FAK tyrosine 407 organized with integrin αVβ5 in Hs578Ts(i)$_8$ advanced triple-negative breast cancer cells

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Abstract. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase known to promote cell migration and invasiveness. Overexpression and increased activity of FAK are closely associated with metastatic breast tumors and are linked to poor prognosis. This study discovered an inverse correlation between FAK activity and migratory and invasive behavior. We show decreased phosphorylation levels of FAK at tyrosine residues 397 and 861, and most prominently at Y407, in the more invasive Hs578Ts(i)$_8$ subclone of the Hs578T breast cancer progression model. There is limited information available on FAK Y407, and here we demonstrate its presence in triple-negative breast cancer (TNBC) cell lines. Furthermore, our studies propose that localization of FAK Y407, rather than FAK expression and overall FAK Y407 phosphorylation levels, is crucial for the control of cell motility. FAK Y407 is found extensively at the cell periphery in focal adhesion-like structures at each end of actin stress fibers and organized with integrin αVβ5 receptors, linking the αVβ5 integrin-mediated migratory behavior of Hs578Ts(i)$_8$ cells to FAK Y407. These data suggest that subcellular localization, next to expression and activity levels, are important for understanding TNBC progression. Such an approach opens new avenues for further studies and may provide novel insight for the classification of TNBC and facilitate the discovery of effective biomarkers for diagnosis and therapy of TNBC.

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Abbreviations: TNBC, triple-negative breast cancer; ECM, extracellular matrix; FAK, focal adhesion kinase; ERK, extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinase

Key words: migration, integrin, FAK Y407, triple-negative breast cancer, localization, organization, spatio-temporal

Introduction

Breast cancer is one of the leading causes of cancer deaths among women worldwide (1). Even with all the attention towards prevention and latest advances in screening, diagnosis, treatment modalities and methods to follow-up, 1 out of 8 US women will have invasive breast cancer, either at the time of diagnosis or developed during disease progression (2,3). Particularly interesting is the diversity and complexity with which advanced breast cancer occurs (4). The intricate variety is not only related to the distinct breast cancer subtypes but is even present within a single subtype. Specifically, triple-negative breast cancers (TNBC), represent a major challenge. The treatment options for this breast cancer subtype, which is classified as estrogen receptor (ER) and progesterone receptor (PR) negative and human epidermal growth factor 2 (HER2) negative, are extremely limited, because of the absence of specific targets and the heterogeneity with which TNBC presents itself (5). While standard systemic chemotherapy may have some positive impact and lead to the eradication of the disease, patients with recurrent disease or other TNBC patients for which this therapy is not effective, may experience an aggressive cancer type and a high incidence of metastases, resulting in overall poor patient outcomes with high mortality (5-7).

Over the years, TNBC has been characterized by high cell proliferation, poor cellular differentiation, several recurrent gene copy number imbalances, and in numerous cases mutations in the p53 tumor suppressor gene and BRCA-1 (8). In addition, more recent studies aiming at the discovery of effective therapies for TNBC, indicated specific roles for poly(ADP-ribose) polymerase-1 (PARP), mainly against *BRCA*-mutant TNBC cancers (9), and other molecular targets involved in growth factor receptor signaling, such as the insulin-like growth factor receptor (IGFR), epidermal growth factor receptor (EGFR), vascular endothelial growth factor-α (VEGF-α) and non-receptor tyrosine kinases src, phosphoinositide 3-kinase (PI3K) and Akt as well as mTOR. While many of these signal transducers play indeed a role in growth factor receptor-mediated signaling, potential roles for these signal transducers in stimulating metastases are described as well (10,11). From the current scientific literature,

it is suggested that the heterogeneity of TNBC is characterized by the activation of distinct signaling pathways and is supported by suggestions, concerning the changes occurring in biological characteristics in primary tumors as compared to their metastatic counterparts. The latter has been ascribed as a consequence of progressed disease or exposure to treatment (12).

Although new avenues have been opened there is a lack of effective targeted therapies for TNBC breast cancer patients. Therefore, an improved and profound understanding of the mechanisms underlying the metastatic and aggressive nature of TNBC, and this at all levels of the invasion-associated cellular behaviors are needed. This will aid the identification of new biomarkers for diagnosis as well as molecular targets for the development of novel and better targeted therapeutic strategies that can reduce the number of deaths related to this cancer subtype, specifically since TNBC is known to affect a younger age group in addition to specific ethnic groups (13). A drawback of the current trend in research studies and clinical trials, and especially in TNBC studies, is the fact that these studies are specifically designed for the overall TNBC patient population, using a variety of cell lines or patient samples. Results are often disappointing and suggest an overall negative effect, which may not always reflect the usefulness of these identified targets given the heterogenic character of TNBC, and rather underscore the value of the new findings for a subset of patients, with particular TNBC characteristics (14).

To address this problem, this study explores the changes in invasion-associated cellular events in the Hs578T TN breast cancer cell line and its more invasive subclone Hs578Ts(i)$_8$, representing an *in vitro* cell system of TNBC breast cancer progression which embodies an elegant experimental model for studying the metastatic nature of TNBC (15) or at least may provide valuable insight on novel predictive biomarkers and therapeutic targets for a subset of TNBC patients. Moreover, with the movement towards personalized therapies, biochemically-oriented comprehensive approaches linking active targets or full aberrant signaling pathways, which do not always signify mutations or amplifications (16), and their (sub)cellular localization to biological functions may propose new directions for classification of metastatic TNBC and consequently may suggest new targeted therapeutic approaches.

Signal transduction molecules, such as the cytoplasmic non-receptor tyrosine kinases (NRTK), focal adhesion kinase (FAK) and src have been found to play an important role in tumorigenesis. Increased catalytic activity of FAK and src has been observed in numerous invasive and metastatic tissues, such as breast, prostate and thyroid cancers and in TNBC as well as in TNBC cell lines (17,18). FAK is linked to signaling events between cells and the extracellular matrix (ECM), thereby affecting cell-adhesion, cell motility and invasion (19) and may be an important prognostic biomarker and therapeutic target for metastatic TNBC tumors.

In this study, we investigated differences in invasion-associated cellular activities, as cell-matrix adhesion and migration, between the mesenchymal-like Hs578T TNBC cell line, which is considered moderately aggressive, and the more invasive and isogenic Hs578Ts(i)$_8$ cells. The invasive Hs578Ts(i)$_8$ cells demonstrated important invasion-associated cellular changes as compared to the parental Hs578T cells. The cellular behavior could be correlated to the organization of integrin αVβ5 receptors in the cell membrane with FAK Y407. Our results revealed an unusual activation pattern, yet a common activation pattern when taking into account the dynamic spatio-temporal organization.

Materials and methods

Antibodies and other reagents. Antibodies directed against the distinct tyrosine phosphorylation sites of FAK (Y397, Y407, Y576, Y861, and Y925), src (Y416, Y527), p-JNK, p-paxillin and p-rac as well as anti-FAK, anti-src, non-phospho-src (Y416 and Y527), anti-rac, anti-JNK and anti-paxillin and anti-β-actin antibodies were from Cell Signaling Technology as well as the mouse and rabbit monoclonal IgG isotype, Alexa Fluor® 555 Phalloidin and Alexa Fluor® antibodies (Danvers, MA, USA). The antibodies against the different integrin subunits and receptors were from EMD Millipore (Billerica, MA, USA). Secondary biotinylated anti-rabbit and anti-mouse, FITC-labeled anti-mouse and FITC-labeled anti-rabbit secondary antibodies and Vectashield mounting medium were obtained from Vector Laboratories (Burlingame, CA, USA). Antibodies against serine phosphorylation sites of FAK (S732 and S910), anti-mouse and anti-rabbit alkaline phosphatase-labeled secondary antibodies and the BCA protein assay reagent kit were from ThermoFisher Scientific (Waltham, MA, USA).

