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Original Article

Unveiling the impact of CD133 and CD105 in MDA-MB-231 cell-derived exosomes on breast cancer cell signaling pathways

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Abstract

Recent studies have revealed the critical role of exosomes in cancer progression, particularly aggressive breast cancers. These findings underscore the requirement for further investigation into the mechanisms of exosome-mediated cancer and emphasize the urgency and critical nature of such studies. In the present study, exosomes of MDA-MB-231 cells were isolated from serum-free media using differential ultracentrifugation. Size distribution was assessed using dynamic light scattering, and exosome morphology was examined using scanning electron microscopy. Flow cytometry analysis showed considerable expression of the metastatic markers CD105 and CD133, although cancer cells exhibited low expression of these markers. Exosomes were labeled with Aco-490 and internalized by MDA-MB-231 and MCF-7 cells. The results indicated that post-sorting, CD133-positive exosomes considerably increased the phosphorylation of AKT and extracellular signal-regulated kinase, although they did not have a notable influence on cyclin D1 levels. This study investigated the effects of exosomes on breast cancer, underscoring the requirement for further studies on exosomes that may potentially impede metastasis and tumor growth.

Keywords: Breast cancer, Endoglin, Exosomes, Extracellular vesicles, Prominin-1

1. Introduction

Breast cancer is the most frequently diagnosed cancer among women worldwide and is the primary cause of cancer-related death. The global prevalence of breast cancer has consistently increased. Consequently, despite advancements in identification and treatment that have resulted in improved survival rates, pursuing novel therapeutic approaches and identifying predictive and prognostic markers remain essential [1, 2]. Exosomes, which are nano-sized vesicles secreted by cells, play a multifaceted role in cancer progression. Recent studies have indicated their involvement in promoting metastasis, a complex process through which cancers spread to distant organs [3, 4]. Exosomes achieve this by transporting bioactive molecules, including cytokines, that modulate the tumor microenvironment. These cytokines can stimulate angiogenesis and formation of new blood vessels, providing a pathway for tumor cells to escape. Furthermore, exosomes suppress immune response to cancer cells, facilitating their survival and proliferation [5-7]. Numerous studies have provided evidence of exosome-mediated signaling in all facets of breast cancer metastasis including enhanced epithelial-mesenchymal transition phenotypes, migration and invasion, drug resistance, viability, and angiogenesis [8-12].

CD133, also called AC133 or prominin-1, a glycoprotein characterized by five transmembrane domains, is a recognized marker of cancer stem cells in colon, liver, and lung cells. It modulates several carcinogenic signaling pathways; therefore, CD133 levels are generally increased in patients with colon cancer, leading to metastasis and recur-

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rence [13-15]. CD105, also called endoglin, a transmembrane glycoprotein, is recognized for its role in mediating angiogenesis and has been investigated as a therapeutic target in tumor microenvironments that exhibit elevated CD105 levels [16, 17]. Specific surface markers on exosomes are potential biomarkers for disease diagnosis and prognosis. CD133 and CD105, which are often linked to angiogenesis and tumor stemness, have been detected in exosomes derived from different cancer types [14, 18-21].

Further investigation of the specific functions of exosomal CD133 and CD105 in various cancers may result in innovative therapeutic strategies for impeding cancer invasiveness. Here, we investigated exosomes secreted from breast cancer cells. We focused on two specific surface markers, CD133 and CD105, which are involved in activities of cancer stem cells and metastasis. We aim to gain valuable insights into the pathogenesis of breast cancer by unraveling the roles of these exosomal proteins.

2. Material and methods

2.1. Cell culture

MDA-MB-231 and MCF-7 breast cancer cell lines were purchased from ATCC and maintained in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Isolation of the MDA-MB-231 breast cancer cell-derived exosomes

