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Original Article

The Role of Akt/Rab5A Signalling in Regulating Cell Migration of MDA-MB-231 Breast Cancer Cell Line

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Abstract

Rab5A and Akt pathways are reported to be responsible for the invasiveness of cancer cells, indicated by the fact that Rab5A activates the downstream Phosphoinositide-3-kinases (PI3K)/Akt signalling pathway, which results in promoting cancer metastasis. However, little attention has been given to the emerging role of Rab5A and Akt signalling pathways in regulating the direction of MDA-MB-231 cell migration. MDA-MB-231 breast cancer cell line was used as a model in this study because it is highly metastatic and motile. Time-lapse microscopy was used to examine the effect of Akt and Rab5A inhibitors on cell migration, proliferation and wound healing. Later, the cells were transfected with GFP-Akt-PH or GFP-Rab5A (used as a biosensor to detect Akt and Rab5A). Therefore, confocal time-lapse images were used to visualize Akt and Rab5A at the front and rear edges of the cells. The recorded data demonstrated that Akt and Rab5A inhibition reduced cell migration, proliferation and wound healing. The results of the current study also demonstrated that Akt localizes at the trailing edge while Rab5A localize more at the leading edge than the trailing edge of cells. This study suggests that Akt and Rab5A inhibition might regulate the direction of breast cancer migration.

Keywords: Akt, Rab5A, Cancer Cell Migration, MDA-MB-231 Cell Line

1. Introduction

Cell migration is an essential, highly complex and coordinated physiological process involved in immune surveillance, embryonic development, wound repair and tissue homeostasis. Unusual cell migration can also lead to the development of various disorders such as atherosclerosis, chronic inflammatory diseases, and even cancer. Many proteins are known to be involved in a successful case of cell migration, including adhesion molecules, actin-binding proteins, various kinases etc. Two proteins, PI3K and Rab5A are discussed here. PI3K is a lipid kinase that regulates cell migration and focal adhesions along with cell growth and survival (1-4). These kinases are known to regulate cell migration in various cell types in two ways, first by causing direct binding of proteins to their lipid products and, secondly by an indirect way, through crosstalk with other pathways, such as RhoGTPase signalling. The direct mode of action of PI3K is, as mentioned above, by generating lipid secondary messengers by phosphorylating the head group of phosphoinositides at the 3' position.

Consequently, the effects of PI3Ks are transmitted through these lipid products, which bind to and regulate downstream protein effectors (5, 6). The indirect method involves positive feedback loops between PI3K and Rho GTPase signalling, which is responsible for integrating and amplifying cell signalling reactions,

both of which are required for efficient cell migration. Recent studies have indicated that PI3Ks are involved at different stages in cell migration, in various capacities, and even within one cell type. Another factor involved here is external stimuli, along with the status of cell signalling. Also, it was observed that different PI3K isoforms possess specific roles in cell polarization and migration.

Rab proteins are small GTPases involved in endosome fusion, tethering, and transport along the cytoskeleton. More than 60 Rab proteins have been recognized, which, based on their structure, function, and localization, have been classified into different sub-families. Of the 60 Rabs, 3 Rab proteins are involved in early endosome functions, namely, Rab5, Rab21 and Rab22. Rab5 is one of the most important members of the Rab family, which has been characterized well functionally. Rab5, like its all other counterparts, oscillates between the activated form, i.e., GTP-bound Rab5 (GTP-Rab5), and the inactivated form, i.e., GDP-bound Rab5 (GTP-Rab5) (7). The activated form binds with its effectors and is known to be involved in vesicular transport, membrane trafficking, and signalling pathways (8). Rab5, in its activated form, tends to regulate the internalization and trafficking of membrane receptors by mediating the regulation of vesicle fusions and also sorts the receptors in early endosomes (9, 10). Rab5a is supposed to be a central regulator of focal adhesions disassembly for promoting coordinated cell migration, established by the fact that the inactive mutated version of Rab5 (Rab5/S34N) failed to perform these activities (11). Rab5a is also involved in actin remodelling (12).

