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Integrin β5 contributes to the tumorigenic potential of breast cancer cells through Src-FAK and MEK-ERK signaling pathways

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Abstract

Cancer progression, response to therapy and metastasis depend on tumor microenvironment. Integrins are cell adhesion receptors that mediate interactions of cells with extracellular matrix (ECM). The αv-β-family of integrins contributes to tumorigenesis, response to therapy and cancer-stem cell biology. Thus, understanding the function of specific integrins in cancer is critical for development of therapeutic approaches targeting integrins. The study investigated the role of integrin β5 in breast carcinomas by depleting integrin β5 using RNA interference and re-expression of integrin β5. Depletion of integrin β5 in triple-negative breast carcinoma cells markedly reduced tumor take, growth, and tumor angiogenesis, while re-expression of integrin β5 rescued this phenotype. Reduction in tumor angiogenesis is associated with lower expression of VEGF-A in integrin β5-depleted tumors. Tumor cells deficient for integrin β5 have lower migration and proliferative capacities. Biochemical assays revealed that integrin β5 mediates Src-FAK and MEK-ERK signaling events that operate independently, and inhibition of these pathways phenocopies integrin β5-deficiency. Breast carcinoma cells express high levels of integrin β5, whereas expression of integrin β3 is limited to stromal compartments and integrin β6 is lost in metastatic cells. Together these findings show a critical role for integrin β5 in the tumorigenic potential of breast carcinoma cells and therapeutic targeting of integrin β5 is especially attractive for triple-negative breast carcinomas, which are refractory to most of the current therapies.

Keywords

Integrin; focal adhesion kinase (FAK); extracellular signal-regulated kinase (ERK); anchorage-independent growth; transforming growth factor β (TGF-β)

INTRODUCTION

The lifetime risk of breast cancer (BC) is currently one in eight women and advanced breast cancers are frequently incurable [1]. Breast cancer is a heterogeneous disease with several subtypes classified by molecular and clinico-pathological characteristics [2]. Specific

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CONFLICT OF INTEREST

None to disclose

therapeutic strategies have been developed for treatment of hormone-receptor positive (ER/PR) and HER2-positive sub-groups [3]. Breast tumors with low levels of HER2, oestrogen and progesterone hormone receptors constitute a group of triple-negative breast cancers (TNBCs) that occur more frequently among young women (<50 years) and in women of African and Hispanic descent [2]. This group of breast cancers does not have a specific targeted therapy due to incomplete understanding of the genetic and epigenetic events causing TNBCs [3]. TNBCs account for up to 17% of all breast carcinomas and patients with TNBCs present a more aggressive disease and have a shorter survival time compared to other sub-groups. Significant research effort is directed on identification of therapeutic targets for TNBCs [4, 5].

Breast cancer progression and response to therapy have been linked to tumor cell interaction with extracellular matrix (ECM). Integrins, heterodimeric trans-membrane matrix receptors, are major mediators of cell adhesion to ECM and ECM-induced intracellular signaling [6, 7]. Several β-integrins of the αv-β-family, including β1, β3 and β5, have been linked to invasion, epithelial-mesenchymal transition and cancer-stem cell biology [7, 8]. Integrin levels are frequently elevated in aggressive tumors, making these proteins attractive targets for therapy, although the function of specific integrins is not fully understood [7]. Recent studies have shown that integrin β5 contributes to the transforming growth factor β (TGF-β)-induced EMT [8], tumor angiogenesis [9] and resistance to radio- and chemotherapy [10, 11]. Integrins lack enzymatic activity and promote intracellular signaling by recruiting and activating integrin-associated kinases such as focal adhesion kinase (FAK) and integrin-linked kinase (ILK) [6, 12]. FAK is a non-receptor tyrosine kinase that is activated at the sites of cell-matrix adhesions and integrin clustering by auto-phosphorylation at Tyr397 and by Src and other tyrosine kinases [13]. FAK can also mediate signaling of various growth factor receptors such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), G-protein coupled receptors (GPCRs) and hepatocyte growth factor receptor c-MET [12]. In particular, the interaction of Src with FAK at Tyr861 is crucial for integrin β5-mediated signaling in response to VEGF [14] and Ras transformation of fibroblasts [15]. In some systems, FAK can activate mitogen-activated protein kinases (MAPK) [16–18]. Breast tumors with higher histologic grade and of a triple-negative sub-type express elevated levels of FAK [19]. Thus, β-integrins and their signaling components might be potential therapeutic targets in breast cancer.

The present work examines the function of integrin β5 in the tumorigenic capacity of advanced-stage breast carcinoma cells by modulating integrin β5 levels using RNA interference and forced expression approaches. The study provides evidence that integrin β5 facilitates cancer cell migration, anchorage-independent growth and tumor angiogenesis. Integrin β5 enables intracellular Src-FAK and MEK-ERK signaling events that operate independently and underlie the tumorigenic potential of carcinoma cells. Thus, integrin β5 is an attractive therapeutic target in aggressive breast cancers.

RESULTS

Integrin β5 is expressed in primary and metastatic breast cancer cells

Previous studies have implicated integrin β5 in pro-tumorigenic activities of TGF-β such as EMT and invasion [8]. To address the role of integrin β5 in breast cancer, we measured expression of αvβ-integrins in cell lines representing triple-negative and luminal breast carcinomas. Semi-quantitative RT-PCR assays showed expression of integrins αv, β1 and β5 in non-tumor and cancer cell lines, whereas integrin β6 is markedly reduced in all cancer cells. Integrin β3 expression is low in triple-negative and absent in ER/PR-positive cell lines. Integrins β5, β3 and αv were induced by TGF-β1 in triple-negative cell lines: MCF10A, MDA-MB-231 and BT549 (Figure 1A). All tested cell lines expressed integrin β5 protein with the highest level in the primary carcinoma Hs578T cells, while integrin β1 is preferentially expressed by triple-negative cells (Figure 1B). These results were validated by the metadata analysis of the Oncomine database (www.oncomine.org). To distinguish epithelial and stromal compartments of breast tumors, we assessed two datasets utilizing a laser microdissection approach for RNA isolation [20, 21]. The Richardson Breast2 dataset of breast epithelium [20] shows high mRNA levels of integrins αv, β1 and β5 in normal breast and ductal carcinomas (Supplemental Figure 1A). Integrin β6 expression is reduced in tumors compared to normal epithelium, similar to tropomyosin-1 (TPM1), a known tumor suppressor [22]. Integrin β3 is not expressed by either normal or tumor epithelial tissues. In contrast, the Finak dataset examining breast stroma [21] reveals elevated levels of integrin β3 in tumor stroma, while integrin β5 is low in this compartment (Supplemental Figure 1A). Protein levels of integrins in breast cancer tissues were assessed using the Human Protein Atlas database (www.proteinatlas.org). This database contains immunohistochemistry (IHC) data and the quality-control information for a genome-wide set of targets. High protein levels of αv, β1, and β5 integrins were found in normal and carcinoma cells, whereas integrin β3 staining was limited to stromal cells (Supplementary Figure 1B), consistent with the Oncomine data. Thus, high levels of integrin β5 is found in breast carcinoma cells, suggesting an important role for this integrin in both triple-negative and ER-/PR-positive cancers.

