



Role of ERK in Modulating MMP 2 and MMP 9 with Respect to Tumour Invasiveness in Human Cancer Cell Line MCF-7 and MDA-MB-231

Shuvojit Moulik, Sekhar Pal, Jaydip Biswas, Amitava Chatterjee

Shuvojit Moulik, Sekhar Pal, Amitava Chatterjee, Department of Receptor Biology & Tumor Metastasis, Chittaranjan National Cancer Institute, 37, S.P Mukherjee Road, Kolkata: 700026, India

Jaydip Biswas, Department of Surgery, Chittaranjan National Cancer Institute, 37, S.P Mukherjee Road, Kolkata: 700026, India

Correspondence to: Amitava Chatterjee, Department of Receptor Biology & Tumor Metastasis, Chittaranjan National Cancer Institute, 37, S.P Mukherjee Road, Kolkata: 700026, India

Email: amitavachatterjee24@gmail.com

Telephone: +91-9830128320

Received: September 26, 2013 Revised: November 4, 2013

Accepted: November 11, 2013

Published online: February 18, 2014

Moulik S, Pal S, Biswas J, Chatterjee A. Role of ERK in Modulating MMP 2 and MMP 9 with Respect to Tumour Invasiveness in Human Cancer Cell Line MCF-7 and MDA-MB-231. *Journal of Tumor* 2014; 2(2): 87-98 Available from: URL: <http://www.ghrnet.org/index.php/JT/article/view/602>

INTRODUCTION

Among the signaling pathways which integrates external clues in Cancer, Mitogen-activated protein kinase (MAPK) cascade is a key intracellular signaling pathway that regulates diverse cellular functions including cell proliferation, cell cycle regulation, cell survival, angiogenesis and cell migration^[1,2]. Disruption of the integrity of the basement membrane is a key histological marker of tumor's transition to invasive carcinoma. The basement membrane forms a cellular support for tumors and is made up of a complex mix of Extracellular matrix (ECM) proteins, including laminin, collagens and proteoglycans. MAPKs have been shown to be involved in regulating the proteolytic enzymes that degrade the basement membrane. In metastatic cells ERK activity was shown to be higher than non-metastatic cancer cells. In response to external stimuli phosphorylated ERK translocates from cytoplasm to the nucleus and in the nucleus ERK activates a host of transcriptional factors including activating protein-1 (AP-1), as a result of the induction of the c-fos gene, via phosphorylation of the ELK-1 ternary complex factor by phospho-ERK1/2. In tumors sustained MAPK activation could lead to enhance induction of proteolytic enzymes in the surrounding environment, leading to destruction of ECM, a key histological marker of invasive carcinoma. Elevated expression of MMP9 is associated with increased metastatic potential in many cancer types including breast, melanoma, prostate and brain cancers. Matrix metalloproteinase-9 (MMP-9) represents the largest and most complex member among the MMPs. MMP-9 has a gelatin binding fibronectin domain, composed of three fibronectin repeats, inserted between the active site domain and Zn²⁺ binding domain. Among the known substrates for MMP-9 are gelatin, elastin, fibronectin, proteoglycans, laminin and type IV, V, VII & X collagens. On the other hand the major substrate for MMP2, is type IV collagen. It has also been implicated in the degradation of type V, VII and X collagens, gelatins, elastin and fibronectin. Beside degradation of ECM, tumor cell invasion and migration rely on the

ABSTRACT

Extracellular signal regulated kinase (ERK) is a key molecule located in the Mitogen activated protein kinase (MAPK) pathway responsible for several cell process in cancer including cell invasiveness which primarily involved breakdown of extracellular matrix. Matrix metalloproteinase facilitates cell invasiveness by degrading Gelatinase which are again differentially regulated by Integrins, a family of receptors. Our study analyses the role of ERK in modulating cell invasiveness. Aims: the role of ERK1/2 in modulating MMP2 and MMP9 and thereby cell migration and invasion. Materials & Methods: The study involves downregulation of ERK through potent inhibitors and ERK1/2 siRNA and its effect on MMP2 and MMP9 and Integrin alpha 5 and beta 1 molecules. Western Blot, Gelatin Zymography, Cell invasion and Cell Migration assays are the primarily used to interpret the effect. Result: There was a considerable change in MMP 9 and MMP 2 after ERK inhibition which was also reflected in the downstream process of the ERK pathway and Integrins alpha 5 and Beta 1. Conclusion: There might be a direct correlation of ERK activity and MMP2 and MMP 9 activity with targeted modulation of ERK. Specific inhibition of ERK also might lead to integrin regulation.

© 2014 ACT. All rights reserved.

Key words: ERK1/2; MMP9; MMP 2; cell invasion; MAPK pathway

receptor dependent attachment and release from the matrix and other cells. MMP activity directly modulates the cell-cell and cell matrix attachment and regulates the process of cell invasion and migration. Integrins play central role in anchorage dependent growth, apoptosis, differentiation, and migration. Integrins differentially regulate the production of MMPs. In human melanoma cell lines, $\alpha 5\beta 1$ integrin and $\alpha V\beta 3$ integrin modulates release of MMP-2 and subsequent invasive behaviour. There is evidence that the sustained activation of the Ras/Raf/MEK/ERK pathway is involved in the expression of $\beta 3$ integrins^[3,4]. Recent studies have shown that significantly increased active ERK was found in primary breast tumors compared with their adjacent matched normal breast tissues and this increase ERK activity correlates with enhanced nodal metastasis. Hence in this study the molecular mechanism of regulation and deregulation of ERK was analysed with respect to MMP 2 and MMP 9 as a factor for tumor invasiveness which is an essential requirement considering the complexity lying beneath^[5]. The study necessarily try to illustrate that if ERK gets modulated the activity of MMP 2 and MMP 9 gets effected directly. The work is conducted over a series of experiments where Fibronectin induced human breast cell lines MCF-7 and MDA-MB-231 is used as a model. If the results are properly transcribed there is huge significance with respect to the molecules involved and the probable direct co-relation between ERK and MMP2/9 can be used for therapeutic prognosis of breast cancer.

MATERIALS AND METHODS

Minimal Essential Medium (MEM), fetal bovine serum (FBS), fibronectin (440 kDa), 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. West Femto substrate for Western Blot was acquired from Thermo scientific Pierce, Rockford, IL USA. All primary antibodies (monoclonal and polyclonal), secondary antibodies, ERK 1 and ERK2 siRNA and negative (scramble) control siRNA were purchased from Santa Cruz Biotechnologies, Santa Cruz, CA, USA. ERK inhibitor (PD98059), MEK inhibitor (U0126) were purchased from Promega, Madison, WI, USA. Lipofectamine 2000 was purchased from Invitrogen, Life Technologies (USA). Costar Transwell Plate was purchased from Corning Incorporated, Corning, New York, USA. Human breast cancer cell lines MCF-7, MDA-MB-231, were obtained from NCCS, Pune.

Cell culture

Cells were grown and maintained in required medium (MEM, DMEM and RPMI) containing 10% FBS in a CO₂ incubator at 37°C. For all the experiments cells were grown as a monolayer on culture plates in absence and in presence and absence of fibronectin (20 μ g/ml) for 2 hours (for MCF-7) and 8 hours (for MDA-MB-231).

Cell Extraction

Cells were initially grown in required medium supplemented with 10% FBS. The cells were collected from culture flasks by trypsinisation and homogenized in Cell Extraction Buffer (Tris-37.5 mM, NaCl-75mM, Triton-X-0.5% and pH adjusted to 7.5) at 15 minutes of intervals for 1-1.5 hours at 4°C. It will then be centrifuged at 15,000 r.p.m for 10 mins at 4°C. The supernatant was collected and the pellet was discarded and the protein estimated by Lowry's Method. (It can also be stored at -86°C till use).

Zymography:

Cells were initially grown in required medium supplemented with

10% FBS in petridishes, treated for the stipulated time period in SERUM FREE CULTURE MEDIUM (SFCM) for 24hours and the respective SFCMs was collected. The culture supernatant was collected by centrifugation at 3000 r.p.m for 5 minutes. The matrix metalloproteinase was separated from SFCM using Gelatin Sepharose 4B beads (75 μ L) for overnight at 4°C. The beads was washed x3 with Tris-buffered saline with Tween-20 (TBST) and suspended in 50 μ L of 1X sample buffer (0.075 gm Tris, 0.2 gm SDS in 10 ml water, pH 6.8). The suspension was incubated for 30 minutes at 37°C and then centrifuged at 3000 r.p.m. for 3 minutes. The supernatant was then subjected to zymography on 7.5% SDS-PAGE co-polymerized with 0.1% gelatin. Gel was washed in 2.5% Triton-X-100 for 30 minutes to remove SDS and was then incubated overnight in reaction buffer (50 mM Tris-HCl pH 7, 4.5 mM CaCl₂, 0.2 M NaCl). After incubation, the gel was stained with 0.5% Coomassie Blue in 30% methanol and 10% glacial acetic acid. The bands was visualized by distaining the gel with water.

