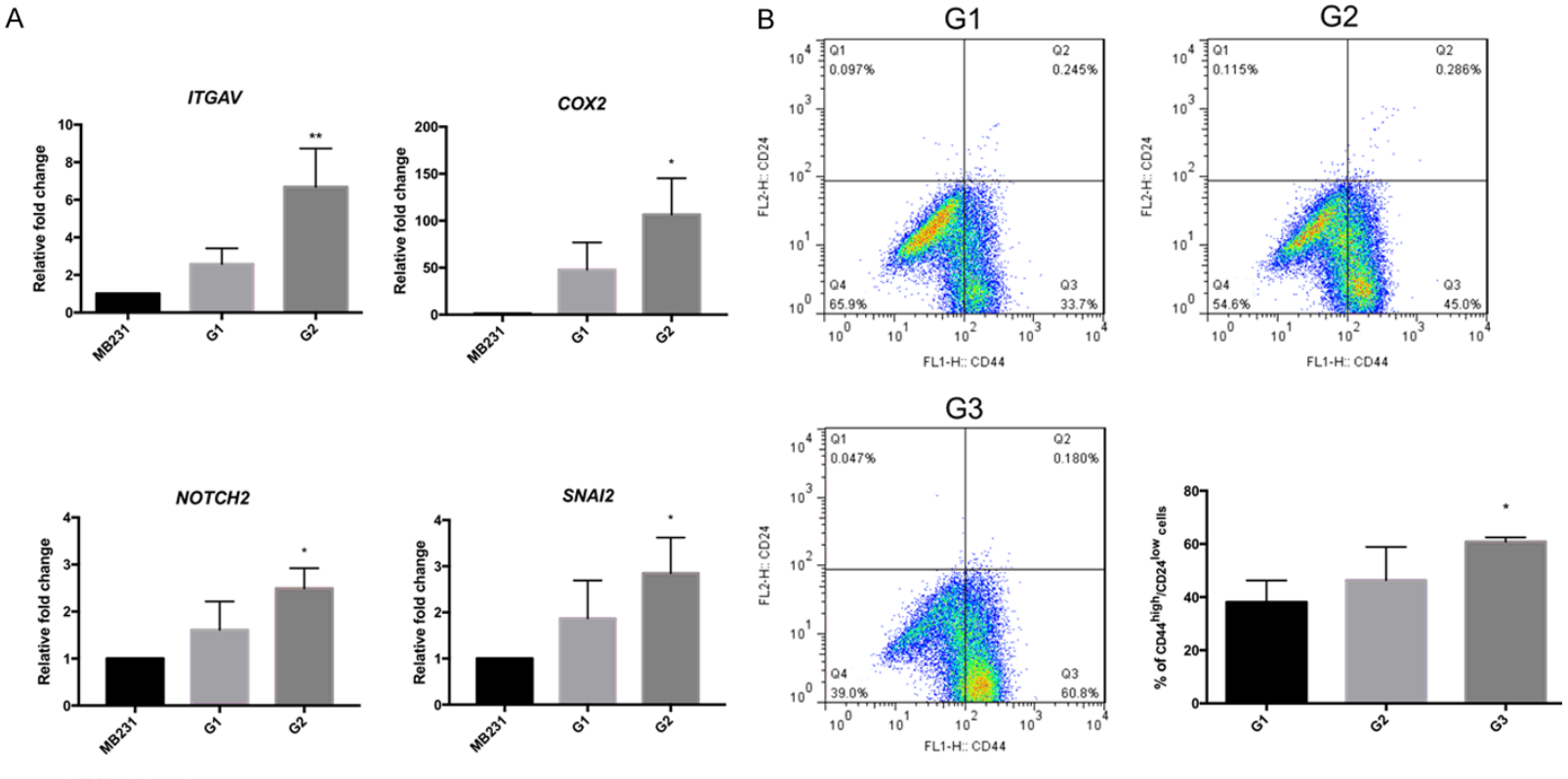


Figure 3. Silencing of *ITGAV* inhibits cell proliferation, invasion and migration of breast cancer cells. A. Expression of *ITGAV* in five breast cancer cell lines. B. Relative expression of *ITGAV* after siRNA silencing in MB231 and T47D cells. C. Immunofluorescence images of MB231 and *ITGAV*-siRNA transfected cells. D. Effect of *ITGAV*-siRNA on cell proliferation of MB231 and T47D cells. E. Effect of Cilengitide on cell proliferation of MB231 and T47D cells. F. Effect of *ITGAV*-siRNA on colony formation abilities of MB231 cells. Representative images of colonies induced from MB231 (left) and *ITGAV*-siRNA transfected cells (right). G. Effect of *ITGAV*-siRNA on cell migration of MB231 cells. Representative images (10× magnification) of invaded cells of MB231 (top) and *ITGAV*-siRNA transfected cells (bottom). All experiments were performed in triplicate. *P<0.05, **P<0.01 and ***P<0.005 are considered statistically significance.