Integrin β5 is important for the anchorage-independent growth of breast carcinoma cells

The role of integrin β5 in breast cancer cells was examined by depleting integrin β5 using RNA interference and testing cell growth in anchorage-independent assays that closely predict the tumorigenic capacity [23]. Protein levels of integrin β5 were effectively reduced in two cell lines representing triple-negative (MDA-MB-231) and ER/PR-positive (MCF7) breast carcinomas (Figure 1C–D). The specificity of siRNA duplexes was validated in previous studies [8]. Depletion of integrin β5 resulted in 30–40% reduction of soft-agar colonies by both cell lines (Figure 1C–D), indicating a critical role of integrin β5 in the tumorigenic potential of breast cancer cells.

Integrin β5 contributes to breast carcinoma cell adhesion and migration

To confirm the siRNA findings, we generated MDA-MB-231 cells with stable suppression of integrin β5 by shRNA targeting the 3′-UTR of human integrin β5 (shRNA-ITGB5, shB5). For validation, the shB5 cells were transduced with a human integrin β5 cDNA construct in

the pBMN-IRES-EGFP bi-cistronic retroviral vector that is not targeted by shRNA-ITGB5. The resulting cells (MDA-MB-231-shB5-oeB5) express EGFP and integrin β5. Control cells were transduced with the EGFP-empty vector. Immunoblotting showed a significant reduction of integrin β5 in the shB5 cells and high integrin β5 levels in the shB5-oeB5 cells (Figure 2A). The shRNA-ITGB5 construct did not affect mRNA levels (Supplementary Figure 2), similar to other micro-RNAs [24]. Depletion of integrin β5 did not reduce basal and TGF-β-induced levels of integrin β1 (Figure 2A).

Soft-agar assays revealed a 75% reduction in colony formation by the shB5 cells compared to the control, whereas cells re-expressing integrin β5 (shB5-oeB5) recovered the anchorage-independent growth (Figure 2B). To assess whether modulation of integrin β5 expression affects surface presentation of integrin β1 or complexes with αv-integrin, we carried out flow cytometry assays with antibodies recognizing surface epitopes of αvβ5 or β1 integrins. Cells depleted of integrin β5 displayed nearly 5-fold fewer αvβ5 integrin on surface compared to control cells, while shB5-oeB5 cells had 50% more than control (Figure 2C). The surface presentation of integrin β1 was comparable across all three cell lines (Figure 2C, bottom panel). Together these findings confirm a critical role of integrin β5 in anchorage-independent growth of breast carcinoma cells.

The contribution of integrin β5 to cell-matrix interactions was examined in adhesion and migration assays. Depletion of integrin β5 impaired cell adhesion to vitronectin (Figure 2D), despite the presence of β1 and β3 integrins that are capable in recognizing of vitronectin [6, 7]. This is consistent with a study using function-blocking antibodies [25]. Adhesion to vitronectin was restored by re-expression of integrin β5 (Figure 2D). As expected, modulation of integrin β5 did not affect adhesion to fibronectin and collagen (Figure 2D). Integrin β5-depleted cells had a reduced spreading capacity on tissue-culture dishes compared to the control and shB5-oeB5 cells, while spreading was recovered on collagen-coated dishes (Supplement Figure 2B). The migration capacity was tested in wound-closure assays on collagen-coated plates. The shB5 cells showed a significant delay in wound closure compared to the control and shB5-oeB5 cells (Figure 2E and Supplementary Figure 3A). Thus, integrin β5 contributes to tumor cell migration in part by facilitating cell-matrix interactions.

Integrin β5 in breast carcinoma cell survival and proliferation

The defect of integrin β5-depleted cells in anchorage-independent growth could be associated with reduction in survival or proliferation [23]. The clonogenic assay, testing an individual cell potential for survival and “unlimited” division [26], showed that integrin β5-depleted cells formed 70% fewer foci than control cells, while re-expression of integrin β5 resulted in a three-fold increase compared to the control (Figure 3A). Cell survival was examined in anchorage-independent conditions by culturing cells in serum-free media over a layer of 1% agarose. Modulation of integrin β5-expression did not impact cell survival (Figure 3B), suggesting that integrin β5 contributes the replicative potential of cells rather than cell death.

Cell proliferation was measured over a four-day period on plastic and collagen-coated surfaces. On tissue-culture dishes, the shB5 cells showed a statistically significant difference

in growth compared to the control and shB5-oeB5 cells (Figure 3D). The growth was not statistically different on collagen-coated dishes (Figure 3E). The cell cycle analysis did not reveal major differences among the three lines on either plastic or collagen (Figure 3C). Thus, the reduction in colony formation by shB5 cells in the clonogenic and soft-agar assays may associate with reduced cell adhesive capacity (see above) or intracellular signaling but not with cell survival or disruption of the cell cycle.

Integrin β5 mediates activation FAK and ERK signaling

To dissect the molecular mechanism underlying the pro-tumorigenic function of integrin β5, we examined intracellular signaling. Integrins bind focal-adhesion kinase (FAK) and mediate FAK activation and phosphorylation at several Tyr residues [13], as well as phosphorylation of Tyr31 in Paxillin, a component of focal adhesions [27]. In turn, integrin-FAK can activate the MEK-ERK axis [16–18], which contributes to cell survival and proliferation [23]. TGF-β is a strong modulator of integrin signaling [8], and can activate MEK-ERK [28–30]. We found that depletion of integrin β5 blocked basal and TGF-β-induced phosphorylation of FAK-Tyr861, Pax-Tyr31 and ERK1/2 without an effect on the total levels of FAK, Pax or ERK1/2 (Figure 4A). Phosphorylation of Smad2, an immediate target of TGF-β receptors, was not affected (Figure 4A, bottom). Phosphorylation levels of FAK-Tyr861, Pax-Tyr31 and ERK1/2 were restored in cells re-expressing integrin β5 (Figure 4A). The auto-phosphorylation Tyr397 site in FAK was not affected by integrin β5 depletion (Supplementary Figure 3B). The effect of integrin β5 on phosphorylation of FAK-Tyr861 and ERK1/2 were further validated in triple-negative BT549 and ER/PR-positive MCF7 and T47D cell lines using siRNA to integrin β5 (Figure 4B). Thus, integrin β5 mediates specific signaling events such as phosphorylation of FAK-Tyr861, Pax-Tyr31 and ERK but does not impact TGF-β-mediated signaling to Smads.