Exosomes of MDA-MB-231 cells were isolated from serum-free medium via differential centrifugation. Conditioned medium was collected after 48 h from 4 × P150 plates containing MDA-MB-231 cells and centrifuged at 1000 ×g for 10 min to remove cells. The supernatant was collected and centrifuged at 2000 ×*g* for 20 min to remove dead cells. For ultracentrifugation (UC), the conditioned medium was centrifuged at 100,000 ×*g* at 4 °C for 120 min using a Sw41 Ti swinging bucket rotor (Beckman Coulter, Life Sciences Division Headquarters, Indianapolis, USA). For SUC-based isolation, the conditioned medium was slowly loaded over 4 mL of 10, 28, 34, 40, and 70% sucrose solution [prepared in 1 × phosphate-buffered saline (PBS)], forming a layer, and centrifuged at 100,000 ×*g* and 4 °C for 16 h using a Sw41 Ti swinging bucket rotor (Beckman Coulter). The third fraction, containing the 34% and 40% interfaces, was collected from the top. The final pellet was resuspended in PBS and stored at -80 °C for further use.

2.3. Quantification of exosome proteins

The protein content of the isolated exosomes was quantified using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Before protein quantification, all exosomes resuspended in PBS were lysed by adding an equal volume of a mixture of radioimmunoprecipitation assay (RIPA) buffer (Pierce RIPA Buffer; Thermo Fisher Scientific) and Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific), followed by sonication for 10 min on ice. The absorbance was measured at 562 nm using a microplate spectrophotometer.

2.4. Dynamic light scattering (DLS) analysis of the exosomes

The size distribution of exosomes was analyzed using a Zetasizer Nano Series apparatus (Malvern Panalytical Ltd., UK) with a He–Ne (4 mW) laser at 633 nm. The exosome preparations were diluted with PBS passed through a 0.22-µm filter and shaken to disaggregate possible exosome clumps. Subsequently, 200 µL of each sample was added to a cuvette having a 10-mm path length. These experiments were conducted in PBS at 25 °C at least five times with three freshly prepared samples. DLS measurements were performed with 15 10-s acquisitions.

2.5. Cancer cell and exosome labeling analysis using nanoflow cytometry

Exosomes (5×10^{10} particles/mL) and cancer cells (2×10^5 cells/mL) were incubated with anti-CD9-APC/Fire 750, anti-CD63-FITC CLBGran/12, anti-CD81-Alexa Fluor 700, anti-CD105-APC, and anti-CD133-Brilliant Violet 421 antibodies (BioLegend, USA) for 30 min at room temperature. The exosome samples were resuspended in PBS and passed through a 0.1-µm filter to a final volume of 200 µL. Data were acquired and analyzed using a CytoFLEX SRT Sorter (Beckman Coulter) equipped with red (638 nm), blue (488 nm), and violet (405 nm) lasers. Distilled water (passed through a 0.2-µm filter) was used as the sheath fluid. The performance of flow cytometry was verified using a standard calibration kit (Nanobead Calibration Kit, Bang Laboratories Inc., Technologies Drive Fisher, Indiana, USA) composed of microspheres with 50- and 100-nm diameters and an internalized fluorescent dye. These beads were used to set the exosome gate and calculate the exosome counts. The optical configuration was set to use side-scatter information from a 405-nm laser (Violet-SSC-A). FSC and Violet-SSC-A were set on a logarithmic scale, and the fluorescence channels were set at a logarithmic gain. PBS passed through a 0.2-µm filter was analyzed before sample acquisition to remove background noise. The analysis was restricted to exosomes based on the characteristic properties of these vesicles in terms of FSC (size) and Violet-SSC-A (complexity). Each sample was analyzed using the low flow rate setting of 10 µL/min. At least 10×10^5 events were acquired for analysis.

2.6. Sorting exosomes using nanoflow cytometry

The exosomes labeled as previously described were mixed at a 1:1 ratio and sorted using a CytoFLEX SRT Sorter (purity mode; drop delay 34.01; side stream mode: Auto; Software version 1.0.3.10011). CD133+/CD105-, CD133+/CD105+, CD133-/CD105+, and CD133-/CD105- exosomes were separately sorted in 5 mL of sheath fluid.

2.7. Assessment of exosome morphology via scanning electron microscopy (SEM)

The exosome preparations were resuspended in PBS passed through a 0.2-µm filter, fixed in 4% glutaraldehyde, and serially diluted with distilled water. The surface was made conductive by applying a 2–5 nm gold-palladium coating by sputtering before imaging using FIB-FE SEM and a Versa 3D model (Field Electron and Ion Company, OR, USA).

