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Exosomes Derived from Metastatic Colon Cancer Cells Induced Oncogenic Transformation and Migratory Potential of Immortalized Human Cells

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Abstract: Tiny vesicles, synthesized from cell membranes through endocytosis, form multivesicular bodies (MVBs). These MVBs can either undergo lysosomal degradation or be released to the extracellular environment. The vesicle that ranges in size from 30-300 nm that were positive for markers such as CD63 or CD9 are known as exosomes. Exosomes are shown to contain various biological macromolecules such as DNA, RNA, miRNA, proteins, and lipids. Releasing exosomes from cells to the extracellular environment is an important strategy to transfer information between the cells. We hypothesize that the exosomes - derived from metastatic colon cancer cells will induce the oncogenic transformation and migratory potential of normal cells. Exosomes from primary colon cancer cells have been shown to induce the oncogenic transformation of normal cells. In this study, we have demonstrated that treating normal cells (HEK) with exosomes derived from the metastatic colon cancer cell line (SW620) increases cell proliferation, allowing the normal cells to grow anchored independently, suggesting the oncogenic transformation of normal cells. In addition, there was an increase in migration and invasion properties of HEK cells exposed to exosomes from SW620 cells. Our results suggest that following the treatment with metastatic colon cancer cell-derived exosomes, the HEK cells underwent an oncogenic transformation and Epithelial-Mesenchymal Transition (EMT) that will allow the cancerous cells to metastasize.

Introduction

The incidence of cancer is increasing worldwide. Though there are many cancer treatments available all over the world, cancer-related deaths are increasing. Global cancer burden statistics showed 19.3 million cases in 2020 and are expected to have 28.4 million cases by 2040. Among all the cancers, breast (11.7%), lung (11.4), and colorectal (10.0%) are the leading cause of cancer. Colorectal cancer is the third leading cause of cancer and the second (9.4%) leading cause of cancer-related death (Sung et al., 2021). To avoid the immense increase in death rate, the medical field demands potential diagnostic/prognostic markers to detect cancer in the early stage. Researchers found various compounds to use as chemotherapeutic drugs to prevent cancer and to increase the patient's vitality. Despite these chemotherapeutic drugs, the rise in deaths is uncontrollable because of treatment failure and tumour relapse. Even after surgery, chemotherapy, and radiotherapy, the cancer cells can able to grow, affect nearby or distant organs, and worsen the patient's life. Moreover, the cancer cells can attain drug resistance and escape the immunosurveillance. This ability of cancer cells to evade the treatment approach and immune mechanism is due to the alterations in the gene regulations involved in various signaling pathways (Marzagalli et al., 2021).

Cell membrane undergoes the endocytosis process and forms early endosomes and late endosomes. The late endosome may undergo lysosomal degradation or be released in the extracellular environment through

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exocytosis. The size of the exosomes is categorized between 30-300 nm and packed with biological macromolecules such as DNA, RNA, Proteins, miRNA, and lipids (Bebelman et al., 2018; Fu et al., 2020). The process of exosome biogenesis is not well known, but based on previous studies, it is known that exosome biogenesis is involved in ESCRT-dependent and independent pathways. Proteins like syndecan, syntenin, and Alix are involved in the membrane budding and exosome release to the extracellular environment. Exosomes isolated from normal and cancer patients show different protein and RNA profiles. As we discussed, the researchers are developing chemotherapeutic drugs targeting cancer cells. However, it is important to inhibit the communication between cancer cells and normal cells (Guo et al., 2017; Zhong et al., 2021). Exosomes are secreted in all body fluids, such as saliva, urine, seminal fluid, and blood (Rajagopal and Harikumar, 2018; Thakur et al., 2022). Xia Wang et al. reported that metastatic cancer cells secret more exosomes than normal cells. Also, they stated the exosome marker CD63 is upregulated in cancer patients, which shows that we can use exosomes to detect the cancer prognosis (Wang et al., 2022). Phosphoproteins in the exosomes can also facilitate distinguishing between healthy and cancer patients (Bidarra et al., 2019; Dai Jie and Yiqun Jiang, 2020; Tsukamoto et al., 2017). Being synthesized from our cells and having natural signals to reach the target cells, we can engineer the exosomes and use them as a nanocarrier to deliver the drug safely without getting affected by the immune cells (Gilligan and Dwyer, 2017; Kamerkar et al., 2017; Pinky et al., 2021; Vakhshiteh et al., 2019). This way, we avoid various side effects of chemotherapeutic drugs and their adverse reactions.