Integrin β5 can synergize with EGFR in intracellular signaling in some cell systems [31]. The EGFR inhibitor AG1478 even at 10μM did not affect phosphorylation of FAK at Tyr397 and Tyr861, and phospho-ERK1/2 (Figure 4C). In comparison, nanomolar concentrations of the EGFR inhibitor completely abolished phosphorylation of ERK1/2 in mammary epithelial MCF10A cells (Figure 4C, bottom panel). To test whether EGF stimulation modulates integrin signaling, MDA-MB-231 cells were treated with EGF and examined for activating phosphorylation of EGFR at Tyr845 and ERK1/2. EGF induced Tyr845-phosphorylation of EGFR and this was blocked by the EGFR inhibitor but neither treatment affected ERK phosphorylation (Figure 4D). The level of phospho-Tyr845-EGFR following EGF stimulation was reduced in the shB5 cells compared to control. Given the contribution of Src to integrins β1- and β3-induced phosphorylation of EGFR [32], we examined the effect of Dasatinib, a selective inhibitor of Src kinase [33]. Nanomolar concentrations of Dasatinib reduced EGF-induced phospho-Tyr845-EGFR (Figure 4E). Thus, in tumor cells integrin β5 mediates activation of FAK (Tyr861) and ERK independently of EGFR signaling; the latter event requires Src.

Integrin β5 signaling to ERK is independent from the Src-FAK signaling axis

The role of FAK, Src and ERK in integrin β5-promoted anchorage-independent growth of breast cancer cells was examined in soft-agar assays using kinase inhibitors. Selective

inhibitors of FAK (PF573228/PF228 [34]) and Src (Dasatinib) reduced colony formation by nearly 50%, while the MEK-ERK inhibitor U0126 was the most effective (Figure 5A). Cell proliferation was reduced by depletion of FAK by siRNA or by pharmacological inhibition of Src, FAK and MEK (Supplement Figure 4A-B-C). Thus, FAK, Src and MEK-ERK contribute to integrin β5-promoted tumor cell growth in both adhesive and anchorage-independent conditions.

Integrin-mediated activation of ERK depends on FAK in some systems [17, 18], while in others it is FAK independent [16, 35]. Treatment of MDA-MB-231 cells with the FAK inhibitor PF228 blocked phosphorylation of FAK at the auto-phosphorylation Tyr397 site but did not reduce phospho-ERK1/2 (Figure 5B). Conversely, the MEK inhibitor effectively reduced phospho-ERK1/2 but did not affect phospho-Tyr397 of FAK (Figure 5B). Neither inhibitor had an effect on phosphorylation of FAK at Tyr861. Thus, in MDA-MB-231 cells, the FAK kinase activity is dispensable for activation of ERK and phosphorylation of FAK at Tyr861, events that are mediated by integrin β5. FAK is known to have a scaffolding function [13]. Suppression of FAK by siRNA had no effect on ERK phosphorylation (Figure 5C), disproving a role of the FAK scaffold function in ERK activation in MDA-MB-231 cells.

The role of Src in integrin β5-mediated signaling was assessed using Dasatinib, a selective Src inhibitor. Dasatinib suppressed phosphorylation of FAK at Tyr861 and Tyr925, consistent with a role of Src in phosphorylation of FAK-Tyr861 in endothelial cells [14] and FAK-Tyr925 in colon cancer cells [36]. Depletion of integrin β5 reduced phosphorylation of FAK-Tyr861 (Figure 4) with no effect on FAK-Tyr925 (Supplementary Figure 3C). Dasatinib blocked phosphorylation of Pax-Tyr31, whereas phosphorylation of ERK and FAK-Tyr397 were not affected (Figure 5D). Thus, Src is required for integrin β5-mediated phosphorylation of FAK-Tyr861 and Pax-Tyr31, as well as integrin β5-independent phosphorylation of FAK-Tyr925.

Integrin β5 in tumor growth in an orthotopic model

The *in vivo* requirements for integrin β5 were tested by inoculating breast carcinoma MDA-MB-231 cells (control, shB5 and shB5-oeB5) into the mammary fat-pad of female SCID mice. Mice in the control group developed palpable tumors at 40 days post-injection and by 64 days all animals had tumors (Figure 6A). Mice injected with the shB5 cells showed a significant delay in tumor development (Figure 6A). At the endpoint of the study (84 days post-injection), nearly 60% of mice in the shB5 group remained tumor-free. Tumor appearance in the shB5-oeB5 group, re-expressing integrin β5, was comparable to the control (Figure 6A).

Tumor growth rates in the control and integrin β5-re-expressing groups were indistinguishable, whereas tumor growth was significantly delayed in the integrin β5-deficient group (Figure 6B). Microscopic examination of histological H&E sections showed that tumors in all groups are located within the mouse mammary gland (Supplementary Figure 5). The shB5 tumors showed a decrease in the Ki67 index compared to the control and integrin β5-re-expressing groups, indicating a defect in tumor cell proliferation (Figure 6C). Evaluation of tumor vasculature with CD31 staining revealed a nearly 70% decrease in

the blood-vessel area in the shB5 tumors compared to control and shB5-oeB5 groups (Figure 6D). Accordingly, large necrotic areas were observed in the shB5 tumors compared to control and shB5-oeB5 tumors (data not shown). Quantitative RT-PCR detecting three major VEGF-A isoforms ($VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$) in MDA-MB-231 cells showed a reduction in VEGF expression in the shB5 tumors compared to the control and integrin β5-re-expressing tumors (Figure 6E). In cultured cells, depletion of integrin β5 reduced VEGF expression compared to control cells, while the MEK inhibitor U0126 reduced VEGF levels in control cells (Figure 6F). Thus, integrin β5 facilitates tumor growth and angiogenesis in part by regulating expression of VEGF via MEK-ERK signaling.

DISCUSSION

The current work demonstrates a critical role of integrin β5 in the tumorigenic potential of breast carcinoma cells, promoting tumor cell migration, growth and tumor angiogenesis. Depletion of integrin β5 in metastasis-capable breast carcinoma cells markedly hinders the tumorigenic capacity *in vitro* and in the orthotopic mouse model. Integrin β5 activates two independently-operating signaling pathways, Src-FAK and MEK-ERK, which contribute to the tumorigenic capacity of carcinoma cells.

The experimental and metadata analysis of the αvβ integrins revealed high levels of αv, β1 and β5 integrins in breast carcinomas and cancer cell lines, whereas integrin β3 is expressed by tumor stroma and TGF-β-responsive triple-negative cancer cells. These findings are supported by previous studies in breast cancer cell lines [25]. A low expression of integrin β6 in breast tumors and cancer cell lines suggests a tumor-suppressor function for integrin β6. This is consistent with integrin β6 role in the activation of TGF-β, a potent tumor-suppressor. Thus, β5 and β1 integrins might facilitate and β6-integrin may restrain the tumorigenic potency of breast carcinoma cells.

In the orthotopic model, depletion of integrin β5 hindered tumor initiation, growth and tumor vasculature (Figure 6), indicating that integrin β5 facilitates tumor angiogenesis in addition to tumor-cell intrinsic function. The angiogenesis effect is likely associated with regulation of VEGF-A by MEK-ERK signaling (Figure 6F). These results are consistent with a role of FAK and MEK in tumor angiogenesis of mouse mammary carcinoma 4T1 cells [37]. Integrin β5 *via* VEGF may exert both paracrine and autocrine activities contributing to tumor vascularization and survival [38]. In addition to endothelial cells, VEGF may promote an intracrine survival loop in BC cells [39] *via* VEGF-receptor-dependent activation of β5-integrin-Src signaling [14, 40]. Low levels of VEGF receptors, FLT1, KDR and FLT4/VEGFR3 in BC cell lines argue against this crosstalk, though.