pared to parental cells by Partek pathway analysis software. Silencing of *ITGAV* resulted in downregulation of *BCL2* in both MB231 and T47D (**Figure 5A**). Notably, *PXN*, which encodes for the focal adhesion protein paxillin, was also downregulated after silencing of *ITGAV* in both MB231 and T47D (**Figure 5B**).

Overexpression of ITGAV promotes lung metastasis in vivo

Expression of *ITGAV* was compared between primary tumors extracted from the mammary fat pad and metastatic tumors isolated from the lung. Higher expression of *ITGAV* and *PXN* was observed in metastatic tissues when compared to primary tumors (**Figure 6A**, **6B**). These results suggest that upregulation of *ITGAV* promotes breast cancer tumorigenesis and metastasis through regulation of *PXN*. Furthermore, treatment with the *ITGAV* antagonist, cilengtide, significantly decreased lung metastatic area percentage and proliferative index in comparison to the control group (**Figure 6C**, **6D**). Taken together, these data suggest that *ITGAV* upregulation plays a key role in the promotion of lung metastasis.


A
ITGAV
Relative fold change
MB231
G1
G2
COX2
NOTCH2
SNAI2
B
G1
G2
G3
Q1
Q2
Q3
Q4
0.097%
0.245%
65.9%
33.7%
0.115%
0.286%
54.6%
45.0%
0.047%
0.180%
39.0%
60.8%
FL1-H:: CD44
FL2-H:: CD24
% of CD44high/CD24low cells