Epithelial-mesenchymal transition is one of the significant processes for the cells to attain certain tissue types by generating different germ layers during embryogenesis. During inflammation or repair mechanisms, the cells must obtain mesenchymal properties by decreasing the cell's polarity and migrating to a distant site to repair the damaged tissue (Bigagli et al., 2016; Mashouri et al., 2019). This course of transformation from epithelial cells to mesenchymal cells is managed by different groups of genes such as E/N-cadherin, vimentin, SNAIL, Slug, FoxD3, occludins, claudins, collagens, and elastins. The cells shed their epithelial markers and gained a migratory phenotype through matrix remodeling and basement membrane degradation (Kalluri and Weinberg, 2009). Jiang et al found that the contents of exosomes regulate the EMT process (Jiang et al., 2022). Primarily the exosomes involved in the preparation of the premetastatic niche enhance the transformation of polygonal shape to spindle shape to favour the blood vessel infiltration and increase the tumour cells progression (Kim et al., 2020; Risha et al., 2020; Wang et al., 2019). Even before the chemotherapeutic drug effect begins, the cancer cells release the exosomes to prepare the metastatic niche for tumour progression. The assembly of a set of EMT genes and its regulation can also trigger the activation of cancer stem cells (Visan et al., 2020).

Limited knowledge of the interaction between cancer cells and normal cells is a hurdle in the development of chemotherapeutic drugs. Since exosomes are freely circulated in body secretions, we can use them as a biomarker for cancer prognosis and diagnosis (Sahebi et al., 2020). Hence it might be helpful to overcome the traditional invasive procedures in the patients. In addition, the biomolecules present in the exosomes reflect the origin of exosomes, this footprint helps us to track the communication between the cells **(**Nam et al., 2020). The research gap in cancer treatment and chemotherapy development can be filled by the study of exosome communication.

Earlier studies have shown that the treatment with cancer patients' serum and primary colon cancer cell line (colo320)-conditioned medium on HEK cells has increased migration, invasion, and tumour progression compared to the untreated HEK cells. Also, they have studied the effect of normal human serum on HEK and found no cell differentiation and tumour progression (Abdouh et al., 2014). We have observed that the condition medium from metastatic colon cancer cells (SW620) has increased the proliferation and migration of normal cells (HEK) (data not shown), therefore we have isolated the exosome from metastatic colon cancer cell line conditioned medium and analysed its effect on oncogenic transformation and cell migration.

Materials

Cells and Reagents

Human embryonic kidney cells (HEK) and metastatic colon cancer cell line (SW620), [all cell lines were purchased from ATCC], were grown in DMEM media, with 10% FBS (Gibco), 1% antibiotic and antimycotic solution (Himedia), and maintained at 37^0C in 5% CO_2. Antibodies CD63 (Thermo Scientific), E-cadherin, and slug (Abcam) were purchased.

Methods

Isolation of exosomes

SW620 cells were cultured in a T25 cell culture flask. After 48 hours, we removed the cell culture medium and

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centrifuged it for 10 mins @37^{0}C at 2000 rpm to remove the macromolecules and cell debris. Again, the culture medium was centrifuged for 30 mins at 10,000 g to remove bigger-sized vesicles. Finally, the culture medium was centrifuged in ultracentrifugation for 70 mins at 100,000 g, then we removed the supernatant, collected the pellet safely resuspended with PBS, and repeated the ultracentrifugation process. All the centrifugation temperatures were maintained at 4^{0}C. The pure exosomes were resuspended in PBS and aliquoted in a fresh tube. [In this paper, we denoted M-exo for the exosomes isolated from metastatic colon cancer cell line (SW620).

TEM

We used HR- TEM JEOL Japan, JEM-2100 plus to confirm the morphology of exosomes. The copper grid was kept in a Petri dish, loaded 1 µl of exosomes on the grid, and allowed to dry for 4 hours and images were taken.

Dynamic Light Scattering Assay

DLS was used to determine the size and size distribution of exosomes. The 20 µl of clear sample without any sedimentation was used, the light scattering on the vesicles provides the particle size distribution of exosomes. The DLS is reliable for the size range from 0.3 nm – 10 µm.

