Reports of Biochemistry & Molecular Biology
Vol.10, No.2, Jul 2021

Original article

www.RBMB.net

Roles of Endocytic Processes and Early Endosomes on Focal Adhesion Dynamics in MDA-MB-231 Cells

Bandar Fahad Alharbi[1], Dhurgham Al-Fahad*[2], Philip Richard Dash[3]

Abstract

Background: Focal adhesion (FA) play a critical role in many biological processes which include cell survival and cell migration. They serve as cellular anchor, allowing cells to stay attached to the extracellular matrix (ECM), and can also regulate cellular transduction. Previously, it has been suggested that vesicles such as endosomes could interact directly with FA or be implicated in their turnover. In this study, we investigated whether there is a relationship between FA and the early endocytic machinery in MDA-MB-231 cells.

Methods: In this study, cell culture, transfection, time laps confocal microscopies, immunocytochemistry, western blotting, Cell fractionation and immunoprecipitation techniques were performed.

Results: Cells acutely treated with Dynasore, an inhibitor of dynamin, or with Pitstop 2, an inhibitor of clathryn-dependent endocytosis showed a reduction in the expression of early endosome biomarkers such as Rab5 and EEA1. Additionally, cells treated with these endocytic inhibitors exhibited an increase number and size of FA, as well as an increase FA turnover duration. This data was consistent with the reduction of the speed of cell migration. We demonstrated that Rab5- and EEA1-positive early endosomes were found to be colocalized with internalized FA.

Conclusions: The present study suggests that there is a link between FA and early endosome markers, which indicates that the early endosomes may be involved in FA dynamics.

Keywords: Cell migration, Early endosome, Endocytosis, Focal Adhesion.

Introduction

Endosomes are intracellular vesicles that transports endocytic materials either toward the lysosomes, where the content is degraded, or toward the plasma membrane, where the content is recycled. Endosomes play a key role in cellular transduction (1). As previously demonstrated, cell signalling mediated by plasma membrane receptors can be diminished following their internalization and transient sequestration in endosomes (2). Conversely, endocytosis defects can result in the accumulation of plasma membrane receptor, which in turn causes the over-activation of their respective signalling pathways. For example, a defect in epidermal growth factor receptor (EGFR) (a protein implicated cellular proliferation internalization) result in its accumulation in the plasma membrane, which in turn may results in the over-activation of cellular proliferation (2). There are three types of endosomes: early, late and recycling, each distinguishable by different protein markers. The early endosome compartment is the first compartment that

1: Department of clinical laboratory, College of Applied Medical Science, University of Hail, Hail, 81411, Saudi Arabia.
2: Department of Pathological Analysis, College of Science, University of Thi-Qar, Thi-Qar, 64001 Iraq.
3: Department of Biomedical, School of Biological Sciences, University of Reading, Reading, RG6 6UB, United Kingdom.
***Corresponding author: Dhurgham Al-Fahad; Tel: +009647736332794; E-mail: dhurgham.alfahad@sci.utq.edu.iq.**
Received: 21 April, 2021; Accepted: 22 April, 2021

labels the incoming cargo and then transports the latter to its proper destination (3,4). The lumen of early endosomes is slightly acidic, usually ranging between pH 5.0 to 6.8 (4,5). Cargoes (e.g., receptors) recruited by early endosomes originate from different endocytic pathways, such as clathrin-dependent, caveolae-dependent, micropinocytosis and ADP-ribosylation factor 6 (ARF6)-dependent pathways (5). Ultrastructure analysis of early endosomes showed that they are made up of vesicular structures and multiple tabulated membranes (6). The vesicular structures are generally 700 nm in diameter, while the tabulated membranes range from 50 to 60 nm in diameter.

Focal adhesions (FA) are multi-protein complexes that connect the cytoskeleton to the extracellular matrix (ECM) (7). Constituents of FA, such as FA proteins (e.g., vinculin, paxillin, talin or zyxin) and kinases (e.g., FA kinase (FAK)) can provide sufficient attachment to ECM (8). FA act as molecular clutches which can promote the formation of cellular protrusion (9). In addition, FA play a critical role in many physiological processes such as cell survival, morphology, proliferation, and cell migration (10). For example, in order to enable cells migration, some FAs undergo a rapid assembly and disassembly, a process referred as FA turnover (11,12).

As previously shown, the inhibition of dynamin and clathrin endocytosis pathways with (inhibitors) results in the reduction of early endosomal compartments and cell migration, suggesting that endocytosis may contribute to cell migration (13,14). This is consistent with the findings showing that the polarization and FA recycling occurred at the leading edge of cells where Rab5, an early endosomal marker, accumulates. FAK and the proto-oncogene tyrosine-protein kinase Src, both implicated in FA turnover, have been found to be regulated by SNARE proteins, a group of endocytic proteins involved in membrane fission and trafficking (15). FA turnover is reduced with the inhibition SNARE proteins, which in turn impairs Src trafficking and FAK phosphorylation (15). To date, the role of endocytic process and Rab5-positive endosomes in FA dynamics is not clearly understood. In this study, we aimed to identify the possible link between FA and the early endosomes.

Materials and Methods

Cell lines and cell culture condition

MDA-MB-231 Human Invasive Breast Cancer Cell Line (ATCC® HTB-26™) were purchased from the American Type Culture Collection's cell bank (Manassas, VA). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) mixed with 10% foetal bovine serum (FBS) (Gibco, A4766801) and 1% penicillin/streptomycin (v/v, 10^4 units/mL penicillin, 10^4 µg/mL streptomycin (Gibco). For optimal cell growth, these cells were incubated at 37 °C, under atmospheric conditions containing: 95% air, 5% CO_2, and approximately 90% humidity. An EZ-PCR Mycoplasma Test kit (Geneflow, K1-0210, UK) was used to test cells regularly for mycoplasma.

