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Role of MAPK Pathway in Epithelial to Mesenchymal Transition, Single and Collective Cell Migration in Asian Head and Neck Cancer Cell Lines

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Abstract

Objective*: The role of Ras-Raf-MEK-ERK pathway was investigated in terms of: cellular protrusion, epithelial to mesenchymal transition (EMT), single cell migration and collective cell migration using MEK inhibitor (PD98059).*

Methods: *Single and collective cell migration were measured by Scatter assay and wound healing assay respectively. Phosphorylation and localization were investigated by using immunofluorescence, SDS- PAGE and Western blotting techniques to study molecular markers of EMT (E-cadherin and Vimentin,) the MAPK (Thr202/Tyr204) and AKT (Ser473) expression.*

Results: *PD98059 completely inhibited cellular protrusion, EMT and single cell migration in all the cell lines used in the project. In contrast, PD98059 completely inhibited collective cell migration in HSG and AZA1 cell lines while partially inhibiting collective migration in HaCaT and TYS cell lines. Inhibition of ERK with PD98059 lowered MAPK (Thr202/Tyr204) phosphorylation while it increased AKT473 phosphorylation.*

Conclusion: *These data suggest that single and collective cell migration use different signaling mechanism. Drugs need to be designed to target both types of cell migration or a dual therapy approach is needed. Blocking the MAPK pathway is not enough to inhibit collective cell migration in certain cell lines and possible involvement of other signaling pathways. Personalized therapy and dual inhibition of pathways inhibitors might overcome the problem.*

Keywords: *MAPK pathway, EMT, cell migration, MAPK inhibitor, single cell migration, collective cell migration.*

Introduction

Head and neck cancer is the sixth most common cancer in the world with highest incidence found in South and South East Asia countries such as India, Pakistan, Sri Lanka, Taiwan [1]. These countries have heavy usage of tobacco, both smoke and smokeless tobacco (Betel quid) [2]. Ras (Rat sarcoma virus) mutation is more common in Asia when compared with the West [3]. This leads to question of whether Ras mutation is caused by specific carcinogen in betel quid or related with ethnicity.

Around 90% of cancer deaths are caused by metastasis. In order to for a cancer to undergo metastasis, cells need to detach from the primary tumour, invade through the basement membrane and into the surrounding tissue [4]. Cell migration is crucial in invasion and the metastasis process. There are three different modes of cancer cell migration (single cell migration, multicellular streaming and collective cell migration). Amoeboid or mesenchymal phenotype are displayed in single cell migration and multicellular streaming [5]. In collective cell migration, the cells can display either mesenchymal or epithelial phenotypes [5].

Epithelial to mesenchymal transition (EMT) is an initial process for the cells to be able to migrate [6]. In EMT closely connected cuboidal shape epithelial cells with apical basal polarity are transdifferentiated into elongated mesenchymal shape cells with front to back leading-edge polarity, motile and therapy resistance [6,7]. In culture dishes, epithelial cells grow as colonies while mesenchymal cells display spindle shape and fibroblast like phenotype [7]. Downregulation of Epithelial markers such as E-cadherin, desmoplakin, cytokeratin and upregulation of mesenchymal markers such as Vimentin, N-cadherin, Snail, Slug were found in cell undergoing EMT [8,9]. E-cadherin is responsible for maintaining cell to cell adhesion and loss of E-cadherin is related with loss of epithelial features [10]. In contrast, Vimentin is responsible for maintaining cellular integrity, stabilization of cytoskeleton, cell morphological changes and cell migration [11,12]. Loss of E-cadherin and acquired Vimentin is correlated with invasion and metastasis [13]. Cells which have undergone EMT are motile, resistance to apoptosis, and have increased production of matrix degradation substances [7,14,15]. Growth factors such as EGF (Epidermal growth factor), TGFα (Transforming growth factorα), bone morphogenetic proteins (BMPs), hypoxia-inducible factor (HIF), WNTs and Notch are known to induce EMT [7]. Signalling pathways such as the TGFβ, Notch, Wnt/βcatenin, Hedgehog and receptor tyrosine kinase signalling have also been implicated in EMT [16,17].

The MAPK pathway is one such pathway that has been reported to be involved in a variety of cellular process such as proliferation, differentiation, migration and apoptosis [18]. The MAPK pathway is activated by various growth factors, cytokines, hormones and also cellular stress. There are four MAPK cascades: extracellular signal-regulated kinase 1 and 2(ERK1/2), c Jun N terminal kinase (JNK), p38 and ERK5. Each cascade consists of three core kinases (MAPKKK, MAPKK, and MAPK) which activate in a series [18,19]. MAP kinase kinase kinase (MAPKKK) is the first phosphorylated which in turn activates downstream MAPK kinase (MAPKK). Then MAPK kinase (MAPKK) in turn phosphorylates and activates MAPKs [18,19]. The Erk1/2 cascade has 3 major components Ras, Raf and MEK [19]. Ligand binding causes receptor activation leading to phosphorylation of tyrosine kinase residues which provide docking sites for adaptor proteins and enzymes such as SHC and GRB2 with phosphotyrosine-binding (PTB) and SH2 domains. SHC recruits GRB2 through its SH2 domain. Son of Sevenless (SOS) binds GRB2 at the plasma membrane resulting in GRB2-SOS complex. SOS then activates the small Ras [20]. Ras is a proto-oncogene, meaning that a mutation of Ras protein leads to oncogenic activity. Ras mutations are found in a variety of cancers. In head and neck cancer, Ras mutation is commonly found in Asian patients compared with Western patients and this is believed to be associated with areca nut chewing habits [3]. Ras has the ability to activate MAPK pathway. Active Ras at the cell membrane binds to and activates Raf (which acts as MAPKKK) and activates (MAPKK) MEK1 and MEK2, which, in turn phosphorylate and activate ERK1 and ERK2. ERK activation is involved in a variety of cellular process promoting proliferation, differentiation, EMT, migration, angiogenesis, disease progression, resistant to apoptosis and drug resistance [18,19,21]. High ERK phosphorylation is related with poor prognosis [22]. Constant ERK activation in the cancer cells induces the production of matrix degrading enzymes such as Matrix Metalloproteinase (MMPs) which have ability to breakdown the basement membrane, disrupt cell adhesion, and allow cancer cells to dissociate from the primary tumour and invade the stroma [23]. ERK activation mediates phosphorylation of other proteins involved in cell cycle progression, cell detachment, membrane protrusion, actin polymerization, cell survival and then eventually leading to proliferation, differentiation, migration, and resistance to apoptosis [24]. Ras-Raf-MEK-ERK pathway is overactive in a variety of cancers as a result of Ras mutation. Inhibition of ERK can be the ideal target for patients for people with Ras mutation. PD98059 is a specific non competitive MEK inhibitor which inhibits MEK and thereby prevents the activation of ERK.