Western Blot Assay

Cells were initially grown in required medium supplemented with 10 % FBS in petridishes. The respective cells were collected. Cell extraction was carried out using the standard extraction protocol and the protein content of the extracts was estimated by Lowry's method. Equal amount of protein was taken and after boiling with 0.1 volumes b-mercaptoethanol for 5-8 minutes at 80-90°C is finally subjected to electrophoresis on 10 % SDS-PAGE. The proteins was transferred on to PVDF membranes by Western Blot at 300 mA for 3 hours. The membranes was blocked with 1% BSA and subsequently washed x3 with TBST. The immunoblots was reacted with required primary antibody. The blots was developed using respective alkaline phosphatase coupled second antibodies. The color was developed using ECL as substrate.

Cell Invasion assay

24 well transwell plate (Corning) with 12 inserts were taken and the lower chamber of each well was poured with 600 mL MEM SFCM. Control and fibronectin treated 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 and 48 h. After 24 and 48 h of incubation, media was pipetted 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.

Wound Healing Assay

Cells were grown as a monolayer on culture plates in absence and in presence and absence of fibronectin (20 μ g/mL). The monolayer was scratched with a sterile pipette tip, followed by washing with SFCM to remove cellular debris. Cell migration across the wound was observed by microscopy and documented by photography at 0 hr, 6 hrs and 24 hrs, 48 hrs.

siRNA transfection

Single transfection procedure. Cells were plates 24 hrs prior to transfection. The cells were incubated at 37°C for 24 hrs under 5%



CO₂. After incubation, the plate was washed with PBS (once), and layered with 1.75 mL OptiMEM. The following mix was prepared and incubated at room temperature for 5 minutes.

Tube 1: siRNA: 100 nM (Stock conc: is 100 μ M, siRNA: 2 μ L); OptiMEM: 198 μ L

Tube 2: Oligofectamine: 4 μ L; OptiMEM : 46 μ L

The content of both the tubes were mixed and incubated for further 30 minutes. siRNA-lipid mix on the cells (dropwise with constant swirling) was layered. The plate was incubated at 37°C for 4 hrs. Fresh media was added after 4 hours incubation and incubate further 24 hrs. Cells were analyzed depending upon the experiment.

Double transfection (combinatorial transfection) procedure

For experiment with two round of siRNA transfection, second round of transfection was done in the similar manner as described above after 24 hours of first round of Transfection. Scramble control for siRNA was similarly prepared.

RESULTS

Effect of fibronectin on activity and expression of MMP2, MMP-9 and ERK1/2

Figure 1 shows the effect of Fibronectin on activity and expression of MMP2 and MMP9 and also depicts the expression of pERK1/2 and ERK1/2. There was appreciable increase in the activity of MMP2 as well as MMP9 with the introduction of 20 μ g Fibronectin in MCF-7 cells (Figure 1A). Activity of MMP9 also got increased in MDA-MB-231 cells. The total protein levels of MMP9/MMP2 didn't change at all in both the cell lines. An appreciable increase of pERK1/2 was noted and both the cell lines, though the increase was higher in MDA-MB-231 cells with respect to MCF-7 cells. The total (dephospho) protein levels of ERK1/2 in both the cell lines had hardly any effect of Fibronectin treatment. This activation corresponded with the activity of MMP2 and MMP9 in both the cell lines and hence showed an indication of a possible correlation between the two events. The internal control Beta tubulin didn't show any change. (we did this experiment to study possible interrelationship of ERK 1/2 and MMP-2/9 activity).

Effect of MEK/ERK inhibitor PD98059 on activity and expression of MMP 2/MMP9 and ERK1/2

Figure 2 depicts the effect of MEK/ERK inhibitor PD98059 on

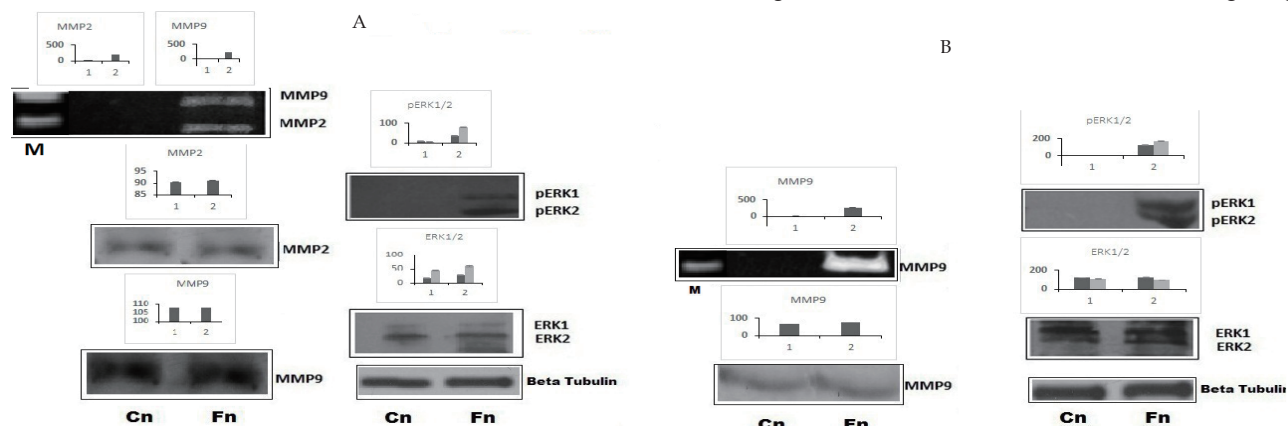


Figure 1 (A: MCF 7, B: MDA231). Effect of fibronectin on activity and expression of MMP2, MMP-9 and ERK^{1/2}. MCF7 and MDAMB231 cells (300,000 cells/mL) were grown in serum free culture medium in absence (control) and in presence of 20 μ g Fibronectin (MCF7: 2hrs, MDAMB231: 8hrs). The respective serum free medium was subjected to Gelatin Zymography and cell extract was subjected to Western Blot by standard protocol. The immunoblots were probed for anti-ERK^{1/2} and anti-MMP2/MMP9 antibody and developed using respective horse redox peroxidase (HRP) coupled second antibodies. Beta tubulin was used as internal control for all the western blots. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using image J Launcher (version 1.4.3.67) and arranged in the similar order as of the lanes. Data are means \pm SEM of three experiments. M represents marker lane.

Moulik S *et al.* Role of ERK in modulating MMP2 and MMP9

activity and expression of MMP2 and MMP9 as well as ERK1/2. There was a sharp decrease of both MMP2 and MMP9 in Zymogram of MCF-7 cells. This also corresponded with the inhibition of pERK1/2 levels in MCF-7 cells. In MDA-MB-231 cells PD98059 effectively inhibited phosphorylation of ERK1/2. There was also a decrease in activity of MMP9 in MDA-MB-231 cells. The levels of total protein of MMP9 and MMP2 in both the cell lines didn't show any change with the introduction of the inhibitor. Dephospho form of ERK1/2 was almost unaffected by inhibitor treatment. (we did this experiment to see the inhibition of ERK 1/2 on MMP-2/9 activity).

Effect of MEK/ERK inhibitor U0126 on activity and expression of MMP 2/MMP9 and ERK1/2

Figure 3 portrays the expression and activity of ERK1/2 in relation to MMP2 and MMP9 after treatment with inhibitor U0126. There was noticeable decrease of pERK1 with the treatment of the inhibitor, though the dephospho protein didn't show any visible change (Figure 3A). Both MMP2 and MMP9 showed reduction in their activity in degrading Gelatin. In MDA-MB-231 cells there was a sharp decrease of pERK1/2 levels which corresponded to the appreciable decrease of MMP9 activity. The total protein levels of MMP2 and MMP9 didn't change (we did this study to see the effect of ERK 1/2 inhibition on MMP-2/9 activity).

Effect of ERK1/2 siRNA on activity of MMP 2/MMP9 and activity and expression of ERK1/2

Figure 4 demonstrates the effect of specific inhibition of ERK1/2 by sequential double transfection of ERK1 and ERK2 siRNA on activity of MMP2 and MMP9. In MCF-7 cells there was an 80% (approx) decrease in the phosphorylation levels of ERK1/2 along with the sharp decrease of the dephospho levels. The MMP9 and MMP2 activity levels showed a sharp decrease after siRNA treatment. In MDA-MB-231 cells along with the decrease of ERK1 levels, the activity of MMP9 also reduced to basal levels. Thus it might be said that in both the cell lines there was a possible correlation between ERK and MMP9 and MMP2. The scramble control didn't show any change throughout. (we did this experiment to study specific ERK 1 or ERK 2 on MMP-2/9 activity).