Chemicals

Dynasore (20 µM) (Selleckchem, S8047) and Pitstop 2 (25 µM) (Abcam, ab120687) were used to inhibit dynamin and clathrin-mediated endocytosis, respectively. The vehicle used in the control consist of 0.1% dimethyl sulphoxide (DMSO).

Transfection

The 35 mm ibidi dishes (Thistle Scientific, IB-81158) were coated with 2 mg/mL rat tail type 1 non-pepsinized collagen diluted in DMEM (pH adjusted to 7.0 with NaOH) (BD Bioscience, 354236). MDA-MB-231 cells were seeded at a density of 1 x 10^5 cells/mL (2 mL) in ibidi dishes, and were incubated overnight, under the cell culture conditions mentioned above. These cells were then transfected with 3 µg plasmid DNA mixed with the TurboFect™ transfection reagent (Thermo Scientific, R0532), at a ratio of 1:1 (w/v, plasmid DNA: TurboFect). These plasmid DNA consist of constructs expressing

either mCherry-zyxin. After 16 h, cells were treated with Dynasore or Pitstop 2 or the vehicle, for 30 min, while live-cell imaging was performed at 100x magnification using confocal microscopy (Nikon A1R).

Endosome-tracking and FA turnover assays

Cells were seeded in a 35 mm ibidi dish at a density of 1 x 10^5 cells/mm^2 and were incubated overnight, under the cell culture conditions. The cells were then transfected with mCherry-zyxin and were transduced with the CellLight™ Early Endosomes-GFP reagent (BacMam 2.0, C10586), which contains a Rab5-GFP construct; the volume of reagent added used for transduction had a concentration 30 particles per cells. After 24 h, these cells were subsequently treated for 30 min with Dynasore or Pitstop 2 or the vehicle (used as control). Live-cell imaging was performed using confocal microscopy (Nikon A1R). The fluorescence of GFP and RFP (or mCherry) were detected at 488 nm/510 nm and 568 nm/590 nm (excitation/emission), respectively. Images were taken every 5 s for 5 min using a 100x objective lens. The duration of FA turnover (defined as the time covering FA assembly and disassembly) was measured using ImageJ. To analyze the number and size of FA, the cells were fixed, and the images were captured digitally using the same method as described above.

Cell migration assay

MDA-MB-231 breast-cancer cells (1 x 10^5 cells/mL) were seeded onto 12-well-plates coated with 10 µg/mL fibronectin (BD Bioscience, 610077); the cells were incubated overnight under the cell culture conditions described above. These cells were placed onto the head stage of a time-lapse microscope (Nikon Eclipse TiE), heated at 37 °C and supplied with 5% CO_2, where they were treated for 24 h with Dynasore or Pitstop 2 or the vehicle, while images were taken every 15 min at 10x magnification, using a Nikon DXM1200 camera controlled with the Nikon Eclipse TE200 running NIS elements software. The MtrackJ tool of the ImageJ software was used to determine the distance covered by individual cells. The speed of migration (expressed in µm/h) was calculated as a total distance covered by individual cells divided by 24 h.

Western blot analysis

Cells (1 x 10^5 cells/mL) treated with Dynasore, Pitstop 2 or the vehicle for 30 min were lyzed and clarified by centrifugation (14,000 x *g*, 10 min). Protein concentration in cell lysates were determined using the Bradford protein assay; the absorbance was measured at 595 nm. The sample lysates containing 30 µg were denatured at 95 °C for 5 min in a loading buffer containing sodium dodecyl sulphate (SDS) and β-mercaptoethanol. Protein separation was performed by SDS-polyacrylamide gel electrophoresis (150 V for 1 h), using a 10 or 12% gel. Proteins in the gel were transferred onto a polyvinylidenedifluoride (PVDF) membrane using a semi-dry transfer. The membranes were incubated for 1 h at room temperature in a blocking solution containing 5% of dry milk dissolved in Tris-Buffered Saline Tween (TBST). Subsequently, the membranes were incubated overnight at 4 °C under agitation with primary antibodies raised against Rab5 (Cell Signalling, #3547) and EEA1 (Cell Signalling, #24115; Cell Signalling, #3288) diluted in 5% dry milk or 5% dry bovine serum albumin (BSA), both dissolved in TBST. The membranes were washed 3 times in TBST (10 min each time). Anti-rabbit (Sigma-Aldrich, AP307P) and anti-mouse (Sigma-Aldrich, AP306P) HRP-conjugated antibodies diluted in 5% dry milk (solubilized in TBST) were added to the membrane and was left to incubate for 1 h at room temperature under agitation. Excess of these secondary antibodies was removed with 3 washes with TBST (10 min each time). The membranes were subsequently washed three times with TBST (10 min each time) and an enhanced chemiluminescence solution was added to the membrane. Images of the immunoreactions were acquired using

the ImageQuant LAS 4000 imager (GE Healthcare Life Sciences) and Image Quant TL software.