A previous study showed that EGF and TGFα induced an EMT phenotype and cell migration in head and neck cancer cell lines derived from Asian patients [25]. The aim of this study was to determine the role of

MAPK pathway in EMT and cell migration in head and neck cancer cell lines. The effect of MEK inhibitor (PD98059) was investigated on EGF and TGFα induced EMT like morphological change, single cell migration, collective cell migration, EGFR localization, AKT phosphorylation and MAPK phosphorylation. The effect of EMT inducers (EGF and TGFα) was investigated on the expression of an epithelial marker (E-cadherin) and a mesenchymal marker (Vimentin) to investigate whether the molecular changes of EMT match with morphological changes of EMT. This would further lead to an evaluation of whether single cell migration and collective cell migration use same signalling mechanism or not.

Materials and Methods

Cell lines and culture

Cancer cell lines of Japanese origin (TYS-from oral adeno squamous cell carcinoma, HSG-from irradiated salivary cells, AZA1-HSG cells treated with 5-Azacytidine) were gifts from Dr Koji Harada and Prof Mitsunobu Sato, University of Tokushima, Japan. Control cell line (HaCaT-from normal adult keratinocyte) was also a gift from Prof.S.L. Schor (Late) DDS, University of Dundee, UK. All cells were cultured at 37'C and 5% CO2 in minimum essential medium (MEM) supplemented with 10% Fetal calf serum and 200 mM L-glutamine.

Reagents, protein, inhibitors, and antibodies

Epidermal growth factor(10-1033-B) and Transforming growth factor (10-1033) (Insight biotechnology), Primary antibodies used were AKT 473 (4060), PhosphoP44/42 MAPK(Erk1/2) (Thr202/tyr204) (D13.14.4E) XP® Rabbit mAb (4370) from Cell signalling technology

E-cadherin (3195) and Vimentin (3932) from Cell signalling technology. Secondary antibodies used were Anti rabbit IgG, HRP linked antibody (7074)(Cell signalling technology) and Goat anti rabbit Ig G(H+L), F(ab")2 Fragment (Alexa Fluor® 488 conjugate) (150077) (Abcam). Inhibitor used was PD98059 (9900) from Cell signalling technology.

Scatter assay and Immunofluorescence

Scatter assay and Immunofluorescence techniques were performed as described earlier [25]. The cells were seeded into 1x105 per dish and were grown until it reaches to mid confluency. The cells were serum starved for 24 hours before incubation with (EGF/TGFα± PD98059). Serum free and PD98059 only treated dishes were used as control. EMT like phenotypical change and individual cell migration were accessed in the cells up to 48 hours. The cells were washed with PBS and photomicrographs were taken at appropriate interval with inverted microscope (IX70) Olympus, Japan (either 100X or 200X magnification) before fixation with with ice-cold methanol for 20 mins. The dishes were washed with PBS for 5mins x 3times and then with 0.2% triton X100 in PBS for 5mins x 1time and then washed again with PBS for 5mins x 3times. The dishes were blocked with 5% Normal goat serum/PBST (PBS+0.1 Tween 20) for 1 hour, then incubated with specific primary antibody diluted in 5% Normal goat serum in PBS T in 4-degree C overnight. Then incubated with secondary antibody Alexa Flour (1:1000) for 1 to 2 hrs. The dishes were again rinse PBS for 5mins x 3 times. The section was covered with mounting medium and a coverslip. Images were taken with Olympus Tokyo Japan IX70 inverted fluorescent micro-scope using 20 X or 40 X. Pictures were then observed with ImageJ software (NIH, Bethesda, MD, USA).

Scratch assay

2D migration assay was performed as described as above [25]. Cells were seeded with 1x105 cells in 30mm dish and grown until 100% confluency. The cells were serum starved for 24 hours, 1mm pipette tip was applied for scraping and the wound was created. The debris were washed with serum free and then incubated with EGF/TGFα±PD98059 for 24 hours. Serum free and PD98059 only dishes were used as control. Pictures were taken at the scratch line across the dishes with camera Olympus Tokyo Japan at either 100X or 200X magnification.

Cell lysis, Protein estimation assay, SDS page and Western blot

Cell lysis was performed as described earlier [26]. The cells were rinsed with cold PBS and lysed in ice cold RIPA buffer (each tablet of protease and phosphatase inhibitor into 10ml RIPA buffer). Gelation of lysates were break down with syringe and needles. Protein estimation assay was carried with Pierce BCA protein Assay Kit (23225). Proteins were separated by SDS-PAGE, followed by transferring onto nitrocellulose membrane or PVDF by Western blot. The blots were then blocked with 1% w/v dried milk

or 5% BSA in 1x TBST followed by incubating with specific primary antibodies anti- phospho p44/42 MAPK (Erk1/2) (Thr202/ Tyr204 1:2000), anti- phospho- akt (Ser473 1:2000) and goat anti- rabbit secondary antibody (1:2000). Blots were developed using BioRad Clarity Western ECL Substrate and Biorad GelDoc system.

Results

Introduction of EGF and TGFα for 48 hours did not change E-cadherin and Vimentin expression

The cells were incubated with different concentration of EGF and TGFα for different time point, Epithelial marker (E-cadherin) and Mesenchymal marker (Vimentin) expression were investigated by Western blot and Immunofluorescence as described in the Materials and Methods.

Western blot analysis indicated (Figure 1b&2b), the control cell line HaCaT expressed high E-cadherin and no Vimentin expression displaying completely epithelial-like characteristics. The TYS, HSG, AZA1 cell lines were negative E-cadherin and positive Vimentin expression indicating a transformed cell line characteristic. Addition of different concentrations of EGF and TGFα for 24 hours did not change the intensity of E-cadherin and Vimentin.

Using Immunofluorescence (Figure1a), HaCaT cell line had strongest E-cadherin expression of all the cell lines showing epithelial characteristic. Incubation with EGF and TGFα for 48 hours did not induce a change in intensity of E-cadherin expression in all the cell lines. Diffuse E-cadherin staining was detected in the HSG and AZA1 cell lines while membranous staining was produced in the TYS cell lines. HaCaT cell line, membranous staining was found in control (serum free) dishes. It is interesting to note some cells in growth factor treated dishes had a reduction in staining and therefore expression of E-cadherin.