Effect of ERK1/2 inhibition on activity and expression of JAK2, STAT3 and PI-3K

To investigate the effect of ERK1/2 inhibition on other signaling

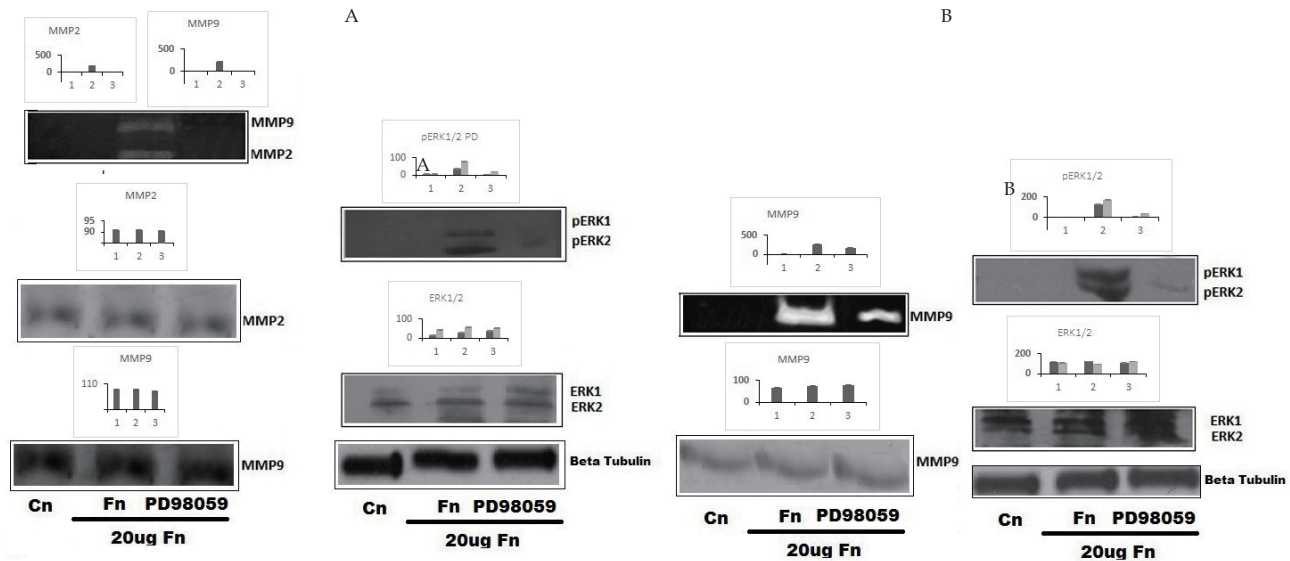


Figure 2 (A: MCF 7, B: MDA231). Effect of MEK/ERK inhibitor PD98059 on activity and expression of MMP 2/MMP9 and ERK1/2. MCF7 and MDAMB231 cells (300,000 cells/ml) were grown in serum free culture medium in absence (control) and in presence of 20ug Fibronectin (MCF7: 2hrs, MDAMB231: 8hrs). The cells were pre-treated with inhibitor PD98059 for 1 hour. The respective serum free medium was subjected to Gelatin Zymography and cell extract was subjected to Western Blot by standard protocol. The immunoblots were probed for anti-ERK1/2 and anti-MMP2/MMP9 antibody and developed using respective horse redox peroxidase (HRP) coupled second antibodies. Beta tubulin was used as internal control for all the western blots. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using image J Launcher (version 1.4.3.67) and arranged in the similar order as of the lanes. Data are means \pm SEM of three experiments.

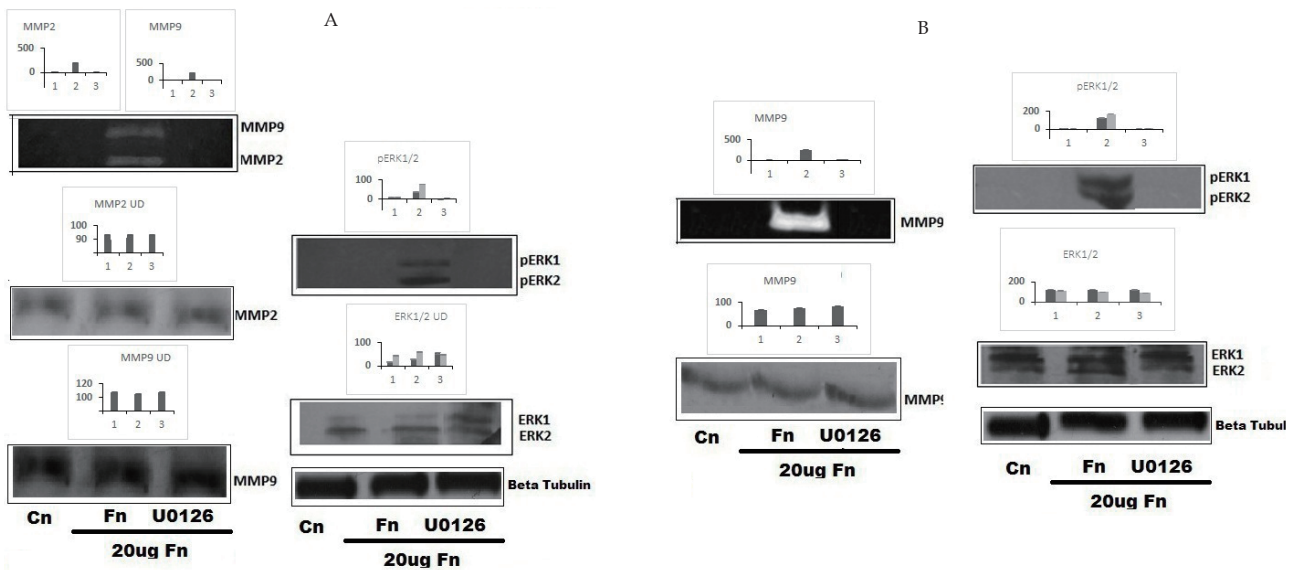


Figure 3 (A: MCF 7, B: MDA231). Effect of MEK/ERK inhibitor U0126 on activity and expression of MMP 2/MMP9 and ERK1/2. MCF7 and MDAMB231 cells (300,000 cells/ml) were grown in serum free culture medium in absence (control) and in presence of 20ug Fibronectin (MCF7: 2hrs, MDAMB231: 8hrs). The cells were pre-treated with inhibitor U0126 for 1 hour. The respective serum free medium was subjected to Gelatin Zymography and cell extract was subjected to Western Blot by standard protocol. The immunoblots were probed for anti-ERK1/2 and anti-MMP2/MMP9 antibody and developed using respective horse redox peroxidase (HRP) coupled second antibodies. Beta tubulin was used as internal control for all the western blots. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using image J Launcher (version 1.4.3.67) and arranged in the similar order as of the lanes. Data are means \pm SEM of three experiments.

molecules the levels of (Janus kinase) JAK2, (Signal transducer and activator of transcription) STAT3 and (Phosphoinositide-3 kinase) PI3K were checked in Figure 5. In MCF-7 cells there was no visible effect of ERK inhibition by inhibitor or siRNA on activity or expression of JAK2 (Figure 5A). The results were also similar in MDA-MB-231 cells. The expression of pSTAT3 in MCF-7 cells showed a decrease when PD98059 was introduced, after a sharp increase in Fibronectin (Fn) treated cells. With U0126 treatment this decrease was similar which further went down significantly with siRNA treatment. In MDA-MB-231 cells the results were

similar with a sharp escalation of STAT3 activity in Fn lane, which went down when MEK/ERK inhibitors were introduced. With the introduction of ERK1/2 siRNA, the pSTAT3 levels went further down suggesting a role of activated STAT3 in this pathway. The expression of STAT3 however remain unchanged in both the cell lines. The phospho PI3K slightly increased with Fn treatment but decreased with administration of PD98059. When MEK/ERK inhibitor U0126 was treated, MCF-7 cells showed a slight decrease in PI3K activity. But the levels didn't change with ERK1/2 siRNA treatment. The expression of PI3K didn't alter in the total protein

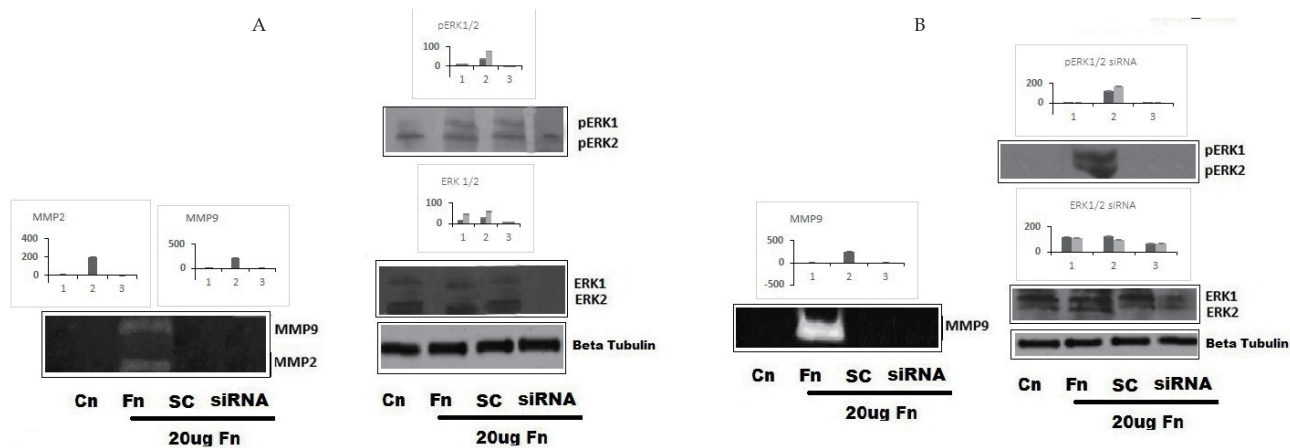


Figure 4 (A: MCF7, B: MDA231). Effect of ERK1/2 siRNA on activity of MMP2/MMP9 and activity and expression of ERK1/2. For the transfection process ERK1 siRNA and ERK2 siRNA (sequential double transfection procedure) and negative control siRNA (SC) were transfected using Lipofectamine™ 2000 following standard protocol. After that MCF7 and MDAMB231 cells (300,000 cells/ml) were grown in serum free culture medium in absence (control) and in presence of 20ug Fibronectin (MCF7: 2hrs, MDAMB231: 8hrs). The respective serum free medium was subjected to Gelatin Zymography and cell extract was subjected to Western Blot by standard protocol. The immunoblots were probed for anti-ERK1/2 antibody and developed using respective horse redox peroxidase (HRP) coupled second antibodies. Beta tubulin was used as internal control for all the western blots. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using image J Launcher (version 1.4.3.67) and arranged in the similar order as of the lanes. Data are means \pm SEM of three experiments.

form. In MDA-MB-231 cells the activity of PI3K remain almost similar and only changed when cells were treated with U0126. (we did this experiment to see the effect of ERK 1/2 inhibition on other signalling pathway).