Immunocytochemistry

MDA-MB-231 cells (1 x 10^5 cells/mL) seeded in 6-well plates, on glass coverslips coated with fibronectin (10 µg/mL) were incubated overnight, under cell culture condition. Some cells were incubated serum free DEMEM containing either transferrin conjugated to Alex Fluor-546 (Thermo Fisher, T13342) (25 µg/mL) for 30 min at room temperature. Prior to immunostaining, all cells were washed with phosphate-buffered saline (PBS); 4% paraformaldehyde (PFA) diluted in PBS was used to fix the cells for 20 min at room temperature. The excess of PFA was removed with PBS. Thereafter, cells were permeabilized at room temperature for 10 min with PBS containing 0.5% Triton X-100. 10% goat serum diluted in PBS was used as blocking agent. Cells were probed with different primary antibodies: anti-EEA1, anti-APPL1 (Cell signalling, #3858), anti-Rab5, anti-paxillin (ThermoFisher, MA5-13356; Abcam, ab32084), and anti-vinculin (Abcam, ab130007; Abcam, ab129007). Excess of these primary antibodies was removed by sequential washes with PBS (3 times, 10 min each time); cells were incubated for 1 h with corresponding anti-IgG antibodies conjugated to Alexa Fluor 488/546 (Cell Signalling, #44085; ThermoFisher, A-11030). Cells were visualized by confocal microscopy and the images were captured digitally at 100x magnification. ImageJ was used to split the captured images from different filters, then processed through background fluorescence subtraction, and analyze for protein localization to determine whether the localization of different proteins was correlated using Spearman rank coefficient.

Cell fractionation

Cells (1 x 10^5 cells/mL) grown overnight in T75 flasks were washed twice with 5 mL of chilled PBS. These cells were removed from flask in the presence of PBS containing Tween20 (0.1%), using a rubber policeman. Cell suspensions were centrifuged at 1500 x *g* for 15 min at 4 °C and the pellets were re-suspended in 4 mL of chilled homogenization buffer (HBA), containing 220 mM Mannitol, 70 mM Sucrose, 5 mM HEPES and 1 mM EGTA (pH adjusted to 7.4 with KOH). Cells were homogenized on ice with a Teflon homogenizer (10 times) on ice. Subsequently, the homogenates were centrifuged at 750 x *g* for 10 min to remove cells debris and large organelles such as the nucleus. The supernatant was added to 90 % Percoll (diluted in HBA, and the pH adjusted to 7.4) to achieve a final concentration of 27% of Percoll in a volume of 8.5 mL. This mixture was layered over a 1 mL 2.5 M sucrose solution and was centrifuged at 29,000 x *g* for 90 min at 4 °C. 8 fractions (whitish color) were observed, but fractions 3 and 8 were the most abundant (Fig. 1). Fraction 3 to Fraction 8 were collected starting with a plastic Pasteur pipette (1 mL for each fraction). Finally, the fractions were lyzed with RIPA buffer and a western blot was performed to detect EEA1, Rab5, vinculin, paxillin, H2B (Abcam, ab52484), Actin (Abcam, ab179467) and GAPDH (Sigma-Aldrich, G8795).

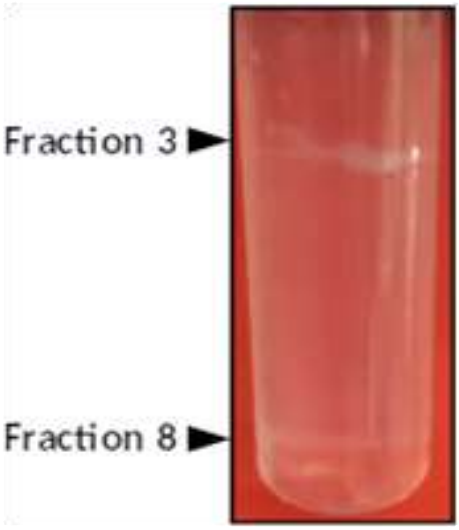

Fig. 1. Appearance of a centrifuge tube following the Percoll-Sucrose gradient centrifugation step. 8 fractions resulted from this subcellular fractionation of which, fraction 3 and fraction 8 were found to be the most visible.

Co-immunoprecipitation assay

The lysate of fraction 3 (containing 200 µg protein) was incubated overnight at 4 °C with either anti-EEA1 or anti-Rab5 or anti-IgG, under agitation. 100 µL of RIPA buffer containing Protein A/G agarose beads (20-50 µL) (Thermo Scientific, 78609) were added to the sample and were incubated for 2 h at 4 °C, under agitation. The samples mixed with Protein A/G agarose beads were then centrifuged at 2000 x *g* for 1 min; the pellet was washed 3 times with 300 µL with RIPA buffer. 100 µL of loading buffer (containing SDS and β-mercaptoethanol) was added to the pellet. The proteins were denatured for 5 min at 95 °C and western blot was performed to detect vinculin.

Statistical analysis

Data obtained from imaging were statistically analyzed with GraphPad prism 5 (GraphPad software, San Diego, CA). One-way analysis of variance (ANOVA) was performed on data comprising three experimental groups. Following a significant difference identified via ANOVA, Dunnett's post-hoc test was used for pairwise comparisons in order to compare each treatment to a single control (vehicle). All results were obtained from at least three independent experiments in triplicate (n= 3). A value of $p \leq 0.05$ was considered statistically significant.