Immunofluorescence data (Figure2a), for cell lines TYS, HSG and AZA1 indicate strong Vimentin expression showing mesenchymal characteristics while the HaCaT cell line showed weak Vimentin expression. Incubation with EGF and TGFα for 48 hours did not induce the change in intensity of Vimentin expression. Diffuse Vimentin expression was found in the TYS, HSG and AZA1 cell lines.

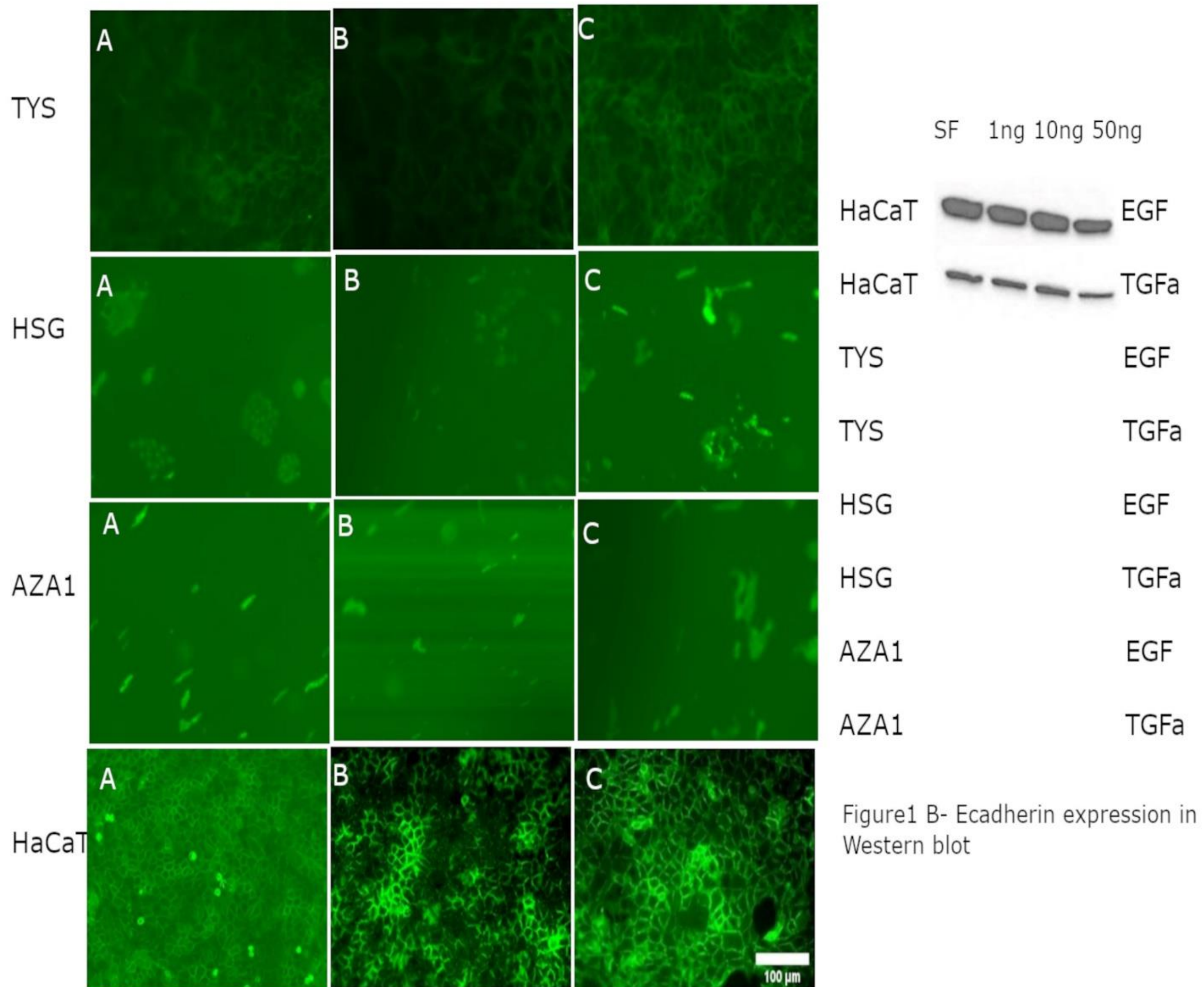

Figure1 B- Ecadherin expression in Western blot

Figure1 A- Ecadherin expression in Immunofluorescence

Figure 1: E-cadherin expression in Immunofluorescence (a) and Western blot (b). For figure 1a, four cell types were plated as described in the Material and Methods (TYS, HSG, AZA1 and HaCaT). Column A) serum free for 48 hours, B) EGF (50ng/ml) for 48 hours, C) TGFα (50ng/ml) for 48 hours, Immunofluorescence was visualized for E-cadherin expression. (All images were captured using an Olympus SC35 digital camera at x100 magnification). It should be noted HaCaT cell line has highest E-cadherin expression. For figure 1b-Western blot experiments for expression of E-cadherin. The Four cell type were plated as described in the Material and Methods (TYS, HSG, AZA1 and HaCaT). Cells were incubated in serum free, (1ng/ml,10ng/ml,50ng/ml) of EGF and TGFα for 24 hours then washed with PBS and lysed. Cell lysates were run on SDS-PAGE and blotted onto nitrocellulose and then probed. Images were captured using GelDoc system.