Zeta potential

The vesicle stability was confirmed by the zeta potential analyser. Exosomes pellet was resuspended in PBS and made up to 200 µl. The solution was spun down and pipetted out to make a uniform solution. The surface electrical charge of the extracellular vesicles is mostly negative.

Western Blotting

Exosomes were isolated through ultracentrifugation and resuspended in PBS. The protein was extracted by RIPA (Radioimmunoprecipitation Assay) buffer and the concentration was estimated by the Bradford method. Proteins (30 µg) were mixed with lamelli buffer and heated for 5 mins at 1000 C before loading onto SDS PAGE. Later the proteins were transferred to nitrocellulose membrane and probed with CD63 antibody. The bands were developed using ECL reagent (Advanta) and observed in ChemiDoc (Bio-Rad).

Proliferation Assay/Cell Viability Assay

In a 96-well plate, we seeded 5000 HEK cells/well and kept them in a CO_2 incubator overnight, the control group was given PBS and the treatment group received 20 µg of M-exo derived from metastatic colon cancer cells and incubated for 48 hours in a CO_2 incubator. After incubation we carefully removed the medium and 100 µl of MTT reagent (0.2%) was added, incubated for 4 hours, the formazan crystals formed were solubilized with DMSO and taken reading at 575 nm.

Colony formation assay

HEK cells were seeded (500 cells/well) in a six-well plate, after 24 hours we added PBS in the control wells and the treatment group received 20 µg of M-exo. After 2 days, we removed the medium, replaced it with a fresh medium, and incubated it for 7 days. Later, the cell plates were removed from the incubator and stained with Giemsa stain. The number of colonies was analysed in ImageJ.

Anchorage- Independent Assay

We created two layers of agarose with different percentages on a six-well plate. 0.6% agarose dissolved in sterile water forms the bottom layer. 0.3% agarose makes up the top layer. The top layer solution was allowed to cool at room temperature, and then 500 HEK cells/well were added, thoroughly mixed, and treated with M-exo, and evenly poured on top of the bottom layer. The colonies grown in the treatment and control groups were examined under a microscope, and the image was capturedafter seven days. Using ImageJ analysis, the colonies' sizeand quantity were determined.

Migration Assay/wound closure assay

In a six-well plate, we seeded 80,000 HEK cells and incubated them for 24 hours in the CO_2 incubator. A scratch was gently made with a 200 µl tip, added serum-free medium, and incubated for 24 hours in CO_2. The migration was observed and photographed in the microscope. The area of the wound covered was measured in ImageJ.

Cell Invasion Assay

In a Boyden chamber, the Matrigel was added and kept in a CO_2 incubator for 4 hours. HEK (500 cells/well) were added to the Matrigel with serum-free medium. The control cells were incubated with PBS and the treatment cells received M-exo. We kept the Boyden chamber in the 12 well plates with the complete medium (with fetal bovine serum [FBS] as a chemo-attractant) in the bottom layer. After 24 hours the invaded cells were washed, stained, observed, and photographed in the microscope and analysed in ImageJ.

Immunofluorescence

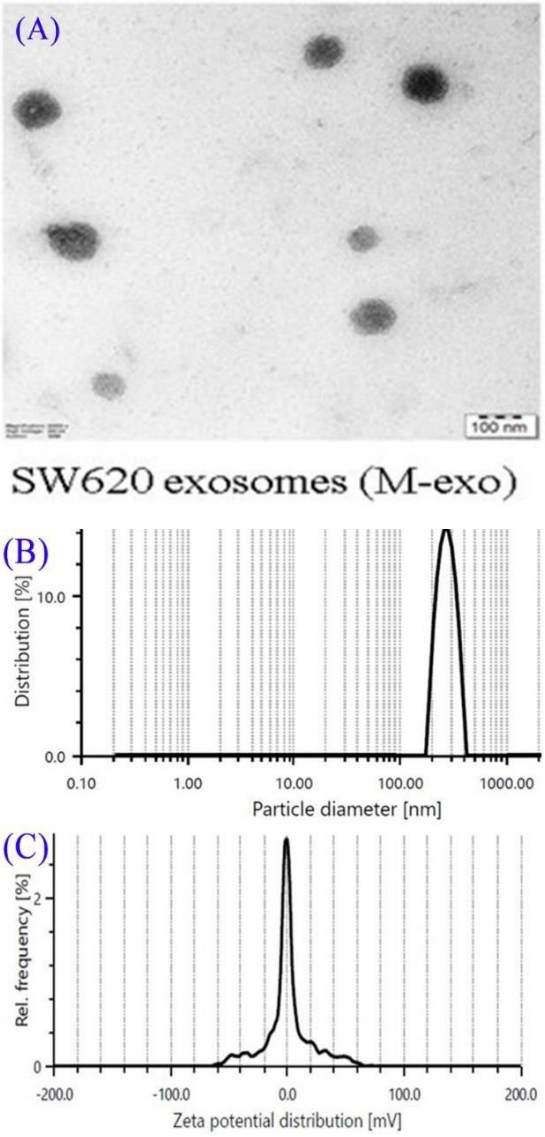
The cells were seeded on the coverslip in a six-well plate and allowed to grow the cells 24 hours, we then added M-exo to the cells and incubated them for 48 hours.Then we fixed the cells with 4% paraformaldehyde and washed them with PBS thrice. We added the primary antibody to the cells and incubated them overnight. Then, the cells were carefully washed with PBS thrice. Then, a secondary antibody was added, and the cells were incubated for 4 hours, then washed with PBS. DAPI was