Rab5a-dependent pathways are also said to be responsible for not only the cell migration but also the invasiveness of cancer cells, indicated by the fact that Rab5a activates the downstream PI3K/Akt signalling pathway, which results in changes in not only the morphology of cells but also promotes the proliferation of the cells, meanwhile increasing the risk of cancer development (13). Akt is a serine/threonine kinase that acts crucially in the body's signal transduction of the PI3-K pathway in the body and helps maintain and regulate various biological functions like cell survival, proliferation, and metabolism. Various groups have reported the role of actuated PI3-K/Akt mechanisms in cancer development and progression, making them potential therapeutic targets. Different isoforms of mammalian Akt are known to regulate metastasis in breast cancer (13). This study examines the emerging role of Akt and Rab5A signalling in regulating the direction of cell migration.

2. Materials and Methods

2.1. Condition of Cell Culture and Cell Line

The American Type Culture Collection cell bank provided the MDA-MB-231 (ATCC® HTB-26TM) (Manassas, VA). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, A4766801) as well as 1 % penicillin/streptomycin (v/v, 104 units/mL penicillin, 104 g/mL streptomycin) (Gibco). All those cells were maintained at 37 °C under 95 % air, 5% CO_2, and roughly 90% humidity for maximum cell growth. To test cells for mycoplasma, an EZ-PCR Mycoplasma Based detection (Geneflow, K1-0210, UK) was utilized on a routine basis.

2.2. Akt and Rab5A Inhibitors

Dynasore (20 mM) (Selleckchem, S8047), as well as Pitstop 2 (25 mM) (Abcam, ab120687), were employed to suppress dynamin, clathrin-mediated endocytosis, API-1 (5 μM) to inhibit Akt (TOCRIS; Cat. No. 3897) and 0.1% of dimethyl sulfoxide (DMSO) was used as a control.

2.3. Cell Tracking Assay

The seeding of MDA-MB-231 breast cancer cells (1 X 10^5 cells/ml) was done on a 6-well plate and incubated overnight under the above-mentioned culture conditions. Subsequently, the cells were subjected to treatment with Dynasore (20 mM), Pitstop 2 (25 mM) and API-1 (5μM) for 20 hours. The migration of the cells was monitored by time-lapse microscopy (Nikon Eclipse TiE), taking snapshots (10X magnification) every 15 minutes for 20 hours using NIS elements

software and a Nikon DXM1200 camera. The speed of migration (μm/hour) of the cells was assessed as the distance mapped by the individual cells per 20 hours determined by the MtrackJ tool of ImageJ software.

2.4. Wound Healing Assay

The seeding of MDA-MB-231 breast cancer cells (1 X 10^5 cells/ml) was done on a 6-well plate and incubated to reach 90% confluence. The culture medium was removed, and multiple wound lines were introduced by scratching the cell monolayer with a 200 ul pipette tip. Following this, the cells were washed and incubated in DMEM containing Dynasore (20 mM), Pitstop 2 (25 mM) and API-1 (5μM). The wound width was recorded immediately after adding the DMEM (t=0) and after 16 hours, and the images were taken at 10X using the Nikon Eclipse TiE microscope. The percentage of wound coverage area after 20 hours was calculated as (Initial average area, t=0 hours) – (Final average area, t=20 hours) X 100.

2.5. Visualization of Rab5 and Akt at the Leading/Trailing Edges of MDA-MB-231 Cells

It is critical to look into the possible relationship between Rab5 and Akt and cell direction. For putting it to the trial, MDA-MB-231 cells were sown on a 6-well plate or even a 1-well glassy ibidi slides, then encouraged to stick to the base of the well for 24 hours. When such wells reached 70% confluency, they were transfected with GFP-Akt-PH (Plasmid #51465) or GFP-Rab5 domain (BacMam 2.0, C10586) and incubated for 24 hours. Cells were planted in a 35 mm ibidi dish with a density of 1 X 10^5 cells/mm^2 and incubated for 24 hours under the same culture conditions as above. Confocal microscopy was used to image live cells (Nikon A1R). GFP and RFP (or mCherry) fluorescence was observed at 488 nm/510 nm and 568 nm/590 nm (excitation/emission), respectively. Using only a 100x objective lens, snapshots were clicked almost every 5 seconds for 10 minutes. This was done to show the estimated amount of Rab5A and Akt that appeared (rate) at the leading and trailing regions. The cells were fixed using paraformaldehyde and imaged using confocal laser scanning microscopy to detect the cell edge and hypothesize the migrational direction of the MDA-MB-231 cells. The region from either margin to the nuclei was chosen to systematically evaluate Rab5A and Akt, including the number and size of Rab5A.