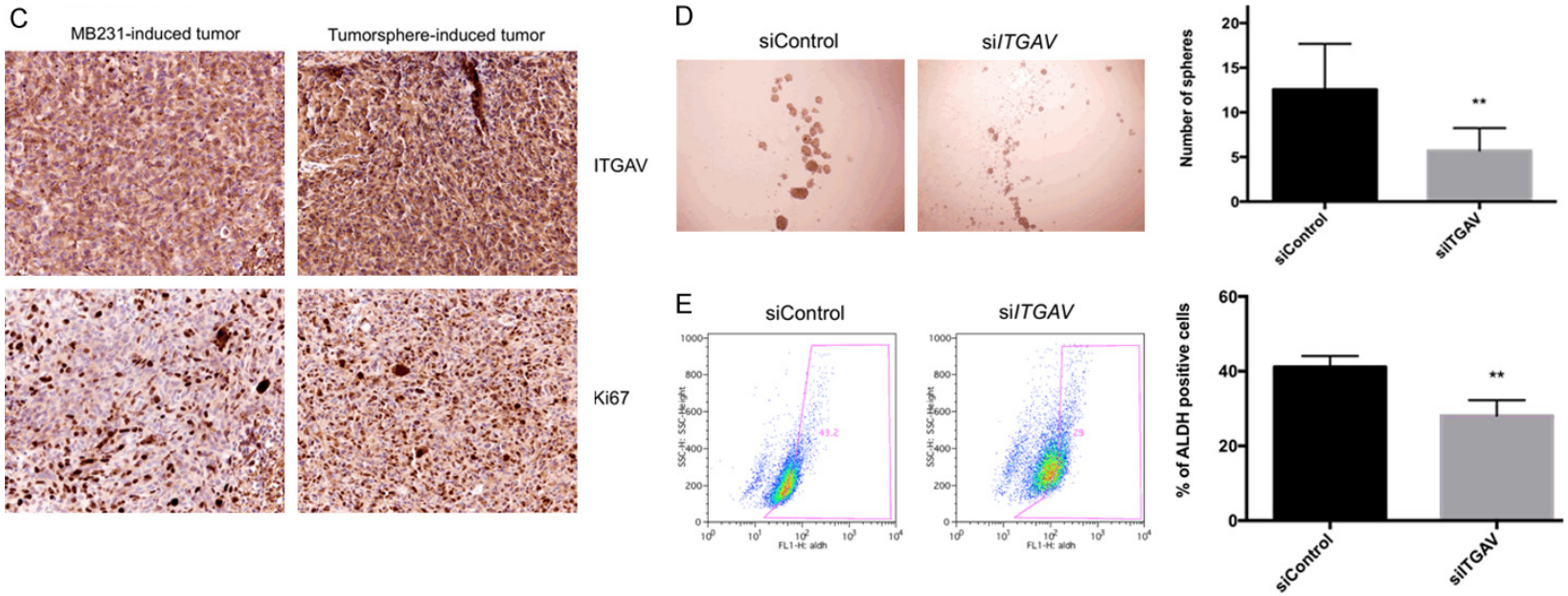


Figure 4. Upregulation of ITGAV in breast cancer tumorigenesis and metastasis. A. Expression of COX2, *ITGAV, NOTCH2 and SNAI2* in tumorspheres. B. Representative images of CD44+/CD24- subpopulation in primary (G1) (top left), secondary (G2) (top right), and tertiary (G3) (bottom left) tumorspheres. C. Representative images of *ITGAV* and Ki67 expressions in tumor tissues induced by MB231 and MB231-induced tumorspheres. D. Effect of *ITGAV*-siRNA on tumorsphere formation. Representative images (40×) of tumorspheres induced from MB231 (left) and *ITGAV* siRNA transfected cells (middle). E. Representative images of ALDH flow cytometry of MB231 (left) and *ITGAV* siRNA transfected cells (right). All experiments were performed in triplicate. *P<0.05 and **P<0.01 are considered statistically significance.

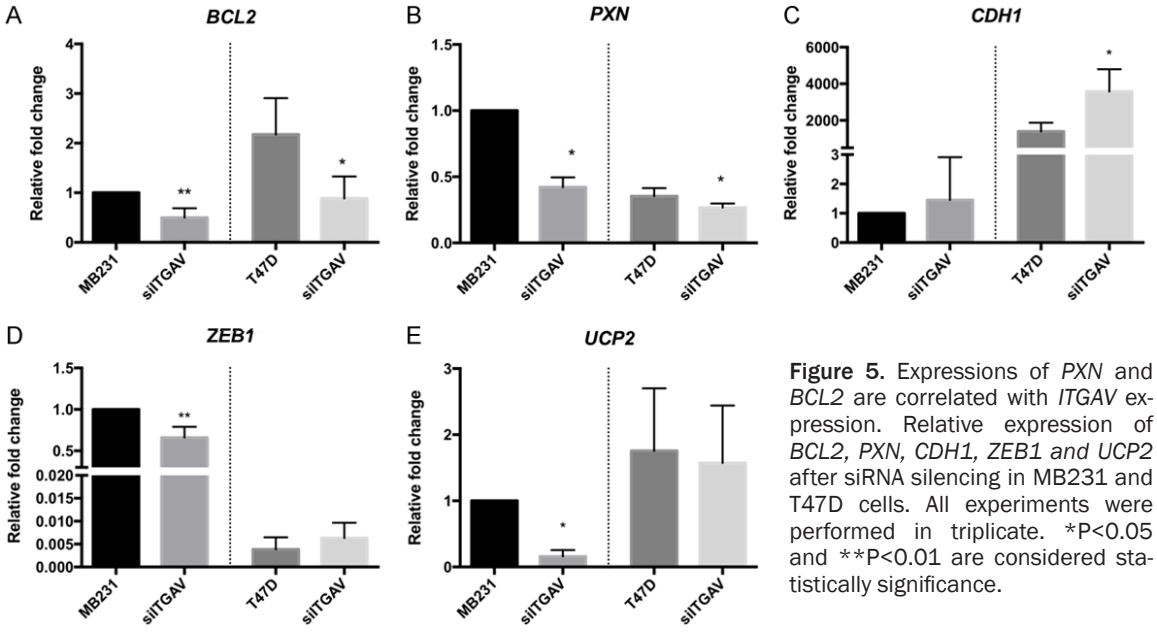


Figure 5. Expressions of *PXN* and *BCL2* are correlated with *ITGAV* expression. Relative expression of *BCL2, PXN, CDH1, ZEB1 and UCP2* after siRNA silencing in MB231 and T47D cells. All experiments were performed in triplicate. *P<0.05 and **P<0.01 are considered statistically significance.