Cell culture. The human mesenchymal breast cancer Hs578T cells and the derivative cell line Hs578Ts(i)$_8$ were a kind gift from Dr S. McDonnell (UCD School of Chemical and Bioprocess Engineering, University College Dublin, Ireland) (15) and were grown in DMEM-medium supplemented with 10% (v/v) FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.1 U/ml bovine insulin (Thermo Fisher Scientific) at 37˚C equilibrated with 5% (v/v) CO_2 in humidified air. The breast cancer cells used in this study were frozen in liquid nitrogen when not in use and were not passaged in our laboratory for >10 weeks.

Cell adhesion assay. Cells were detached with 0.2% (w/v) EDTA, to avoid proteolytic degradation of cell surface proteins, and washed with serum-free medium. Next, 5x10^5 cells were resuspended in 1 ml DMEM supplemented with 2% (v/v) FBS. Cell suspensions (100 μl), in the presence or absence of function blocking antibodies (5-10 μg/ml) were added to collagen I, collagen IV, fibronectin and laminin precoated 96-well plates (BD Biosciences, San Jose, CA, USA), and centrifuged for 1 min at 115 g. After 90-min incubation at 37˚C, wells were washed four times with PBS to remove non-adherent cells. The adherent cells were then detected and quantified by measuring the acid phosphatase activity, through solubilization of the remaining cells with 0.2% Triton X-100 and by the addition of the substrate, PNPP (*p*-nitrophenyl phosphate; Sigma, St. Louis, MO, USA). Absorbance values of the lysates were determined on a microplate reader at 405 nm (Biotek, Synergy H1, Winooski, VT, USA) and expressed as relative absorbance (%). Mouse and rabbit IgG isotype control antibodies were included to estimate the non-specific binding.

Wound healing assay. Cells were grown in 24-well plates until confluency and washed twice with PBS. A scratch was made using a P-200 pipette tip and 3 ml of medium in the presence or absence of function blocking integrin antibodies (5-10 μg/ml) was added. Cell migration was monitored and images were collected, after 10 and 17 h, with a Leica DMIL microscope with a CCD camera (Leica, Buffalo Grove, IL, USA). Leica software was used to estimate the cell free area of the wounds. The distances over which the cells migrated were measured and expressed as migratory velocity (μm/h) or as % compared to control Hs578T cells.

Flow cytometry analysis. Cells were detached with 0.2% (w/v) EDTA, to avoid proteolytic degradation of cell surface proteins, neutralized with cold PBS and resuspended in PBS containing 0.1% (w/v) BSA (Sigma). Next, $2.5x10^5$ cells were incubated with the relevant primary antibodies for 90 min at 4˚C, followed by secondary FITC or Alexa Fluor®-labeled antibodies for 45 min at 4˚C. After washing, $1x10^4$ stained cells were analyzed for fluorescence using the Cell Lab Quanta™ SC MPL (Beckman Coulter, Miami, FL, USA) and BD FACSCalibur™ (BD Biosciences). Stainings without primary antibodies and IgG isotype antibodies were used as controls.

Western blotting. Cell lysates were made from 80 to 90% confluent cultures using 0.5 ml lysis buffer containing 1% (v/v) Triton X-100, 1% (v/v) Nonidet P-40 substitute (Roche, Indianapolis, IN, USA) and the following inhibitors: aprotinin (10 μg/ml), leupeptin (10 μg/ml), PMSF (1.72 mM), NaF (1 mM), $NaVO_3$ (500 μM), and $Na_4P_2O_7$ (500 μg/ml) (Sigma). Aliquots of lysates, containing 30 μg of protein, were boiled for 5 min in SDS-PAGE sample buffer containing 5% (v/v) β-mercaptoethanol, electrophoresed on 7.5 or 10% TGX™ precast gels and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). After transfer, membranes were blocked and incubated with relevant primary antibodies followed by incubation with secondary biotinylated antibodies and developed by using an ECL (Vectastain ABC-AmP) detection kit. Some of the membranes were stripped at 50˚C for 30 min in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.8), and reblotted with appropriate antibodies, for control of equal loading. Membranes were imaged on the BioChemi System and analysis software (UVP, Upland, CA, USA). Alternatively, the proteins of interest were detected using alkaline phosphatase-labeled secondary antibodies and developed using NBT/BCIP substrate (Roche, 1:50 in 0.1 M Tris-HCl, 0.05 M $MgCl_2$ and 0.1 M NaCl at pH 9.5).

Fluorescence immunostaining. Cells were grown on glass cover slips (diameter, 12 mm) placed in 24-well plates. The glass cover slips were removed, washed and fixed with ice-cold methanol or 4% paraformaldehyde (EMS, Hatfield, PA, USA). Next, fixed cells were washed, blocked and incubated with Alexa Fluor® 555 Phalloidin or primary antibodies, followed by incubation with Alexa Fluor®-labeled secondary antibodies or for intracellular staining, prior to the antibody incubation, cells were treated with Triton X-100 (2%). Stained cells were mounted with Vectashield mounting medium and images were acquired using a Leica DMI 3000B fluorescence microscope and a DFC310 FX digital color camera (Leica). Control stainings were performed without primary antibodies and with IgG isotype control antibodies to measure possible cross-reaction or non-specific binding.

Co-immunoprecipitation of FAK Y407. Cells at 80-90% confluency were lysed, as described in '*Western blotting*'. Lysates, containing 1,000-1,500 μg protein, were mixed with protein G-Sepharose beads (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) to preclear non-specific binding. Site-specific p-FAK Y407 antibody (1:500) was added to the collected supernatant and rotated at 4˚C overnight. Subsequently, protein G-Sepharose beads were added to recover the immunocomplexes. The immunoprecipitates were resolved in 150 μl SDS-PAGE sample buffer and heated to 95˚C for 5 min. The supernatants were then electrophoresed on 7.5 or 10% TGX™ precast gels and transferred to PVDF membranes. After transfer, the membranes were analyzed and developed as described in '*Western blotting*'.

Statistics. All treatments were matched and carried out at least five times. Data were analyzed using Excel, for determination of mean, SD, and Student's t-test (95%). Intensity of the immunoblotted bands was quantified by densitometry, using statistical software Scion image (Scion Corp., Frederick, MD, USA).

Results

Differences in cell-matrix adhesive and migratory behavior. The group of S. McDonnell established this isogenic Hs578T/$Hs578Ts(i)_8$ highly invasive triple-negative breast cancer model. Such a matched breast cancer cell line pair, with the same genetic background, is a unique preclinical model and allows for the investigation of the underlying mechanisms involved in the tendency these cells have to form metastasis, in this subtype of breast cancer. Their previous study showed that the $Hs578Ts(i)_8$ cell line is 3-fold more invasive and 2.5-fold more migratory in the BD Matrigel™ invasion chamber assay than the parental Hs578T cells and produces tumors in mice (15). Here, we show additional differences in invasion-associated cellular behaviors. Fig. 1A illustrates that the invasive subclone, $Hs578Ts(i)_8$, interacts significantly more with several extracellular matrices, including collagen I and IV, fibronectin and laminin. Furthermore, Fig. 1B displays results of wound-healing assays under normal growth conditions, and reveals that the $Hs578Ts(i)_8$ migrate twice as fast compared to the parental Hs578T cells which is in line with the previously published increased migratory capacity of the more invasive $Hs578Ts(i)_8$ cell line (15). Overall, these results indicate noteworthy differences between the two cell lines related to increased metastatic behavior and provide a solid basis for further investigation into the underlying mechanisms.

Identification of mediators of cell-matrix adhesion and migration. To identify the mediators involved in the observed increased adhesion to extracellular matrix proteins and migratory capacity of the $Hs578Ts(i)_8$ cells, we analyzed the potential roles of integrins, the major class of cell-matrix adhesion molecules mediating cell-matrix adhesion and migration.