2.6. The Impact of Nutrient Deficiency and Starvation on RabA5 and Cell Migration

Cells were allowed to be seeded in a 12-well plate and incubated for 20 hours in reduced glucose medium (MEM) with 10% FBS. The following day, the media was changed to 0.50% FBS, transfected with GFP-Rab5, and cultured for an extended 20 hours. After the culture incubation duration, the fixation of cells was done using paraformaldehyde. Following that, it was assessed if the prolonged deprivation affected the migration of cells. Therefore, the overall distance among both places of emigrated cells was measured every other 30 minutes for 20 hours to detect and differentiate a migration shift in both FBS concentrations: FBS at 10% and 0.5 %.

3. Results

3.1. Akt and Rab5A Inhibition Reduces the Cancer Cell Migration, Proliferation and Wound Healing

In order to study the emerging role of Akt and Rab5A in regulating the direction of breast cancer migration, it is important to examine the effect of Akt and Rab5A on cell migration, proliferation and wound healing. The cells were treated with Dynasore (20 mM), Pitstop 2 (25 mM) and API-1 (5μM) for 20 hours. The speed of migration (μm/hour) of the cells was assessed as the distance mapped by the individual cells per 20 hours determined by the MtrackJ tool of ImageJ software. The proliferation was also calculated. Our results showed that Akt and Rab5A inhibitors significantly reduce cell migration and proliferation (Figure 1A). Our results also demonstrated that Akt and Rab5A inhibitors significantly reduce wound healing (Figure 1B).

