ORIGINAL ARTICLE

Focal Adhesion Kinase Induces Matrix Metalloproteinase-2 by Involving α5β1-Mediated Signaling in Breast Cancer Cell, MCF-7

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ABSTRACT

Introduction: Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that plays a pivotal role in cell invasion. Matrix metalloproteinases (MMPs) are implicated as the key players in cancer cell invasion. Hence, the role of FAK in MMP regulation is very important in understanding tumor progression. **Materials and Methods:** Here, we studied the role of FAK, its association with other signaling kinases and involvement in the α5β1 integrin receptor-mediated regulation of MMP-2 activity and expression in human breast cancer cell line MCF-7. **Results:** Immuno blot analysis revealed that FN treatment causes phosphorylation of FAK and FAK gets localized at the cell attachment focal point of MCF-7 cells. FN treatment did not change the mRNA status of FAK but enhanced mRNA level of MMP-2 and MT1-MMP, also caused downregulation of TIMP-2. Co-imunoprecipitation and inhibitor studies revealed the association of FAK with α5β1, Paxillin, PI3K and ERK. siRNA studies revealed that FAK is critical in regulation of activity and expression of MMP-2 and downstream signaling kinases. **Conclusion:** The interaction of α5β1 integrin with FN initiates a signaling cascade with FAK as its central player. FAK gets phosphorylated and in turn associates with tyrosine kinases like PI3K and ERK. FAK also activates PI3K and ERK that serve as very crucial mediators of the signaling pathway leading to induction of MMP-2 activity and resulting invasion of breast cancer cell, MCF-7.

Keywords: Breast cancer, Focal Adhesion Kinase, Integrin, Matrix metalloproteinase, Metastasis, Signaling

INTRODUCTION

Phosphorylation and dephosphorylation of tyrosine residues are critical to the signal transduction pathways that regulate tumor growth and invasion. Binding of extracellular matrix ligands to the integrin receptors triggers multiple cellular responses and an outside-in signaling pathway. Integrins are heterodimeric receptors that transmit signals between extracellular matrix ligands and intracellular signal transduction pathways[1] to support adhesion and invasion.[2] Many of the responses triggered by integrin attachment are mediated through tyrosine phosphorylation-based activation of Focal Adhesion Kinase (FAK). FAK is a non-receptor tyrosine kinase and a critical player in integrin-mediated migration and signaling[3]. Initially, phosphorylation of FAK occurs in its major autophosphorylation site, Tyr 397. Phosphorylation of Tyr 397 initiates a signaling cascade that results in the phosphorylation of subsequent tyrosine kinase residues, including Tyr 576, 577, 861 and 925, which render the molecule as a fully active kinase.[4] When FAK is autophosphorylated at the Tyr 397, it binds to the SH2 domain of PI3K,[5] thereby bringing the catalytic subunit of PI3K to the membrane, where the PI3K is phosphorylated and activated. FAK-PI3K association, induced by integrin activation is seen in both platelets and fibroblasts.[6] PI3K plays an important part in the invasion by various types of cancer.[7-9] FAK has been shown to activate ERK signaling pathway, either directly or through the activation of PI3K signaling pathway. ERK activation stimulates the activation of a number of transcription factors, which in turn induce the expression of many genes which play a crucial role in cancer cell proliferation and migration. A large number of reports show an enhanced protein expression of FAK in a number of human cancers, including breast

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cancer.[10] Furthermore, FAK overexpression has been associated with the invasive potential of tumor cells. One of the critical steps in tumor invasion is the destruction of ECM catalyzed by matrix metalloproteinases (MMPs).[11] MMPs such as MMP-2 and MMP-9 are a family of neutral proteinases secreted from cells as inactive zymogens and require proteolytic cleavage for their activation.[12] There are earlier reports which suggest that FAK often plays a pivotal role in MMP-2 induction and activation.[13,14]

In the present communication, we have observed the role of FAK and its related signaling molecules in $\alpha 5\beta 1$-mediated responses in human breast cancer cell line, MCF-7. We have particularly tried to establish the role of FAK in $\alpha 5\beta 1$-induced expression and activity of MMP-2 in MCF-7 cells.

RESULT

FN Induces the Phosphorylation of FAK in MCF-7 Cells

In Figure 1. A. immunoblot analysis reveals that FN treatment did not change the protein expression of FAK even after treatment up to 2 hours (upper panel) but FN treatment appreciably induced the phosphorylation of FAK (Y397) in MCF-7 cells in a time-dependant manner (lower panel).

FN Induces mRNA Expression of MMP-2 and MT1-MMP in MCF-7 cells

Figure 1. B. FN treatment did not cause any appreciable change in the mRNA expression level of FAK in MCF-7 cells upto 2 hours treatment. FN stimulated mRNA expression of MMP-2 at 20 µg/ml treatment for 2 hours as determined by real time RT-PCR. FN treatment also caused an appreciable increase in the mRNA level of MT1-MMP in MCF-7 cells. FN treatment downregulated the mRNA level of TIMP-2 within 1 hour.

FN Causes the Localization of FAK at the Sites of Focal Adhesions

Figure 1. C. Immunocytochemical analysis revealed that FN treatment at a concentration of 20 µg/ml for 2 hours caused the localization of FAK at the sites of cell adhesions. FN treatment caused FAK to get localized as focal points in MCF-7 cells.

FAK Association Studies and the Effect of FN Treatment on the Association of FAK with Other Proteins

In Figure 2. Co-immunoprecipitation study reveals that $\alpha 5$ integrin receptor is physically associated with FAK and the association increases with FN treatment of 20µg/ml for 2 hours (upper panel). Co-immunoprecipitation also shows that paxillin is a part of the $\alpha 5$-FAK complex as