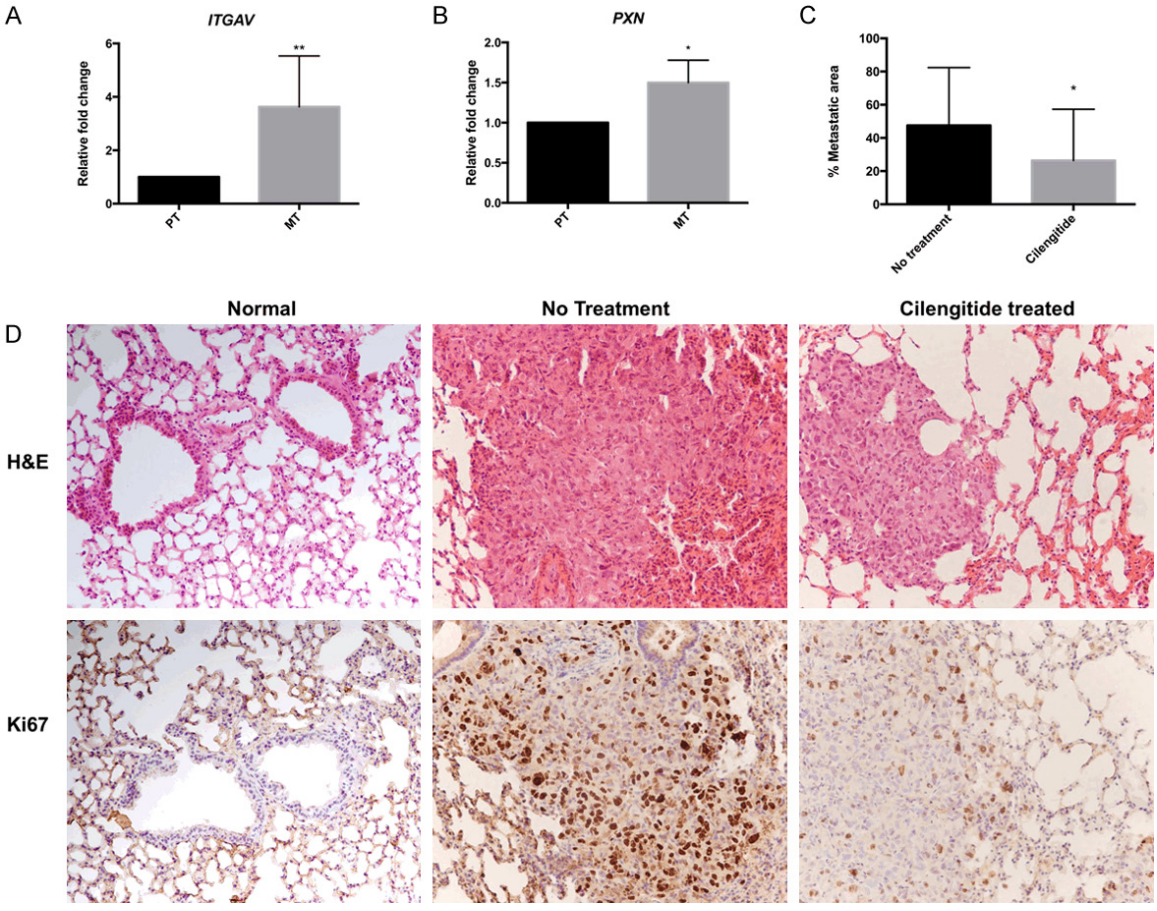


Figure 6. Overexpression of *ITGAV* mediates lung metastasis in vivo. (A, B) Relative expression of (A) *ITGAV* and (B) *PXN* in metastatic tissues (MT) when compared with primary tumors (PT). (C) Percent metastatic area in the lung was quantified in control mice (n=4) and mice treated with cilengitide (n=4) after tail vein injection of MB231 cells (5 visual fields per mice). (D) Representative photographs of H&E-stained and expression of ki67 of lung sections (20×). *P<0.05 is considered statistically significance.