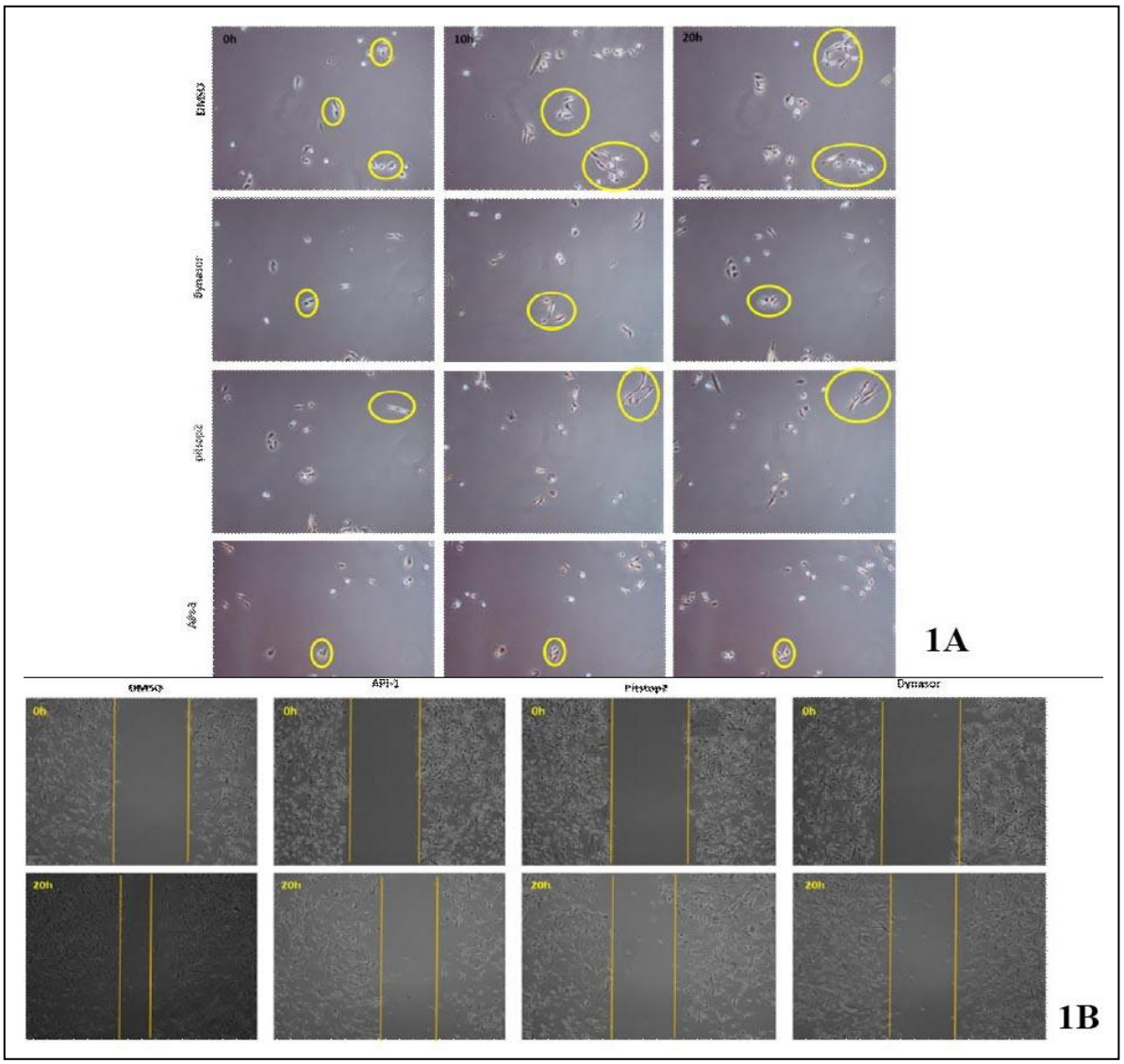

Figure 1. Monitoring the effect of Dynasore, Pitstop 2 and API-1 inhibitors on the proliferation, cell migration and wound healing of MDA-MB-231 cells. The cells were treated with the used inhibitors for 20 hours. **A:** Live cell tracking by time-lapse microscopy images representing the migration and proliferation of the cells monitored after 20 hours. The bar plot represents the quantitative analyses of cell migration speed and proliferation. **B:** The corresponding wound healing assay images for the MDA-MB-231 cells treated with Dynasore (20 mM), Pitstop 2 (25 mM) and API-1 5 (µM), taken at time 0 and 20 hours of wound closure (the lines show the wound edges). The bar plot represents the quantitative analysis of the total area covered by the cells after 20 hours of scratching. Three independent experiments with the analysis of 40 cells each were done in this study. Dunnett's test for comparison was performed following a real statistical difference using one-way ANOVA. *P<0.05, ** P<0.01 and *** P<0.01. The results are displayed as mean±SEM

3.2. Akt and Rab5A Localization

It is critical to look into the possible relationship between Rab5 and Akt and cell direction. We tried to investigate the localization of Akt and Rab5A in the migrating and highly metastatic cell line MDA-MB-231. The cells were transfected with a GFP-Akt-PH or GFP-Rab5, and observed the live samples with laser onfocal microscopy. Interestingly, we found the constant localization of the GFP-Akt at the trailing edge, while Rab5A at the leading and trailing edge of the migrating MDA-MB-231cells (Figure 2A and 2B).

3.3. Influence of Nutrient Deficiency and Starvation on RabA5 and Cell Migration

Earlier studies have shown that inhibiting the Clathrin internalization route reduces the expression of initial endosome proteins, resulting in decreased cell motility. Such findings lead researchers to speculate that addressing endocytosis routes through alternative means, instead of inhibiting endocytotic routes with blockers, could be used to control Rab5. Endocytosis has been linked to glucose as well as a nutritional deficiency. As a result, it was determined if chronic hunger alters the size and number of Rab5-containing endosomes. Our data showed no significant differences between 0.50% and 10% of glucose (Figure 3A and 3B).