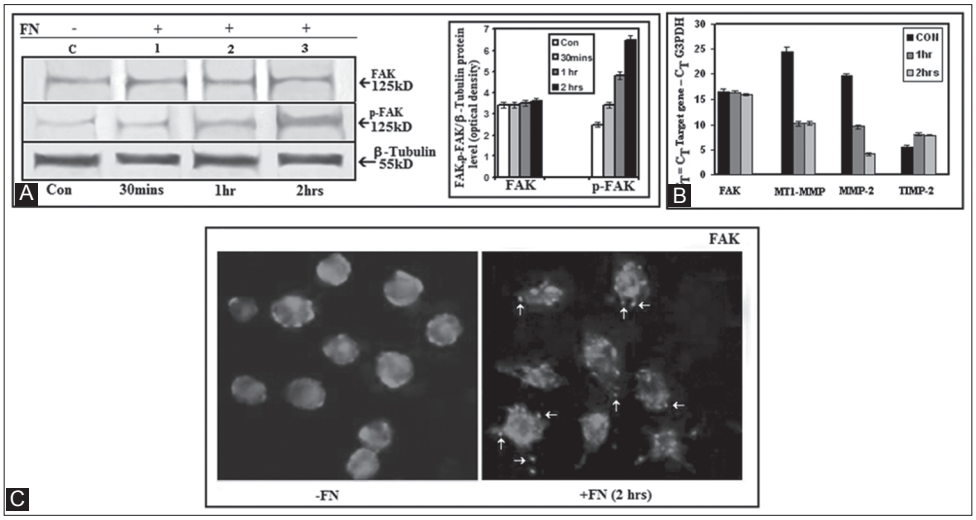

Figure 1: (A) Effect of FN treatment on the expression of FAK and p-FAK in MCF-7 cells: MCF-7 cells (300,000 cells/ml) were grown in SFCM in absence (C), in presence of 20 µg/ml FN coated for 30 mins (1), 1 hour (2) and 2 hours (3). The cells were collected; extracted and equal protein (100 µg) was subjected to western blot analysis with anti-FAK (upper panel) and anti-p-FAK (lower panel) antibody (1:1000 dilution for 1½ hrs at 37°C). β-tubulin was used as internal control and done in parallel to all the blots. The accompanying graphs represent the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means±SEM of three experiments. (B) Determination of gene expression of MMP-2, FAK, TIMP-2 and MT1-MMP by Quantitative Real-Time RT-PCR: MCF-7 cells (300,000 cells/ml) were grown in absence (C) and in presence 20 µg/ml FN coated for 1 hour and 2 hours in SFCM. Total RNA was extracted from control and experimental MCF-7 cells (1×10^6 cells). 2 steps RT-PCR was done with equal amounts of total RNA. 2 µl of cDNA was subjected to Real-Time quantitative RT-PCR with SYBR Green as a fluorescent reporter. Relative levels of expression of MMP-9, FAK, TIMP-2 and MT1-MMP and the control G3PDH in control and experimental sets was measured by quantitative real time RT-PCR by calculating the C_T value. The calibrator used in our experiments is the control FN untreated (C) MCF-7 cells and the samples are the 1hour (1) and 2 hours (2) FN-treated cells. In the given graph the C_T value is inversely proportional to the mRNA expression of the samples. (C) Immunocytochemical analysis of the status of FAK in fibronectin treated MCF-7 cells: MCF-7 cells were grown on coverslips in absence (control -FN) and presence of fibronectin (20 µg/ml) for 2 hours (+FN 2 hours). Immunocytochemistry was preformed with anti-FAK antibody (1:1000 dilution for 1½ hrs at 37ºC) and then incubated with FITC-labeled respective secondary antibody. Coverslips were observed under a fluorescence microscope (40X)

immunoblot of paxillin after immunoprecipitation with α5 shows bands; the intensity of which and hence association is enhanced after FN treatment of 20 μg/ml for 2 hours (lower panel). Figure 2. B. reveals that FAK is physically associated with PI-3K (upper panel and ERK1/2 (lower panel) in MCF-7 cells and the association increases with FN treatment of 20 μg/ml for 2 hours.

Integrin receptor α5 is crucial for FAK phosphorylation and the downstream effects

Immunoblot assay of Figure 2. C shows that FN treatment of 20 μg/ml for 2 hours causes appreciable phosphorylation of FAK (Y397) in MCF-7 cells. However, blocking the cells with α5 monoclonal antibody prior to FN treatment causes inhibition of the FN-induced phosphorylation of