Discussion

Metastasis is one of the major causes of death of breast cancer patients even for those diagnosed at an early stage. Finding effective molecular targets to prevent/treat metastasis will help to improve the prognosis for these patients. Integrins are adhesion molecules that play a pivotal role in cancer progression by modulating cell-cell and cell-ECM interactions. Cancer cells that have undergone EMT show increased motility and reduced cell adhesion, suggesting a critical role for integrins in cancer progression. As such, integrins are an appealing target for cancer therapy.

High expression of the $\alpha_V\beta_3$ integrin has been demonstrated in tumors and angiogenic cells [11]. Overexpression of the $\alpha_V\beta_3$ integrin has been reported in various types of cancers including glioblastoma, melanoma, ovarian, pancreatic, prostate, and breast cancer [10, 11, 23, 25-27]. With regard to *ITGAV*, only limited reports have evaluated expression levels and the functional role of *ITGAV* in cancer progression. The integrin α_V subunit pairs with multiple beta-subunits to form a number of integrin receptors. A previous *in vivo* study demonstrated that $\alpha_V\beta_3$ promoted organ-specific breast cancer metastasis to bone and lung [28]. Herein, breast cancer *ITGAV* overexpression conferred stem-cell like properties *in vitro* and lung tissue metastasis *in vivo,* indicating a potential role for *ITGAV* in breast cancer progression. Knockdown of *ITGAV* in breast cancer cells inhibited proliferation, invasion, and colony formation, consistent with previous results for colorectal cancer, laryngeal, and hypopharyngeal carcinoma [15, 29]. Waisberg *et al.* reported overexpression of *ITGAV* to be associated with higher progression and spread of colorectal cancer. A direct correlation between *ITGAV* and Ki67 was also reported [15]. Further, *ITGAV* overexpression is an unfavorable prognostic marker for several other cancers including stomach, liver, and pancreatic cancer [30, 31]. Herein, *ITGAV* silencing downregulated *BCL2* and *PXN in vitro* and treatment with cilengitide reduced lung metastasis and expression of Ki67 *in vivo*. KM analysis using publicly available data demonstrated a strong correlation of *ITGAV* overexpression and poor prognosis for breast cancer patients. The relationship between *ITGAV* and *PXN* expression and the association of that relationship with breast cancer metastasis requires further investigation.

Integrin inhibitors have been investigated for therapeutic application in both preclinical and clinical studies of various types of solid cancers. For example, LM609 is a mouse monoclonal antibody that targets the human $\alpha_V\beta_3$ integrin, inhibiting angiogenesis [32]. It was the first integrin antagonist assessed in a preclinical study as a therapeutic cancer intervention [10]. LM609 inhibits angiogenesis by suppressing FGF-2 and TNF-a signaling [33]. Further, inhibited tumor invasion, tumor growth, and organ-specific metastasis to bone have been demonstrated for LM609 with breast cancer and melanoma [32, 34]. Due to the success of LM609 in preclinical studies, humanized versions of LM609, such as Vitaxin and etaracizumab have been developed to investigate potential clinical applications for cancer treatment [35]. Vitaxin was the first humanized form of LM609 tested in a clinical setting [36] and was later reformulated as etaracizumab (Abegrin) [11]. Etaracizumab is an $\alpha_V\beta_3$ antagonist that blocks the binding of vitronectin and osteopontin to integrins, inhibiting angiogenesis and metastasis [33]. Combinations of etaracizumab and anti-angiogenetic agents have produced promising outcomes in ovarian cancer [27]. The therapeutic potential of etaracizumab is under investigation in several clinical trials for treatment of solid tumors as well as treatment of psoriasis and rheumatoid arthritis [11]. Other integrin α_V antagonists such as CNTO 95 and GLPG0187 also have antitumor activity in breast [17], prostate [16], and bladder cancer [37].

Cilengitide is a cyclic arginylglycylaspartic acid (RGD) pentapeptide that specifically targets $\alpha_V\beta_3$ and a_Vb_5 integrin receptors [38]. Cilengitide is the most advanced integrin inhibitor, initially used in preclinical studies of melanoma and glioblastoma [32, 38]. Subsequent preclinical studies of various types of cancer have investigated cilengitide as a therapeutic cancer treatment modality. Cilengitide has been shown to inhibit metastasis of breast cancer [23, 25, 26] and osteosarcoma [39]. Consistent with these previous findings, our results demonstrate cilengitide to inhibit breast cancer proliferation in a dose-dependent manner, inhibit lung cancer metastasis, and reduce Ki67 expression.