Results

Effect of endocytotic pathways inhibitors on the expression of early endosome markers and the organization of FA

To assess the effect of Dynasore and Pitstop 2 on the level of early endosome proteins, western blots were performed to determine their effect on Rab5 and EEA1 expression. The inhibition of endocytic pathways with Dynasore and Pitstop 2 reduced the expression of Rab5 and EEA1, both expressed in % ratio normalized to Actin (Fig. 2). The expression of Rab5 decreased from 51.1±1.0 (in vehicle-treated cells) to 37.2±3.3 ($p< 0.05$) and 28.0±3.9 ($p< 0.01$) in Dynasore and Pitstop 2 treated cells, respectively (Fig. 2A). EEA1 expression decrease from 92.0±2.6 (in vehicle-treated cells) to 61.2±7.6 ($p< 0.05$) and 34.0±6.8 ($p< 0.05$) in cells treated with Dynasore and Pitstop 2, respectively (Fig. 2B). Next, we assessed the effect of Dynasore and Pitstop 2 on the number and the size of vinculin-positive FA by immunocytochemistry. The results showed that both endocytic inhibitors increased the number of FA containing vinculin, as the number increased from 37±1 per cell (in vehicle-treated cells) to 43±1 per cell ($p< 0.05$) and 43±0 per cell ($p< 0.05$) in cells treated with Dynasore and Pitstop 2, respectively. Dynasore and Pitstop 2 were shown to increase the size of vinculin-positive FA from 0.83±0.04 μm^2 to 1.38±0.07 μm^2 ($p< 0.05$) and 1.37±0.09 μm^2 ($p< 0.05$), respectively (Fig. 2C).

Effect of endocytotic pathways inhibitors on the duration FA turnover and cell migration

We subsequently evaluated the effect of Dynasore and Pitstop 2 on the duration of zyxin-positive FA turnover. Dynasore increased the duration of zyxin-positive FA turnover from 54.5±3.5 s (in vehicle-treated cells) to 80.9±2.1 s ($p< 0.05$) (Fig. 3A). Cells treated with Pitstop 2 displayed a higher duration of FA turnover (108.3±5.7 s, $p< 0.01$) in comparison to that of vehicle-treated cells. The assessment of the effect of Dynasore and Pitstop 2 on cell migration showed that both inhibitors reduced the speed of cell migration; Dynasore and Pitstop 2 decreased cell migration from 25.5±0.9 µm/h ($p< 0.05$) (in vehicle-treated cells) to 0.6±0.3 µm/h ($p< 0.05$) and 19.7±0.8 µm/h ($p< 0.05$), respectively (Fig. 3B and Fig. 3C).

Relationship between endosomes and focal adhesions

In order investigate the potential link between early endosomes and FA, we began by assessing whether early endosomes and FA were colocalized in MDA-MB-231 cells. Our result showed that the localization of Rab5 and EEA1 were highly correlated (0.9±0.0) (Fig. 4A). Cells labelled with paxillin and Rab5 (0.6±0.2) showed that the localizations of both

proteins were correlated in the same manner as the localizations paxillin and EEA1 (0.6±0.2) (Fig. 4A). Since the internalization of transferrin receptors is a well-established method to visualize endosome transport and recycling, we sought out to determine whether transferrin can be enriched around or within the FA and early endosome markers. Our result showed that the localizations of paxillin and transferrin, which was colocalized to EEA1 were found to be correlated (0.6±0.1). Similarly, the localizations of paxillin and transferrin, which was colocalized with Rab5 were also found to be correlated (0.6±0.1) (Fig. 4A). Subsequently, we investigated whether FA were localized in early endosomes in cells co-transfected with mCherry-zyxin and GFP-Rab5 constructs Live-cell imaging performed over a period of 5 min, showed that 40% of endosomes contained zyxin, with a correlation coefficient of 0.7±0.1, while 32% of zyxin-positive FA was colocalized with Rab5, with correlation coefficient of 0.7±0.0 (Fig. 4B). This observation was further confirmed through the analysis of Rab5 and zyxin co-localization over time. This was performed by taking 1 image every 5 s for 20 min. The results showed that the average live time of colocalization between GFP-Rab5 and mCherry-zyxin shown to be around 3 min then disappear or collapse (Fig. 4B), suggest that FAs are targeted by early endosomes.