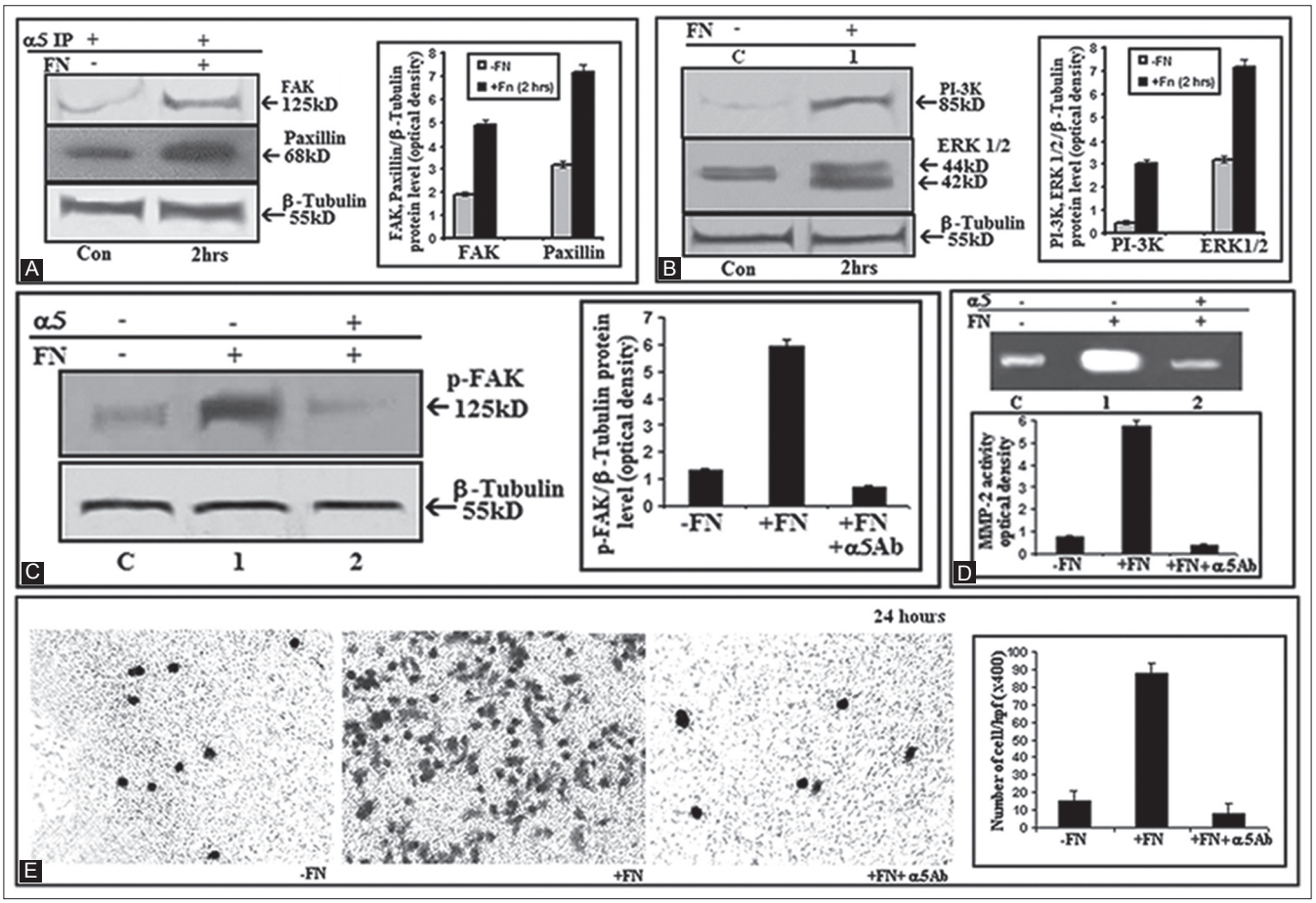

Figure 2: (A) Effect of FN treatment on the association of integrin receptor α1 with FAK and Paxillin in MCF-7 cells: MCF-7 cells (300,000 cells/ml) were grown in SFCM in absence (C), in presence of 20 μg/ml FN coated for 2 hours (1). The cells were collected; extracted and equal protein (100 μg) was immunoprecipitated with anti-α1 antibody and subjected to western blot analysis with anti-FAK (upper panel) and anti-paxillin (lower panel) antibody (1:1000 dilution for 1½ hrs at 37°C). β-tubulin was used as internal control and done in parallel to the blots. The accompanying graphs represent the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means±SEM of three experiments. (B) Effect of FN treatment on the association of FAK and downstream signaling kinases like PI-3K and ERK in MCF-7 cells: MCF-7 cells (300,000 cells/ml) were grown in SFCM in absence (C), in presence of 20 μg/ml FN coated for 2 hours (1). The cells were collected; extracted and equal protein (100 μg) was immunoprecipitated with anti-FAK antibody and subjected to western blot analysis with anti-PI-3K (upper panel) and anti-ERK (lower panel) antibody (1:1000 dilution for 1½ hrs at 37°C). β-tubulin was used as internal control and done in parallel to the blots. The accompanying graphs represent the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means±SEM of three experiments. (C) Involvement of integrin α5β1 on fibronectin-induced p-FAK level in MCF-7 cells: MCF-7 cells (300,000 cells/ml) were grown in absence of fibronectin (C) and presence and presence of fibronectin (20 μg/ml for 2 hours) (1) and in presence of 1μg/ml anti-α5 antibody (2) for 1 hour prior to treatment with 20 μg/ml fibronectin for 2 hours. The cells were collected; extracted and equal protein (100 μg) was subjected to western blot analysis with anti-p-FAK antibody (1:1000 dilution for 1½ hrs at 37°C). β-tubulin was used as internal control and done in parallel to the blot. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means±SEM of three experiments. (D) The SFCM of control (C), FN treated (1) and FN and α5 antibody treated (2) sets were collected. The culture supernatants were collected and gelatin zymography was performed by using a 7.5% SDS-PAGE co-polymerized with 0.1% gelatin. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means±SEM of three experiments. (E)Transwell Chamber Assay: MCF-7 cells untreated (-FN) and treated with fibronectin treated (+FN), treated with both fibronectin and α5 monoclonal antibody (+FN+α5 Ab) were seeded into transwell insert and were allowed to grow for 24 hrs. Figure shows MCF-7 cell migration through membrane after 24 hrs in FN-non treated, FN-treated and FN+α5 antibody treated sets. The accompanying graph shows the number of control (-FN), fibronectin treated (+FN) and both fibronectin and α5 monoclonal antibody (+FN+α5 Ab) treated MCF-7 cells migrated through transwell insert were counted per high power field after 24 hrs of cell seeded. Data are means±SEM of three experiments

FAK. Zymographical analysis in Figure 2.D. shows that FN treatment of 20 μg/ml for 2 hours causes induction of the gelatinolytic activity of MMP-2 in MCF-7 cells. When the cells are blocked with α5 monoclonal antibody prior to FN treatment then FN-induced MMP-2 activity was appreciably reduced. Hence, abrogation of α5 integrin receptor causes inhibition of FAK phosphorylation and the activity of MMP-2. In Figure 2 E. when control (-FN) and fibronectin treated (+FN) MCF-7 cells were seeded on the membrane of transwell insert, the fibronectin treated cells were observed to be migrated through the membrane after 24 hrs. The accompanying graph shows that after an incubation of 24 hrs total number of 85±5 fibronectin treated cell (+FN) were migrated, compared to control (-FN) cells (14±1). When MCF-7 cells were treated with α5 monoclonal antibody prior to FN treatment and when these cells were seeded on the membrane of transwell insert the invasion of the antibody treated cells were much less than the only the FN treated sets. The accompanying graph shows the migration of the antibody treated set to be around 6±3.

Role of FAK in expression and activity of MMP-2 in MCF-7 cells

Zymographical analysis of Figure 3. A. shows that treatment of MCF-7 cells with FAK siRNA noticeably inhibited the MMP-2 activity of control FN-untreated MCF-7 cells and also FN-induced MMP-2 activity in MCF-7 cells. Control siRNA did not show any such effect on the MMP-2 activity. Control as well as FN-mediated MMP-2 protein expression level (Figure 3.D.b) in addition to mRNA expression level (Figure 3.C) was appreciably inhibited in FAK siRNA treated MCF-7 cells. Thus, the blockade of FAK abrogated the FN-mediated MMP-2 induction, confirming the role of FAK in MMP-2 stimulation.

Involvement of FAK in FN-mediated signaling events

Figure 3. B. FAK siRNA blocked endogenous FAK mRNA expression in control FN-untreated MCF-7 cells and also in FN-treated MCF-7 cells. FAK siRNA blocked endogenous FAK mRNA expression and also the level of p-FAK in MCF-7 cells (Figure 3. D.a). Treatment of MCF-7 cells with FAK siRNA appreciably downregulated the expression of p-ERK (Fig.3.D.c) and p-PI3K (Fig.3.D.d) indicating position of FAK upstream of both ERK PI-3K in the signaling pathway and the pivotal role of FAK in FN-mediated intracellular signaling.

Role of FAK in the invasive potential of MCF-7 cells

In Figure 3.E. when control (-FN) and fibronectin treated (+FN) MCF-7 cells were seeded on the membrane of transwell insert, the fibronectin treated cells were observed to be migrated through the membrane after 24 hrs. The accompanying graph shows that after an incubation of 24 hrs total number of 87±3 fibronectin treated cell (+FN) were migrated, compared to control (-FN) cells (12±2). When MCF-7 cells were treated with FAK siRNA prior to FN treatment and when these cells were seeded on the membrane of transwell insert the migration/invasion of the FAK siRNA treated cells were much less than the only the FN treated sets. The accompanying graph shows the migration of the antibody treated set to be around 4±2. The cells treated with control siRNA how ever did not show any change in migration as compared to FN treated sets. Thus abrogation FAK inhibited the invasive potential of the MCF-7 cells and this property can be attributed to the regulation of MMP-2 by FAK.

Role of PI3K in FN-mediated responses

The immunoblot analysis of Figure 4. A. shows that treatment of control MCF-7 cells with PI3K inhibitor caused an inhibition of phosphorylation of PI3K (panel a) and also the phosphorylation of FAK (pane; b) and ERK 1/2 (panel c). When the cells were treated with FN, it caused enhanced phosphorylation of PI3K, FAK and ERK 1/2, when the cells were treated with PI3K inhibitor before treatment with FN, then PI-3K inhibitor caused an appreciable decrease in the FN-induced phosphorylation of PI3K, FAK and ERK. Hence, inactivation or abrogation of PI3K activation abrogated the FN-induced activation of FAK and ERK1/2.