Conclusion

In conclusion, high levels of *ITGAV* expression were demonstrated in cancer metastatic tissue and in cancer cells with stem-cell like proper-

ties. As such, *ITGAV* should be considered as a potential therapeutic target for treatment of metastatic breast cancer as well as primary breast tumors with high *ITGAV* expression. Such treatment may improve therapeutic efficacy and treatment outcomes.

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Disclosure of conflict of interest

None.

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References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.

[2] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015; 136: E359-86.

[3] Kennecke H, Yerushalmi R, Woods R, Cheang MC, Voduc D, Speers CH, Nielsen TO and Gelmon K. Metastatic behavior of breast cancer subtypes. J Clin Oncol 2010; 28: 3271-7.

[4] Singh A and Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene 2010; 29: 4741-51.

[5] Polyak K and Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer 2009; 9: 265-73.

[6] Frantz C, Stewart KM and Weaver VM. The extracellular matrix at a glance. J Cell Sci 2010; 123: 4195-200.

[7] Hood JD and Cheresh DA. Role of integrins in cell invasion and migration. Nat Rev Cancer 2002; 2: 91-100.

[8] Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P. Integrins. In molecular biology of the Cell, 4th edition. New York: Garland Science; 2002.

[9] Takada Y, Ye X and Simon S. The integrins. Genome Biol 2007; 8: 215.

[10] Desgrosellier JS and Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. Nat Rev Cancer 2010; 10: 9-22.

[11] Weis SM and Cheresh DA. αV integrins in angiogenesis and cancer. Cold Spring Harb Perspect Med 2011; 1: a006478.

[12] Avraamides CJ, Garmy-Susini B and Varner JA. Integrins in angiogenesis and lymphangiogenesis. Nat Rev Cancer 2008; 8: 604-17.

[13] Aoudjit F and Vuori K. Integrin signaling in cancer cell survival and chemoresistance. Chemother Res Pract 2012; 2012: 283181.

[14] van der Horst G, Bos L and van der Pluijm G. Epithelial plasticity, cancer stem cells, and the tumor-supportive stroma in bladder carcinoma. Mol Cancer Res 2012; 10: 995-1009.

[15] Waisberg J, De Souza Viana L, Affonso Junior RJ, Silva SR, Denadai MV, Margeotto FB, De Souza CS and Matos D. Overexpression of the ITGAV gene is associated with progression and spread of colorectal cancer. Anticancer Res 2014; 34: 5599-607.

[16] van der Horst G, van den Hoogen C, Buijs JT, Cheung H, Bloys H, Pelger RC, Lorenzon G, Heckmann B, Feyen J, Pujuguet P, Blanque R, Clément-Lacroix P and van der Pluijm G. Targeting of alpha(v)-integrins in stem/progenitor cells and supportive microenvironment impairs bone metastasis in human prostate cancer. Neoplasia 2011; 13: 516-525.

[17] Chen Q, Manning CD, Millar H, McCabe FL, Ferrante C, Sharp C, Shahied-Arruda L, Doshi P, Nakada MT and Anderson GM. CNTO 95, a fully human anti alphav integrin antibody, inhibits cell signaling, migration, invasion, and spontaneous metastasis of human breast cancer cells. Clin Exp Metastasis 2008; 25: 139-148.