A critical role of integrin β5 in anchorage-independent growth of tumor cells reflects the integrin β5 function in the cell replicative potential, a hallmark of cancer cells [41]. This activity of integrin β5 is likely mediated by Src-FAK and MEK-ERK signaling pathways (Figure 4, 5). It appears that in MDA-MB-231 cells, expressing constitutive Ras-ERK signaling [42], these pathways are regulated *via* independent signaling events that are disengaged from receptor-tyrosine kinases (RTKs) (Figure 4). In other systems, such as endothelial, kidney and colon epithelial cells, a crosstalk of integrin β5 and RTKs is required

for activation of Src-FAK and Ras-ERK signaling [14, 43]. FAK can be phosphorylated at various Tyr residues by the adhesion-mediated integrin clustering or by the integrin-RTK crosstalk [7]. Integrin β5 mediates Src-induced phosphorylation of FAK at Tyr861 but not at Tyr925 in MDA-MB-231 cells. This is a significant finding as FAK-Ty861 phosphorylation promotes FAK interaction with integrin β5 [14] and Ras-mediated tumorigenesis [44]. Integrins β1 or β3 might promote FAK-Tyr925 phosphorylation, as seen in smooth-muscle cells where the integrin β3-PDGFRβ crosstalk facilitates phosphorylation of FAK at Tyr925 and Tyr397 [45].

Our results argue that although integrin β5 mediates ERK signaling in MDA-MB-231 cells, this event is independent of FAK kinase and scaffolding functions (Figure 5). This is consistent with integrin β1-mediated signaling in fibroblasts [16]. Depletion of integrin β5 may prevent the recruitment to the membrane of nucleotide-exchange factors activating the Ras-Raf-MAPK cascade [46]. Alternatively, integrin β5 could regulate MEK-ERK through PAK4 that directly interacts with integrin β5 and activates MAPKs [47]. Additionally, integrins may interact with tetraspanin proteins in the organization of membrane compartments and Ras-ERK signaling [48]. It is also conceivable that the interaction of Src-FAK and Ras-ERK pathways in the context of αvβ-integrin signaling may depend on K-Ras, PTEN, PIK3CA and ERBB2 pathways.

In summary, the current work provides novel insights into the integrin β5 function in breast cancer. Integrin β5 promotes tumor growth and angiogenesis by facilitating matrix adhesion and signaling *via* Src-FAK and Ras-ERK pathways that may operate independently in tumor cells (Figure 7). These findings suggest alternative strategies to the current therapeutic approaches targeting extracellular domains of β5 and β3-integrins [7]. The inhibition could be achieved by disabling the intracellular link of integrin β5 to Src-FAK and MEK-ERK or by inactivating of both pathways simultaneously. These approaches are particularly advantageous for treatment of recurrent disease that is resistant to RTK inhibitors or triple-negative breast cancers, which are refractory to most of the current therapies.

MATERIALS and METHODS

Cell culture

Human mammary epithelial MCF10A and MCF12A, and breast cancer Hs578T, MDA-MB-231, BT549, and T47D cell lines were from American Tissue Culture Collection (ATCC, Manassas, VA). MCF7 cells were from BD Biosciences (Palo Alto, CA). MDA-MB-231, MCF10A, and MCF12A cells were cultured as recommended by ATCC. All other cells were maintained in Dulbecco's Modified Eagles's medium (DMEM) (Invitrogen, Carlsbad, CA) as described in previous studies [8, 49]. MDA-MB-231 cells expressing shRNA to integrin β5 were cultured in flasks coated with rat type I collagen (BD Biosciences, San Jose, CA).

Antibodies and other reagents

TGF-β1 was from R&D Systems (Minneapolis, MN). The following antibodies were used: mouse monoclonal to α-tubulin (Sigma-Aldrich, St. Louis, MO); rabbit polyclonal to

GAPDH, integrin β5, EGFR, FAK, and phospho-FAK Y925 (Santa Cruz, Biotechnology, Santa Cruz, CA); rabbit polyclonal to phospho-paxillin Y31, phospho-FAK Y861 and phospho-EGFR Y845 (BioSource International Invitrogen), rabbit polyclonal to integrin β1 and mouse monoclonal to human integrin αvβ5, clone P1F6 (Millipore, Temecula, CA); mouse monoclonal to phospho-ERK, rabbit polyclonal to phospho-Smad2, and rabbit polyclonal to phospho-FAK Y397 (Cell Signaling, Beverly, MA); rabbit polyclonal to ERK (Promega, Madison, WI); mouse monoclonal to paxillin (BD Biosciences). Anti-rabbit or anti-mouse IgG antibodies conjugated to Horseradish Peroxidase (HRP) were from GE Healthcare (Piscataway, NJ). APC-conjugated mouse to human integrin β1 and PE-conjugated rat to mouse IgG were from BD Biosciences. Propidium iodide was purchased from Sigma. The FAK inhibitor PF573228 was from Tocris (Ellisville, MO); U0126 and AG1478 were from EMD Bioscience-Calbiochem (La Jolla, CA); Dasatinib was a gift of Dr. Yahao Bu (Kinex Pharmaceutical, Buffalo, NY).

Short Interference RNA

Experiments were done as previously described [8]. Non-silencing scramble siRNA conjugated to Rhodamine was from Qiagen (Valencia, CA). siRNA pools against β5-integrin were from Santa Cruz Biotechnology. siRNA to FAK was a gift of Dr. Irwin Gelman (RPCI, Buffalo, NY).

Short Hairpin RNA

A retroviral vector encoding short-hairpin RNA (shRNA) to human integrin β5 was obtained from Open Biosystems through the RPCI shRNA core facility. Preparation of retroviruses, infection and selection of puromycin-resistant cell populations are described elsewhere [49].

Integrin β5 over-expression

A retroviral pCX-EGFP expression vector encoding human integrin β5 cDNA was a kind gift from Dr. Raymond Birge (New Jersey Medical School, NJ). Integrin β5 open reading frame (ORF) was excised with BamHI/NotI and sub-cloned into the retroviral pBMN-IRES-EGFP vector. Preparation of retroviruses, infection and selection of GFP-positive cells are detailed elsewhere [49].

Cell survival in suspension

Tissue culture plates were coated with agarose diluted to 1% in growth medium. Cells were plated on top of the agarose-coated wells and cultured in serum-free medium for three days. The cells were collected by centrifugation and counted with Trypan blue. Experiments were done in triplicates and repeated at least twice.

Cell adhesion

The adhesion assays were done in 96-well plates coated with 1μg/ml vitronectin, 10μg/ml fibronectin (Sigma), or 10μg/ml rat tail collagen type I as previously described [8]. The results are presented as the mean value ± standard deviation from two independent experiments.

Cell proliferation

Cell proliferation assays were done in 96-well plates either pre-coated or not with rat Collagen Type I (10 μg/ml) in five-replicates and repeated at least twice as described elsewhere [8].