Role of ERK in FN-mediated responses

The immunoblot analysis of Figure 4. B. shows that treatment of control MCF-7 cells with ERK inhibitor caused an inhibition of phosphorylation of ERK 1/2 (panel a). However, treatment of ERK inhibitor did not cause any noticeable effect on the phosphorylation of FAK or PI3K. When the cells were treated with FN, it caused enhanced phosphorylation of ERK 1/2, FAK and PI3K, when the cells were treated with ERK inhibitor before treatment with FN, then ERK inhibitor did not cause any appreciable effect on the FN-induced activation of FAK or PI3K. Hence, inactivation or abrogation of ERK activation did not have any effect on the FN-induced activation of FAK and PI-3K.

Inhibitor assay of Figure 4.C. revealed that MMP-2 gelatinolytic activity was considerably decreased with treatment of the cells with PI-3K inhibitor (LY 294002) and ERK inhibitor (PD 98059), whereas no appreciable effect was observed upon treatment with MEK inhibitor (U0126) and p38 inhibitor (SB 203580) at their respective concentrations.

Enhanced FAK expression in breast tumour tissue sections

Immunohistochemistry of Figure 5 clearly showed that FAK is located in the cytosol of the cells in the tissue sections.

IHC also reveals that normal breast tissue section had low level of cytosolic FAK expression which was appreciably enhanced in breast tumour tissue sections. Hence, FAK is over expressed in tumour samples as compared to normal breast tissues.

DISCUSSION

FAK overexpression in breast carcinoma has been found in all stages of the cancer progression and was associated with the presence of lymph node metastasis, thus suggesting

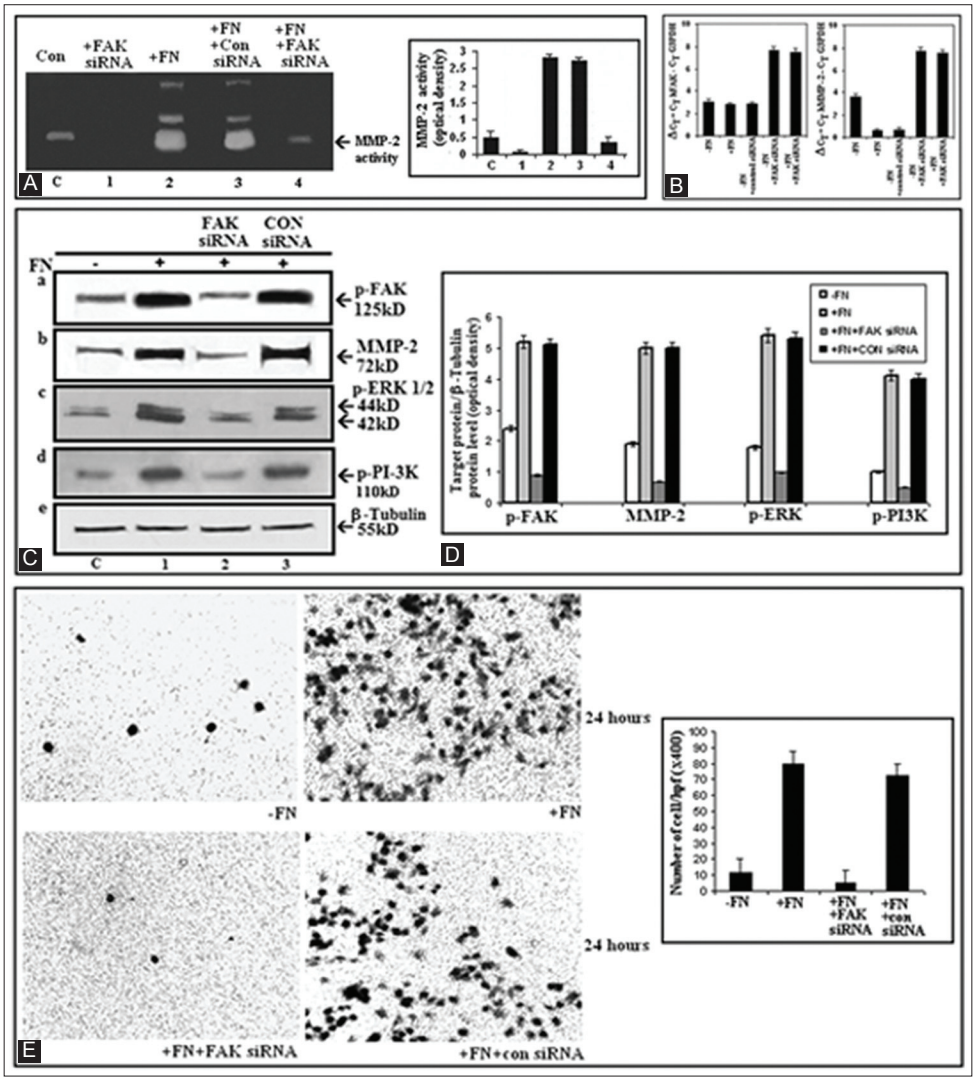

Figure 3: Effect of blocking FAK signals on FN-induced MMP-2 activity and expression by gelatin zymography and Quantitative Real-Time RT-PCR: MCF-7 cells were grown in SFCM for 24 hours, transfected with control (lane 4) and FAK siRNA (lane 4) (100 nM each) for 48 hrs before treating the cells with 20 µg/ml FN coated for 2 hours. (A) The culture supernatants were collected and gelatin zymography was performed by using a 7.5% SDS-PAGE co-polymerized with 0.1% gelatin. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means±SEM of three experiments. Total RNA was extracted from control, FN treated and siRNA treated MCF-7 cells ($1 \subseteq 10^6$ cells). 2 µl of cDNA from each sample was subjected to Real-Time quantitative RT-PCR as before using specific primer for FAK. (B) and MMP-2. (C) Relative levels of expression of FAK, MMP-2 and the control G3PDH in control, FN treated (20 µg/ml for 2 hours) and siRNA (control siRNA and FAK siRNA) treated MCF-7 cells as measured by quantitative real time RT-PCR by calculating the C_T value. The calibrator used in our experiments is the control untreated (-FN) MCF-7 cells and the samples are the FN treated (20 µg/ml, 2 hours) (+FN) and siRNA treated (+FN+ control siRNA or +FN+FAK siRNA) MCF-7 cells. In the given graph the CT value is inversely proportional to the mRNA expression of the samples. (D) Effect of blocking FAK signals on FN-induced expression levels of MMP-2, p-PI3K and p-ERK: MCF-7 cells were grown in SFCM for 24 hours, transfected with control siRNA (lane 3) and FAK siRNA (lane 2) (100 nM each) for 48hrs before treating the cells with 20 µg/ml FN coated for 2 hours. Control cells were untreated with FN (C). The cells were collected; extracted and equal protein (100 µg) was subjected to western blot analysis with anti-FAK (panel a), anti-MMP-2 (panel b), anti-p-ERK1/2 (panel c) and anti-p-PI3K antibody (panel d) antibody (1:1000 dilution for 1½ hrs at 37ºC). β-tubulin was used as internal control and done in parallel to the blots. The accompanying graphs represent the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means±SEM of three experiments. (E) Transwell Chamber Assay: MCF-7 cells untreated (-FN) and treated with fibronectin treated (+FN), treated with both fibronectin and FAK siRNA (+FN+FAK siRNA), treated with fibronectin and control siRNA (+FN+con siRNA) were seeded into transwell insert and were allowed to grow for 24 hrs. Figure shows MCF-7 cell migration through membrane after 24 hrs in FN-nontreated, FN-treated, FN+FAK siRNA and FN+con siRNA treated sets. The accompanying graph shows the number of control (-FN), fibronectin treated (+FN), both fibronectin and FAK siRNA (+FN+FAK siRNA) treated and both fibronectin and control siRNA (+FN+con siRNA) treated MCF-7 cells migrated through transwell insert were counted per high power field after 24hrs of cell seeded. Data are means±SEM of three experiments