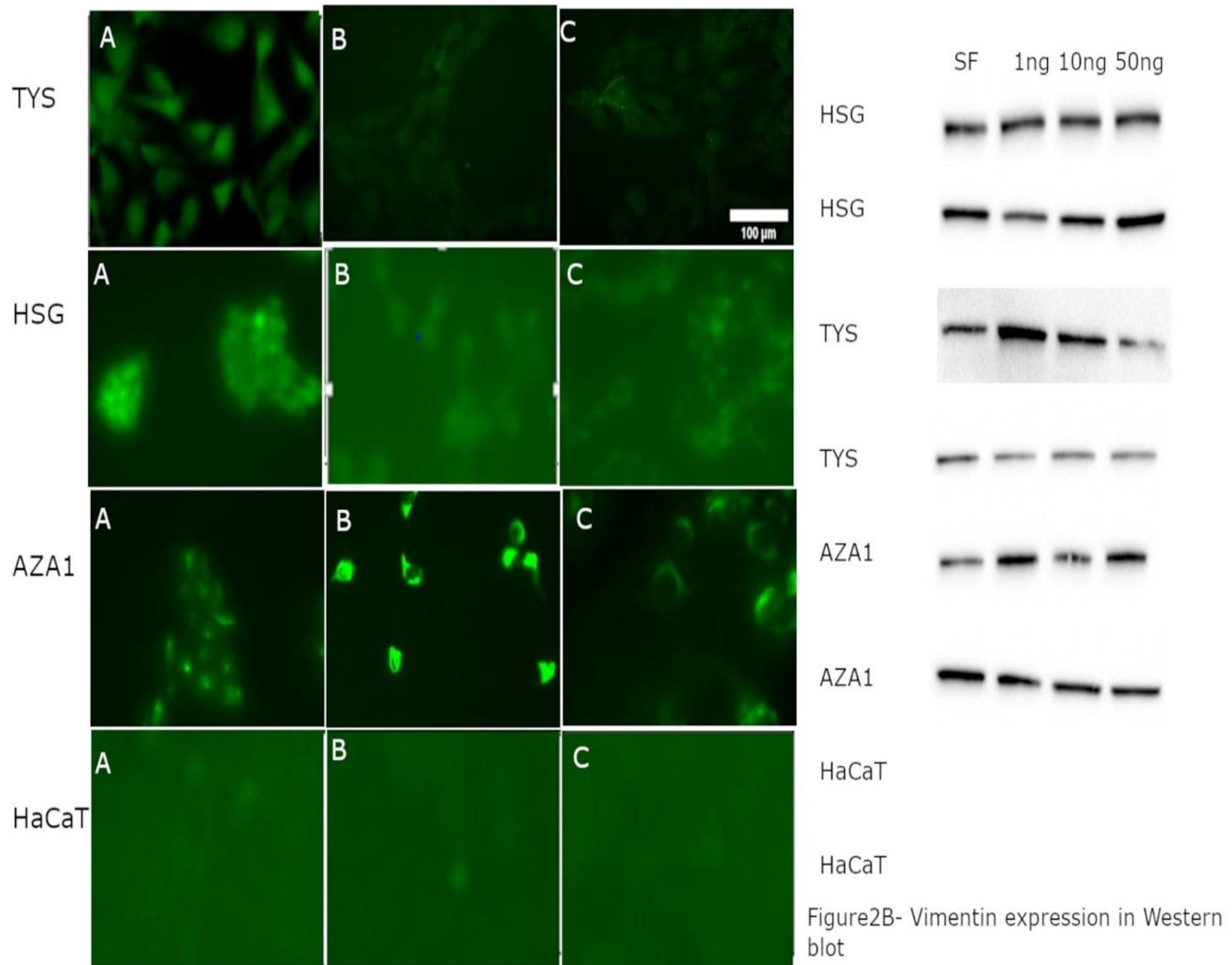

Figure 2: Vimentin expression in Immunofluorescence (a) and Western blot (b).

2a- 4 cell types were plated as described in the Material and Methods (TYS, HSG, AZA1 and HaCaT). Column A) serum free for 48 hours, B) EGF (50ng/ml) for 48 hours, C) TGFα (50ng/ml) for 48 hours, Immunofluorescence was visualized for Vimentin expression. (All images were captured using an Olympus SC35 digital camera at x100 magnification). It should be noted HaCaT cell line has least Vimentin expression. For figure 2b-Western blot experiments for expression of Vimentin protein. The Four cell type were plated as described in the Material and Methods (TYS, HSG, AZA1 and HaCaT). Cells were incubated in serum free, (1ng/ml,10ng/ml,50ng/ml) of EGF and TGFα for 24 hours then washed with PBS and lysed. Cell lysates were run on SDS-PAGE and blotted onto nitrocellulose and then probed. Images were captured using GelDoc system.

One-hour pre-treatment with MEK inhibitor (PD98059) inhibit EGF and TGFα induced EMT like phenotype with cellular projection and cell scattering/single cell migration

A previous study showed EGF and TGFα induce EMT like phenotype (dendritic and spiky cellular projection) and single cell migration. In this study, we used EGF and TGFα as positive control. A pilot study using three concentrations of PD98059 (5 µM ,25 µM and 50 µM) was performed (data not shown). In all the cell lines, one hour pre-treated with 25 µM PD98059(MEK inhibitor) before addition of growth factors completely inhibited EGF and TGFα induced EMT like morphological changes, cellular projections, and cell scattering (Figure3-D&E). The targeting of the MAPK pathway alone was enough to inhibit EMT and single cell migration for the cell lines used in this study.