Flow Cytometry for surface expression

Cells were collected by trypsinization, washed once with PBS and resuspended in cold FACS buffer (1X PBS, 2%FBS, 0.1% NaN_3, 1mM $CaCl_2$, 1mM $MgCl_2$). Cells were incubated for 15min on ice with normal mouse IgG (200μg/ml final) then aliquoted in 5ml polystyrene round-bottom tubes (2×10^5 cells/tube). Cells were washed twice with FACS buffer, spun at 1,200rpm, 5min, 4°C and incubated with the primary antibody in 100μl (P1F6, 1:250 dilution; APC-CD29, 1:10 dilution) for 30 min on ice. Cells were washed twice and fixed with 2% PFA in PBS, 30min on ice, then washed and spun twice and, when needed, incubated with the PE-conjugated secondary antibody (5μg/ml) in 100μl. After additional washes, cells were resuspended in 1ml of FACS buffer and 1×10^4 events were acquired with a FacsCalibur instrument. The results were analyzed with the FCS Express program.

Flow Cytometry for cell cycle

Asynchronous cell cultures were grown on either plastic or collagen I-coated dishes, harvested by trypsinization and centrifuged at 850rpm, 10min, RT. 2ml of ice-cold ethanol were added drop-wise to the cells while vortexing and cells were incubated at 4°C overnight. Cells were collected by centrifugation, re-suspended in 0.5% of BSA in PBS and spun again. Propidium iodide solution (0.1% sodium citrate, 0.02 mg/ml RNase A, 0.2% NP-40, 0.05mg/ml propidium iodide in water) was add to each sample and after 5h incubation at 4°C samples where acquired with a FacsCalibur instrument. The results were analyzed with the Modfit software.

Reverse Transcription-PCR

Cells were treated with 2 ng/ml of TGF-β1. Preparation of total RNA and subsequent cDNA synthesis, amplification reactions and agarose gel assays are detailed elsewhere [8]. Primer sequences are in Supplement Table.

Quantitative Reverse-Transcription PCR

Amplification reactions were carried out in triplicates with 1μl of the cDNA reaction using FIREPol EvaGreen qPCR Mix Plus (ROX) (Oak Biotechnologies, Mountain View, CA) and ABI-PRISM 7900HS Sequence Detection System (Applied Biosystem, Carlsbad, CA). Results were analyzed with SDS2.3 software. The threshold cycle (Ct) value was normalized using the mean Ct for the reference gene, beta2 microglobulin (B2M). The normalized mRNA level was defined as ΔCt = Ct (test gene) − Ct (mean for the reference gene). The final data were presented as the fold-difference between the test sample and the control sample, which was defined as 2^(ΔCt test − ΔCt control). Primer sequences are in Supplement Table.

Immunoblotting

Whole-cell extracts were prepared using NP-40 buffer and analyzed as detailed elsewhere [8].

Colony formation in soft agar

Experiments were done in triplicates and repeated at least twice as described in a previous study [8].

Clonogenic assay

Cells (500/well) were seeded in 6-well plates in complete media and grown for 12 days. Media was replenished every 3–4 days. Foci were stained with 0.5% methylene blue in water/methanol (50:50) for 1 hour at RT. Plates were rinsed in ddH_2O, dried and the foci in each well were counted. Experiments were done in triplicates and repeated at least twice.

Wound closure assay

Experiments were done in triplicates and repeated at least twice using 6-well plates coated with type I Collagen (10μg/ml) and detailed elsewhere [8].

Animal studies in the orthotopic model

Female SCID/CBl7 mice, 8-weeks old, were from a colony of SCID/Bl7 mice at the Department of Laboratory Animal Resources (DLAR) facility at RPCI, Buffalo, NY. Tumor growth and histological studies were performed as described elsewhere [49, 50].

Statistical analysis

Statistical analysis was done using the Student *t*-Test. P value ≤ 0.05 was assigned for the level of significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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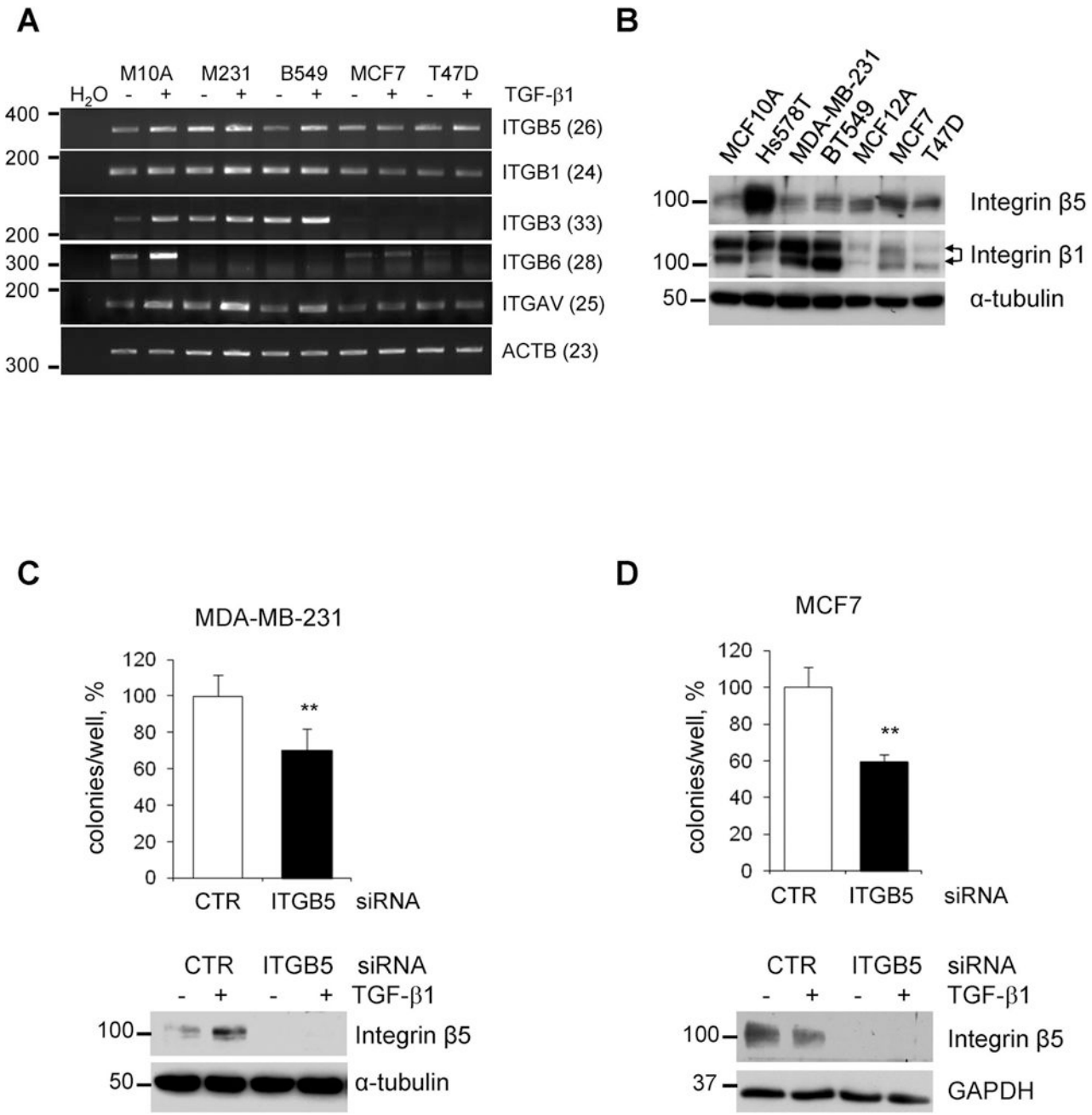