added to the cells to stain the nucleus and washed with PBS. The cells are imagined under a fluorescent microscope for protein expression.

Results and Discussions

The exosomes isolated from the metastatic colon cancer cell lines (SW620) through the ultracentrifugation method were characterized using TEM, DLS, and zeta potential. Transmission electron microscopy (TEM) shows the morphology of the exosomes, the scale bar shows 100 nm (Fig 1A). The DLS shows the particle size distribution, the average size of the exosomes was 295 nm Zeta potential of the exosomes shows a negative value of -0.6 mV, which represents that the colloidal stability of the exosomes were stable in the ultracentrifugation method (Fig 1B). Furthermore, in Fig 1 C, the presence of exosome marker CD63 through western blot assay confirms the exosome isolation.

Characterization of M-exo (Metastatic colon cancer cell line- derived exosomes)



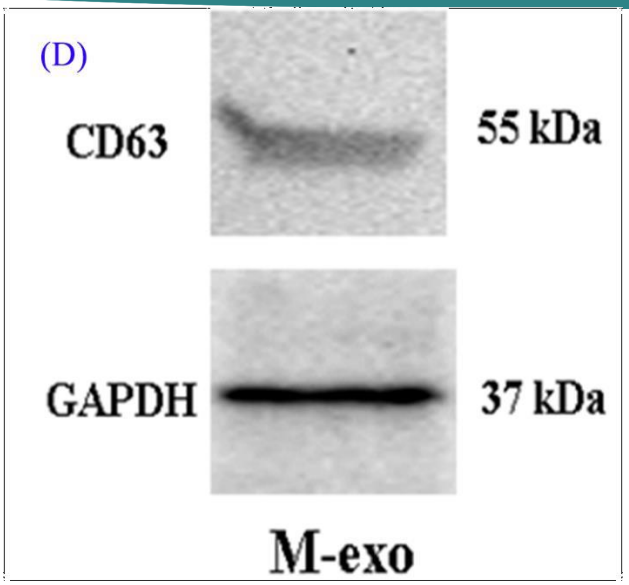
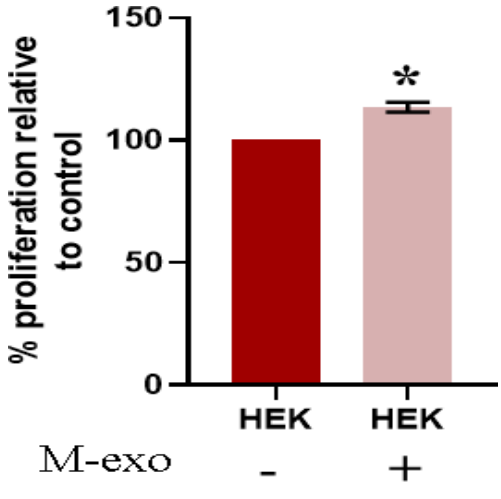


Figure 1. Characterization of Exosomes: (A) TEM analysis of SW620 exosomes (M-exo) after ultracentrifugation to confirm the morphology. The scale bar represents 100 nm. (B) Particle size distribution of exosomes shows an average size between 30 and 300 nm. (C) The zeta sizer determines the zeta potential of exosomes. The surface charge of the exosomes shows their stability. (D) Western blotting of M-exo for the confirmation of exosome marker CD63.