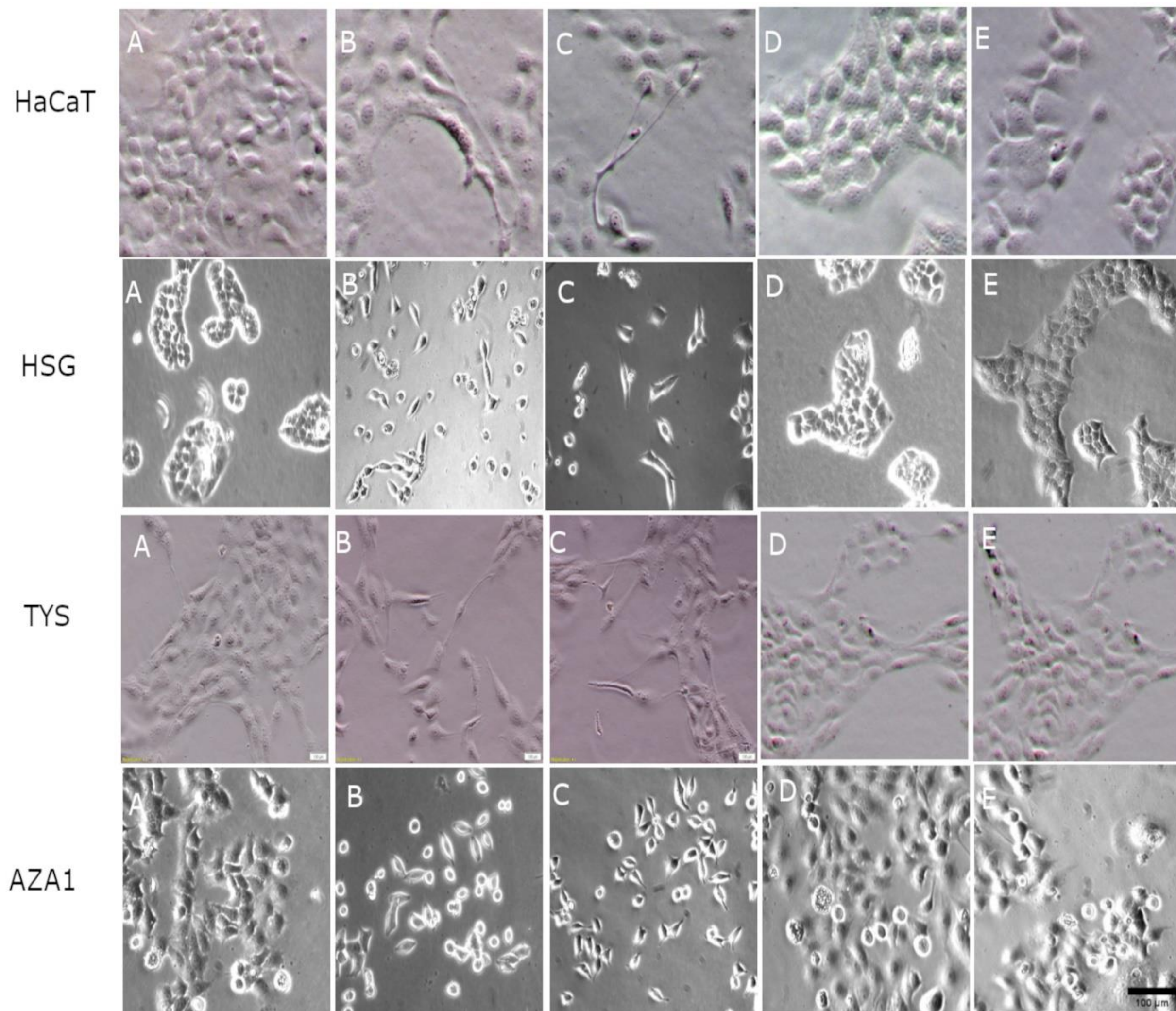

Figure3: Scatter Assay

Figure 3: Scatter Assay: Four cell types (HaCaT, HSG, TYS and AZA1) were plated in the dishes as described in the Material and Methods. Column A) serum free for 48 hours, B) EGF (50ng/ml) for 48 hours, C) TGFα (50ng/ml) for 48 hours, D) One-hour pre-incubation with PD98059 (25µM) before incubation with EGF (50ng/ml) for 48 hours, E) One-hour pre-incubation with PD98059 (25µM) before incubation with TGFα (50ng/ml) for 48 hours. Growth factors treated cells (B-C) displayed dendritic and/or spiky projection, and mesenchymal phenotype while PD98059 treated cells had no morphological change(D-E). (All images captured using an Olympus SC35 digital camera at x40 magnification).

Role of MEK inhibitor (PD98059) on collective cell migration is cell line dependent

Previous study found out EGF and TGFα induced collective cell migration and intensity of cell migration between cell lines is different. In this study, we used EGF and TGFα as positive control to stimulate collective cell migration. One hour pre-treated with 25µM PD98059 completely inhibited EGF and TGFα induced collective cell migration in HSG and AZA1 cell lines. In contrast, partial inhibition of collective cell migration was found in TYS and HaCaT cell lines. (Figure4-D&E).