Figure 1. Expression of αv-β-integrins in breast carcinoma cell lines

(A) RT-PCR of integrins αv, β1, β3, β5, and β6 in a panel of non-tumorigenic mammary epithelial and breast carcinoma cells treated with TGF-β1 (2–5ng/ml for 24h). In brackets is indicated the number of PCR cycles performed.**(B)** Immunoblot analysis of integrin β1 and integrin β5 in a panel of non-tumorigenic mammary epithelial and breast carcinoma cells. α-Tubulin is used as loading control. Anchorage-independent growth analysis of MDA-MB-231 **(C)** and MCF7 **(D)** treated with 100nM siRNA to integrin β5. Immunoblot analysis shows the effective suppression of integrin β5 expression.

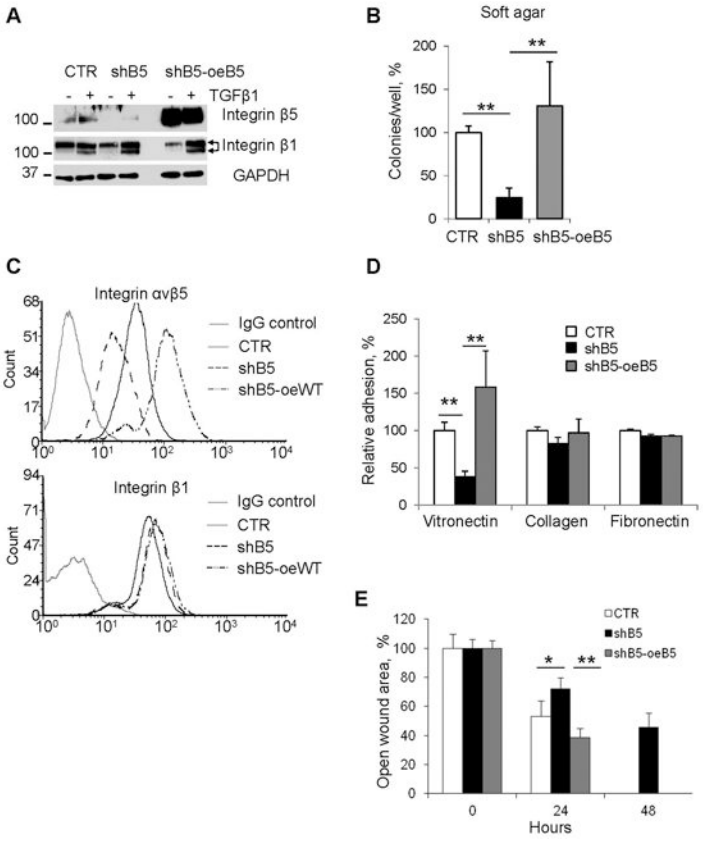

Figure 2. Integrin β5 is important for tumor growth in anchorage-independent condition

(A) Immunoblot analysis of whole-cell protein extracts from control, shRNA-ITGB5 (shB5) and shRNA-ITGB5-over-expressing integrin β5 (shB5-oeB5) MDA-MB-231 cells, probing for integrin β5 and integrin β1. GAPDH probing shows equal loading. **(B)** Anchorage-independent growth analysis of MDA-MB-231 cells control, expressing shRNA to integrin β5 or over-expressing integrin β5. Statistical analysis by Student *t*-Test is provided (** $p<0.001$). **(C)** Flow cytometry analysis for cell surface presentation of integrins αvβ5 and β1 in control, sh-ITGB5, and sh-ITGB5-over-expressing integrin β5 MDA-MB-231 cells. **(D)** Adhesion of control, shB5, and shB5-oeB5 MDA-MB-231 cells on vitronectin-, collagen type I-, and fibronectin-coated wells. Adherent cells were fixed and stained with 0.5% methylene blue in water/methanol (50:50), rinsed with ddH_2O, and solubilized with 1% SDS in PBS. The absorbance was read with a fluorimeter. The experiments were performed in six replicates and repeated at least twice. Results are expressed as percentage of adhesion compared to control. Statistical analysis by Student *t*-Test is provided (** $p<0.001$). **(E)** Wound closure assay was performed on collagen type I-coated wells for control, shB5, and shB5-over-expressing integrin β5 MDA-MB-231 cells. Area of open wound is expressed as percentage of the freshly made wound (0 h).

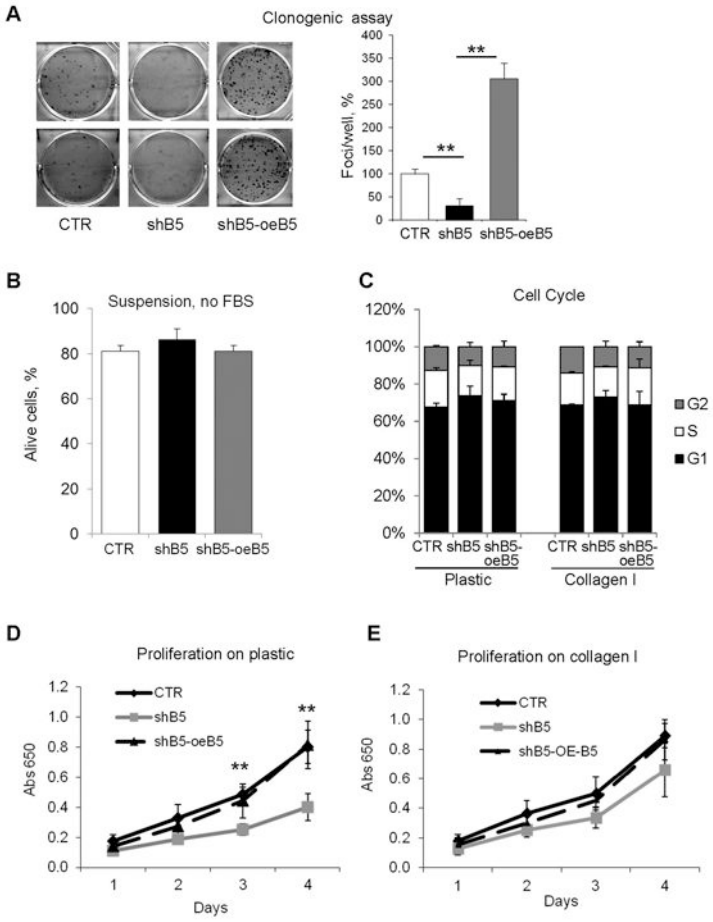

Figure 3. Integrin β5 in breast carcinoma cell survival, proliferation and migration