Effect of M-exo on HEK cell proliferation

The HEK cells were treated with exosomes isolated from SW620 cells. The MTT assay result shows an increase in the proliferation of cells in the treated group compared to the control (Figure 2A). The long-term effect of the short-term treatment with exosomes on HEK cells were analysed by colony formation assay. We treated the cells with M-exo for 48 hours and then removed the medium and added fresh serum-free medium. After 7 days of incubation in a CO_2 incubator, we counted the number of colonies formed in the wells. We found an increased number of colonies in the treated cells compared to the control (Fig. 2B).



(A)

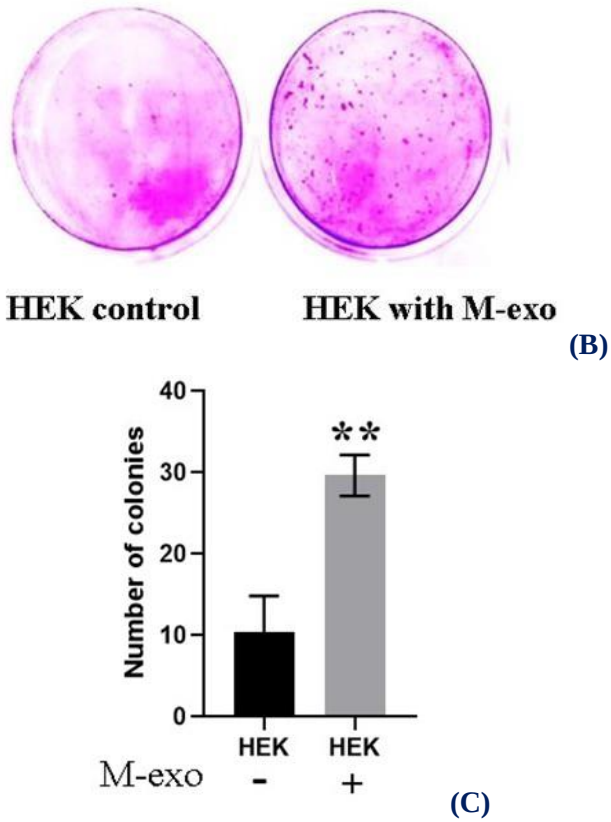


Figure 2. M-exo increased the proliferation of HEK cells. (A) Proliferation of HEK cells were increased following treatment with M-exo compared to control. (B) An increased number of colonies formed following the treatment with M-exo. (C) The number of colonies counted using ImageJ, the average was taken from triplicate and the assay was repeated three times. The graphs represent statistical data (±SEM) with significant values, *p<0.05.

M-exo induced the anchorage-independent growth of HEK cells

The HEK cells were seeded on the soft agar along with 20 µg of M-exo and after 7 days of incubation, we counted the number and size of colonies in the microscope. The results (Fig. 3A) show more colonies grown in an anchorage-independent manner. The HEK cells require matrix/anchorage to grow, however, after the treatment with M-exo, the HEK cells can able to grow in the soft agar (anchorage-independent) coated plates. This shows that the component of exosomes induces the anchorage-independent growth of HEK cells.

M-exo treatment increased the migration and invasion potential of HEK cells.

We analysed the wound closure capacity of the HEK cell by scratch assay, after the M-exo treatment the scratch area is almost completely covered in the treated cells compared to the untreated cells. This effect of increased wound closure rate is due to the regulation of the EMT marker by M-exo (Fig 4A). To study the impact of the M-exo in the mechanism of invasion of HEK cells, we performed a Trans well invasion assay/Boyden chamber assay. The control group received only PBS, and the treatment group received 20 µg of M-exo, after 48 hours, there was a significant number of cells invaded in the M-exo-treated cells compared to the PBS-treated cells (Fig 4B).

Expression of Epithelial-Mesenchymal markers through immunofluorescence assay

We performed an immunofluorescence assay using specific antibodies to evaluate the protein expression in the HEK cells after M-exo treatment. The HEK cells when exposed to exosomes isolated from metastatic colon cancer cell lines show down-regulation of E-Cadherin and up-regulation of slug (Fig 5). The regulation of EMT marker expression by M-exo revealed that the HEK cells lost their epithelial morphology and transformed to mesenchymal. The red fluorescence shows the presence of proteins probed (E-Cadherin and Slug). In Figure 5, there is more red fluorescence for E-cadherin in control cells compared to the M-exo treated cells where there was decreased expression of E-cadherin in the treated cells. Similarly, there is more red fluorescence in the cells treated with M-ego compared to the control, which indicates that there was an increased expression of slug protein.