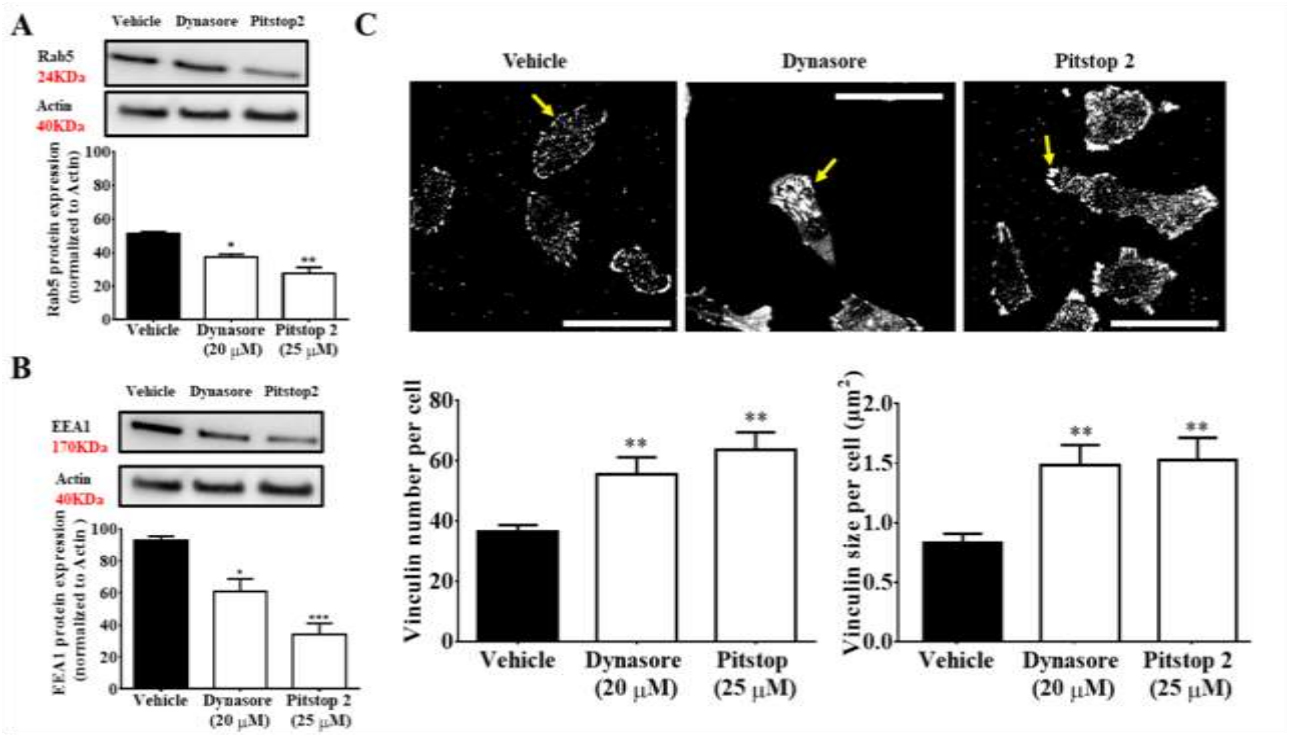

Fig. 2. Effect of Dynasore and Pitstop 2 on the expression of early endosomal proteins and FA in MDA-MB-231 cells. Cells were treated for 30 min with the dynamin inhibitor Dynasore (20 µM) or the inhibitor of clathrin-mediated endocytosis Pitstop 2 (25 µM). The effects of these inhibitors were compared to that with the vehicle (0.1% DMSO). **A)** Western blot analysis showing the reduction of Rab5 expression in Dynasore or Pitstop 2 treated cells. B) Western blot analysis showing the reduction of EEA1 expression in Dynasore or Pitstop 2 treated cells. C) Immunocytochemistry analysis showing an increase number and size of vinculin-positive FA in Dynasore or Pitstop 2 treated cells. The scale bar represents 50 µm. The graphs represent the mean±standard error of 24 cells (100 FA) from three independent experiments. Dunnett's multiple comparison test was performed following One-way ANOVA. Statistical significance differences were accepted at **p< 0.01.

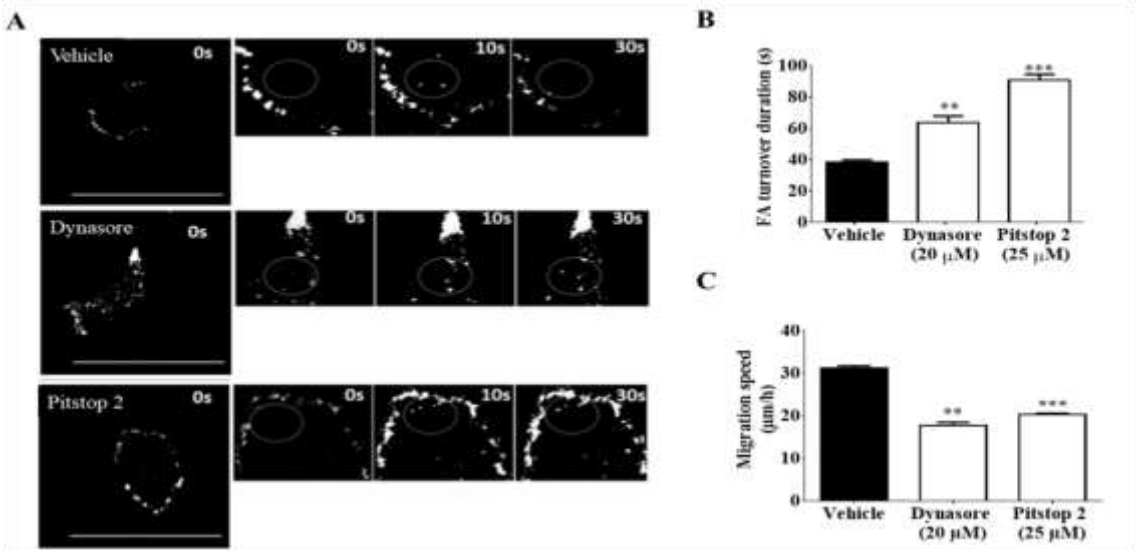

Fig. 3. Effect of Dynasore and Pitstop 2 on FA turnover and cell migration in MDA-MB-231 cells**.** Cells were transfected with mCherry-zyxin then were treated 24 h post-transfection with the vehicle, Dynasore or Pitstop 2. A) Live cell images of mCherry-zyxin positive MDA-MB-231 cells treated with the vehicle, Dynasore or Pitstop 2. B) Pitstop2 and Dynasore increase the duration of FA turnover. C) Pitstop2 and Dynasore reduce the speed of cell migration. The white circles show the region where the turnover of zyxin-positive FA was analyzed. The scale bar represents 50 µm. The graphs represent the mean±standard error of 24 cells (100 FA) from three independent experiments. Dunnett's multiple comparison test was performed following One-way ANOVA. Statistical significance differences were indicated as **p< 0.01 and ***p< 0.001.