(A) Clonogenic assay of control, shB5, and shB5-over-expressing integrin β5 MDA-MB-231 cells. Cells (500/well) were grown for 12 days, then stained with 0.5% methylene blue in water/methanol 50:50, rinsed in water and the foci in each well were counted. Two representative wells for each cell line are shown. Statistical analysis by Student *t*-Test is provided (* $p<0.05$; ** $p<0.001$). **(B)** Survival in suspension conditions. Control, shB5, and shB5-over-expressing integrin β5 MDA-MB-231 cells were cultured over a layer of 1% agarose in serum-free media for 72h. Cells were then collected by centrifugation and counted by Trypan blue exclusion. The percentage of alive cells was calculated. Experiments were performed in triplicates and repeated at least twice. **(C)** Cell cycle analysis of control, shB5, and shB5-over-expressing integrin β5 cells through propidium iodide (PI) staining. Experiments were repeated twice. Control, shB5, and shB5-over-expressing integrin β5 MDA-MB-231 cells were tested for proliferation on plastic **(D)** or collagen type-I **(E)**. The data presented represents the average and standard deviation of four independent experiments, performed in six replicates.

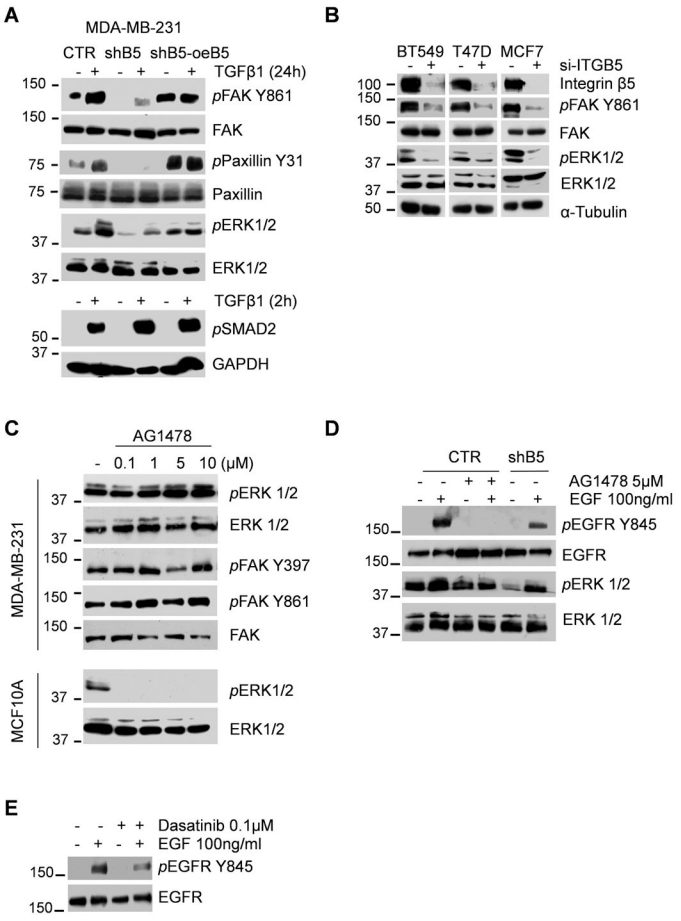

Figure 4. Integrin β5 mediates activation FAK and ERK signaling

(A) Immunoblot analysis of whole-cell protein extracts from control, shB5 and shB5-over-expressing integrin β5 MDA-MB-231 cells treated with TGF-β1 (2ng/ml) for 24h (upper panel) or 2h (lower panel). Membranes were probed with antibodies for FAK, phospho-FAK-Tyr861, phospho-paxillin-Ty31, paxillin, ERK1/2, phospho-ERK1/2, phospho-Smad2, and GAPDH as loading control. **(B)** Immunoblot analysis of whole-cell protein extracts from control and siRNA-ITGB5-treated BT549, MCF7, and T47D cells. Membranes were probed with antibodies for integrin β5, phospho-FAK-Tyr861, FAK, ERK 1/2, phospho-ERK1/2 and a-tubulin. **(C)** MDA-MB-231 (upper panel) and MCF10A (lower panel) cells were treated with the EGFR inhibitor AG1478 for 2h at the indicated concentrations. Immunoblot analysis was performed on whole-cell extracts probing for FAK, phospho-FAK-Tyr397, phospho-FAK-Tyr861, ERK1/2, and phospho-ERK1/2. **(D)** Immunoblot analysis of whole-cell protein extracts from control and shB5 cells, treated with EGF (100ng/ml) for 2h. Where indicated, a 1h pre-treatment with AG1478 (5μM) was performed. Membranes were probed for phospho-EGFR-Tyr845, total EGFR, phospho-ERK1/2, and total ERK1/2. **(E)** Immunoblotting of whole-cell protein extracts from control cells, treated with 100ng/ml EGF for 2h. Where indicated, a 1h pre-treatment with 100nM Dasatinib was done. Membranes were probed for phospho-EGFR-Tyr845 and EGFR.

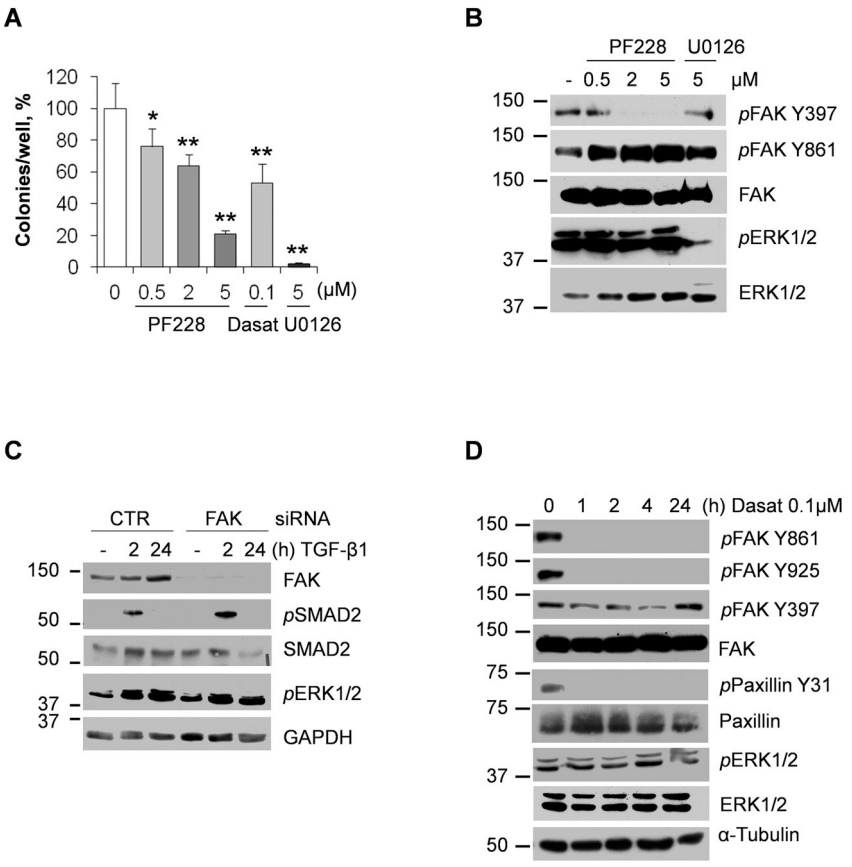

Figure 5. Integrin β5-mediated signaling to ERK is independent from the Src-FAK signaling axis