In this study, we report the oncogenic transformation of the HEK cells following the treatment of metastatic colon cancer cell line-derived exosomes (M-exo). The exosomes isolated through ultracentrifugation were characterized by TEM, DLS, Zeta potential and western blot (Huang et al., 2023; Martins et al., 2018). we isolated the exosome through the ultracentrifugation method and the yield of exosomes is higher in this method compared to other isolation methods like kit and acetone precipitation method (Manish Dash et al., 2021). The exosome marker CD63 was checked in western blotting to confirm the exosome isolation. The HEK cells when treated with M- exo increase the cell proliferation and colony formation (Liet al., 2022). Generally, the cancer cells can grow anchorage independently, this characteristic feature of cancer cells helps them to grow without any adhesion support and translocate from the primary tumour site to neighbouring or distant organs (Lobos-González et al., 2020). The major challenge in the current cancer therapiesis that the cancer cells can move from one place to another,and escape from our immune cells and/or chemotherapeutic drugs (Kim et al., 2016). To acquire thisunique character, cancer cells undergo various mechanisms to grow independently without cell-cell adhesion, then undergo intravasation and extravasation to reach the favourable tumour microenvironment.

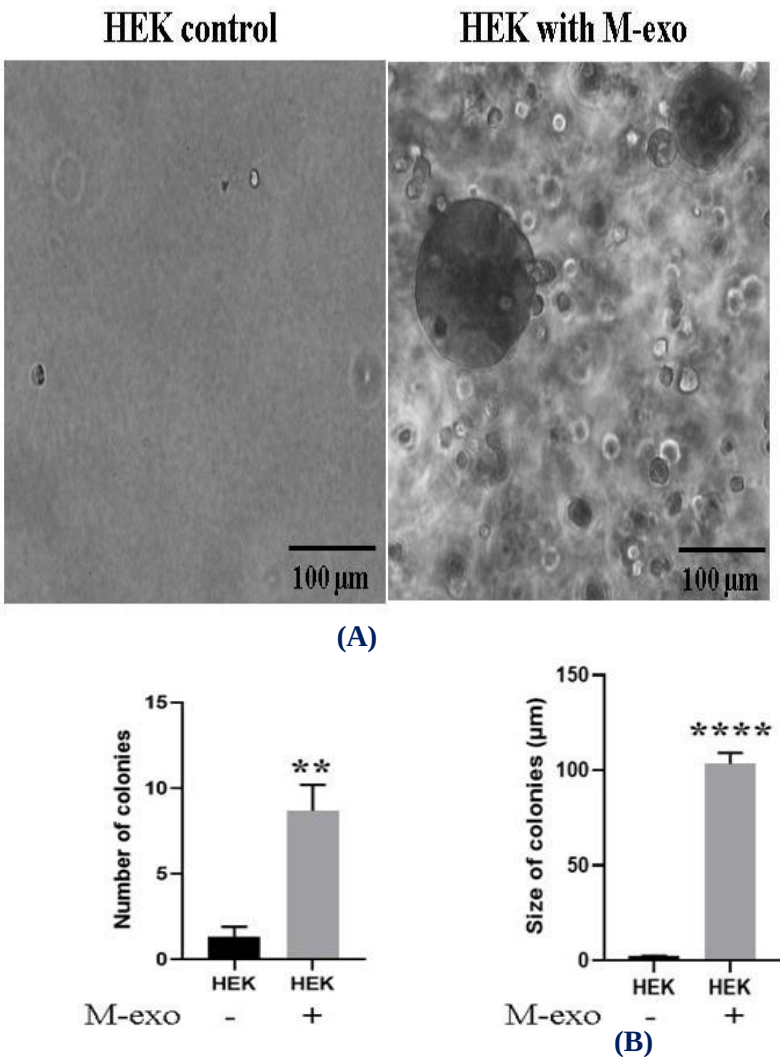


Figure 3. M-exo induced anchorage-independent growth of HEK cells. (A) Only cancer cells can grow in an anchorage-independent manner; however, the HEK cells were grown on soft-agar after M-exo treatment, which was evident after 10 days. (B) The graph shows the number and size of colonies formed, following the M-exo treatment. The average was taken from triplicate and repeatedthe assay three times. The graphs represent statistical data (±SEM) with significant values, **p <0.01, ***p< 0.001, ****p< 0.0001.