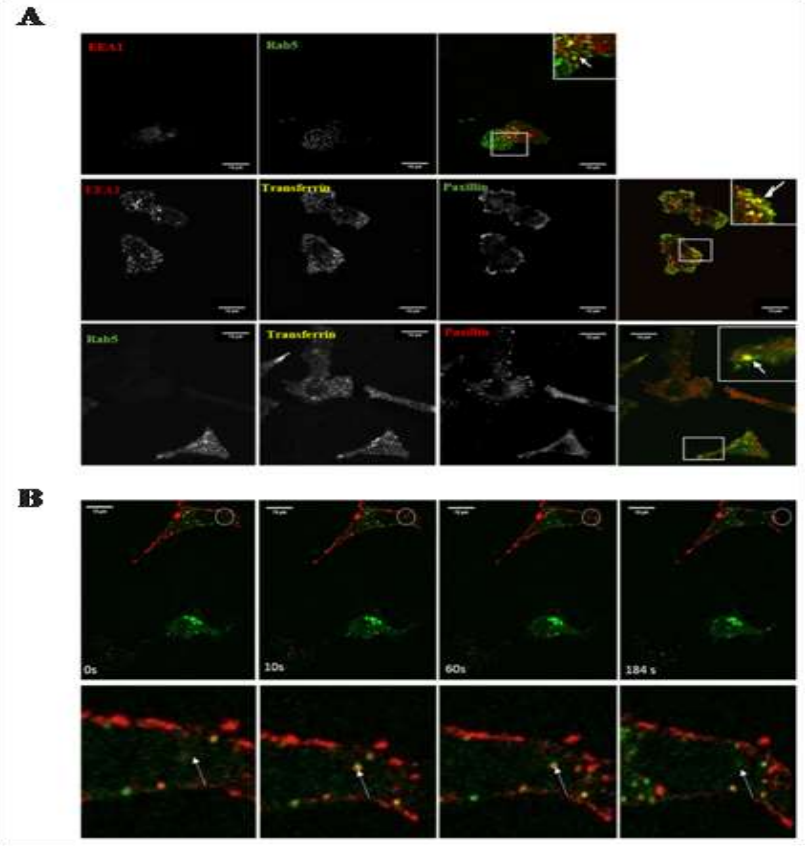

Fig. 4. Analysis of the cellular localization of Rab5, EEA1 and paxillin-positive FA in MDA-MB-231 cells. A) Fluorescence immunostainning showing the colocalization of Rab5 and EEA1. Cells incubated for 30 min with Transferrin-conjugated Alexa Fluor-546 (25 µg/mL) show that Transferrin, EEA1, Rab5 and paxillin are localized. B) Live cell images of mCherry-zyxin and GFP-Rab5 positive cells shows that colocalized zyxin (red) and Rab5 (green) present identical turnover kinetic for both proteins over a 5-min period. The white circle shows the region where the turnover of mCherry-zyxin and GFP-Rab5 were analyzed. The scale bar represents 19 µm. Three independent experiments were performed on at least 3 cells for each experiment.

Detection of FA proteins in endosomal subcellular fractions

To confirm whether FA proteins are localized in early endosomes, MDA-MB-231 cells were fractionated into 8 fractions in a medium containing isotonic Percoll layered above a sucrose solution. The content of proteins was analyzed via western blot. Our result showed that all endosomal organelles were present in fraction 3 and 4 since it contained both Rab5 and EEA1. Both fractions were devoid of nuclei as no H2B was detected. FA proteins such as vinculin and paxillin were also found in fraction 3 and 4 together with the cytosolic proteins GAPDH and actin (Fig. 5A). Vinculin was found in other fractions aside from fraction 3 and 4, although the level of expression was higher in the latter fractions. Actin was also shown to be present in fraction 5. Next, we evaluated whether Rab5 and EEA1 from fraction 3 binds to vinculin. Vinculin was found amongst the materials resulting from Rab5 and EEA1 immunoprecitation (Fig. 5B).

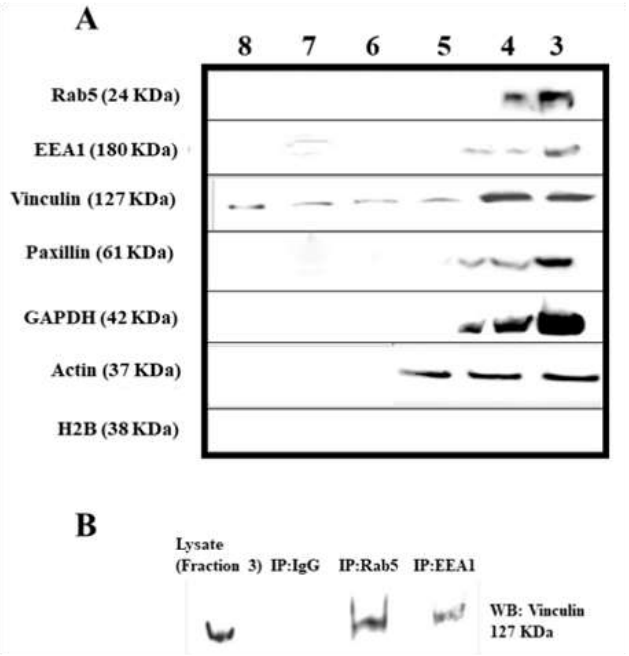

Fig. 5. Distribution of proteins in different fractions separated following subcellular fractionation. A) Western blot analysis showing the distribution EEA1, Rab5, vinculin, paxillin, GAPDH, Actin and H2B in different subcellular fractions. B) Western blot analysis showing the presence vinculin in samples co-immunoprecipitated with anti-Rab5 and anti-EEA1 antibodies from fraction 3.

Discussion

In this study, we investigated the role of endocytosis in FA regulation. The effect of Dynasore and Pitstop 2 on early endosome markers and FA dynamics were examined in MDA-MB-231 cells. FA are essential in clathrin-mediated endocytosis process through scission of newly formed vesicle from the membrane (16,17). Our results showed that both inhibitors decreased EEA1 and Rab5expression levels. The number and the size of FA was found to be increased in cells treated with both Dynasore and Pitstop 2.