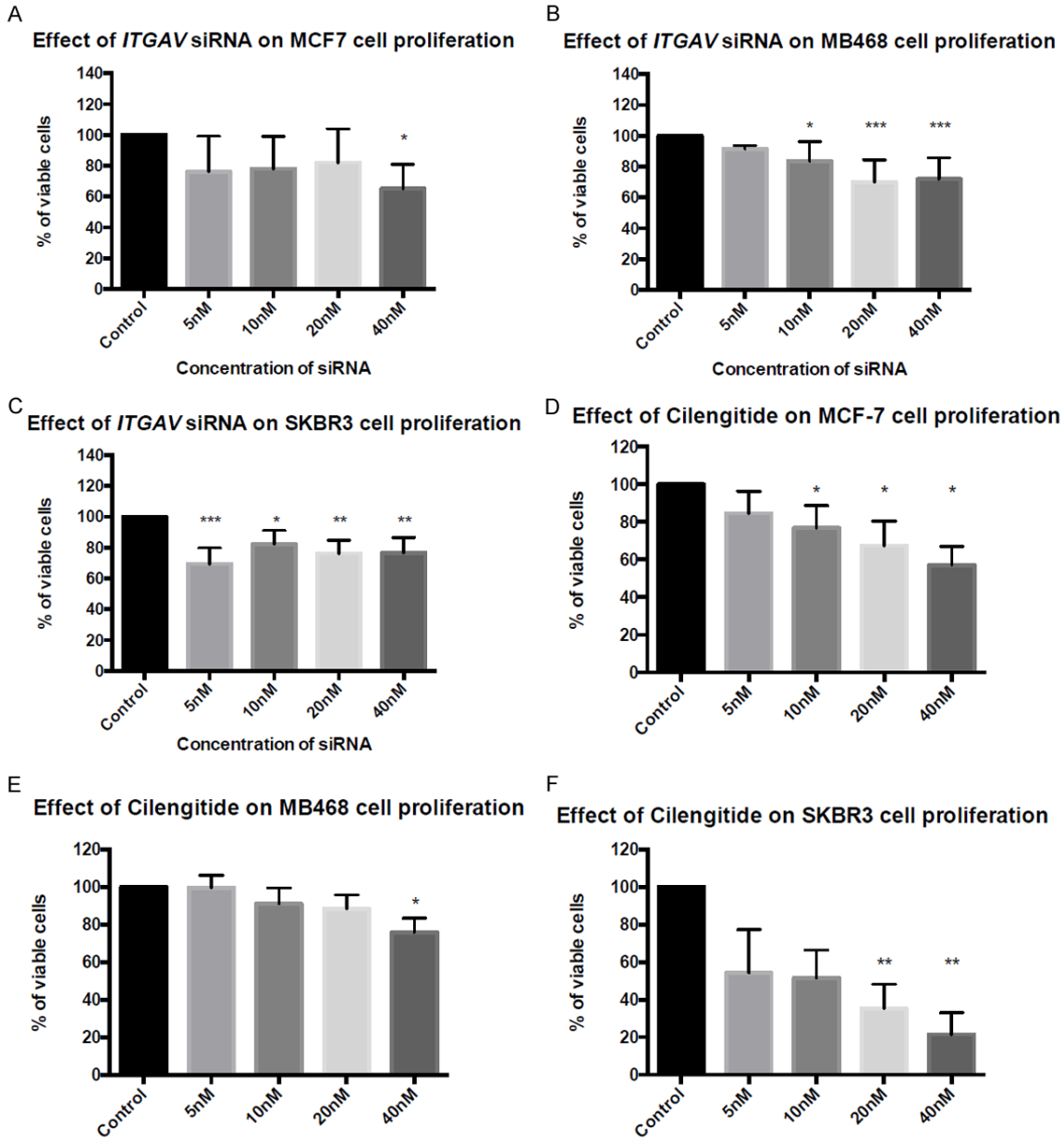
[18] Chen DL, Wang ZQ, Ren C, Zeng ZL, Wang DS, Luo HY, Wang F, Qiu MZ, Bai L, Zhang DS, Wang FH, Li YH and Xu RH. Abnormal expression of paxillin correlates with tumor progression and poor survival in patients with gastric cancer. J Transl Med 2013; 11: 277.

[19] Turner CE. Paxillin and focal adhesion signalling. Nat Cell Biol 2000; 2: E231-236.

[20] Wu DW, Wu TC, Wu JY, Cheng YW, Chen YC, Lee MC, Chen CY and Lee H. Phosphorylation of paxillin confers cisplatin resistance in non-small cell lung cancer via activating ERK-mediated Bcl-2 expression. Oncogene 2014; 33: 4385-95.

[21] Mas-Moruno C, Rechenmacher F and Kessler H. Cilengitide: the first anti-angiogenic small molecule drug candidate design, synthesis and clinical evaluation. Anticancer Agents Med Chem 2010; 10: 753-68.