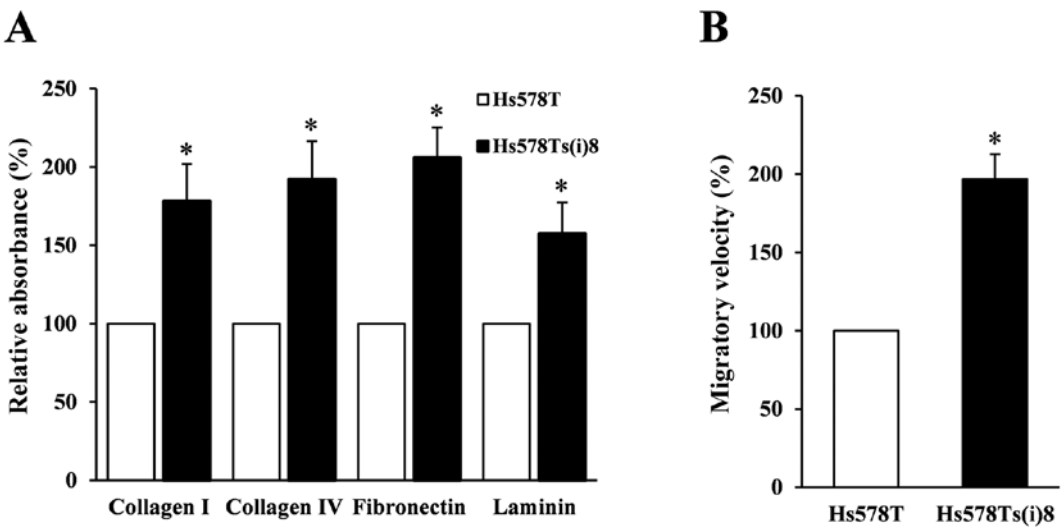

Figure 1. Adhesion and migration of Hs578T and Hs578Ts(i)$_8$ breast cancer cells. (A) Hs578Ts(i)$_8$ cells show increased adhesion to extracellular matrix proteins. Hs578T cells (open bars) and Hs578Ts(i)$_8$ cells (closed bars), at a density of 10^4 cells/100 μl were seeded in a collagen I and IV, fibronectin and laminin precoated 96-well plate and incubated for 90 min. The adherent cells were washed and determined by measuring acid phosphatase activities. The interaction and adhesion to the different extracellular matrix proteins are represented as relative absorbance (%), as compared to control Hs578T cells, after subtracting non-specific binding. Twenty-four wells were analyzed in each experiment. (B) Migratory differences between Hs578T and Hs578Ts(i)$_8$ cells. Migration of Hs578T (open bars) and Hs578Ts(i)$_8$ cells (closed bars). The cells were grown in 24-well plates until confluency, wounded and allowed to grow for 10 or 17 h. The distances over which the cells migrated were measured and results are expressed as migratory velocity (in %) as compared to control Hs578T cells. Twelve wells were used for each experiment. Asterisks indicate statistical difference from parental Hs578T cells ($p<0.05$). All data are means and SD from at least five independent experiments.

Using a panel of available integrin function blocking antibodies, at concentrations suggested by the manufacturer, ranging from 5 to 10 μg/ml, we found that the antibody against the integrin α5β1 receptor was the sole antibody significantly inhibiting the adhesion of Hs578Ts(i)$_8$ cells to fibronectin (Fig. 2A), while little or no reduced effect was observed when using other function blocking integrin antibodies in adhesion experiments using collagen I and IV or laminin (data not shown). Furthermore, the integrin αVβ5 and α5β1 antibodies, where found to reduce the migratory velocity of Hs578Ts(i)$_8$ cells by almost 50 and 40%, respectively, while no such effect was observed on the parental Hs578T cells (Fig. 2B). Next, we examined the cell surface and total expression levels of the identified integrin receptors by western blotting and flow cytometry. Fig. 2C shows the cell surface expression levels of the α5β1 and αVβ5 integrin receptors, detected using a Cell Lab Quanta™, and confirmed on a BD FACSCalibur™. A slight decrease of α5β1 integrin receptors at the cell surface of the more invasive subclone Hs578Ts(i)$_8$ was found, while no significant differences were observed for the integrin αVβ5 receptor at the surface of the two cell lines. The total expression level experiments, with quantification, displayed in Fig. 2D, reveal that the expression levels of the integrin α5 and β1 subunits, as well as the α5β1 integrin receptor are significantly reduced in the invasive subclone as compared to the expression levels in the parental Hs578T cells, whereas no significant differences were found for the total expression levels of the integrin αV and β5 subunits and integrin αVβ5 receptors.

Phosphorylation of FAK and its additional sites. It is well known that integrin receptor engagement results in the activation of FAK. FAK becomes phosphorylated at Y397 upon integrin ligation. Subsequent binding of FAK to src leads to the formation of an active and transient signaling complex, which promotes further FAK kinase activity and signaling through phosphorylations on several tyrosine sites, including Y407 near the N-terminal FERM (band 4.1-ezrin-radixin-moesin) domain, the kinase domain activation loop (Y576 and Y577), as well as phosphorylation of FAK at the C-terminal domain residues, Y861 and Y925. Subsequently, this results in the activation of downstream signaling events (17). Moreover, recent studies have shown upregulated FAK and src expression as well as FAK Y397 phosphorylation in tumor samples of patients with advanced breast cancer, including triple-negative breast cancer (18). We therefore examined and compared the total expression and phosphorylation levels of FAK and src in Hs578T and Hs578Ts(i)$_8$ cells by western blotting. Fig. 3 demonstrates that both cell lines express similar protein levels of FAK and src, while differences were found in levels of phosphorylated FAK at tyrosine residues Y397, Y407 and Y861 and src Y416, but not at FAK Y576-577 and Y925. Interestingly, we observed a pronounced increase in FAK Y397, Y407 and Y861 activation in the parental Hs578T cell line, while it was expected in the more invasive Hs578Ts(i)$_8$ cells, as increased FAK activation correlates with advanced stages of breast cancer (18). The src phosphorylation studies on the other hand, showed increased phosphorylation levels in the Hs578Ts(i)$_8$ cells at Y416 in the activation loop of the kinase domain and is associated with increased src activity, which is in agreement with previous studies demonstrating increased src activity in human breast cancer tissues (13), and the corresponding decreased phosphorylation levels at Y527 in the carboxy-terminal domain, associated with decreased