The cells must change their morphology from epithelial to mesenchymal state (EMT) to attain the invasion property. This phenotypic change induces the cell migration to reach the target area, and again, they change their morphology from mesenchymal to epithelial state (MET) to adhere to the target cells. From the immunofluorescence assay, it is evident that the M-exo was involved in the loss of E-cadherin expression and an increase in the slug expression to attain the mesenchymal property of the HEK cells. The function of E-Cadherin in the cells is not completely explored, this protein can act as a tumour suppressor and supports the proliferation and metastasis along with different signalling pathways (Park et al., 2017). All these intentional processes of cancer cells are to escape from the site of origin. The researchers have developed several strategies to kill the cancer cells and curtail their spreading, however, the cancer cells release more exosomes as messenger molecules that enable tumour progression and metastasis (Dong et al., 2022). Thus, the cancer cells can deliver the signals to nearby or distant organs through exosomes. The communication of signals from the cancer cells to the normal cells increases the burden in development of cancer treatments. Even though we enhanced the treatment processes like targeted therapy by synthesizing nanoparticles, immunotherapy, and hormonal therapy, the cancer cells silently transfer the information through exosomes and transform the normal cells into cancerous cells. Hence, the researchers are urgedto study how the exosomes are synthesized, loaded with cargo, and released into the extracellular environment(Becker et al., 2016). Furthermore, it is equally important to study how the exosomes reach the target cells or organswithout degraded by the immune cells or chemotherapy (Yang et al., 2020).

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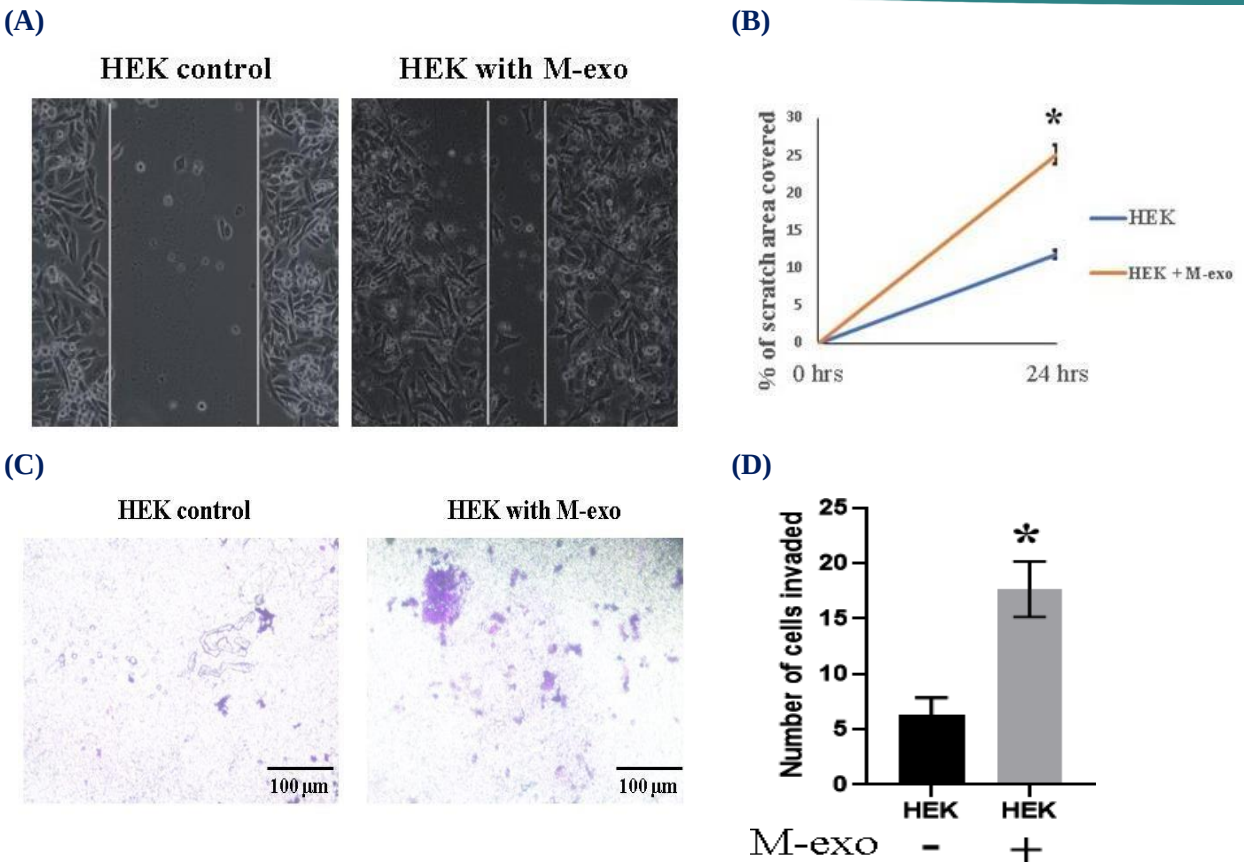