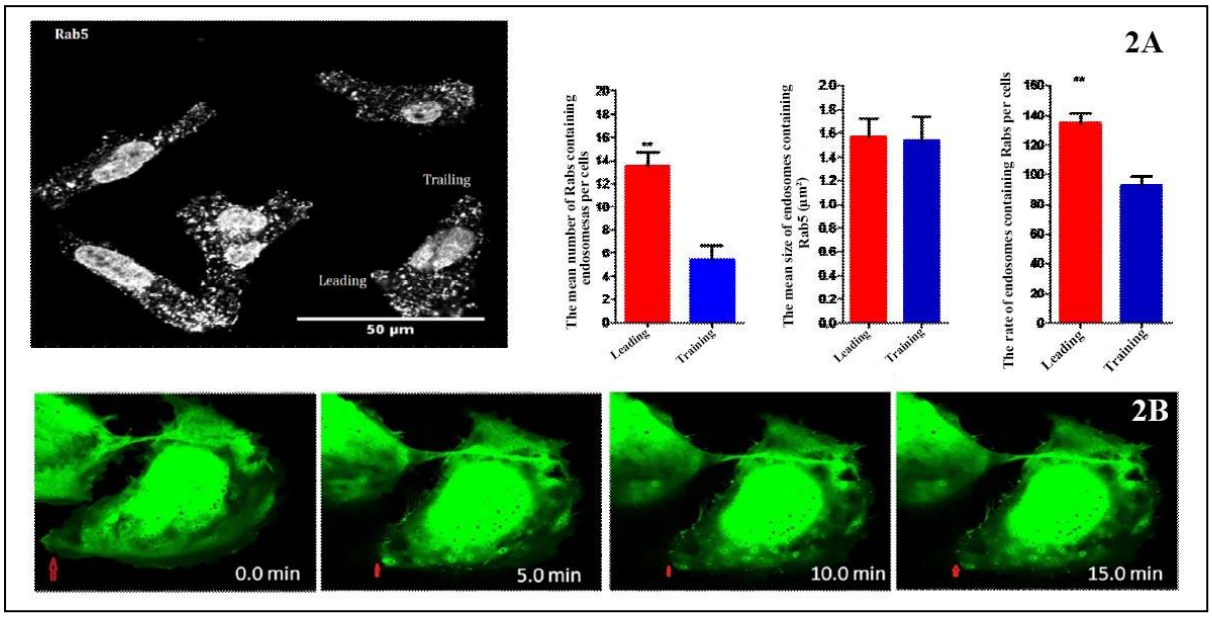

Figure 2. Localization of Akt and Rab5A on the edges of the migrating MDA-MB-231 cells. **A:** The distribution of Rab5A at the leading-trailing edges of the cells. The bar plots displayed that Rab5A is more concentrated at the leading edges than the trailing edges. **B:** The localization of Akt at the trailing edges of the migrating cells. The red arrows mark the disruption in localization of Akt at the trailing edge and reduced over time of imaging after being treated by (5 µM) API-1

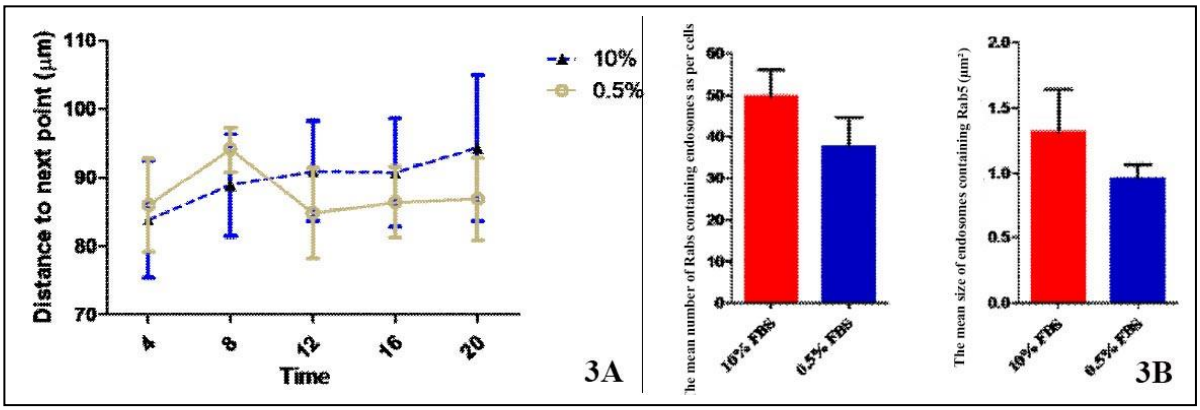

Figure 3. Monitoring the effect of nutrient deprivation on the cell tracking assay, number and size of Rab5A containing endosomes in MDA-MB-231 cells. **A:** The graph displays the distance traversed by the individual cells in 20 hours. **B:** The live tracking of the cells incubated in 10% FBS or 0.5% FBS was done using time-lapse microscopy. One-way and Two way ANOVA tests were done. No significant statistical differences in the results were observed, as represented in the graph (mean±standard error). Three independent experiments were carried out in which 40 cells were taken for analyses in each case