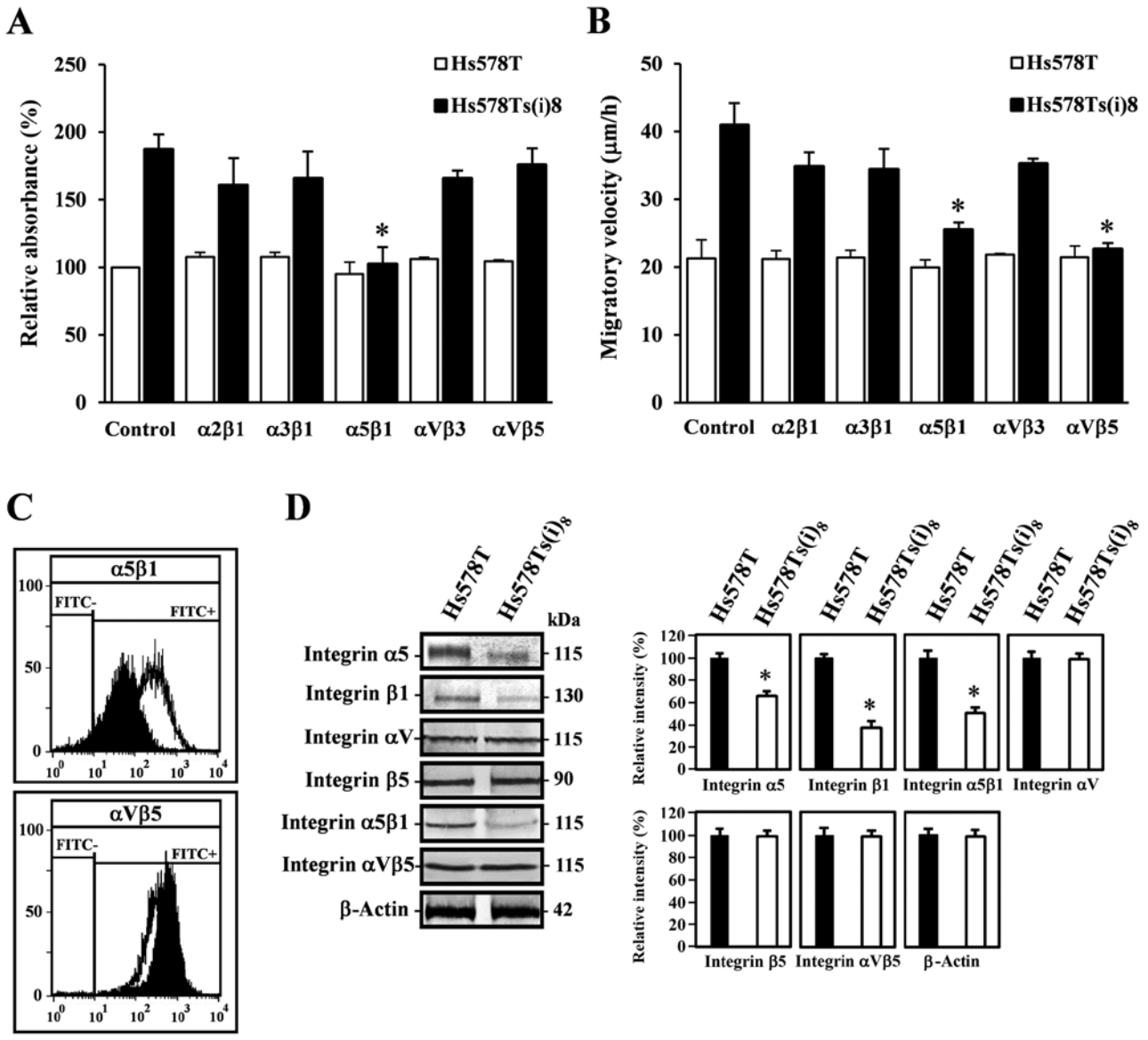

Figure 2. Integrins mediate cell-matrix adhesion and migration of Hs578Ts(i)$_8$ cells. (A) Blocking the α5β1 integrin receptor in Hs578T (open bars) and Hs578Ts(i)$_8$ cells (closed bars), reduced the binding of Hs578Ts(i)$_8$ to fibronectin. 10^4 cells/100 μl were seeded in fibronectin precoated 96-well plates, in the presence (5-10 μg/ml) or absence of integrin function blocking antibodies and incubated for 90 min. The adherent cells were washed and determined by measuring acid phosphatase activities. The adhesion of these cells to fibronectin is represented as relative absorbance (%), after subtracting non-specific binding. Mouse IgG isotype control antibodies were included to estimate non-specific binding. Eight wells were analyzed for each condition, in each experiment and compared to Hs578T cells, under similar conditions. (B) Migratory differences between Hs578T and Hs578Ts(i)$_8$ cells. Cells grown in 24-well plates until confluency, were wounded and allowed to grow in the absence or presence of function blocking integrin (5-10 μg/ml) antibodies. After 10 and 17 h, the distances over which the cells migrated were measured and results are expressed as migratory velocity (μm/h) as compared to the respective Hs578T and Hs578Ts(i)$_8$ cells, and the particular conditions. Asterisks in (A) and (B) indicate statistical difference from control Hs578Ts(i)$_8$ cells ($p<0.05$). (C) Cell surface expression levels of integrin α5β1 (upper panel) and αVβ5 receptors (lower panel) in Hs578T (open area) and Hs578Ts(i)$_8$ cells (closed area). Single cell suspensions of Hs578T and Hs578Ts(i)$_8$ cells were incubated with relevant primary integrin receptor antibodies, followed by FITC-labeled secondary antibodies and analyzed on the Cell Lab Quanta™ or BD FACSCalibur™. Control stainings were performed without primary antibody or with mouse IgG isotype control antibodies. (D) Western blot analysis for total expression levels of integrin α5, β1, αV and β5 subunits and α5β1 and αVβ5 receptors in Hs578T and Hs578Ts(i)$_8$ cells. Cell lysates, containing 30 μg protein, were analyzed by 7.5% TGX™ precast gels, transferred to PVDF membranes and blotted with the corresponding primary antibodies (left panel). Scion image densitometry analysis of bands comparing the expression levels of the subunits or receptors in Hs578Ts(i)$_8$ cells against the control Hs578T cells (right panel). β-actin was used as a loading control. Bar graphs are means ± SD from at least five independent experiments. Asterisks indicate statistical difference from control Hs578T cells ($p<0.05$).

src activity. Furthermore, levels of src when dephosphorylated at Y416 and Y527 were found elevated in the parental Hs578T and more invasive Hs578Ts(i)$_8$ cells respectively, confirming the src results described above. Additionally, we noted that downstream signal transducers, such as JNK and ERK, that correlate with the adhesive and migratory behavior were also more phosphorylated in the less invasive Hs578T cells, which again represents an unexpected activation pattern and this in an opposite way (data not shown). Given that numerous studies indicate FAK activation during epithelial