Effect of ERK1/2 inhibition on cell migration

Figure 6 shows the effect of ERK1/2 inhibition on cell migration with the upper lanes showing MCF-7 cells and the lower lanes showing MDA-MB-231 cells. In MCF-7 cells there was increase of cell migration in cells treated with PD98059, which slowed down with U0126 treatment and was almost comparable with control cells with ERK1/2 siRNA treatment. In MDA-MB-231 cells both the inhibitors slowed down the rate of migration, but the combinatorial transfection of ERK1/2 siRNA showed a remarkable slowdown of migrating cells. This gives an idea of the importance of ERK1 modulation along with ERK2. (we did this experiment to study the inhibition of ERK 1/2 on cell migration & invasion).

Effect of ERK1/2 inhibition on cell invasion

The rate of cell invasion after ERK1/2 inhibition is shown in Figure 7. The invasion rate of MCF-7 cells (Figure 7A) went higher with Fibronectin treatment, but decelerated with PD98059. The invasion rate of MCF-7 cells went further down with U0126 treatment and became similar as control cells with ERK1/2 siRNA treatment. The results were similar in MDA-MB-231 cells but with siRNA treatments it showed the highest effect on the invasion rate of MDA-MB-231 cells.

Effect of ERK1/2 inhibition on activity and expression of RKIP, MEK1/2 and cFos

To further investigate the role of ERK1/2 the activity and expression of RKIP (Raf kinase inhibitor protein), MEK1/2 and cFos were checked after ERK1/2 modulation (Figure 8). In MCF-7 cells with the introduction of Fibronectin the activity of RKIP reduced considerably which slightly increased with inhibitor treatment (PD98059 and U0126) and went further up with ERK1/2 siRNA treatment. The expression of RKIP also followed the same pattern with siRNA treated cells having the highest band intensities. The results of pRKIP in MDA-MB-231 cells were similar. The total protein levels

of RKIP almost remained unaltered with only Fibronectin treated cells showing a sharp decrease in the band intensities. The activity of MEK1/2 in MCF-7 cells remained unaltered although the lanes with only an appreciable increase in Fn treated cells. The total protein levels remained unaltered. In MDA-MB-231 cells Fn treated cells showed increased levels of pMEK1/2, which remained constant with the inhibitors and siRNA treatments. There was hardly any change in the expression of MEK1/2. In MCF-7 cells with only ERK1/2 siRNA treatment the levels of pFos went down considerably which was otherwise constant. The total protein levels of cFos remained unaltered. When MDA-MB-231 cells were treated with Fibronectin, there was a sharp increase of pFos activity which didn't change with PD98059 treatment. With the administration of MEK/ERK inhibitor U0126, the levels of pFos went down and further decreased with ERK1/2 siRNA. The expression of cFos showed an appreciable decrease with U0126 treatment while the band intensities went down further with the administration of ERK1/2 siRNA.

Effect of ERK1/2 inhibition on the expression of Integrin alpha 5, Integrin Beta 1, FAK and Paxillin

Figure 9 shows the effect of ERK1/2 inhibition on the expression of integrin alpha v, integrin beta1, FAK (Focal adhesion kinase) and paxillin. In MCF-7 cells alpha 5 increased with Fn treatment, remained constant with PD98059 and U0126 treatment but showed a considerable change in band intensities with ERK1/2 siRNA treatment. In MDA-MB-231 cells (Figure 9 B) there was a sharper decrease of alpha 5 levels with siRNA treatment. In MCF-7 cells Fn treated cells showed an increase in beta1 band intensities, which went down with the administration of U0126 and reached basal level with ERK1/2 siRNA. Beta1 showed a sharp fall with ERK1/2 siRNA, but otherwise remained constant in MDA-MB-231 cells. This suggest ERK1/2 and Integrin alpha5 beta1 might have direct interaction between the two. In both the cell lines the activity of FAK went up with the treatment of Fibronectin, didn't change with the administration of PD98059 or U0126 and had an appreciable decrease with transfection of ERK1/2 siRNA. The expression of FAK had hardly any visible change in both the cell lines with only one exception in MCF-7 cells where the total protein level went down by 30% in U0126 treated lanes. The expression of paxillin in

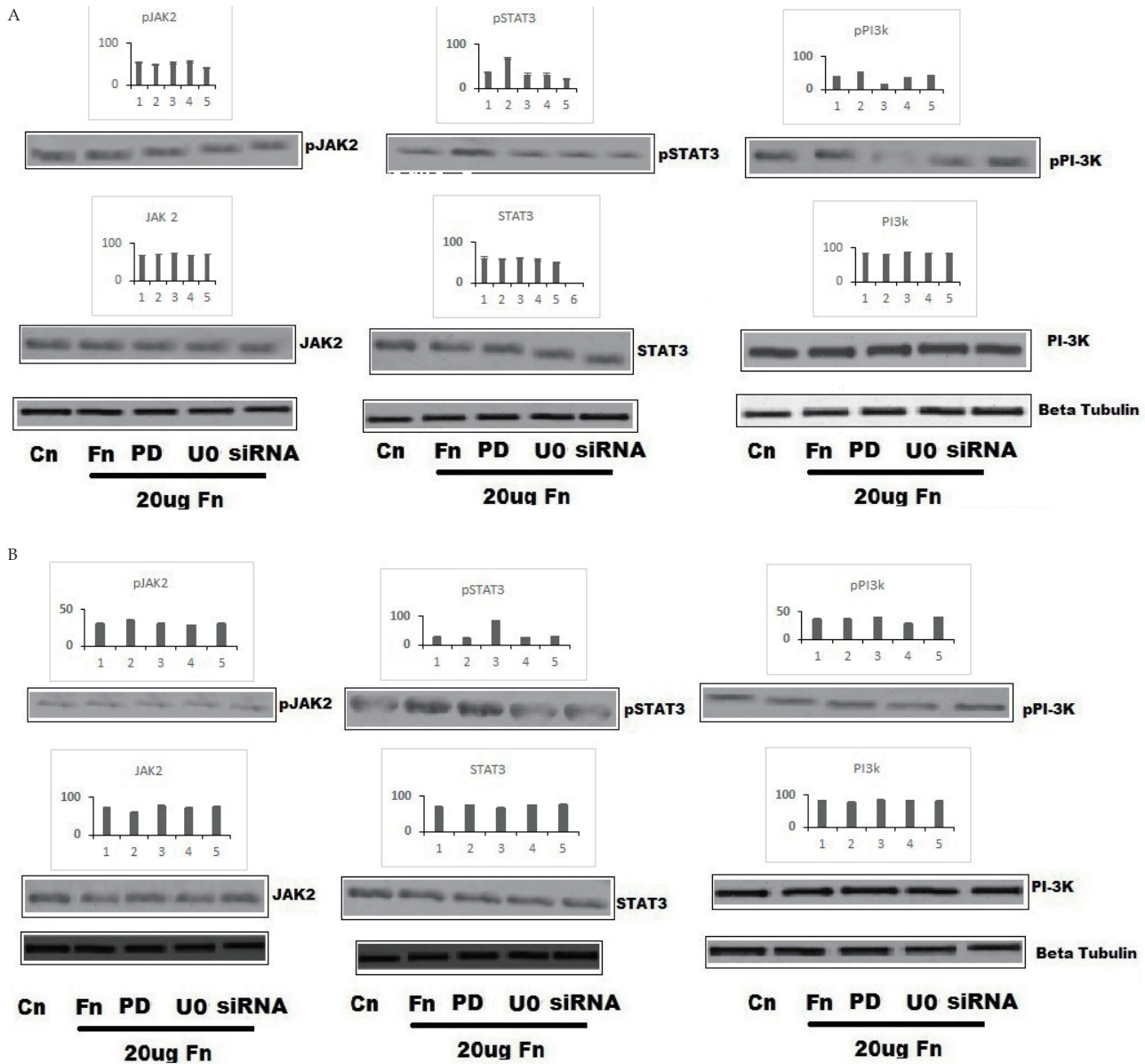


Figure 5 (A: MCF 7, B: MDA231). Effect of ERK1/2 inhibition on activity and expression of JAK2, STAT3 and PI-3K. For the transfection process ERK 1 siRNA and ERK 2 siRNA (sequential double transfection procedure) and negative control siRNA (SC) were transfected using Lipofectamine™ 2000 following standard protocol. Pre-treatment for 1 hour of Inhibitors PD98059 and U0126 were done to both the cell lines. After that MCF7 and MDAMB231 cells (300,000 cells/ml) were grown in serum free culture medium in absence (control) and in presence of 20ug Fibronectin (MCF7: 2hrs, MDAMB231: 8hrs). The respective cell extract was subjected to Western Blot by standard protocol. The immunoblots were probed for anti pJAK2/JAK2/ pSTAT3/ STAT3/pPI-3K/PI-3K antibody and developed using respective horse redox peroxidase (HRP) coupled second antibodies. Beta tubulin was used as internal control for all the western blots. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using image J Launcher (version 1.4.3.67) and arranged in the similar order as of the lanes. Data are means \pm SEM of three experiments.