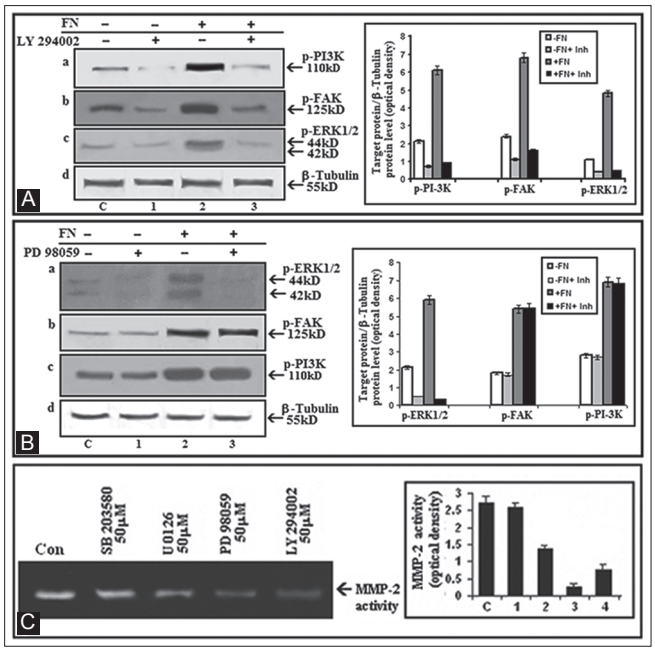

Figure 4: (A) Effect of blocking PI-3K signals on FN-induced expression levels of p-FAK and p-ERK1/2: MCF-7 cells (300,000 cells/ml) were grown in absence (lane C) and presence of PI-3K inhibitor (LY 294002) (lane 1) for 1 hour. MCF-7 cells (300,000 cells/ml) were grown in absence (lane 2) and presence of PI-3K inhibitor (LY 294002) (lane 3) for 1 hour then both the control and experimental sets were treated with fibronectin (20 μg/ml) for 2 hours in SFCM. The cells were collected; extracted and equal protein (100μg) was subjected to western blot analysis with anti-p-PI3K (panel a), anti-p-FAK (panel b), and anti-p-ERK1/2 (panel c) antibody (1:1000 dilution for 1½ hrs at 37 ºC). β-tubulin was used as internal control and done in parallel to the blots. The accompanying graphs represent the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means±SEM of three experiments. (B) Effect of blocking ERK signals on FN-induced expression levels of p-FAK and p-PI-3K: MCF-7 cells (300,000 cells/ml) were grown in absence (lane C) and presence of ERK inhibitor (PD 98059) (lane 1) for 1 hour. MCF-7 cells (300,000 cells/ml) were grown in absence (lane 2) and presence of ERK inhibitor (PD 98059) (lane 3) for 1 hour then both the control and experimental sets were treated with fibronectin (20 μg/ml) for 2 hours in SFCM. The cells were collected; extracted and equal protein (100μg) was subjected to western blot analysis with anti-p- ERK1/2 (panel a), anti-p-FAK (panel b), and anti-p- PI3K (panel c) antibody (1:1000 dilution for 1½ hrs at 37°C). β-tubulin was used as internal control and done in parallel to the blots. The accompanying graphs represent the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means±SEM of three experiments. (C) Identification of signaling molecules involved in constitutive MMP-2 secretion in MCF-7 cells- MCF-7 cells (300,000 cells/ml) were grown in absence (lane C) and presence of p38 inhibitor (SB 203580) (lane1), MEK inhibitor (U0126) (lane 2), ERK inhibitor (PD 98059) (lane 3) and PI-3K inhibitor (LY 294002) (lane 4) at a concentration of 50 μM for 1 hour in SFCM. The culture supernatants were collected and gelatin zymography was performed by using a 7.5% SDS-PAGE co-polymerized with 0.1% gelatin. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using Image J Launcher (version 1.4.3.67). Data are means±SEM of three experiments.

that the deregulation of FAK may have an active role in the progression of breast tumors.

We demonstrate that culture of MCF-7 cells on FN-coated surface (20 μg/ml) for 2 hours in serum starved condition induces phosphorylation of FAK (Y397) in a time dependant manner. Immunocytochemical analysis revealed that FN treatment causes a distinct localization of FAK at the cell contact sites. FN treatment causes FAK to appear as condensed foci at the cell membrane denoting the site of cell-cell, cell-ECM contact. The interaction of the cells with the adhesive glycoprotein fibronectin (FN) has become a paradigm of a specific cell-ECM recognition system mediated by cell surface receptors. We report that α5β1-FAK association in MCF-7 cells gets noticeably enhanced upon FN treatment. We also report that paxillin associated with α5β1-FAK and the level of association increases with increasing FN treatment.

The present study also reports that, exposure of MCF-7 cells to fibronectin causes phosphorylation of FAK and FAK-PI3K p85 subunit association. We also studied the effect of FN on PI3K phosphorylation. We found that FN treatment not only causes enhanced FAK-PI3K p85 subunit association but also causes an appreciable phosphorylation of PI3K itself. Another interesting observation was the association of FAK and ERK upon FN treatment in MCF-7 cells. We observed appreciable enhancement of the expression of FAK-associated 44kD and 42kD of ERK 1 and 2 when treated with FN. FN also caused enhanced phosphorylation of ERK 1/2 in MCF-7 cells. Earlier reports have demonstrated that ERK1/2 has very important role in breast cancer cell proliferation and invasion. Integrin-mediated adhesion to fibronectin leads to activation of ERK signaling cascade in different cell types.[15-18] So, our study confirms that integrin-mediated signaling cause FAK-mediated activation and association of ERK 1 and 2 in breast cancer cells, MCF-7.