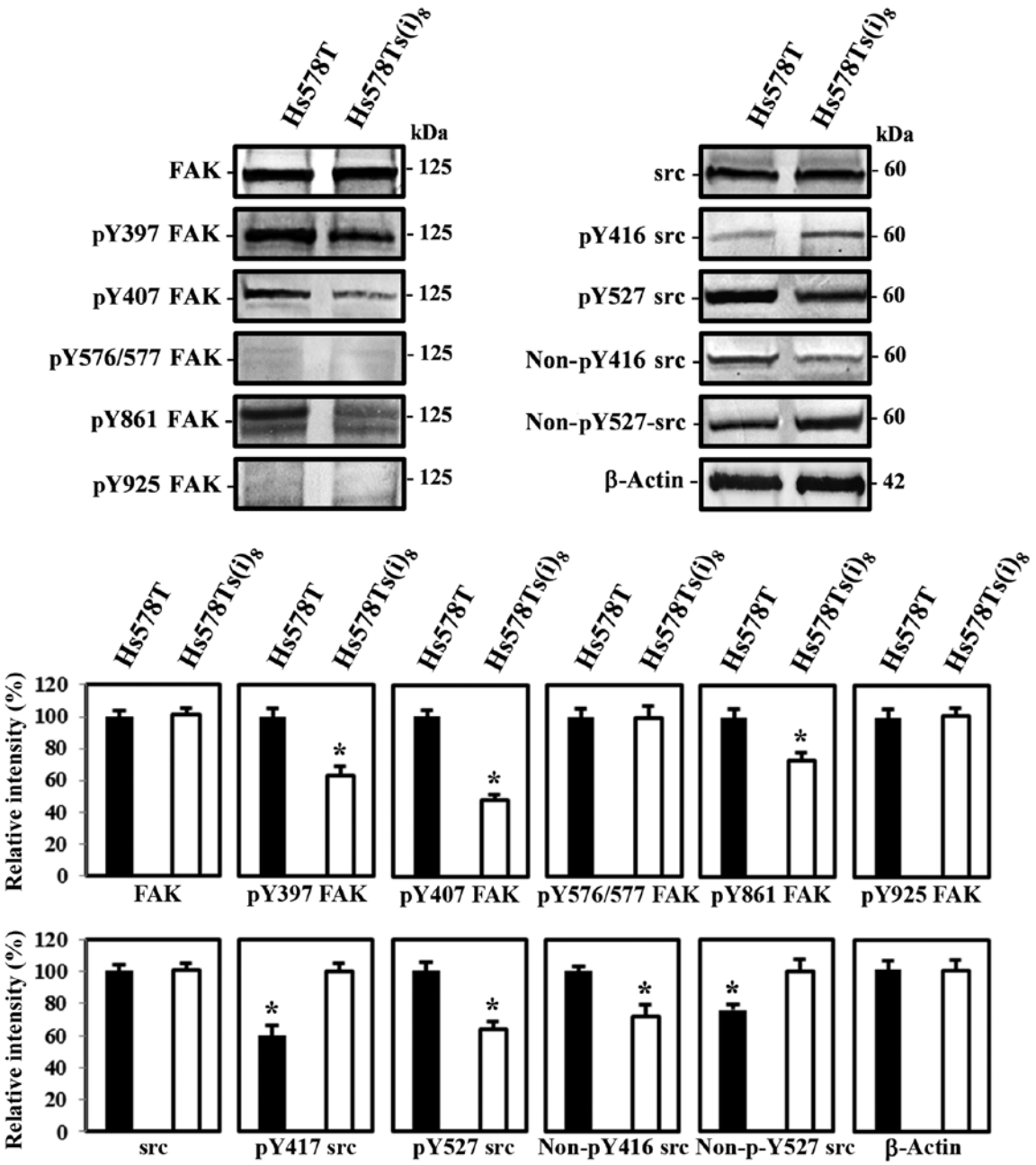

Figure 3. Phosphorylation of FAK and src and their additional sites. Western blot analysis of FAK and p-FAK Y397, Y407, Y576/Y577, Y861 and Y925 (left panel) and src and p-src Y416 and Y527 as well as non-p-src Y416 and Y527 (right panel). Cell lysates containing 30 μg protein were analyzed by 7.5 and 10% TGX^{TM} gels, transferred to PVDF membranes and used with the corresponding primary antibodies. The membranes were reblotted after stripping with anti-FAK antibody for total FAK expression or anti-src antibody for total src expression and β-actin was used as a loading control. Scion image densitometry analysis of bands comparing the expression levels of FAK and src and their activity levels in Hs578T and Hs578Ts(i)$_8$ cells. Bar graphs are means ± SD from at least five independent experiments, asterisks indicate statistical differences ($p<0.05$).

to mesenchymal transition (EMT) and further augmentation during cell migration, one particular study by Nakamura *et al* (19) suggest that FAK Y407 is a unique phosphorylation site to FAK and demonstrated that this tyrosine phosphorylated form is predominantly localized to focal adhesions and the cell periphery in motile cells. Given our observations, we opted in this study to continue further and try to elucidate the unanticipated decreased FAK Y407 activation as well as the phosphorylation of tyrosine residues 397 and 861 in the migratory subclone Hs578Ts(i)$_8$.

Localization of FAK Y407 at focal adhesion sites in more invasive Hs578Ts(i)$_8$. Our previously described results revealed that FAK is well phosphorylated at Y397, Y407 and Y861 in Hs578T cells, as compared to the more invasive Hs578Ts(i)$_8$ cells. The autophosphorylation site of FAK, Y397, has been shown to be activated and to function primarily at focal adhesions. Several studies describe the importance of FAK Y861 and invasiveness (17), however, limited studies demonstrate a role for FAK Y407. We therefore, examined the (sub)cellular localization of FAK and src as well as their tyro-

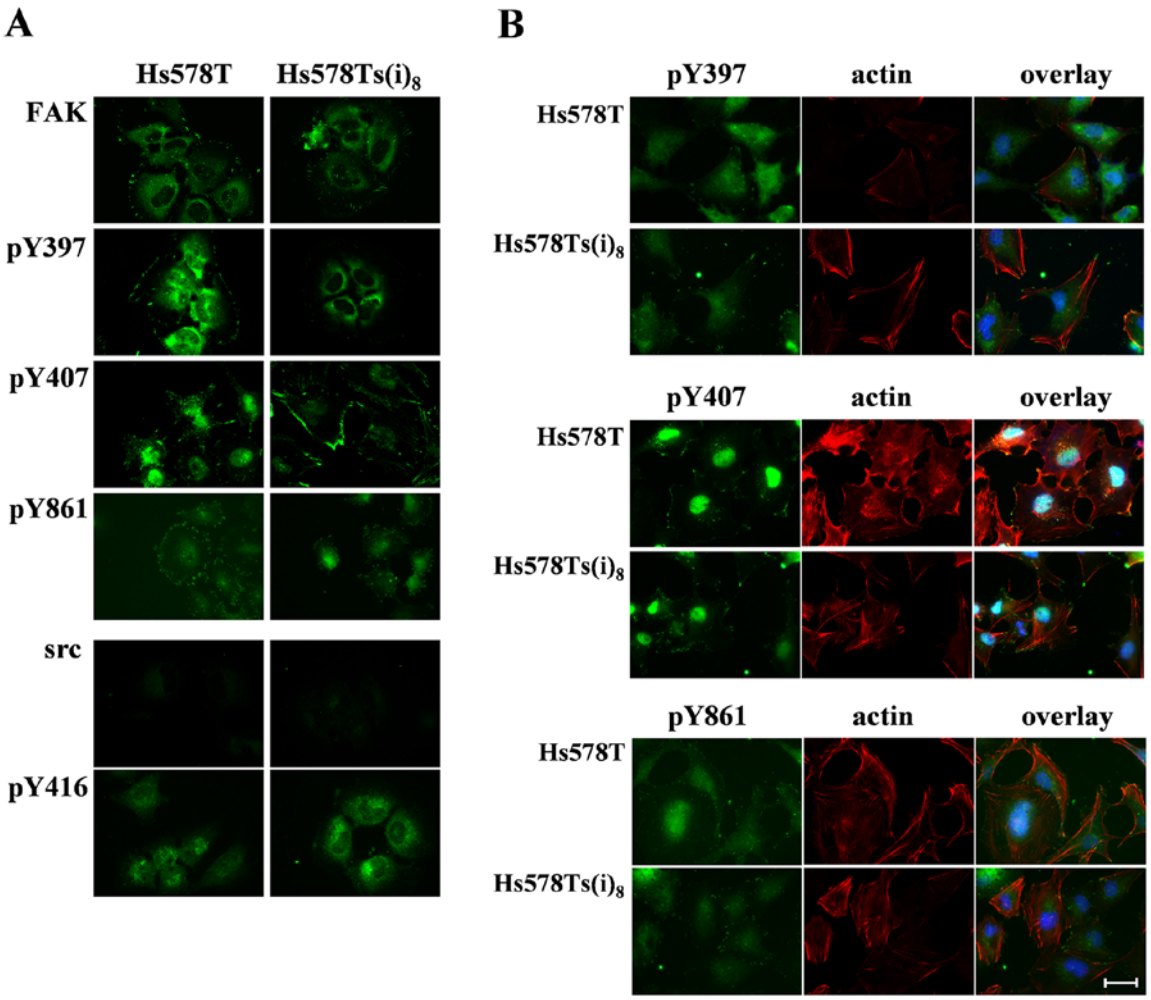

Figure 4. Localization of FAK, src and phosphorylated FAK, src. (A) Comparison of the organization of FAK and phosphorylated FAK (Y397, Y407, Y861) and src and phosphorylated src (Y416) in Hs578T and Hs578Ts(i)$_8$ cells by indirect fluorescence. Cells were grown on glass cover slips and stained by FAK and site-specific p-FAK antibodies as well as src and p-src Y416 antibodies, and Alexa Fluor®-labeled secondary antibodies. (B) Distribution of FAK and microfilaments in Hs578T and Hs578Ts(i)$_8$ cells by indirect fluorescence. Cells were grown on glass cover slips and double stained by site-specific p-FAK antibodies, Alexa Fluor® 555 Phalloidin and Alexa Fluor®-labeled secondary antibodies. Control stainings were performed without primary antibody and with IgG isotype rabbit antibodies (data not shown); scale bar, 10 μm. Experiments were performed at least five times.