4. Discussion

The commonly used assays to study the collective direction of cell migration are wound healing, cell migration and proliferation assays. In the current study, we analyzed the effect of low concentrations of Dynasore (20 mM), Pitstop 2 (25 mM) and API-1 (5µM) on the MDA-MB-231cells which effectively inhibited them and reduced their migration as evidenced by wound healing assay. Our results are congruous with the previous study, which showed a significant reduction in the migration of pancreatic cancer cell lines by knocking down the dynamin2 protein (14). A significant effect on wound closure was observed when incubating the cells with the Pitstop2 inhibitor for 15 minutes compared to the control group. This is in line with the data that pointed to the role of clathrin-mediated endocytosis on T-cell migration using the dominant-negative clathrin mutants. The synergistic effect of Dynasore and Pitstop2 in combination has been observed on wound closure compared to individual Dynasore and Pitstop2 (15). This study also tried to explore the effect of nutrient deprivation on the endocytosis pathways. Nutrient deprivation has been shown to influence protein degradation, as well as the structure and functions of the endocytotic pathways and also showed that the starved cells displayed reduced rates of endocytosis (16). Based on this, it can be hypothesized that nutrient starvation might reduce cell migration. Our result was observed without any change in the size and a slight reduction in the number of endosomes.

This falls further in line with the previous studies that observed different protein and phosphoprotein levels due to starvation stress in 12 cell lines. The migration pattern of the serum-starved cells was analyzed by monitoring the distance traversed by the cell over the time of the assay. No significant differences in motility attributes were observed between the starved and non-starved cells, even after 8 hours of starvation. This implies that the simulated starvation conditions did not affect the cell migration under the study's time frame.

There is a well-established relationship between dynamin and clathrin concerning actin polymerization. However, this association might have some other unexplored effects in the cell (17, 18). Many studies suggested the association between reduced cell migration mediated by clathrin pathways and alterations in the early endosome formation and size. Compared to the normal tissue and primary cancer cells, the upregulated expression of early endosome markers (Rab5 and EEA1) in the more aggressive cancer cells is a characteristic feature and is used as a prognostic marker to identify cancer metastasis (19). These cells show hallmark features of cancer like increased nutrient uptake, uncontrolled cell division and altered signalling pathways (20). Hence, the altered levels of EEA1 and Rab5 may play a crucial role in cell migration through a pathway that is not fully understood. The possible explanations for the role of early endosomes in cell migration include the cytoskeletal organization modulated by the interaction of early endosomes with actin. The dynamic F-actin has been reported to assist in the invagination and removal of vesicles from the plasma membranes (21, 22), which is in line with the observation by previous studies that showed the removal of vesicles from the cell membrane as a result of the association between dextran and actin tail. They also showed that the actin tail structures associate mostly with the early endosomes rather than the late endosomes (23). Another study found that inhibition of actin dynamics results in the formation of large endosomes, which alters the transport of endosomal proteins from early endosomes to recycling endosomes (24). Based on these observations, it can be hypothesized that inhibiting either Dynasore or Pitstop2 results in large endosome formation, and the probable disruption of F-actin might alter the branching of the actin network resulting in reduced cell migration.

Another exciting study revealed the accumulation of Rab5-positive early endosomes with higher clathrin-mediated endocytosis rates at the leading edge of the

new-forming protrusion compared to the trailing edge. This might induce the cells to form lamellopodia at the protrusion site (25). Our results with Rab5 are consistent with the previous findings that used Dynamin2, clathrin and transferring in their study (26).

Authors' Contribution

D. A., M. A. and M. C. experimented. D. A. wrote the manuscript with support from M. A. and D. A. conceived the original idea and supervised the project.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