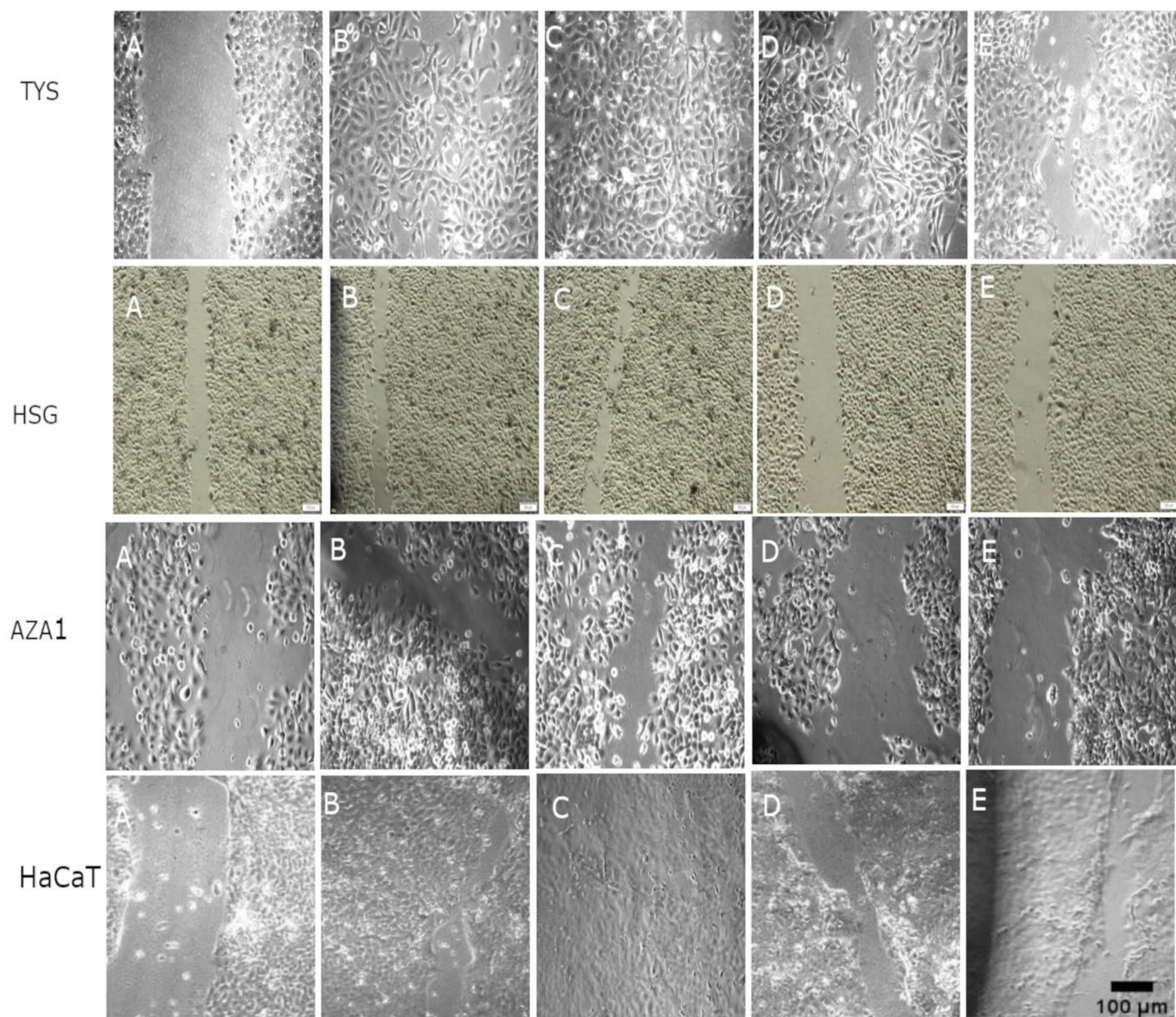

Figure 4 Scratch Assay: Four cell type were plated as described in the Material and Methods (TYS, HSG, AZA1 and HaCaT). Column A) serum free for 48 hours, B) EGF (50ng/ml) for 48 hours, C) TGFα (50ng/ml) for 48 hours, D) One-hour pre-incubation with PD98059 (25µM) before incubation with EGF (50ng/ml) for 48 hours, E) One-hour pre-incubation with PD98059 (25µM) before incubation with TGFα (50ng/ml) for 48 hours. Images have displayed variable migratory behavior between variable conditions All cell lines responded to 50ng/ml of EGF and TGFα over the 48-hour treatment (B-C). (All images were captured using an Olympus SC35 digital camera at x40 magnification). Columns D & E show that the PD98059 fully block the growth factor stimulated collective cell migration in HSG and AZA1 cell lines.

One-hour pre-treatment with MEK inhibitor (PD98059) inhibit EGF and TGFα induced EGFR internalization

Membranous staining of EGFR was detected in serum free treated cells. EGFR internalization was found in cells after incubation with EGF and TGFα for 48 hours. Nuclear staining was found in HSG cell line while cytoplasmic staining was found in TYS, AZA1 and HaCaT cell lines. In contrast, PD98059 treated cells maintained membranous staining (Figure5).

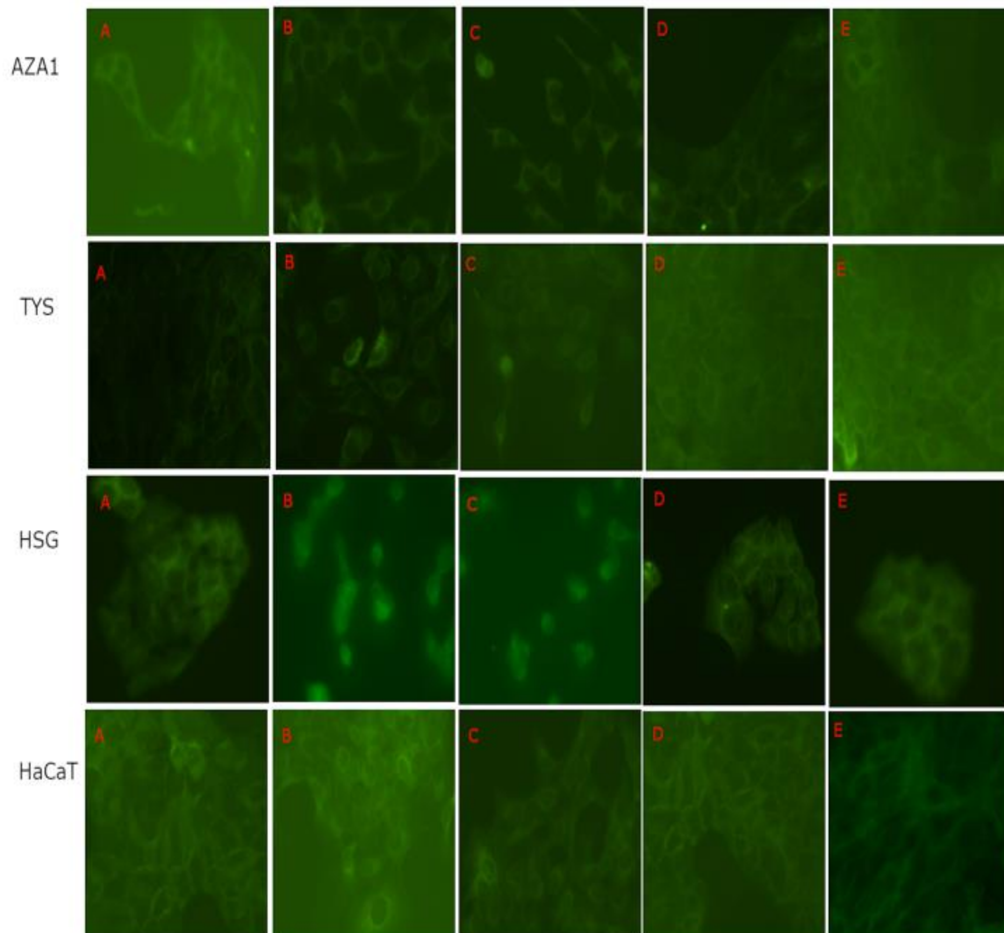

Figure 5-EGFR internalization

Figure 5 EGFR internalization: Four cell types (TYS, HSG, AZA1 and HaCaT) were plated as described in the Material and Methods. Column A) serum free for 48 hours, B) EGF (50ng/ml) for 48 hours, C) TGFα (50ng/ml) for 48 hours, D) One-hour pre-incubation with PD98059 (25µM) before incubation with EGF (50ng/ml) for 48 hours, E) One-hour pre-incubation with PD98059 (25µM) before incubation with TGFα (50ng/ml) for 48 hours. Immunofluorescence was visualised for total EGFR protein. Immunofluorescence was visualised for phosphorylated MAPK202/204. (All images were captured using an Olympus SC35 digital camera at x100 magnification).

One-hour pre-treatment with MEK inhibitor (PD98059) lower MAPK202/204 expression

In Immunofluorescence (Figure 6) with HSG and AZA1 cell lines, PD98059+EGF/TGFα completely inhibit MAPK202/204 expression induced by EGF and TGFα treatment. In contrast, MAPK202/204 is still expressed in PD98059+EGF/TGFα treated cells in TYS cell line.

HaCaT cell line did not display MAPK202/204 expression in all condition.