MCF-7 cells remain unaltered throughout the lanes only decreasing sharply in siRNA transfected cells. In Figure 9B the levels of paxillin increased in Fn induced cells but went down when inhibitor treatment (PD98059/U0126) was introduced and reduced to trace amounts with ERK1/2 siRNA transfection. (we did this study to see the effect of ERK 1/2 inhibition on FAK and paxillin)

DISCUSSION

The role of ERK1/2 in cell invasiveness has been elucidated in many recent evidences. Cell invasiveness primarily involves the breakdown of extra cellular matrix. The family of zinc dependent endopeptidases, which are involved in the breakdown of extra cellular matrix, normal tissue remodeling and repair process are known as matrix metalloproteinase (MMP). In cancer they play

a critical role to facilitate cell migration by removing physical barriers promoting angiogenesis. Among MMPs, MMP2 and MMP9 (gelatinase A and gelatinase B) have found to be related in major cases of tumor invasion and metastasis with their ability to degrade collagen type IV, a major component basement membrane^[9,10]. Integrins are the principal cell surface adhesion receptors, which mediates cell adhesion to the ECM, transduce intra cellular signal and thus regulates proliferation, survival and motility of cells. Integrins differentially regulate the production of MMPs^[6]. Many recent studies in our lab have shown the cross talk between integrins and MMPs. These cross talks were best-shown using ligand dependent activation of MMP9 and MMP2. These ligands include fibronectin, laminin, vitronectin which are substrates of MMPs^[6,7,8]. Many recent evidences have been linked with the activation of ERK1/2 pathway

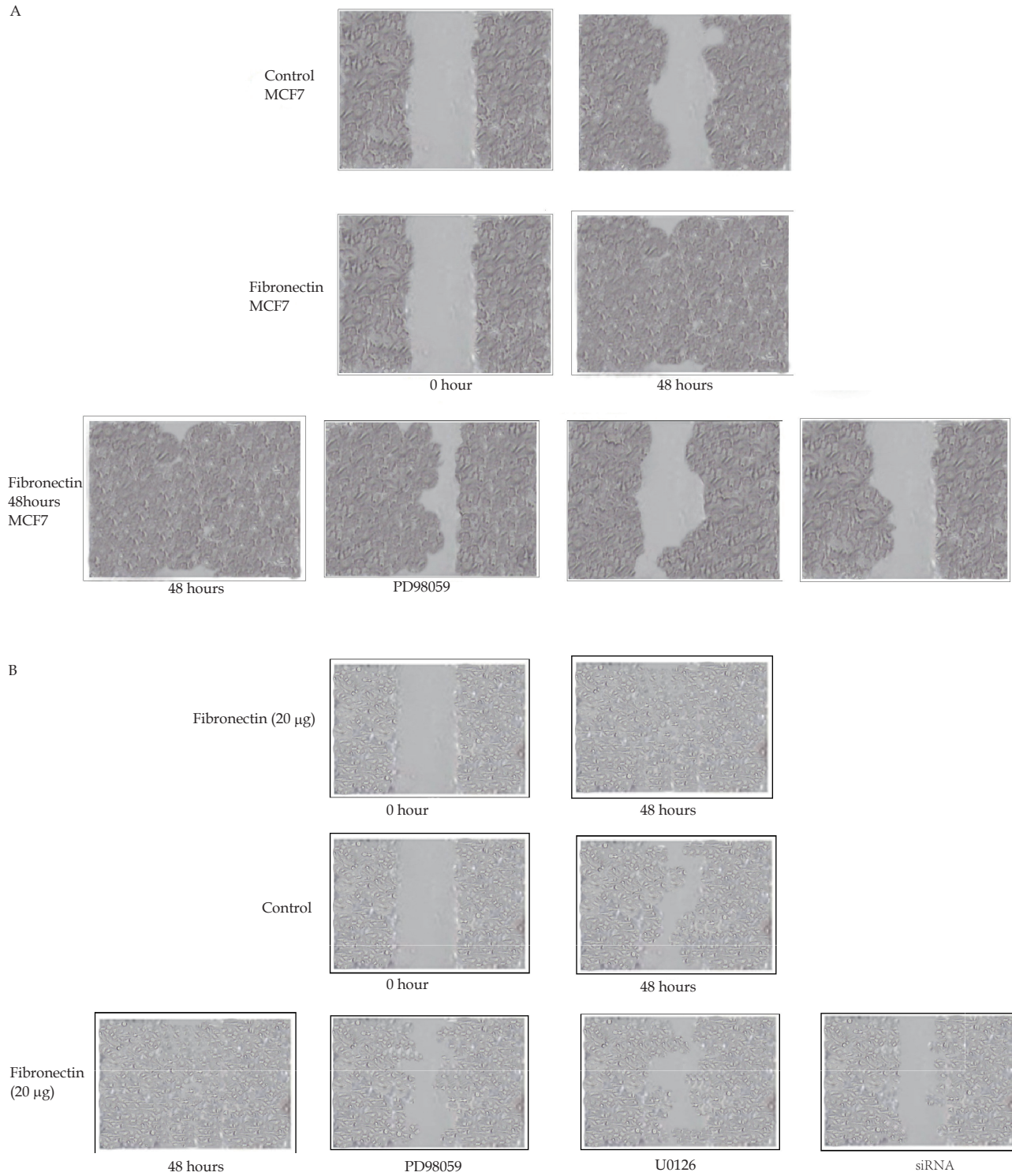


Figure 6 (Upper lane: MCF 7 Lower Lane: MDA231). Effect of ERK1/2 inhibition on cell migration. MCF7 cells and MDAMB231 (300,000 cells/ml) were grown in serum free culture medium in absence (control) and in presence of Fibronectin (20ug per/ml). For the transfection process ERK 1 siRNA and ERK 2 siRNA (sequential double transfection procedure) and negative control siRNA (SC) were transfected using Lipofectamine™ 2000 following standard protocol. Pre-treatment for 1 hour of Inhibitors PD98059 and U0126 were done to both the cell lines. The monolayer was scratched with a sterile pipette tip, followed by washing thrice with SFCM to remove cellular debris. The cells were maintained in fresh SFCM and cell migration was observed by microscopy and documented by photography at 0 hr 24 hr and 48 hr. The cells were maintained in fresh SFCM and cell migration was observed by microscopy and documented by digital photography at 0 hr and 48 hr.

with MMPs. Specific ERK inhibition could inhibit MMP2 and MMP9 expression in human gallbladder cell line^[9]. In this present study we aimed to look at the role of ERK1/2 in modulating MMP2 and MMP9 in a fibronectin induced system. The study also takes a sneak peek in the possible signaling molecules contributing in the modulation with respect to cell invasion and migration.