sine phosphorylated forms in Hs578T and Hs578Ts(i)$_8$ cells cultured on glass substrate under normal growth conditions. Fig. 4A, shows that there are no significant differences in FAK location between the two cell lines. FAK was predominantly found in the perinuclear region and partially at the focal adhesions. FAK Y397, on the contrary, was more present in the perinuclear area and the cytosol in the invasive Hs578Ts(i)$_8$ cells and weakly at the focal adhesions, while it was found in the nucleus and to a lesser extent in the perinuclear region and focal contact sites in Hs578T cells. Furthermore, FAK Y407 staining occurred weakly in the nuclear region, and was more pronounced at the cell periphery particularly at focal adhesions, at the leading and trailing edges of the more invasive Hs578Ts(i)$_8$. In addition to this major staining, a filamentous pattern that extended into the cytosol was also detected. Slight differences in distribution were observed for FAK Y861 fluorescence, which was seen in a weak punctate pattern throughout the cytosol of Hs578Ts(i)$_8$ cells and more intensely at the cell periphery in Hs578T cells. Next, the organization of src and src Y416 was examined. The src fluorescence staining was weak and showed a diffuse distribution throughout the cytosol and concentrated in a one-sided perinuclear pattern in both cell lines. src Y416 staining was punctate at the cell periphery and extended in the cytoplasm in both cell lines, with a more prominent one-sided perinuclear pattern in the more invasive Hs578Ts(i)$_8$ cells.

Given the significant differences in distribution of the phosphorylated forms of FAK in the two cell lines, we investigated the organization of actin with each of these phosphorylated FAKs in Hs578Ts(i)$_8$ cells. Results in Fig. 4B reveal that Y407-phosphorylated FAK, which was already shown in Fig. 4A, to be mainly localized to the focal adhesions in Hs578Ts(i)$_8$ cells, is prevalent at each end of actin stress fibers in this more invasive subclone as compared to the location in the parental counterpart. Moreover, fluorescence stainings of the organization of actin and FAK Y397 or Y861, indicate that there are no major dissimilarities between the parental and more invasive cell lines, which correlates with the minor changes found when both cell lines were compared for distribution of FAK Y397 and Y861 alone. Here, the more diffuse and punc-

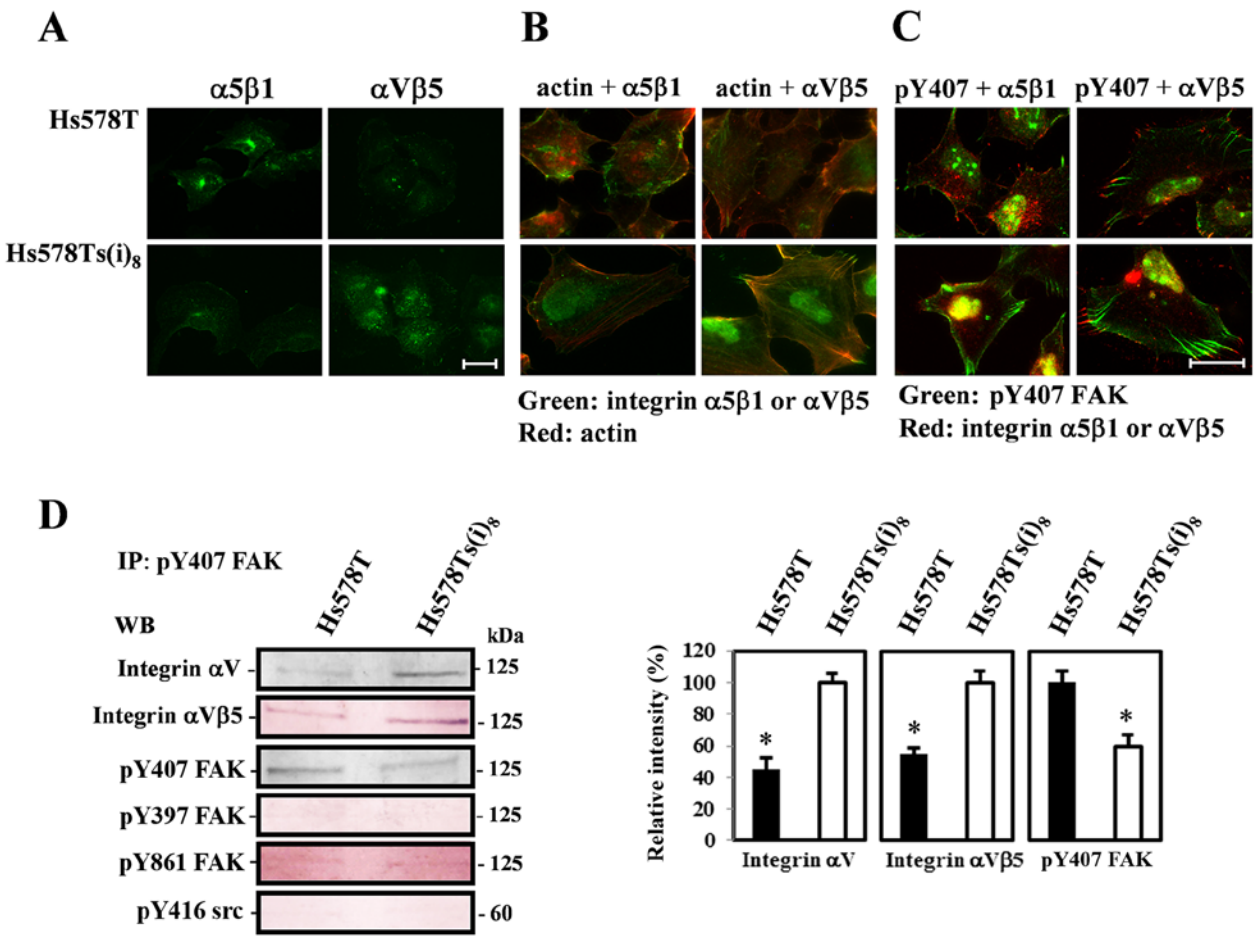

Figure 5. Localization and organization of integrins and FAK. (A) Distribution of integrins α5β1 (left panel) and αVβ5 (right panel). (B) Organization of microfilaments and α5β1 (left panel) and αVβ5 integrins (right panel). (C) Organization of pFAK Y407 and α5β1 (left panel) and αVβ5 integrins (right panel) in Hs578T (upper panel) and Hs578Ts(i)$_8$ cells (lower panel) by indirect fluorescence. Cells were grown on glass cover slips stained with α5β1 and αVβ5 integrin antibodies or double stained by α5β1 and αVβ5 integrin antibodies and Alexa Fluor® 555 Phalloidin and Alexa Fluor®-labeled secondary antibodies. Control stainings were performed without primary antibody and with IgG isotype rabbit antibodies; scale bar, 10 μm. Experiments were performed at least five times. (D) Different levels of integrin receptors αVβ5 and subunits αV organized with pFAK Y407 in Hs578T versus Hs578Ts(i)$_8$ cells (left panel). Aliquots of immunoprecipitates of pFAK Y407 were analyzed by western blotting using the respective αVβ5 and αV antibodies and site-specific antibodies against p-FAK and p-src, including pFAK Y407 as control. Densitometry analysis of the bands (right panel). Bar graphs are means ± SD from at least five independent experiments, asterisks indicate statistical differences ($p<0.05$).

tate staining pattern in the cytosol suggests that, unlike Y407 phosphorylation, phosphorylation of Y397 and Y861 can take place in the cytosol, and that this cytosolic phosphorylation is not associated with the formation of focal adhesions and stress fibers in Hs578Ts(i)$_8$ cells.