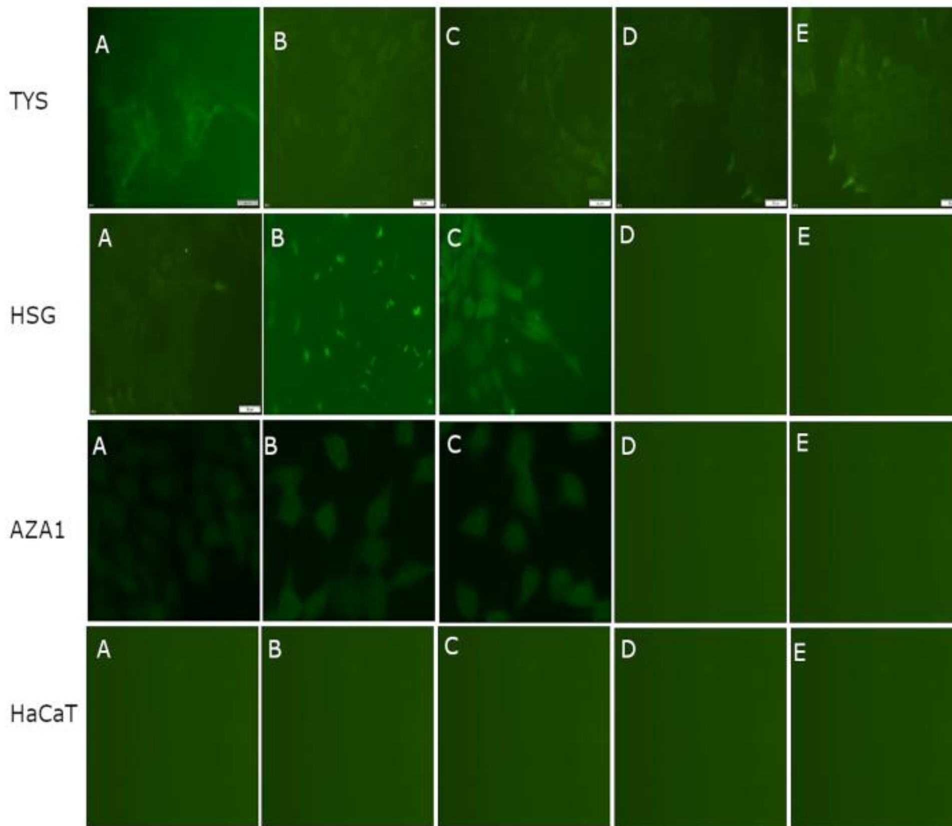

Figure 6: Immunolocalisation of phosphorylated MAPK202/204. Four cell types (TYS, HSG, AZA1 and HaCaT) were plated as described in the Material and Methods. Column A) serum free for 48 hours, B) EGF (50ng/ml) for 48 hours, C) TGFα (50ng/ml) for 48 hours, D) One-hour pre-incubation with PD98059 (25µM) before incubation with EGF (50ng/ml) for 48 hours, E) One-hour pre-incubation with PD98059 (25µM) before incubation with TGFα (50ng/ml) for 48 hours. Immunofluorescence was visualised for phosphorylated MAPK202/204. (All images were captured using an Olympus SC35 digital camera at x100 magnification). Note only 3 cell types expressed MAPK202/204 (TYS, HSG and AZA1). It should be noted addition of PD98059 (D-E) inhibited pMAPK in HSG and AZA1 cell line but not in TYS cell line.

Using Western blotting (Figure 7a), cells treated PD98059+EGF/TGFα(Lane 4 & Lane 5) had lower MAPK 202/204 expression than control (Lane 1 for serum free , Lane 2 for EGF and Lane 3 for TGFα).

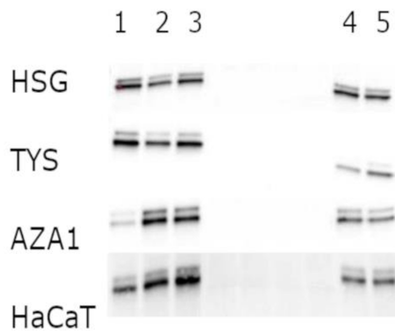

Figure 7a-phosphorylation of MAPK 202/204

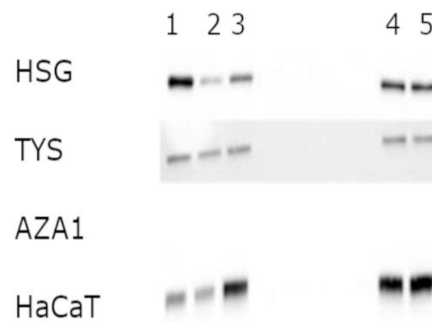

Figure7b-Phosphorylation of AKT 473

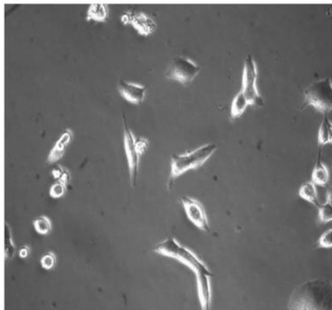

Figure7c-single cell migration

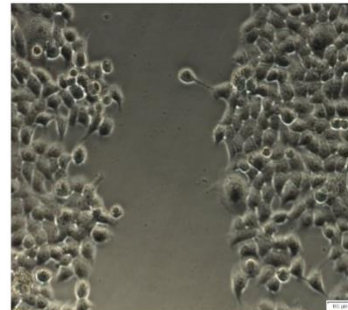

Figure7d-collective cell migration

Figure 7a: Phosphorylation of MAPK 202/204 protein. the cells were lysed after treating in experimental conditions for 24 hours. Cell lysates were run on SDS- PAGE and blotted onto nitrocellulose. The nitrocellulose was probed as described above. Lane 1) serum free for 24 h, 2) EGF (50 ng/ml) for 24 h, 3) TGFα (50 ng/ml) for 24 h, 4) One- hour pre- incubation with PD98059 (25 µM) before incubation with EGF (50 ng/ml) for 24 h, 5) One- hour pre- incubation with PD98059 (25 µM) before incubation with TGFα (50 ng/ml) for 24 h. Images were captured using GelDoc system.

Figure 7b: Phosphorylation of AKT 473, the cells were lysed after treating in experimental conditions for 24 hours. Cell lysates were run on SDS- PAGE and blotted onto nitrocellulose. The nitrocellulose was

probed as described above. Lane 1) serum free for 24 h, 2) EGF (50 ng/ml) for 24 h, 3) TGFα (50 ng/ml) for 24 h, 4) One- hour pre- incubation with PD98059 (25 µM) before incubation with EGF (50 ng/ml) for 24 h, 5) One- hour pre- incubation with PD98059 (25 µM) before incubation with TGFα (50 ng/ml) for 24 h. Images were captured using GelDoc system.

Figure 7c: Single cell migration showing mesenchymal and amoeboid phenotype

Figure 7d: Collective cell migration showing leader and follower cell.