The cell line MCF-7 and MDA-MB-231 cells were chosen very carefully as the expression of ERK1/2 in both the cell lines were prominent enough in fibronectin induced system. In presence of 20 ug fibronectin there was an appreciable activation of pERK1/2 in MCF-7 and MDA-MB-231 cells in 2 hours and 8 hours respectively. The concentration and the activation time were chosen after series

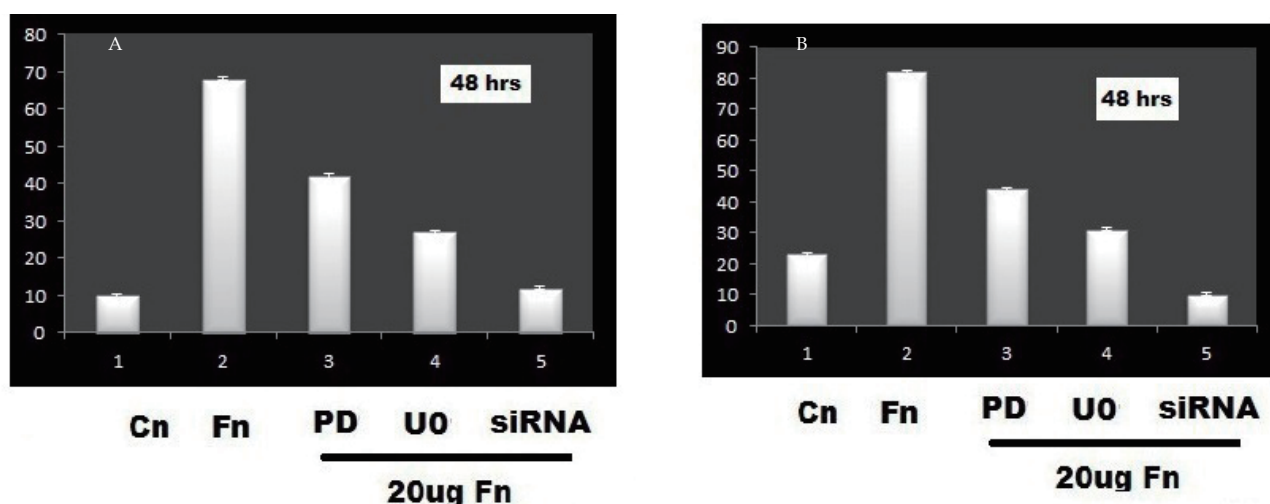


Figure 7 (A: MCF 7 B: MDA231). Effect of ERK1/2 inhibition on cell invasion. Sequentially transfected ERK1+ERK2 siRNA, inhibitor PD98059 and U0126 treated cells were cultured in transwell chambers in triplicate in absence and presence of fibronectin (20 mg/ml). The chamber was inserted in DMEM containing 5% FBS as chemo-attractant and grown for 48 hrs. The chambers were then removed, washed. The cells migrated on the membrane were observed under microscope. Number of cells migrated through transwell insert were counted per microscopic power field. Figure 8 A, Figure 8B represents the number of invaded MCF7 and MDAMB231 cells respectively.

of time and dose dependent experiments with fibronectin. This activation corresponded with the activity of MMP2 and MMP9 in both the cell lines and hence showed an indication of a possible correlation between the two events.

ERK1/2 holds a tight selectivity on their activation mechanism and only get phosphorylated by MEK1/2 making them excellent targets for the development of therapeutic agents against cancer. PD98059, one of the non-ATP competitive inhibitors of MEK1/2 had an appreciable effect on the activation of ERK1/2 along with down regulation of MMP2 and MMP9. This suggested a possible role of ERK1/2 in the modulation of MMP. Interestingly, the total protein levels seem unaffected indicating the importance of activated protein in transducing signals in the cell.

To investigate further effects of ERK1/2 inhibition, another specific selective inhibitor U0126 was introduced. The results of MCF-7 and MDA-MB-231 cells again indicated a direct correlation between ERK1/2 inhibitor and gelatin degrading activity of both MMP2 and MMP9. While PD98059 treated MDA-MB-231 cells showed an appreciable decrease in MMP9 activity the levels went further down in U0126 treated cells signifying the selective role of the inhibitory potency of this non-competitive therapeutic compound.

Though the inhibitors were potent to inhibit ERK phosphorylation in our study, the results of invitrokinase assays in other studies using purified proteins suggested that PD98059 and U0126 inhibited the phosphorylation and activation of MEK1/2 but not the ability of MEK1/2 to phosphorylate ERK1/2. Hence we opted for more targeted approach by small interfering RNA. With sequential double transfection method ERK1 siRNA and ERK2 siRNA was transfected in both the cell lines. The results again confirmed the role of ERK inhibition in diminishing MMP9 and MMP2 activity. These results were in accordance with various other recent evidences, which suggests that specific blockade of ERK pathway inhibits invasiveness of tumor cells by down regulation of matrix metalloproteinase^[4]. Our study also indicated that this regulation was probably dependent on the activated protein as the total protein levels of ERK1/2 showed very less variation throughout.

To understand the effects of ERK inhibitors and siRNA on other signaling pathways and investigate more about the role of ERK signaling, specific signaling molecules from different signaling

pathways were checked. Recent studies have shown the co-operation of ERK1/2 pathway with JAK/STAT pathways involving JAK2 and STAT3 molecules. In our Fn induced system JAK2 didn't show much change in band intensities until ERK1 siRNA was introduced, which might have effected JAK2 activation. The results of STAT3 activity were in accordance with recent studies^[10,11], which were inhibited by treatment by MEK/ERK inhibitors. The decrease levels of pSTAT3 with siRNA transfection also indicated the selective regulation of JAK/STAT pathway, which might have added to the post-transcriptional modulation of MMP2/MMP9 activity. The sudden decrease of PI3K levels in the activated form with the treatment of PD98059 might indicate the non-specificity of the inhibitor with respect to ERK1/2 inhibition.

The phenotypical assays were performed to see the direct efficacy of ERK1/2 modulation on cell migration and cell invasion. The result corresponded with MMP activity showing clear indication of increased cell migration and invasion rate with ERK1/2 activation and the subsequent down regulation with ERK1/2 inhibition. The striking decrease in the rate of cell invasion as well as cell migration with ERK1/2 siRNA transfection again implied the importance of targeted inhibition.

Raf-1 kinase inhibitor protein (RKIP) was originally identified physiologic inhibitor of MAPK/ERK pathway, but was later also recognized as clinically relevant metastasis suppressor genes^[5]. In many recent discoveries it has been shown that RKIP protein expression is down regulated in metastatic cancer cells of breast, prostate and melanoma origin^[10,12,13]. We examined RKIP expression and activity in our model system and found that, there was a sharp decrease of RKIP/pRKIP levels which slowly increased with inhibitor treatments and became comparable to the control cells (in both the cell lines). This suggests that the inhibition of ERK1/2 (by inhibitors/siRNA) might have induced a feedback loop to normalize RKIP levels. The possibilities of feedback mechanism are well discussed in recent evidences^[14,15]. Needless to mention here that RKIP is an upstream molecule in the MAPK pathway. We wanted to check the levels of sole activator of ERK1/2 that is MEK1/2. The immunoblots showing the expression of MEK1/2 didn't show any visible change though the activated MEK1/2 (pMEK1/2) increased with Fn treatment, implying that Fn can activate MEK1/2 and subsequently