We have found that abrogation of α5 integrin receptor prevents FN-induced phosphorylation of FAK (Y397). Blocking of α5 appreciably reduced the gelatinolytic activity of MMP-2 in the culture supernatant. Blocking of α5 also prevents FN-induced invasion of MCF-7 cells as observed by transwell chamber assay. These results suggest the importance of integrin receptor α5β1 in FN-mediated responses. FN by binding to α5β1 activates α5β1 to induce phosphorylation of FAK at Y397 that promote FAK-paxillin, FAK-PI3K p85 and FAK-ERK association. This brings the catalytic subunit of PI3K to the membrane, where it gets phosphorylated. ERK1/2 is phosphorylated either directly by FAK activation or via phosphorylation of PI3K. This triggering of downstream signaling cascade regulates the activity of MMP-2 and thus the invasive potential of the cells. It was reported that integrin signaling through FAK leads to the regulation of cell proliferation and invasion.[19]

We confirmed the importance of FAK in FN-induced MCF-7 cellular responses by using the technique of siRNA to downregulate FAK expression specifically. We found that FAK siRNA treatment prevents FN-induced MCF-7 cell invasion as seen by Transwell chamber assay, whereas non-specific siRNA had no effect. We also observed that FAK siRNA treatment abrogated the FN-induced phosphorylation

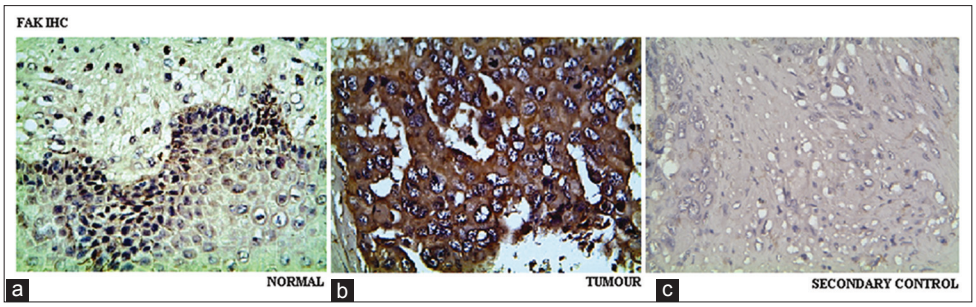

Figure 5: Representative photomicrographs of tissue sections immunostained for FAK. (A) FAK was detected in very low amount in the cytoplasm of normal breast tissue section. FAK overexpression was detected in primary breast cancer tissue section (B). This case was regarded as FAK-overexpression. High-power view of the immunohistochemistry FAK was detected in the cytoplasm of cancer cells (×400). Secondary control (C) (without FAK primary antibody) was done to exclude the possibility of non-specific expression

of PI3K and ERK1/2 establishing the requirement of FAK in FN-induced activation of these kinases.[17]

The present work demonstrated that FAK-mediated signaling is instrumental in breast cancer metastasis by regulation of MMP-2 expression and activity. MMP-2 has been reported to be of immense clinical significance in breast cancer.[18-21] MMP-2 is a marker for tumor progression in many types of cancer including breast cancer[22] and high level of MMP-2 expression correlate with poor prognosis. FN treatment induces the gelatinolytic activity and expression of MMP-2 in MCF-7 cells. Earlier report from the same lab has shown that FN treatment aids rapid expression of MMP-2 in MCF-7 cells.[23,24] In the present study FN treatment enhanced MMP-2 protein levels in both the lysate of MCF-7 cells and also in the culture media. We also show that FN treatment upregulated the mRNA expression of MMP-2 and MT1-MMP and downregulated the mRNA expression of TIMP-2. FN also appreciably increased the protein expression level of MT1-MMP in MCF-7 cells. MT1-MMP has been shown to be a potent activator of pro-MMP-2 by cleaving the propeptide, a process that also promotes gelatinase-A autoproteolytic activation.[24-26] Earlier reports have shown the involvement of FAK in FN-induced upregulation of MMP-2 by modulating MT1-MMP and TIMP-2 in other cell systems.[24,27] Treatment of MCF-7 cells with FAK siRNA appreciably blocked the FN-induced protein expression of MMP-2. FAK siRNA also blocked the FN-induced mRNA level of MMP-2 in MCF-7 cells. Treatment of non-specific siRNA however did not show any effect. Thus, FAK activation mat be crucial for FN-mediated upregulation of MMP-2 expression. Our finding is in agreement with a number of earlier evidences suggesting the role of FAK in MMP-2 secretion and activation in other cancer models.[13,14,28,29]

The specific inhibitors LY294002 and PD98059 prevent FN-induced MMP-2 secretion demonstrating the role of PI3K and ERK1/2 in FN-mediated MCF-7 responses and MMP-2 regulation. FAK siRNA inhibited accumulation of MMP-2 in the lysate in FN-treated MCF-7 cells, whereas non-specific siRNA had no effect. Thus, our results show the requirement of not only FAK but also PI3K and ERK 1/2 in FN-induced MMP-2 activity. This is consistent with earlier reports which suggest the pivotal role of PI3K and ERK in MMP-2 function.[27,30] Interestingly ERK 1/2 has a very important role in regulating transcriptional activation of NFκB and AP-1. So, the transactivation of these transcription factors might activate MMP-2 gene expression.

We have further studied the role of PI3K and ERK on the phosphorylation of FAK itself. Treatment of MCF-7 cells with specific chemical inhibitor, LY294002, noticeably down regulated the phosphorylation of FAK Y397 and also phosphorylation of ERK1/2. Treatment of PD 98059 however failed to cause any appreciable effect on the phosphorylation of FAK or PI3K. Our results thus suggest the requirement of PI3K in FAK phosphorylation and thus FAK-promoted responses in MCF-7 cells. This result confirms earlier findings which report the necessity of PI3K in FAK-related responses,[17-19,31] and shows that the treatment of PI3K inhibitor causes downregulation of FAK phosphorylation at Y397.[32] The present results strongly suggest that PI3K binding is required for FAK to promote its responses and that PI3K plays an important role in the activation of FAK itself. We therefore propose a model for the role of FAK and related signaling molecules in FN-mediated regulation of MMP-2 in MCF-7 cells.

The present study suggests that FN by binding to $\alpha 5\beta 1$ induces phosphorylation of FAK (Y397), PI3K, ERK 1/2, and also causes their association in MCF-7 cells. Treatment of FN caused an appreciable upregulation of the invasive property of MCF-7 cells which gets greatly diminished upon blockage of either FAK or $\alpha 5$ integrin receptor. The study also shows the requirement of FAK, PI3K and ERK1/2 in FN-induced upregulation of MMP-2. Moreover, FAK pY397 is essential for the association of FAK-PI3K p85 subunit[5] and FAK-ERK 1/2. We also report that activation of PI3K is needed for FAK-related responses.[17-19] Inhibition of PI3K decreased both FAK autophosphorylation and MMP-2 secretion. So, FAK and PI3K are essential components of integrin mediated

MMP-2 upregulation. Inhibition of ERK though caused an inhibition of the secreted MMP-2 but did not have any effect on FAK phosphorylation itself suggesting its role as the downstream effecter of FAK in induction of MMP-2. Our results report that FN-mediated FAK phosphorylation leads to upregulation of MMP-2 and MT1-MMP. Abrogation of FAK causes an inhibition of both FN-induced MMP-2 protein and mRNA level. We also demonstrate the pivotal role of PI3K and ERK in FN-induced MMP-2 activity.