Furthermore, we demonstrated that both inhibitors increased the duration of FA turnover and slowed down the speed of cell migration. Early endosome proteins act as station to direct upcoming molecules; their expressions is increased in aggressive cancer cells compared to normal tissue and primary cancer tissue; thus, they serve as prognostic markers for cancer progression (18,19). It is plausible that the inhibition of early endosome protein expression may impair endosome trafficking (20). Previously, it has been shown that the inhibition of endocytosis causes alterations on FA formation (16,17).

This might be due to the role of the GTPase activity of dynamin on the maturation of clathrin-coated vesicle (early and late stage of clathrin formation) (21). For example, at early stage, dynamin self-assembled around the endocytotic pit, and at late stage, present the catalytic activity that sections the plasma membrane (21). The efficiency at which this switch of dynamin function may occurred may also affect the speed of clathrin maturation and the recruitment FA complex. Pitstop 2 has previously been shown to induce failure clathrin assembly (22,23). It is plausible that this may lead to the failure of FA assembly as well as their disassembly.

Our data showed that early endosome markers, internalized transferrin and FA marker were colocalized, suggesting that endosomes may also play a regulatory role in FA dynamics. This is consistent with another study, which showed a direct interaction between dynamin 2 and FAK through the N-terminal FERM domain of FAK (24). Clathrin and paxillin have also been shown to interact (25). Scaffolding proteins such as paxillin are comprised of many binding motifs: LD motifs (N-terminal), LIM (C-terminal) and SH2 domains (24). The LD2 domain of Paxillin was demonstrated to bind clathrin heavy chain (26). A proteomic analysis demonstrated the presence of Rab5 and EEA1 within isolated integrin adhesion complexes (27). Furthermore, vinculin, another scaffolding protein has been found to be associated with endosomal vesicles and clathrin-mediated endocytosis (28,29). This is consistent with a study that demonstrated a colocalization between FA and clathrin adaptors (Dab2, AP-1 and numb), which may be found in the plasma membrane and early endosomes (17). Clathrin coated pits reportedly occurs in the vicinity of FA (30). Therefore, these complexes may be delivered to early endosomal compartments. This is in agreement with our findings, which showed the colocalization of a FA protein with early endosomes markers and transferrin taken up into the cells.

It has been suggested FA requires 3 to 5 min to be transferred from the plasma membrane to early endosomes or recycling compartments. Rab5 and zyxin were found to be colocalized during the FA turnover. Our findings showed that average time of colocalization between zyxin and the endosome marker was approximately 3 min. Giving that the total lifetime for clathrin coated pits is shorter, varying between 40 and 330 s (31), compared to the average lifetime (tracking duration) of GFP-Rab5, which was 3.7 min, a value in agreement with previous studies (32), this suggests that Rab5 is most likely regulated by clathrin-mediated endocytosis in MDA-MB-231 cells. This scenario is possible, as paxillin has been reported to be transported through late endosomes and degraded via autophagy (33,34). Integrins are recycled by the endosomal system every 30 (35). It has been shown that endocytosis of integrins mediates FA disassembly, whereas recycling integrins can help the formation and reassembly of FA (36). The results provided by this study offers additional evidence that FA can be taken into cells through endocytosis and shows a link between FA and the early endosomes.

Our study showed that the inhibition of endocytotic pathway reduced FA dynamics and cell migration. In addition, the localization of early endosome markers was found to be partially correlated with that of FA proteins, indicating that some FAs are colocalized with part of early endosomes. Taken together, our results showed the potential involvement of early endosomal compartment in the regulation FA turnover and cell migration in MDA-MB-231 cells, through clathrin-, dynamin- and Rab5 GTPase-dependent pathways.

Acknowledgements

We thank the Saudi Arabia ministry of higher education and the University of Reading for providing the financial support and the facilities needed to carry out this study.
The authors declare they have no conflict of interest.