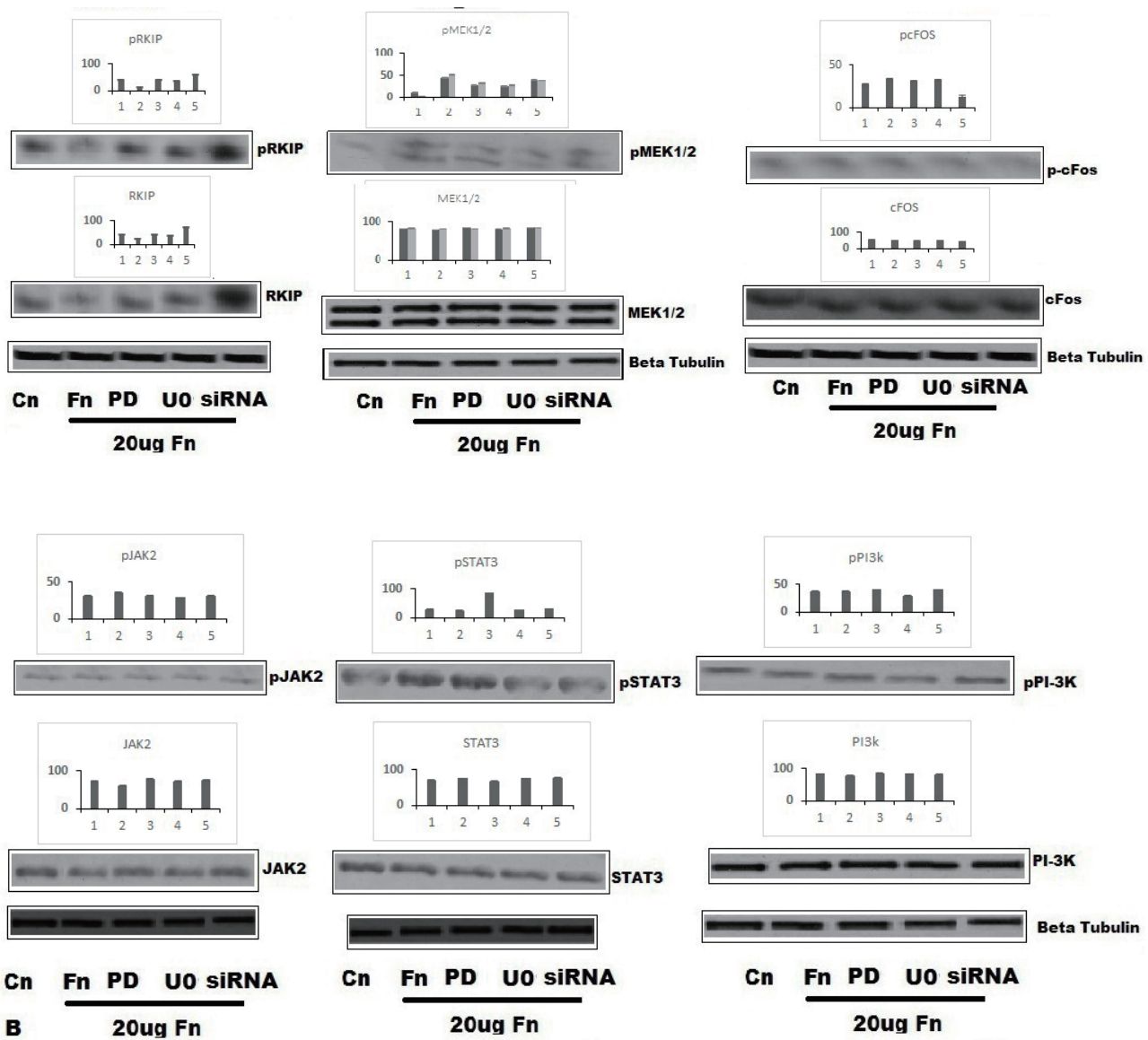


Figure 8 (A: MCF 7, B: MDA231). Effect of ERK1/2 inhibition on activity and expression of RKIP, MEK1/2 and cFos. For the transfection process ERK 1 siRNA and ERK 2 siRNA (sequential double transfection procedure) and negative control siRNA (SC) were transfected using Lipofectamine™ 2000 following standard protocol. Pre-treatment for 1 hour of Inhibitors PD98059 and U0126 were done to both the cell lines. After that MCF7 and MDAMB231 cells (300,000 cells/ml) were grown in serum free culture medium in absence (control) and in presence of 20ug Fibronectin (MCF7: 2hrs, MDAMB231: 8hrs). The respective cell extract was subjected to Western Blot by standard protocol. The immunoblots were probed for anti pRKIP/RKIP/pMEK1/2/MEK1/2/p-cFos/cFos antibody and developed using respective horse redox peroxidase (HRP) coupled second antibodies. Beta tubulin was used as internal control for all the western blots. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using image J Launcher (version 1.4.3.67) and arranged in the similar order as of the lanes. Data are means ± SEM of three experiments.

ERK1/2. The obvious decrease of pMEK1/2 levels with MEK/ERK inhibitor was normalized with ERK1/2 siRNA transfection showing the specificity of the knockdown without affecting any other molecule. One possible mechanism by which sustained MAPK activation could result in MMP9 or MMP2 induction is through regulation of an essential transcription factor cFos^[16]. Evidences suggest that phosphorylation of cFos by ERK1/2 enhances MMP9 expression (Chen *et al.*). There are evidences that MMP2/MMP9 down regulation in human leukemia cells is mediated by ERK-cFos pathway^[17]. When we checked the expression and activity of cFos, the results indicated that both the expression and the activity dropped down to basal level with siRNA treatment suggesting the reason behind negative regulation of MMP9/MMP2. One of the possible mechanisms of regulating MMP9 involves transfection factor AP-1 that has a consensus sequence in MMP9 promoter region and can be

regulated directly or indirectly by MAPK pathway through cFos and ELK1^[18]. Though MMP2 doesn't share the conserved proximal AP1 site but recent studies^[19] reported regulation of MMP2 by functional AP1 binding site.

Integrins which control a variety of signal transduction pathways functional to cell survival, cell proliferation and differentiation are known to have altered expression levels in many types of cancer. The majority of Integrins are multiple ligand binding apart from the only receptor alpha5 beta1 which has a unique ligand specificity of Fibronectin. Implication of this receptor in malignant progression is well reported. Evidences suggest Integrin alpha5 beta1 can control invasion of human breast carcinoma cells by direct and indirect modulation of MMP2 activity^[20]. alpha5 beta1 are also known to be functional in Fn induced MMP9 expression and activity when MCF-7 cells were probed for alpha5, there was a significant decrease

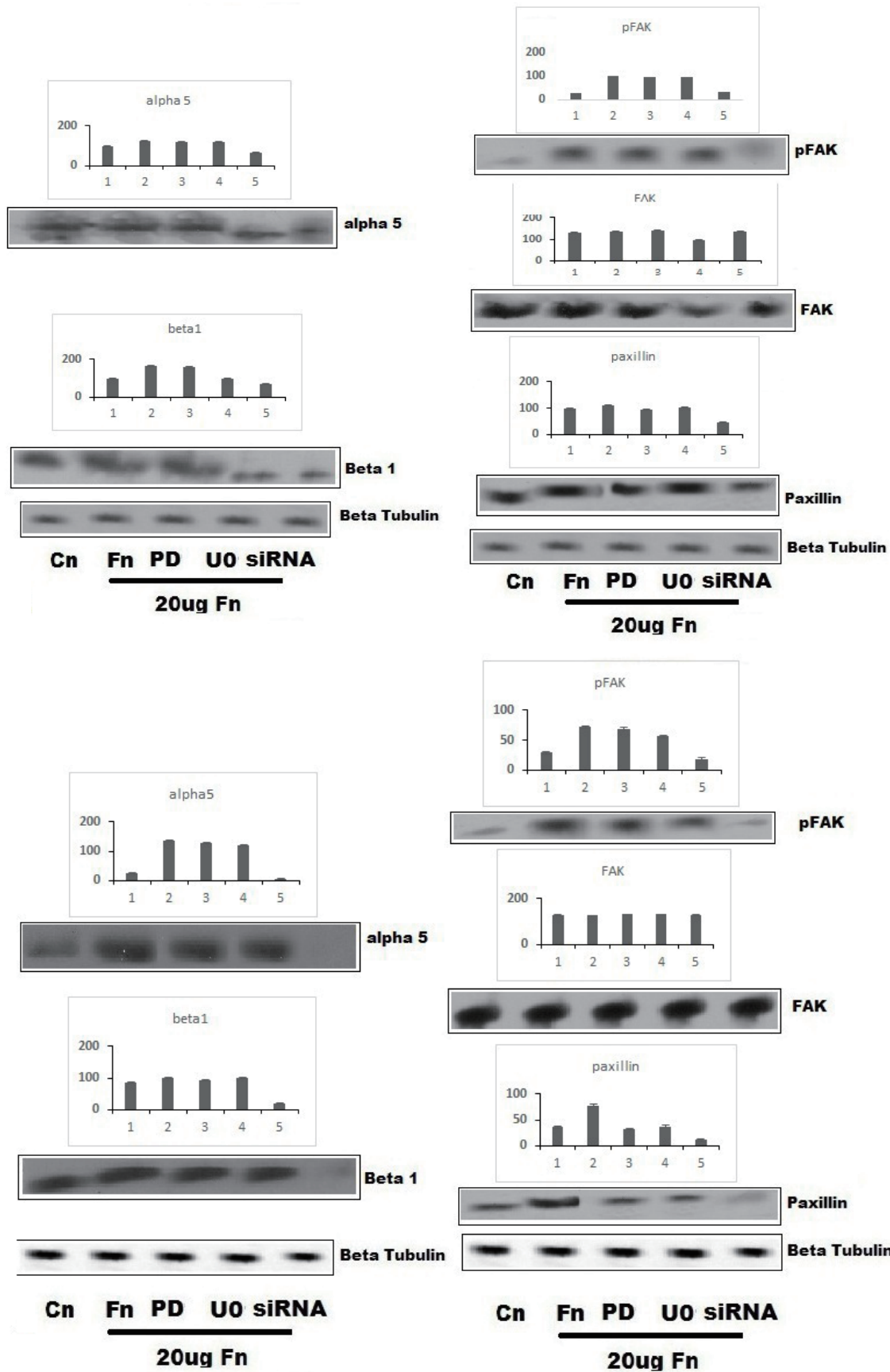


Figure 9 (A: MCF 7 B: MDA231). Effect of ERK1/2 inhibition on the expression of Integrin alpha 5, Integrin Beta 1, FAK and Paxillin. For the transfection process ERK 1 siRNA and ERK 2 siRNA (sequential double transfection procedure) and negative control siRNA (SC) were transfected using Lipofectamine™ 2000 following standard protocol. Pre-treatment for 1 hour of Inhibitors PD98059 and U0126 were done to both the cell lines. After that MCF7 and MDAMB231 cells (300,000 cells/ml) were grown in serum free culture medium in absence (control) and in presence of 20ug Fibronectin (MCF7: 2hrs, MDAMB231: 8hrs). The respective cell extract was subjected to Western Blot by standard protocol. The immunoblots were probed for anti-alpha V/ Beta 1/pFAK/FAK/Paxillin antibody and developed using respective horse redox peroxidase (HRP) coupled second antibodies. Beta tubulin was used as internal control for all the western blots. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using image J Launcher (version 1.4.3.67) and arranged in the similar order as of the lanes. Data are means \pm SEM of three experiments.



with ERK1/2 siRNA transfection which otherwise got higher with Fibronectin treatment. This suggested a correlation between ERK1/2 and alpha5. Integrin beta1 also behaved similarly. The decrease in the levels of integrins (alpha5 beta1) in MDA-MB-231 cells were even steeper than MCF-7 indicating a strong dependency on ERK inhibition. This was in accordance with works, which reported a blockade in the expression of integrins with MEK/ERK inhibition^[21]. The work also reported alpha5 beta1 Integrin expression with sustained activation of ERK pathway, which might also holds true in our study where activation of ERK1/2 by Fibronectin enhanced Integrin expression. This correlation between ERK1/2 and Integrin alpha5 beta1 might involve direct interaction between the two like in case of Integrin beta6, which directly associates with ERK2^[22]. Focal adhesion kinase (FAK) is a major non-receptor kinase activated after Integrin mediated adhesion to ECM proteins like Fibronectin. FAK lies at the crossroad of ERK1/2, PI3K/ AKT (protein kinase B) and JAK/STAT pathways. Paxillin actually connects the ERK and FAK pathways. The binding of ERK2 paxillin induces the binding of FAK to paxillin resulting in further downstream activation^[23]. In our study when we probed for paxillin in both the cell lines, there was an increase with Fn treatment, which went down to basal level with specific ERK inhibition by siRNA supporting the correlation of ERK1/2 with paxillin. The decrease in paxillin levels corresponded with the decrease in the activity of FAK which might indicate a dependence on ERK pathway with paxillin which coordinates the focal adhesion turnover and migration functioning as a regulated scaffold for a localized connection between ERK/MEPK and FAK signaling pathways.