Figure 4. Migration and invasion of HEK cells. (A&B) The HEK cells treated with M-exo show a higher percentage of wound area covered than control HEK cells. (C&D) The M-exo-treated cells increased the invasion of HEK cells compared to the control. The average was taken from triplicate and the assay was repeated three times. All the graphs represent statistical data (±SEM) with significant values, *p < 0.05.

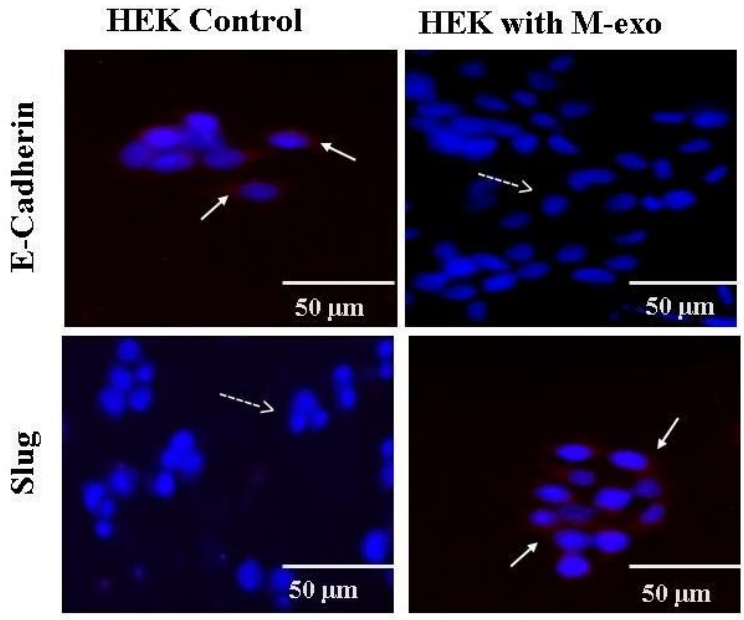

Figure 5. Immunofluorescence Analysis for protein expression. There is a decreased E-cadherin and increased slug expression in M-exo-treated cells. The decrease in cell adhesion molecule E-cadherin expression provides mesenchymal property to the non-cancerous cells, and the increase in slug expression increases the migration of the cells.

[⟶ arrow indicates fluorescence ----> and arrow indicates without fluorescence].

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Our earlier study has shown that there was an increase in cancer stem cell marker expression following the treatment of metastatic colon cancer cell-line-derived exosomes in HCT116 cells (primary colon cancer cells) (Yasodha et al., 2023). Overall, our results comprised that the M-exo-treated HEK cells show increased tumour progression and metastasis.

Conclusion

Exosome biogenesis and its communication is one of the important biological processes that has been shown to play a role in the malignant transformation of normal cells and to increase cancer cell proliferation and migration. In this study, we have shown that the metastatic cell line-derived exosomes induced the oncogenic transformation and increased the migration and invasion of HEK cells. Furthermore, we have determined that the exosomes from metastatic colon cancer cells are involved in the induction of Epithelial-Mesenchymal Transition. However, further studies are required to identify cell-cell communication through exosomes, and it is necessary to analyse the cargo present in the exosomes to extrapolate the potential significance of exosomes in such communication. The study of exosomes can provide more insights into cancer progression and metastasis and could help to design novel therapeutic approaches.

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Ethical approval

This article contains no studies with human participants or animal experiments performed by authors.

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