References

1. Murphy JE, Padilla BE, Hasdemir B, Cottrell GS, Bunnett NW. Endosomes: a legitimate platform for the signaling train. Proc Natl Acad Sci U S A. 2009;106(42):17615-22.
2. Tomas A, Futter CE, Eden ER. EGF receptor trafficking: consequences for signaling and cancer. Trends Cell Biol. 2014;24(1):26-34.
3. Maxfield FR, McGraw TE. Endocytic recycling. Nature Reviews Molecular Cell Biology. 2004;5(2):121-132.
4. Helenius A, Mellman I, Wall D, Hubbard A. Endosomes. Trends in Biochemical Sciences. 1983;8(7):245-50.
5. Mayor S, Pagano RE. Pathways of clathrin-independent endocytosis. Nature Reviews Molecular Cell Biology. 2007;8(8):603-612.
6. Huotari J, Helenius A. Endosome maturation. EMBO J. 2011;30(17):3481-3500.
7. Wozniak MA, Modzelewska K, Kwong L, Keely PJ. Focal adhesion regulation of cell behavior. Biochimica et Biophysica Acta. 2004;1692(2-3):103-19.
8. Burridge K, Fath K, Kelly T, Nuckolls G, Turner C. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu Rev Cell Biol. 1988;4:487-525.
9. Giannone G, Mège R-M, Thoumine O. Multi-level molecular clutches in motile cell processes. Trends Cell Biol. 2009; 19(9):475-86.
10. Meighan CM, Schwarzbauer JE. Temporal and spatial regulation of integrins during development. Curr Opin Cell Biol. 2008;20(5):520-524.
11. Webb DJ, Parsons JT, Horwitz AF. Adhesion assembly, disassembly and turnover in migrating cells – over and over and over again. Nature Cell Biology. 2002;4(4):E97-100.
12. Alfahad D, Alharethi S, Alharbi B, Mawlood K, Dash P. PtdIns(4,5)P2 and PtdIns(3,4,5)P3 dynamics during focal adhesions assembly and disassembly in a cancer cell line. Turk J Biol. 2020; 44(6):381-392.
13. Meister M, Zuk A, Tikkanen R. Role of dynamin and clathrin in the cellular trafficking of flotillins. FEBS J. 2014;281(13):2956-76.
14. Lees JG, Gorgani NN, Ammit AJ, McCluskey A, Robinson PJ, O'Neill GM. Role of dynamin in elongated cell migration in a 3D matrix. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2015;1853(3):611-618.
15. Skalski M, Sharma N, Williams K, Kruspe A, Coppolino MG. SNARE-mediated membrane traffic is required for focal adhesion kinase signaling and Src-regulated focal adhesion turnover. Biochim Biophys Acta. 2011;1813(1):148-58.
16. Chao W-T, Kunz J. Focal adhesion disassembly requires clathrin-dependent endocytosis of integrins. FEBS Letters. 2009;583(8):1337-43.
17. Ezratty EJ, Bertaux C, Marcantonio EE, Gundersen GG. Clathrin mediates integrin endocytosis for focal adhesion disassembly in migrating cells. J Cell Biol. 2009;187(5):733-47.
18. Sadowski L, Pilecka I, Miaczynska M. Signaling from endosomes: Location makes a difference. Exp Cell Res. 2009;315(9):1601-9.
19. Johnson IRD, Parkinson-Lawrence EJ, Keegan H, Spillane CD, Barry-O'Crowley J, Watson WR, et al. Endosomal gene expression: a new indicator for prostate cancer patient prognosis?. 2015;6(35):37919-37929.
20. Ohashi E, Tanabe K, Henmi Y, Mesaki K, Kobayashi Y, Takei K. Receptor Sorting within Endosomal Trafficking Pathway Is Facilitated by Dynamic Actin Filaments. PLoS One. 2011;6(5):e19942.
21. Mettlen M, Pucadyil T, Ramachandran R, Schmid SL. Dissecting dynamin's role in clathrin-mediated endocytosis. Biochem Soc Trans. 2009;37(5):1022-6.
22. Edeling M, Smith C, Owen D. Life of a clathrin coat: Insights from clathrin and AP structures. Nat Rev Mol Cell Biol. 2006;7(1):32-44.
23. Von Kleist L, Stahlschmidt W, Bulut H, Gromova K, Puchkov D, Robertson MJ, et al. Role of the clathrin terminal domain in regulating coated pit dynamics revealed by small molecule inhibition. Cell. 2011;146(3):471-84.

24. Wang Y, Cao H, Chen J, McNiven MA. A direct interaction between the large GTPase dynamin-2 and FAK regulates focal adhesion dynamics in response to active Src. Mol Biol Cell. 2011;22(9):1529-1538.
25. Brown MC, Turner CE. Paxillin: Adapting to Change. Physiol Rev. 2004;84(4):1315-39.
26. Côté J-F, Turner CE, Tremblay ML. Intact LIM 3 and LIM 4 Domains of Paxillin Are Required for the Association to a Novel Polyproline Region (Pro 2) of Protein-Tyrosine Phosphatase-PEST. J Biol Chem. 1999;274(29):20550-60.
27. Horton ER, Byron A, Askari JA, Ng DHJ, Millon-Frémillon A, Robertson J, et al. Definition of a consensus integrin adhesome and its dynamics during adhesion complex assembly and disassembly. Nat Cell Biol. 2015;17(12):1577-1587.
28. Márquez MG, Brandán YR, Guaytima E del V, Paván CH, Favale NO, Sterin-Speziale NB. Physiologically induced restructuring of focal adhesions causes mobilization of vinculin by a vesicular endocytic recycling pathway. Biochim Biophys Acta. 2014;1843(12):2991-3003.
29. Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T. Dynasore, a Cell-Permeable Inhibitor of Dynamin. Dev Cell. 2006;10(6):839-50.
30. Cleghorn WM, Branch KM, Kook S, Arnette C, Bulus N, Zent R, el at. Arrestins regulate cell spreading and motility via focal adhesion dynamics. Mol Biol Cell. 2015;26(4):622-635.
31. Yoshida A, Sakai N, Uekusa Y, Imaoka Y, Itagaki Y, Suzuki Y, el at. Changes of plasma membrane and protein assembly during clathrin-mediated endocytosis. PLoS Biol. 2018;16(5):e2004786.
32. Rink J, Ghigo E, Kalaidzidis Y, Zerial M. Rab Conversion as a Mechanism of Progression from Early to Late Endosomes. Cell. 2005;122(5):735-49.
33. Schiefermeier N, Scheffler JM, de Araujo MEG, Stasyk T, Yordanov T, Ebner HL el at. The late endosomal p14–MP1 (LAMTOR2/3) complex regulates focal adhesion dynamics during cell migration. J Cell Biol. 2014;205(4):525-40.
34. Sharifi MN, Mowers EE, Drake LE, Collier C, Chen H, Zamora M, el at. Autophagy Promotes Focal Adhesion Disassembly and Cell Motility of Metastatic Tumor Cells through the Direct Interaction of Paxillin with LC3. Cell Rep. 2016;15(8):1660-72.
35. Roberts M, Barry S, Woods A, Sluijs P van der, Norman J. PDGF-regulated rab4-dependent recycling of αvβ3 integrin from early endosomes is necessary for cell adhesion and spreading. Curr Biol. 2001;11(18):1392-402.
36. Caswell P, Norman J. Endocytic transport of integrins during cell migration and invasion. Trends Cell Biol. 2008;18(6):257-63.