Next, we investigated the distribution of the α5β1 and αVβ5 receptors in this cell model of breast cancer progression, in an attempt to link the observed integrin-mediated changes in cellular behavior to altered FAK phosphorylation and in particular the phosphorylation of FAK Y407. The fluorescence images in Fig. 5A demonstrate a weaker staining pattern for α5β1 receptors at the cell periphery of Hs578T cells and a more intense staining in the perinuclear region, as compared to the Hs578Ts(i)$_8$ cells, in which also a very faint network of α5β1 integrin receptors was observed. In contrast, αVβ5 receptors were found at the cell periphery of both cell lines, with a more pronounced staining in the focal adhesions and a punctate cytosolic pattern with a vague filamental distribution, in the more invasive cell variant. Dual fluorescence stainings of actin and integrins (Fig. 5B) disclosed that the distribution of integrin α5β1 receptors did not localize to actin stress fibers in Hs578T and Hs578Ts(i)$_8$ cells, while co-staining of actin and αVβ5 integrin receptors revealed that these integrin receptors colocalized with actin in Hs578Ts(i)$_8$ cells. Such a noticeable pattern was absent in Hs578T cells. Moreover, integrin αVβ5 receptor staining was prominent at each end of the actin stress fibers. Visualization of Y407-phosphorylated FAK and these integrin receptors further indicate that in Hs578Ts(i)$_8$ cells, FAK Y407 staining extends inward from αVβ5 receptors at the cell periphery in focal adhesions, as much more highly bundled fiber-like structures. Similar stainings in the parental Hs578T cells show fewer focal adhesions to which integrin αVβ5 receptors are localized and are less organized with FAK Y407. Furthermore, such an organizational pattern was not observed for α5β1 integrin receptors and FAK Y407 in these cells, rather they showed limited focal complexes containing α5β1 integrin receptors and phosphorylated FAK Y407 (Fig. 5C).

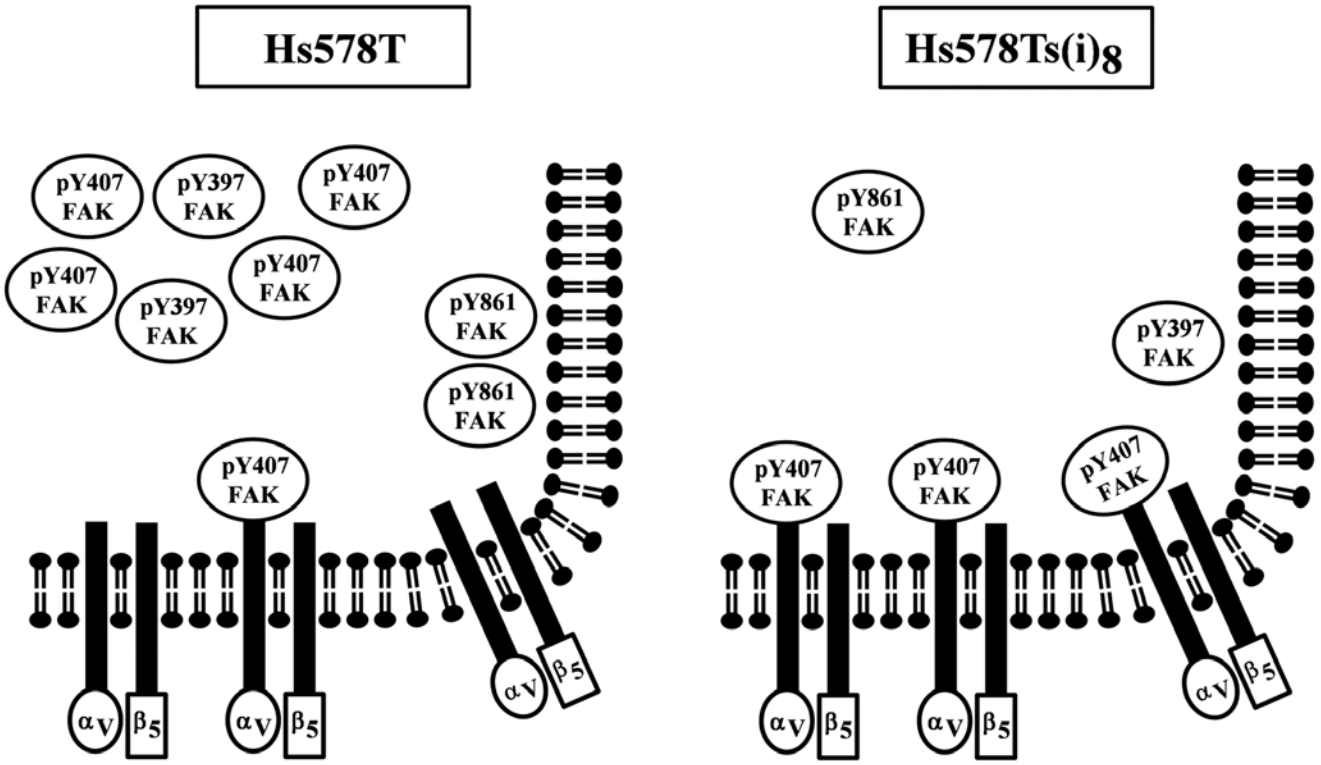

Figure 6. Suggested schematic of phosphorylated FAK localization in Hs578T and Hs578Ts(i)$_8$.

In conclusion, these observations suggest that for each phosphorylated form of FAK, there seems to be some differences in intensities of the signal, depending on the subcellular localization, in particular for Y407 phosphorylation which can be correlated to the migratory behavior and organization of microfilaments and integrin αVβ5 receptors. Subsequently, co-immunoprecipitation experiments using FAK Y407 antibody, confirmed the increased phosphorylation at FAK Y407 in the parental Hs578T cell line and the distribution and close connection of FAK Y407 with integrin αV subunits and αVβ5 receptors, but not with the β5 subunits, in the invasive Hs578Ts(i)$_8$ cells (Fig. 5D). Moreover, the levels of FAK Y397 and Y861 as well as src Y416 were almost not detectable. These results indicate that increased phosphorylation of FAK Y407 in the parental Hs578T cell line does not correlate with the formation of focal adhesions and subsequently its behavior.

Discussion

Several studies and reviews have shown the importance of FAK in processes as tumor cell adhesion, cell motility, invasion and ultimately metastases, in several types of cancers, including breast cancer and also in the triple-negative subtype (17,18). Although the knowledge on FAK has increased over the last years, there is still much knowledge to gain. In this study, we present important findings on differences between the Hs578T triple-negative breast cancer cell line and the more invasive variant Hs578Ts(i)$_8$, a model which represents the metastatic nature of TNBC and eliminates confounding results due to cell line heterogeneity (15). We document for the first time the occurrence of FAK when phosphorylated at Y407 in triple-negative breast cancer cell lines, and provide a rationale for the controversial data previously described in the literature (19-24) and in the findings presented in this report.