CONCLUSION

The present communication demonstrates the role of FAK in invasion of breast cancer cells. Our results suggest that the interaction of $\alpha 5\beta 1$ integrin with FN initiates a signaling cascade with FAK as its central player. FAK gets phosphorylated and in turn associates with other signaling kinases like PI3K and ERK. FAK also activates downstream kinases that serve as very crucial steps of the intricate signaling pathway leading to MMP-2 induction and resulting invasion of breast cancer cell-line, MCF-7. This study elucidates an important signaling cascade in breast cancer cells. Considering the importance of MMP-2 in breast cancer metastasis, elucidation of a novel activation mechanism can be an important future therapeutic target.

MATERIALS AND METHODS

Materials

Minimal Essential Medium (MEM), fetal bovine serum (FBS), fibronectin (440kDa), Protease Inhibitor Cocktail Tablets (complete, mini, EDTA-free), Protein G agarose were purchased from Roche, Germany. Gelatin Sepharose 4B beads was purchased from GE Healthcare Bio-Sciences AB, Uppsala, Sweden. All primary antibodies (monoclonal and polyclonal), secondary antibodies (FITC, HRP coupled and biotinylated), FAK siRNA and negative control siRNA were purchased from Santa Cruz Biotechnologies, Santa Cruz, CA. USA. SYBR Green JumpStart™ Taq Readymix™ was purchased from Sigma-Aldrich, St. Louis, MO, USA. Primers (MMP-2, FAK, TIMP-2, MT1MMP, and GAPDH) were synthesized by Operon, Germany. RNAqueous 4 PCR (Total RNA isolation kit) and Retroscript (RT-PCR Kit) were purchased from Ambion, Austin, TX, USA. ERK inhibitor (PD 98059), PI-3K inhibitor (LY 294002), p38 inhibitor (SB 203580), MEK inhibitor (U0126) were purchased from Promega, Madison, WI. Lipofectamine™ 2000 was purchased from Invitrogen, Life Technologies (USA). Vectastain ABC Elite kit was purchased from Vector Laboratories Inc., Burlingame, CA, USA. SuperSignal West Pico Chemiluminescent Substrate kit and DAB substrate was purchased from Pierce, Thermo Fisher Scientific Inc. Rockford, USA). Costar Transwell Plate was purchased from Corning Incorporated, Corning, New York, USA.

Methods

Cell culture

MCF-7 (human breast adenocarcinoma cell line) was obtained from National Centre for Cell Sciences (NCCS), Pune, India. The cells were grown and maintained in MEM containing 10% FBS in a 5% CO_2 incubator at 37°C. All experiments with MCF-7 were performed at early passages of the cell line (8-12 passages).

Gelatin Zymography

MCF-7 cells (300,000 cells/ml) were grown in absence and presence of FAK siRNA in required concentrations and time periods in serum free culture medium (SFCM). MCF-7 cells (300,000 cells/ml) were grown in presence of FN (20 μg/ml) for 2 hours and then were kept with or without $\alpha 5$ antibody for an additional 1 hour. Gelatin zymography was performed by using a 7.5% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) co-polymerized with 0.1% gelatin as previously described.[24]

Inhibitor Assay

MCF-7 cells (300,000 cells/ml) were grown in absence (control) and presence of ERK inhibitor (PD 98059) (50 μM), PI-3K inhibitor (LY 294002) (20 μM), p38 inhibitor (SB 203580) (10 μM), MEK inhibitor (U0126) (20 μM) for 1 hour in SFCM. The Gelatin zymography was performed by using a 7.5% SDS-PAGE co-polymerized with 0.1% gelatin as previously described.[24] The cells were taken and immunoblot was performed with different antibodies as described below.

Immunocytochemistry

MCF-7 (300,000 cells/ml) cells were allowed to grow on coverslips coated with fibronectin (20 μg/ml SFCM) for 2 hrs and without fibronectin (control) at 37°C in a CO_2 incubator. The coverslips were then washed in PBS, fixed with 3.5% formaldehyde, treated with 0.5% Triton- X100 and nonspecific sites were blocked with 1% BSA. The cells were then incubated with anti-FAK primary antibody (1:1000 dilution) for overnight at 4°C followed by wash and incubation with FITC-coupled second antibody (1:1000 dilution) at 37°C for 1.5 h in a humidified chamber. After washing with PBS, the coverslips were mounted with glycerol on glass slides and observed under a fluorescence microscope (Leica).

Quantitative Real-Time RT-PCR

RNA was extracted from 1x 10^6 cells/ml MCF-7 cells either grown in absence and in presence of fibronectin (20 μg/ml) for 1 hour and 2 hours; or grown in absence or presence of FAK siRNA and con siRNA prior to treatment with fibronectin (20 μg/ml) for 2 hours. Cells were washed in PBS and total RNA was extracted (RNaqeous, Ambion, USA) as previously discussed.[24] Real-Time quantitative

RT-PCR using relative quantitation by the comparative C_T method was used to determine mRNA expression. 2 μl of cDNA was subjected to Real-Time quantitative RT-PCR using SYBR Green as a fluorescent reporter. The specific gene primers (MMP-9, FAK, MT1MMP, TIMP-2 and the internal control gene G3PDH) were amplified in separate reaction tubes. Threshold cycle number (C_T), of triplicate reactions, was determined using the ABI-7500 (ABI-7500, Foster City, CA USA) software and the mean C_T of triplicate reactions was determined using the protocol previously described.[24] The calibrator used in our experiments is the control MCF-7 cells and the samples are fibronectin treated (20 μg/ml, 2 hours) MCF-7 cells. The ΔC_T value is inversely proportional to the mRNA expression of the samples. No primer dimers were obtained for either the target genes or G3PDH as assessed by melt curve analysis. The specificity of the products was also confirmed by melt curve analysis. The reaction conditions and the primer sequences are given below. The PCR cycles in all cases were started with Taq activation at 94°C for 5 mins and followed by final extension of 72ºC for 7 mins.

Primer sequences, PCR cycles and conditions: hFAK:5′-CGCTGGCTGGAAAAAGAGGAA-3′ (forward), 5′-TCGGTGGGTGCTGGCTGGTAGG-3′ (94°C-30secs, 60°C-30 secs, 72°C-90 secs); hMMP-2: 5′ - GTATTTGATGGCATCGCTCA - 3′ (forward), 5′ - CATTCCCTGCAA AGAACACA - 3′ (reverse) (94°C- 30 secs, 56°C-90 secs, 72°C-30 secs); hMT1-MMP: 5′ –CCCTATGCCTACATCCGTGA– 3′ (forward), 5′ –TCCATCCATCACTTGGTTAT – 3′ (reverse) (94ºC- 30 secs, 56°C-90 secs, 72°C-30 secs); hTIMP-2: 5′ –GTTTTGCAATGCA GATGTAG-3′ (forward), 5′-ATGTGGAGAAACTCCTGCTT-3′ (reverse), (94°C- 30 secs, 56°C -90 secs, 58°C - 30 secs).