1. Al-Fahad D. Regulation of focal adhesions by PtdIns (4, 5) P2 and PtdIns (3, 4, 5) P3 in cancer cell migration: University of Reading; 2018.
2. Alfahad D, Alharethi S, 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):381-92.
3. Dhurgam A-F. The possible role of PtdIns (4, 5) P2 and PtdIns (3, 4, 5) P3 at the leading and trailing edges of the breast cancer cell line. Iberoam J Med. 2021;3(1):26-32.
4. Vanhaesebroeck B, Ali K, Bilancio A, Geering B, Foukas LC. Signalling by PI3K isoforms: insights from gene-targeted mice. Trends Biochem Sci. 2005;30(4):194-204.
5. Al-Fahad D, Al-Harbi B, Abbas Y, Al-Yaseen F. A Comparative Study to Visualize Ptdins (4, 5) P2 and Ptdins (3, 4, 5) P3 in MDA-MB-231 Breast Cancer Cell Line. Rep Biochem Mol Biol. 2022;10(4):518.
6. Oak J, Matheu M, Parker I, Cahalan M, Fruman D. Lymphocyte cell motility: the twisting, turning tale of phosphoinositide 3-kinase. Biochem Soc Trans. 2007;35(5):1109-13.
7. Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: conserved structure and molecular mechanism. Nature. 1991;349(6305):117-27.
8. Olchowik M, Miaczyńska M. Effectors of GTPase Rab5 in endocytosis and signal transduction. Postepy Biochem. 2009;55(2):171-80.
9. Al-Fahad D, Alharbi BF, Bih CI, Dash PR. Nitric oxide may regulate focal adhesion turnover and cell migration in MDA-MB-231 breast cancer cells by modulating early endosome trafficking. Med J Cell Biol. 2021;9(2):60-72.
10. Alharbi BF, Al-Fahad D, Dash PR. Roles of Endocytic Processes and Early Endosomes on Focal Adhesion Dynamics in MDA-MB-231 Cells. Rep Biochem Mol Biol. 2021;10(2):145.
11. Mendoza P, Ortiz R, Díaz J, Quest AF, Leyton L, Stupack D, et al. Rab5 activation promotes focal adhesion disassembly, migration and invasiveness in tumor cells. J Cell Sci. 2013;126(17):3835-47.
12. Spaargaren M, Bos JL. Rab5 induces Rac-independent lamellipodia formation and cell migration. Mol Biol Cell. 1999;10(10):3239-50.
13. Chamberlain MD, Chan T, Oberg JC, Hawrysh AD, James KM, Saxena A, et al. Disrupted RabGAP function of the p85 subunit of phosphatidylinositol 3-kinase results in cell transformation. J Biol Chem. 2008;283(23):15861-8.
14. Eppinga RD, Krueger EW, Weller SG, Zhang L, Cao H, McNiven MA. Increased expression of the large GTPase dynamin 2 potentiates metastatic migration and invasion of pancreatic ductal carcinoma. Oncogene. 2012;31(10):1228-41.
15. Samaniego R, Sánchez-Martín L, Estecha A, Sánchez-Mateos P. Rho/ROCK and myosin II control the polarized distribution of endocytic clathrin structures at the uropod of moving T lymphocytes. J Cell Sci. 2007;120(20):3534-43.
16. Schoenfeld JD, Sibenaller ZA, Mapuskar KA, Wagner BA, Cramer-Morales KL, Furqan M, et al. O2·− and H2O2-mediated disruption of Fe metabolism causes the differential susceptibility of NSCLC and GBM cancer cells to pharmacological ascorbate. Cancer Cell. 2017;31(4):487-500. e8.
17. Levin VA, Panchabhai SC, Shen L, Kornblau SM, Qiu Y, Baggerly KA. Different changes in protein and phosphoprotein levels result from serum starvation of high-grade glioma and adenocarcinoma cell lines. J Proteome

Res. 2010;9(1):179-91.
18. Pirkmajer S, Chibalin AV. Serum starvation: caveat emptor. Am J Physiol Cell Physiol. 2011;301(2):272-9.
19. Johnson IR, 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? Oncotarget. 2015;6(35):37919.
20. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
21. Merrifield CJ, Moss SE, Ballestrem C, Imhof BA, Giese G, Wunderlich I, et al. Endocytic vesicles move at the tips of actin tails in cultured mast cells. Nat Cell Biol. 1999;1(1):72-4.
22. Welch MD, Mullins RD. Cellular control of actin nucleation. Annu Rev Cell Dev Biol. 2002;18(1):247-88.
23. Gauthier NC, Monzo P, Gonzalez T, Doye A, Oldani A, Gounon P, et al. Early endosomes associated with dynamic F-actin structures are required for late trafficking of H. pylori VacA toxin. J Cell Biol. 2007;177(2):343-54.
24. 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):19942.
25. Rappoport JZ, Simon SM. Real-time analysis of clathrin-mediated endocytosis during cell migration. J Cell Sci. 2003;116(5):847-55.
26. Palamidessi A, Frittoli E, Garre M, Faretta M, Mione M, Testa I, et al. Endocytic trafficking of Rac is required for the spatial restriction of signaling in cell migration. Cell. 2008;134(1):135-47.