Our study, which investigates the possible role of ERK1/2 in modulating MMP2 and MMP9, suggests that there might be a direct correlation between ERK1/2 activity and MMP2 and MMP9 activity which might involve STAT3 and cFos. This is the key point of this study. The study also throws a light on the involvement of other signaling molecules like MEK1/2, RKIP in this respect. MMP2 and MMP9, which were taken as an indicator of tumor invasiveness along with other phenotypical parameters like cell invasion and cell migration were shown to be regulated by ERK1/2 modulation. Interestingly this regulations were mostly through the activated form of the concerned proteins and dependent on specific or targeted inhibition of ERK1/2. Another key point the study elucidates is the role of ERK1/2 in regulating alpha5 beta1 integrins, which differentially regulates MMP2 and MMP9 adding the importance of ERK signaling in cell proliferation, differentiation and survival. With additional transcriptional study on these molecules, which was beyond the scope of our work, novel mechanisms can be identified which might help in targeted multi-therapeutic approaches for cancer prognosis and survival.

ACKNOWLEDGMENTS

We acknowledge Department of Science and Technology, New Delhi (DST, SR/SO/HS-93/2010) for financial assistance and Director, Chittaranjan National Cancer Institute (CNCI) for academic and infrastructural facilities.

CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

REFERENCES

1 Fujioka A, Terai K, Itoh RE, Aoki K, Nakamura T, Kuroda S et al. Dynamics of the Ras/ERK MAPK cascade as moni-

tored by fluorescent probes. *J Biol Chem* 2006; **281**: 8917-8926.

2 Lopez-Bergami P, Huang C, Goydos JS, Yip D, Bar-Eli M, Herlyn M et al. Rewired ERK-JNK signaling pathways in melanoma. *Cancer cell* 2007; **11**: 447-460.

3 May LT, Hill SJ. ERK phosphorylation: spatial and temporal regulation by G protein-coupled receptors. *Int J Biochem Cell Biol* 2008; **40**: 2013-2017.

4 Tanimura S, Asato K, Fujishiro SH, Kohno M. Specific blockade of the ERK pathway inhibits the invasiveness of tumor cells: down-regulation of matrix metalloproteinase-3/-9/-14 and CD44. *Biochem Biophys Res Commun* 2003; **304**: 801-806.

5 Hagan S, Al-Mulla F, Mallon E, Oien K, Ferrier R, Gusterson B et al. Reduction of Raf-1 kinase inhibitor protein expression correlates with breast cancer metastasis. *Clin Cancer Res* 2005; **11**: 7392-7397.

6 Das S, Banerji A, Frei E, Chatterjee A. Rapid expression and activation of MMP-2 and MMP-9 upon exposure of human breast cancer cells (MCF-7) to fibronectin in serum free medium. *Life Sci* 2008; **82**: 467-476.

7 Sen T, Moulik S, Dutta A, Choudhury PR, Banerji A, Das S et al. Multifunctional effect of epigallocatechin-3-gallate (EGCG) in downregulation of gelatinase-A (MMP-2) in human breast cancer cell line MCF-7. *Life Sci* 2009. **84**: 194-204.

8 Sil H, Sen T, Chatterjee A. Fibronectin-integrin (alpha5beta1) modulates migration and invasion of murine melanoma cell line B16F10 by involving MMP-9. *Oncol Res* 2011; **19**: 335-348.

9 Horiuchi H, Kawamata H, Furihata T, Omotehara F, Hori H, Shinagawa Y et al. A MEK inhibitor (U0126) markedly inhibits direct liver invasion of orthotopically inoculated human gallbladder cancer cells in nude mice. *Clin Cancer Res* 2004; **23**: 599-606.

10 Schuierer MM, Bataille F, Hagan S, Kolch W, Bosserhoff AK. Reduction in Raf kinase inhibitor protein expression is associated with increased Ras-extracellular signal-regulated kinase signaling in melanoma cell lines. *Cancer Res* 2004; **64**: 5186-5192.

11 Plaza-Menacho I, van der Sluis T, Hollema H, Gimm O, Buys CH, Magee AI et al. Ras/ERK1/2-mediated STAT3 Ser727 phosphorylation by familial medullary thyroid carcinoma-associated REmutants induces full activation of STAT3 and is required for c-fos promoter activation, cell mitogenicity, and transformation. *J Biol Chem* 2007; **282**: 6415-6424.

12 Chatterjee D, Bai Y, Wang Z, Beach S, Mott S, Roy R et al. RKIP sensitizes prostate and breast cancer cells to drug-induced apoptosis. *J Biol Chem* 2004; **279**: 17515-17523.

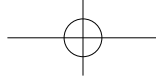
13 Fu Z, Smith PC, Zhang L, Rubin MA, Dunn RL, Yao Z et al. Effects of raf kinase inhibitor protein expression on suppression of prostate cancer metastasis. *J Natl Cancer Inst* 2003; **95**: 878-889.

14 Mirzoeva OK, Das D, Heiser LM, Bhattacharya S, Siwak D, Gendelman R et al. Basal Subtype and (MAPK)/ERK Kinase (MEK)-Phosphoinositide 3-Kinase Feedback Signaling Determine Susceptibility of Breast Cancer Cells to MEK inhibition. *Cancer Res* 2009; **69**: 565-572.

15 Cirit M, Wang CC, Haugh JM. Systematic Quantification of Negative Feedback Mechanisms in the Extracellular Signal-regulated Kinase (ERK) Signaling Network. *J Biol Chem* 2010; **285**: 36736-36744.

16 Lakka SS, Gondi CS, Yanamandra N, Olivero WC, Dinh DH, Gujrati M et al. Inhibition of cathepsin B and MMP-9 gene expression in glioblastoma cell line via RNA interference reduces tumor cell invasion, tumor growth and angiogenesis. *Oncogene* 2004; **23**: 4681-4689.

17 Liu WH, Chang LS. Caffeine induces matrix metallopro-



Moulik S *et al.* Role of ERK in modulating MMP2 and MMP9

- teinase-2 (MMP-2) and MMP-9 down-regulation in human leukemia U937 cells via Ca²⁺/ROS-mediated suppression of ERK/c-fos pathway and activation of p38 MAPK/c-jun pathway. *J Cell Physiol* 2010; **224**: 775-785.
- 18 O-charoenrat P, Wongkajornsilp A, Rhys-Evans PH, Eccles SA. Signaling pathways required for matrix metalloproteinase-9 induction by betacellulin in head-and-neck squamous carcinoma cells. *Int J Cancer* 2004; **111**: 174-183.
 - 19 Bergman MR, Cheng S, Honbo N, Piacentini L, Karliner JS, Lovett DH . A functional activating protein 1 (AP-1) site regulates matrixmetalloproteinase 2 (MMP-2) transcription by cardiac cells through interactions with JunB-Fra1 and JunB-FosB heterodimers. *Biochem. J* 2004; **369**: 485-496.
 - 20 Morozevich G, Kozlova N, Cheglakov I, Ushakova N, Ber-man A . Integrin alpha5beta1 controls invasion of human breast carcinoma cells by direct and indirect modulation of MMP-2 collagenase activity. *Cell Cycle* 2009; **8**: 2219-2225.
 - 21 Smalley KS .A Pivotal Role for ERK in the Oncogenic Behaviour of Malignant Melanoma? *Int J Cancer* 2003; **104**: 527-532.
 - 22 Ahmed N, Niu J, Dorahy DJ, Gu X, Andrews S, Meldrum CJ et al. Direct Integrin $\alpha v\beta 6$ -ERK binding: implications for tumor growth. *Oncogene* 2002; **21**: 1379-1380.
 - 23 Kolch W .Coordinating ERK/MAPK signaling through scaffolds and inhibitors. *Nat Rev Mol Cell Biol* 2005; **6**: 837-838.

Peer reviewer: Zhenyi Ma, Professor, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Tianjin Medical University, Room 402 South Building, 22 Qixiangtai Road, Heping District, Tianjin, 300070, China.