Immunoprecipitation

MCF-7 (300,000 cells/ml) cells were allowed to grow without or with fibronectin (20 μg/ml) for 2 hrs in SFCM. For immunoprecipitation cells were lysed with NP40 buffer (50 mM Tris, 150 mM NaCl, 1% NP40, pH 8, Protease inhibitor cocktail tablets from Roche, 1 mM sodium Orthovanadate and 1nM Sodium fluoride). Preclearing of lysate was done with Protein G agarose and anti- human IgG at 4°C using for 1 hr, followed by protein estimation by Lowry method. Immunoprecipitation was performed form 100 μg of protein with 1μg of anti-integrin α5 (H-104) sc10729 or anti FAK (A-17): sc 557 antibody (Santa Cruz Biotechnology, USA) followed by precipitation with Protein-G-Agarose (Roche) for 2 hr at 4°C, then the beads were washed with NP40 buffer (without Protease inhibitor cocktail, sodium orthovanadate and sodium fluoride). The samples were prepared for SDS-PAGE with the addition of Laemmli buffer and elution at 100°C for 4 min. Western blotting was performed as described below.

Western blot

MCF-7 (300,000 cells/ml) cells were grown in SFCM with treatment as required in the experimental condition. Then the cells were washed with ice cold PBS and were scraped into lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40, 0.1 % SDS, 0.5% Deoxycholate, Protease inhibitor cocktail tablets from Roche, 1 mM sodium orthovanadate and 1 nM Sodium fluoride) on ice and clarified by centrifugation. Protein concentrations were determined using a Lowry method. The samples were then subjected to electrophoresis on SDS-PAGE and the proteins were transferred onto PVDF membrane (Millipore) by Western blot. Nonspecific binding sites on the membrane were preblocked in 4% BSA. Blots were incubated with anti-FAK, anti-pFAK (Tyr397), anti-PI3K, anti-pPI3K, anti-ERK, anti-pERK, anti-MMP-2 and anti- paxillin (Santa Cruz) antibodies. Following incubation in horseradish peroxidase (HRP)-conjugated secondary, detection was performed with the Super Signal West Pico Chemiluminescent Substrate kit (Pierce, USA) following the manufacturer's protocol. All blots were reprobed with anti β-tubulin antibody as internal control.

Focal Adhesion Kinase Small Interfering RNA (siRNA) Treatment

Human breast cancer cell line MCF-7 were seeded in 35 mm dishes and grown to 50% confluence. For the transfection process, FAK siRNA and negative control siRNA were transfected using Lipofectamine™ 2000 following the manufacturer's protocol. The transfection agent (Lipofectamine 2000) was incubated with serum-free culture medium for 10mins at room temperature. Subsequently, respective siRNA (FAK siRNA (h), sense- GCAUGUGGCCUGCUAUGG; antisense- CCAUAGCAGGCCACAUGC) mixed with serum-free culture medium was added to it and incubated at room temperature for additional 15 mins. The mixtures were then diluted in serum-free culture medium and added to each dish so that the final concentration of the siRNA in each plate was 100 nM. The mixture was overlaid on the cells in full media without serum and without antibiotic and incubated at 37°C in the presence of 5% CO_2. After 24 h, the transfection mixture was replaced with fresh media supplemented with 10% FBS and antibiotic and the transfected cells allowed to grow for another 48 h. Cells were exposed to FN for 2 hours and later collected for western blot, gelatin zymography and real-time RT-PCR assays. The cells after FN treatment were also collected for transwell chamber assay.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tumor sections were obtained from participating hospitals' pathology. The experiments were undertaken with the understanding and

written consent of the subject. The study methodologies conformed to the standards set by the Declaration of Helsinki and the study was approved by the local ethics committee. Immunohistochemical staining was performed by the standard streptavidin–biotin (SAB) method. Briefly, each 4-μm tissue section was brought to room temperature, deparaffinised with xylene, and then re-hydrated through graded ethanol series. After re-hydration, sections were subjected to antigen retrieval by 10mM citrate buffer (pH 6.0) with 0.05% Triton X100 at boiling water for 10min. Then after bringing the sections at room temperature those were incubated in fresh 0.3% H_2O_2 in methanol for 30 min at room temperature. Nonspecific binding sites were preblocked in 2% BSA. The sections were incubated at 4°C overnight with anti-FAK antibody at a dilution of 1:500 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. The sections were then washed in PBS and incubated with biotinylated secondary antibody for 1hr at room temperature. Further processing was carried out using the Vectastain ABC Elite kit (Vector Laboratories Inc., Burlingame, CA, USA) according to the manufacturer's protocol, followed by staining with DAB substrate (Pierce, Thermo Fisher Scientific Inc. Rockford, USA).

Transwell migration assay

24 well transwell plate (Corning) with 12 inserts were taken and the lower chamber of each well was poured with 600 μl MEM SFCM. Control and experimental MCF-7 cells (100,000 cells/insert) were seeded in triplicate on membrane in the upper chamber of the insert. Cells were then allowed to grow for 24 hrs. After 24 hrs of incubation, media was pipette out from membrane. SFCMs from lower chambers were collected and centrifuged at 3000 rpm for 3 min. The membranes of the inserts were washed thrice with PBS. Cells were then fixed with 4% formaldehyde solution, followed by washing with PBS. Cells were then stained with Gill's hematoxylin for 10 min. Membranes were then washed thoroughly in running water. The upper side of the membranes was scraped with buds; membranes were then cut and mounted with glycerol. The cells migrated through the membrane pore were observed under microscope.

Quantification of the results

Bands of zymography, western blots and RT-PCR were quantitated using Image J Launcher (version 1.4.3.67).

Statistical analysis

All experiments were repeated at least three times. All data collected from gelatin zymography, western blot, real-time RT-PCR were expressed as mean ± S.D. The data presented in some figures are from a representative experiment, which was qualitatively similar in the replicate sets. Statistical significance was determined by Student's t test (two-tailed) comparison between two groups of data sets. $P < 0.05$ was considered significant.

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Abbreviations

AP-1- Activator protein 1, DAB- 3,3'-Diaminobenzidine, ECM- extracellular matrix, ERK- Extracellular regulated kinase, FAK- Focal Adhesion Kinase, FBS – fetal bovine serum, FITC – fluorecin isothiocyanate, FN-fibronectin, GAPDH– Glyceraldehyde phosphate dehydrogenase, HRP- horseradish peroxidase, MEK- Mitogen activated protein kinase, MMP- matrix metalloproteinase, MT1-MMP- Membrane type 1 metalloprotease, NF-κB -nuclear factor-kappa, PI-3K- Phosphatidyl inositol 3 kinase, SFCM – serum free culture media, siRNA- small interfering RNA, TIMP: Tissue inhibitor of metalloproteinases, Tyr- Tyrosine

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