[22] Stupp R, Picard M and Weller M. Does cilengitide deserve another chance?-Authors' reply. Lancet Oncol 2014; 15: e585-e586.
[23] Bauerle T, Komljenovic D, Merz M, Berger MR, Goodman SL and Semmler W. Cilengitide inhibits progression of experimental breast cancer bone metastases as imaged noninvasively using VCT, MRI and DCE-MRI in a longitudinal in vivo study. Int J Cancer 2011; 128: 2453-62.
[24] Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q and Szallasi Z. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat 2010; 123: 725-731.
[25] Lautenschlaeger T, Perry J, Peereboom D, Li B, Ibrahim A, Huebner A, Meng W, White J and Chakravarti A. In vitro study of combined cilengitide and radiation treatment in breast cancer cell lines. Radiat Oncol 2013; 8: 246.
[26] Burke PA, DeNardo SJ, Miers LA, Lamborn KR, Matzku S and DeNardo GL. Cilengitide targeting of alpha(v)beta(3) integrin receptor synergizes with radioimmunotherapy to increase efficacy and apoptosis in breast cancer xenografts. Cancer Res 2002; 62: 4263-4272.
[27] Landen CN, Kim TJ, Lin YG, Merritt WM, Kamat AA, Han LY, Spannuth WA, Nick AM, Jennnings NB, Kinch MS, Tice D and Sood AK. Tumor-selective response to antibody-mediated targeting of alphavbeta3 integrin in ovarian cancer. Neoplasia 2008; 10: 1259-1267.
[28] Sloan EK, Pouliot N, Stanley KL, Chia J, Moseley JM, Hards DK and Anderson RL. Tumor-specific expression of alphavbeta3 integrin promotes spontaneous metastasis of breast cancer to bone. Breast Cancer Res 2006; 8: R20.
[29] Lu JG, Li Y, Li L and Kan X. Overexpression of osteopontin and integrin alphav in laryngeal and hypopharyngeal carcinomas associated with differentiation and metastasis. J Cancer Res Clin Oncol 2011; 137: 1613-8.
[30] Uhlen M, Zhang C, Lee S, Sjöstedt E, Fagerberg L, Bidkhori G, Benfeitas R, Arif M, Liu Z, Edfors F, Sanli K, von Feilitzen K, Oksvold P, Lundberg E, Hober S, Nilsson P, Mattsson J, Schwenk JM, Brunnström H, Glimelius B, Sjöblom T, Edqvist PH, Djureinovic D, Micke P, Lindskog C, Mardinoglu A and Ponten F. A pathology atlas of the human cancer transcriptome. Science 2017; 357.
[31] Human Protein Atlas [www.proteinatlas.org].
[32] Huang R and Rofstad EK. Integrins as therapeutic targets in the organ-specific metastasis of human malignant melanoma. J Exp Clin Cancer Res 2018; 37: 92.
[33] Weber G. Anti-Angiogenesis. In: editors. Molecular therapies of cancer. Springer, Cham; 2015. pp. 353-371.
[34] Kwakwa KA and Sterling JA. Integrin αvβ3 signaling in tumor-induced bone disease. Cancers (Basel) 2017; 9.
[35] Foubert P and Varner JA. Integrins in tumor angiogenesis and lymphangiogenesis. Methods Mol Biol 2012; 757: 471-486.
[36] Stupack DG and Cheresh DA. Integrins and angiogenesis. Curr Top Dev Biol 2004; 64: 207-38.
[37] van der Horst G, Bos L, van der Mark M, Cheung H, Heckmann B, Clement-Lacroix P, Lorenzon G, Pelger RC, Bevers RF and van der Pluijm G. Targeting of alpha-v integrins reduces malignancy of bladder carcinoma. PLoS One 2014; 9: e108464.
[38] Reardon DA and Cheresh D. Cilengitide: a prototypic integrin inhibitor for the treatment of glioblastoma and other malignancies. Genes Cancer 2011; 2: 1159-65.
[39] Gvozdenovic A, Boro A, Meier D, Bode-Lesniewska B, Born W, Muff R and Fuchs B. Targeting alphavbeta3 and alphavbeta5 integrins inhibits pulmonary metastasis in an intratibial xenograft osteosarcoma mouse model. Oncotarget 2016; 7: 55141-55154.



Supplementary Figure 1. A. Effect of *ITGAV*-siRNA on cell proliferation of MCF7 cells. B. Effect of *ITGAV*-siRNA on cell proliferation of MB468 cells. C. Effect of *ITGAV*-siRNA on cell proliferation of SKBR3 cells. D. Effect of Cilengitide on cell proliferation of MCF7 cells. E. Effect of Cilengitide on cell proliferation of MB468 cells. F. Effect of Cilengitide on cell proliferation of SKBR3 cells. All experiments were performed in triplicate. *p<0.05, **p<0.01 and ***p<0.005 are considered statistically significance.