One-hour pre-treatment with MEK inhibitor (PD98059) increased AKT473 phosphorylation

Using Western blot (Figure 7b), In HSG and HaCaT cell line. PD98059+EGF/TGFα had higher AKT473 phosphorylation than growth factor treated cells. In TYS cell line, AKT 473 phosphorylation is same level with growth factor treated cells. AZAT cell line showed no AKT phosphorylation. AKT473 phosphorylation was not found in Immunofluorescence (data not shown).

Discussion

In normal homeostasis, cells are connected to each other by cell-to-cell adhesions and cell to extracellular matrix (ECM) adhesion [4]. As described in the introduction, activation of ERK induced cell motility is by the production of matrix degrading substances. In general, cells undergoing the process of EMT tend to migrate by one of two main mechanisms: single cell migration and collective cell migration. Single cell migration is the migration of cells as individuals with no cell-to-cell interaction. There are two different phenotypes in single cell migration (amoeboid and mesenchymal phenotype) Figure7c. Amoeboid phenotype cells are rounded in shape and have a number of different variants. The mesenchymal phenotype cells have an elongated cell body with longer protrusions. The cells differ in terms of contractility: amoeboid showing increased contractility (under the influence of the Rho signalling) while the mesenchymal phenotype expresses low contractility [5]. Single cell migration through an interstitial tissue displays a five-step process occurring through a cyclical process: Step 1-Protrusion of leading edge (such as filopodia, lamellipodia, podosomes and invadopodia). Step 2- adhesion force generation. Step 3- focalized proteolysis. Step 4- Actin-myosin contraction, and Step 5- rear retraction and path release [27].

Although, single cell migration has higher initial velocity, they tend to change direction quickly and be less persistent [28].

Multicellular streaming is between single and collective behaviour. The cells are moving in same direction with no cell-to-cell contact. They also display amoeboid or mesenchymal phenotype [5].

Collective cell migration is the migration of cells as a group or sheet. They also display five cyclical processes previously mentioned. In collective cell migration, cells are moving in the same direction and at similar speed. Although collective cell migration is slower than single cell migration, it is more efficient than single cell migration. There are two types of cells in collective cell migration based on their relative position between cell clusters. The first type of cell are leader cells which lead and second is follower cells (Figure7d). The leader cell has the ability to sense the microenvironment, interact with ECM, and is responsible for direction and speed of migration of the whole cluster. The follower cells have the ability to control their leader and are important in polarization and chemotaxis. A successful coordination of the leaders and followers induces collective cell migration. In both vivo and vitro, leaders and followers' cells can exchange their places and roles [28,29]. In collective cell migration, the cells can display mesenchymal or epithelial phenotype. Cells with amoeboid morphology tend to use single cell migration or multicellular streaming while cells with epithelial phenotype tend to use collective cell migration. Cells with a mesenchymal phenotype have ability to switch between single cell migration, multicellular streaming and collective migration modes [5].

In EMT process, the cells morphologically transformed from colonies to spindle shape and fibroblast-like phenotype together with molecular changes (down regulation of epithelial markers and upregulation of mesenchymal markers) [7]. EMT like morphological transformation was found in incubation with 50ng/ml EGF and TGFα after 48 hours [25]. However, significant changes in molecular level of EMT (down regulation of E-cadherin and up regulation of Vimentin) was not found in both Western blot (24hr incubation) and Immunofluorescence techniques (48hrs incubation). This is an unusual finding and data may look negative. However, one study reported a down regulation of E-cadherin after 72 hours incubation with EGF [30]. This leads us to speculate that the amount of time needed to induce EMT at molecular level is longer than morphologically EMT in certain cell lines.

In this study, EMT-like morphological change, cellular projection, cell scattering/ or single cell migration induced by EGF and TGFα was completely inhibited by PD98059 (MEK inhibitor). The results suggest MAPK pathway might be only pathway responsible for these changes.

EGF and TGFα also induce collective cell migration in HSG, TYS, AZA1 and HaCaT cell line with closure of the scratch line. In contrast, PD98059 inhibit collective cell migration in HSG and AZA1 but not in HaCaT and TYS cell line. This data suggest role of MAPK pathway in collective cell migration is cell line dependent and other pathways might involve in it. There is limited information available on why single cell migration and collective cell migration use different signalling pathway in same cell types. These are still to be investigated

In MDA-231 cell line, MEK inhibitor PD98059 does not have ability to inhibit EGF induced cell migration [31]. Some study reported that activation of MAPK pathway alone is not sufficient to induce EGF stimulated cell migration and PLCγ is also required [32]. In high EGFR expressing breast cancer cell lines MDA-468, combine inhibition of ERK and PKC completely block TGFα induced cell migration. In MCF-7 cell line with high EGFR expression, early stage of cell migration (up to 4 hours) is regulated by ERK pathway while later stage (after 4 hours and up to 12 hours) is controlled by PKC γ pathway. This suggests the use of other signalling pathway inhibitors in future experiment.

In contrast, MCF-7 cell line with low EGFR expression, blocking of MAPK pathway alone is necessary for inhibition of cell migration [33].

Localization of EGFR is important in evaluating prognosis of patients. EGFR membranous staining is associated with good prognosis while cytoplasmic staining was associated with poor prognosis [34]. In contrast, EGFR Nuclear staining is related with transcription factor activity of EGFR [35]. In our results, PD98059 + growth factors treated cells-maintained EGFR membranous staining encouraging potential use of prognosis marker.

Western blot data reported MAPK202/204 expression in EGF and TGFα treated cells were more prominent than EGF/TGFα ±PD98059 treated cells.

In Immuofluorescence data, MEK inhibitor (PD98059) inhibit MAPK 202/204 expression in HSG and AZA1 cell lines but could not block its expression in TYS cell line. No sign of MAPK expression was found in HaCaT. Since Immunofluorescence data represents the certain area of the dishes, it is not reliable as Western blot which represent the total protein expression.

In this study, we found out MEK-inhibitor PD98059 increase AKT 473 phosphorylation in multiple cell lines. Other studies have shown similar report even through underlying mechanism is not clearly understand [36]. This suggests dual inhibition of AKT and MAPK pathways to overcome the problem.

Conclusions

Role of MAPK signalling pathway in cell migration is cell line dependent. In HSG and AZA1 cell line, Ras-Raf-MEK-ERK pathway is only pathway the cells used to induce cellular projections, EMT, cell scattering/single cell migration and collective cell migration. In TYS and HaCaT cell lines, there is a clear separation of signalling pathways between single and collective cell migration. Ras-Raf-MEK-ERK pathway is only pathway to induce EMT and single cell migration while other additional signalling pathways might involve in collective cell migration. These encourage personalized therapy and use of dual pathway inhibitors to completely inhibit both types of cell migration in the future.

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