Our results demonstrate that the increased cell-matrix adhesion to fibronectin and the migratory behavior of the Hs578Ts(i)$_8$ cells, as compared to the Hs578T cells, can be ascribed to the action of integrin receptors, α5β1 and αVβ5, respectively. Cell surface and total expression levels of these integrin receptors revealed that the enhanced cellular activities could not be correlated to their expression levels, but rather could be attributed to the location in the more invasive cells, with some integrin α5β1 receptors at the cell's periphery and αVβ5 integrin receptors more pronounced at focal adhesions. While this may be a weak justification at this point, it is the most plausible explanation, given the limited accessibility of commercially available antibodies that block the function of integrin receptors. The latter may also be brought in connection to our findings on other extracellular matrix proteins, for which we were not able to identify a potential role for integrin receptors interacting with their corresponding ECM proteins (data not shown). Moreover, it should be noted that all the experiments involved in this study, except for the cell matrix adhesion assays, were performed with cells grown under normal growth conditions, on either plastic or glass substrates, allowing for the detection of differences between the two cell lines, displaying their specific features and properties, and thus maintaining their phenotypes as previously published (15). Furthermore, the complexity expands given the overlap in substrate specificity (25) and the growing numbers of integrin receptors or other classes of cell surface proteins and lipids involved in cell matrix interactions, such as glycosphingoli-

pids (GSLs) (26-28). These GSLs have been linked to the regulation of cell motility and invasion, by themselves or via particular organizations with integrin receptors. Interestingly, these GSL-assemblies may positively or negatively influence integrin-associated signaling pathways (27,28). This may also provide a justification for the subtle differences in expression levels of the integrin receptors we detected despite their observed role in the adhesion and migration experiments.

One of the major signaling events linking the role of integrin receptors to cell adhesion and motility is the activation of focal adhesion kinase (16). In this study, we show that there are significant differences in tyrosine phosphorylation levels on multiple sites of FAK, but not in the levels of the known serine phosphorylations sites (data not shown), between the Hs578T and Hs578Ts(i)$_8$ cells. Our results demonstrate that the autophosphorylation site Y397 and two additional tyrosine sites (Y407 and Y861) are major activation sites and that phosphorylation of Y576/Y577 in the kinase loop and Y925 in the C-terminal domain, were at the detection limit. Moreover, we found higher activity levels at tyrosine residues 397, 407 and 861 in Hs578T cells, whereas it was expected in the more invasive Hs578Ts(i)$_8$ cell line. The observed phosphorylation of tyrosine residues of FAK in Hs578T is likely attributed to the mesenchymal-like character of these cells and can be related to changes in epithelial to mesenchymal transition, as is the case for MDA-MB-231 and BT-459 cells, representing cell line models for advanced TNBC (28). Generally, increased FAK activity levels are linked to enhanced metastatic behavior (17,18). Despite the fact that Hs578Ts(i)$_8$ cells display a more invasive phenotype as compared to Hs578T cells (15), such a correlation was not detected in this study. To the best of our knowledge, no information is published supporting decreased expression and activity of FAK in advanced disease stages. There are, however, varieties in levels of FAK mRNA and activities in different types of cancer, including breast cancer, for which increased expression and activity is linked to a poor prognosis (18). Interestingly, a recent report demonstrated that src homology phosphotyrosyl phosphatase 2 (SHP2) promotes cell migration by dephosphorylating FAK Y397. Moreover, this study showed that FAK Y407 was not a substrate for SHP2, and further provided evidence that SHP2 not just acts on FAK, but likely targets additional proteins to promote cell motility (30).

Of particular interest in this study are the levels of FAK when phosphorylated at tyrosine 407. Yet, there are limited studies available and some of them indicate a negative correlation, demonstrating increased FAK Y407 phosphorylation under conditions which normally lower FAK Y397 activity, such as serum starvation and cell cycle arrest (21,22). Other studies describe increased Y407 phosphorylation during endothelial cell migration, induced by vascular endothelial growth factor (VEGF) and requires the association of vascular endothelial growth factor receptor 2 (VEGFR2) and heat shock protein (HSP)90. Furthermore, the latter study reports that Y407 phosphorylation is insensitive to src inhibition by the pharmacological inhibitor, PP-2 (20), suggesting that phosphorylation of FAK at Y407 is independent of src, a phenomenon that was also observed in KM12C colon cancer cells expressing kinase deficient src proteins (31). On the contrary, src-dependent phosphorylation of Y407 has been described as well (32,33). In addition, Ciccimaro *et al* implied an important future role for FAK Y407, as an additional autophosphorylation site and measure of FAK activity, next to FAK Y397 (33). Our results, at first sight, also suggested a negative correlation, particularly concerning the decreased FAK tyrosine kinase activities and the migration and invasion-associated cellular activities of the Hs578Ts(i)$_8$ cell line. Furthermore, the inverse relation between phosphorylation of FAK Y407 and src Y416, could be interpreted as FAK activation occurs independently of src or may propose that there are other, thus far unknown, FAK regulatory mechanisms at play. In regards to this, recent reports indicate that FAK activity is regulated by an intra-molecular interaction between the N-terminal FERM and the catalytic domains, which may alter Y397 autophosphorylation and its catalytic activity. These studies highlight the importance of the FERM domain, since truncation increases the FAK kinase activity, and additionally demonstrate that interactions with phosphoinositide lipid and the ECM, induce conformational changes in FAK, influencing its activity and association with tetraspanins and growth factor receptors (34,35). Additionally, it has been shown that FAK activity can be affected by indirect interactions, between the C-terminal focal adhesion targeting (FAT) domain and integrins at focal adhesions. These linkages involve either FAK Y925 or Y861, and are associated with paxillin and p130Cas or FAK S910 activation (36). Our results, however, did not confirm the constitutive activation of the involved FAK Y861 and Y925 or S910, nor paxillin and p130Cas in Hs578Ts(i)$_8$ cells, yet again increased phosphorylation levels of paxillin at Y31 and Y118 phosphorylation and at Y410 of p130Cas were detected in Hs578T cells (data not shown).

Hs578T and Hs578Ts(i)$_8$ cells showed a different subcellular localization of the tyrosine phosphorylated FAKs. In the more invasive Hs578Ts(i)$_8$ cells, FAK Y407 was mainly present at the cell periphery in focal adhesion-like structures at each end of actin stress fibers and organized with integrin αVβ5 receptors. These results link the αVβ5 integrin-mediated migratory behavior in these cells to FAK Y407. Furthermore, our data suggest that localization, rather than FAK expression and overall FAK Y407 phosphorylation levels, could be a FAK-mediated regulatory mechanism to control migratory behavior. In previous studies, such a mechanism has been proposed for src, with its translocation to the cell membrane, phosphorylation and activation of signaling pathways influencing cell adhesion and migration (37). In our studies, this would mean that, in the more invasive Hs578Ts(i)$_8$ cells, αVβ5 integrin receptors at the cell periphery recruit FAK from the cytoplasm, resulting in perhaps autophosphorylation or indeed src-dependent phosphorylation of FAK Y407 and its organization with these integrin receptors, to regulate cellular migration, and this in the absence of its ligand vitronectin. Further investigations, using vitronectin, are necessary to detect the effect of integrin αVβ5-vitronectin engagement on FAK phosphorylation levels. This will allow us to further explore the atypical decrease in FAK activation observed in the Hs578Ts(i)$_8$ cells.

Many FAK studies focus on expression levels and to a lesser extent on activity levels and localization. Our data demonstrate FAK Y407 in TNBC cell lines and suggest that the subcellular localization is more important than the overall Y407 activity levels. We propose that this spatio-

temporal expression of FAK Y407 is the key to the regulation of the αVβ5 integrin receptor-mediated cell motility in the Hs578Ts(i)$_8$ cells (Fig. 6). Moreover, our results hint that FAK as well as the other site-specific phosphorylated FAKs are also temporally and spatially controlled and that this regulation affects their involvement in signaling pathways controlling other cellular functions. Our results add to some existing evidence, supporting the importance of measuring activity levels and determining the subcellular localization of signaling molecules (38). The latter two evaluations may provide a better understanding of the underlying biochemical disease mechanisms and may more accurately reflect tumor progression which all together represents a potentially more suitable way for the identification of novel biomarkers for diagnosis and therapy. In conclusion, our findings propose new directions and ideas for studying disease mechanisms in TNBC and warrant the need for expanding this idea to other TNBC cell lines and metastatic tissues. These future studies may provide valuable insight for the classification of metastatic TNBC and the development of targeted therapeutic approaches.

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