(A) Anchorage-independent growth analysis of MDA-MB-231 cells treated with the FAK inhibitor PF573228, the Src inhibitor Dasatinib, or the MEK inhibitor U0126 at the indicated concentration. Statistical analysis by Student *t*-Test is provided (* $p < 0.05$; ** $p < 0.001$). **(B)** MDA-MB-231 cells were treated with the FAK inhibitor PF573228 or the MEK inhibitor U0126 for 24h at the indicated concentrations. Immunoblotting was performed on whole-cell extracts probing for FAK, phospho-FAK-Tyr397, phospho-FAK-Tyr861, ERK1/2, and phospho-ERK1/2. **(C)** Immunoblot analysis of whole-cell protein extracts from control and siRNA to FAK treated MDA-MB-231 cells that were treated with 2ng/ml TGF-β1 for 2h and 24h. Membranes were probed with antibodies for FAK, phospho-Smad2, Smad2, phospho-ERK1/2, and GAPDH as loading control. **(D)** MDA-MB-231 cells were treated with the Src inhibitor Dasatinib at 100nM for the indicated times. Immunoblot analysis was performed on whole-cell extracts probing for FAK, phospho-FAK-Tyr397, phospho-FAK-Tyr861, phospho-FAK-Tyr925, phospho-paxillin-Tyr31, paxillin, ERK1/2, phospho-ERK1/2 and α-tubulin.

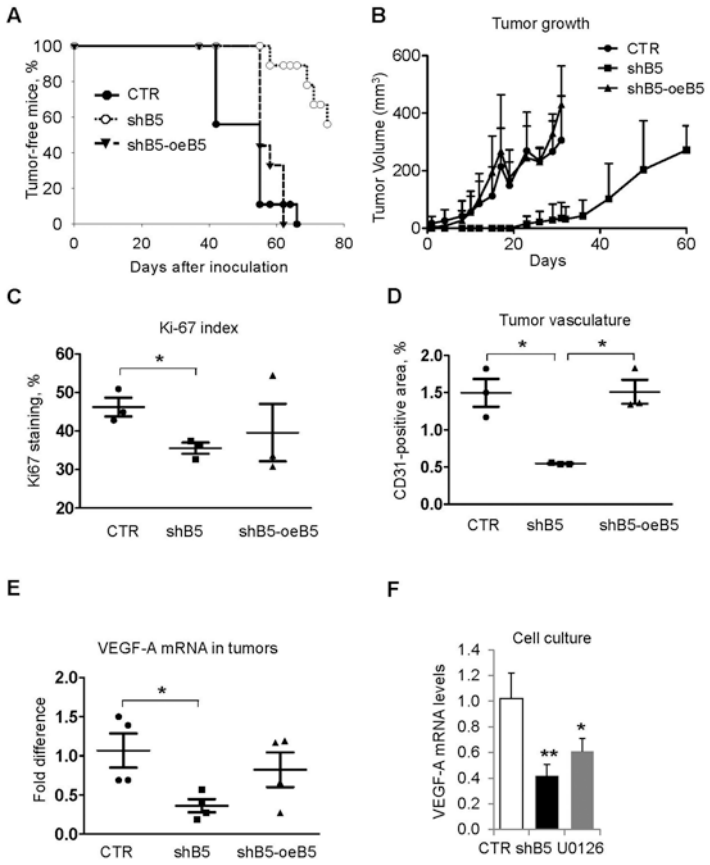

Figure 6. Integrin β5 in tumor growth in the orthotopic model

(A) MDA-MB-231 control, shB5 and shB5-over-expressing integrin β5 cells (1×10^6 cells/mouse, 9 mice/group) were injected in the mammary fat-pad of 8-week-old SCID female mice. Mice were evaluated every second day for tumor appearance by palpation. **(B)** Tumor growth was monitored by serial caliper measurements. The means of tumor volume measures of each group of mice during the study are reported. The volumes were calculated using the equation: tumor volume = $\pi/6$(length × width^2). **(C)** Three tumors per group were stained for Ki67. Nine representative sections for each tumor were acquired at 400X magnification. 400 nuclei/field were counted and scored for Ki67-positivity. The percentage of Ki67-positive nuclei is reported. **(D)** Three tumors per group were stained for CD31. Nine representative sections for each tumor were acquired at a 200X magnification and the percentage of CD31-positive area was quantified using Image J software. **(E)** Quantitative RT-PCR analysis for VEGF-A in total RNA extracted from four tumors/treatment. Levels of VEGF-A mRNA in each samples were normalized on B2M and the fold difference values were calculated as previously described in Materials and Methods. **(F)** Quantitative RT-PCR analysis for VEGF-A in total RNA extracted from MDA-MB-231 control, shB5 and U0126-treated control cells (5μM). Statistical analysis by Student *t*-Test is provided (* $p<0.05$, ** $p<0.001$).

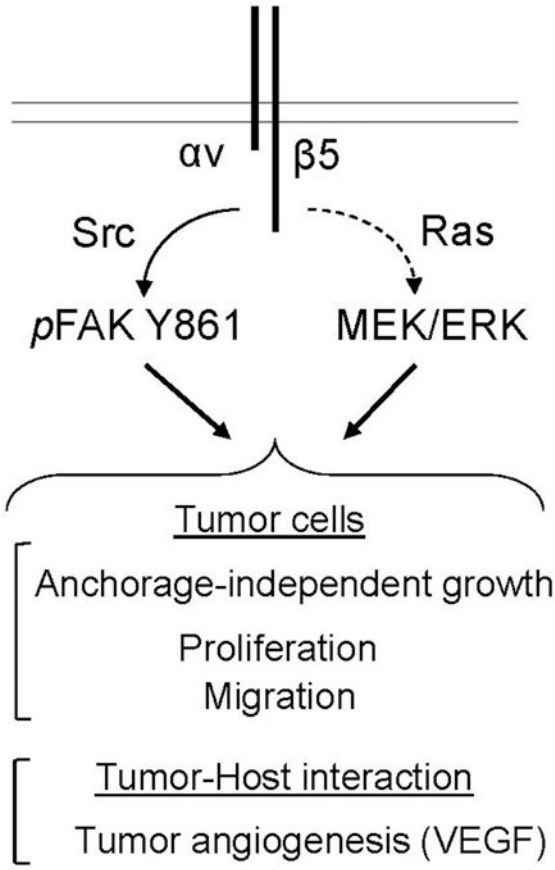

Figure 7. Model of integrin β5 function in breast cancer

Integrin β5 promotes tumor growth and angiogenesis by facilitating matrix adhesion and signaling *via* Src-FAK and Ras-ERK pathways that may operate independently in carcinoma cells.