2.8. Exosome uptake analysis

Exosomes (corresponding to 10 µg protein) were sus-

pended in 100 µL PBS and mixed with 100 µL Acoerela Aco-490 (Chameleon Science, Singapore). The mixture was diluted in 5 mL PBS, and the exosomes were pelleted by ultracentrifugation on a 25% sucrose cushion at 100,000 ×*g* for 70 min at 4 °C. The pellet was washed with PBS and suspended in 100 µL PBS, and exosomes were quantified using DLS. Exosome internalization was determined using confocal microscopy. For confocal microscopy, 1×10^3 MDA-MB-231 cells were seeded per well in an 8-well Lab-Tek chamber slide (Nalge Nunc). The following day, 100 labeled exosomes per cell were added and incubated for 4 h at 37 °C. The slides were subsequently stained with 2 µM CellTracker Red CMTPX (Life Technologies), and images were captured with a 100× objective of Confocal Leica Stellaris (Leica Microsystems).

2.9. Western blot analysis

MCF-7 cells were treated with exosomes derived from MDA-MB-231 cells. Cells were then rinsed with ice-cold PBS and lysed using RIPA buffer supplemented with a protease inhibitor cocktail. The homogenate was centrifuged, and the protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific). Immunoblotting was performed using a Fluorescence Separation Module (Bio-Techne, MN, USA). Briefly, the protein lysate was mixed with a fluorescent master mix and denatured for 5 min at 95 °C. Biotinylated ladder, samples, antibody diluent, specific primary antibodies, including anti-pAkt, anti-Akt, anti-pERK, anti-ERK, anti-cyclin D1, and anti-β-actin (all antibodies procured from Cell Signaling, MA, USA), secondary antibody, and luminol-peroxide mixture were loaded onto the detection module, and immunoblotting was performed using a Jess Automated Western Blot System (Bio-Techne).

2.10. Migration assay

MCF-7 and MDA-MB-231 cells were cocultured with the respective exosome and seeded at a density of 2×10^5 cells/well in a 24-well plate. Cells were then incubated at 37 °C under a water-saturated 95% air–5% CO_2 atmosphere. Cells were scraped with a P200 pipette tip, washed with PBS, and the medium was replaced with a serum-free medium. Cells were then photographed using a live-cell imaging system (Celloger Mini Plus) at 5× magnification at 0, 24, and 48 h.

2.11. Statistical analysis

The results are presented as mean ± standard deviation (SD). A two-tailed *t*-test was used to identify significant differences between two samples, and one-way analysis of variance (ANOVA) was performed to compare more than two groups. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Characterization of MDA-MB-231 breast cancer cell-derived exosomes

Exosomes were prepared from MDA-MB-231 cell-conditioned serum-free medium using differential UC. The isolated exosomes were subjected to DLS, which revealed their size distribution (Figure 1A). All extracellular vesicle (EV) preparations revealed an acceptable size range (40–140 nm). Further analysis of the exosome concentration showed a large number of particles (2×10^{11}–10^{13} particles/mL). Additionally, the morphology of the exosomes, a round shape with a uniform and one-modal size distribution, was characterized using SEM (Figure 1B). Nanoflow cytometry demonstrated notable tetraspanin expression, specifically CD9, CD63, and CD81, with percentages of 4.26%, 24.06%, and 19.15%, respectively (Figure 1C). These findings indicated that exosomes were successfully extracted using UC, based on their sizes and surface markers, for downstream analyses.

3.2. Metastatic marker identification on cancer cells and cancer cell-derived exosomes

Flow cytometry analysis showed that the exosomes derived from MDA-MB-231 cells expressed the metastatic markers CD105 and CD133. Notably, the cancer cells exhibited low expression of these markers (Figure 2).

3.3. Identification of MDA-MB-231 breast cancer cell-derived exosomes and uptake by breast cancer cells

MDA-MB-231 breast cancer cell-derived exosomes were labeled with Acoerela Aco-490 and incubated with MDA-MB-231 and MCF-7 cells stained with CellTracker Red CMTPX. Fluorescently labeled exosomes were observed in the cytoplasm, indicating that the exosomes were taken up by breast cancer cells (Figure 3).

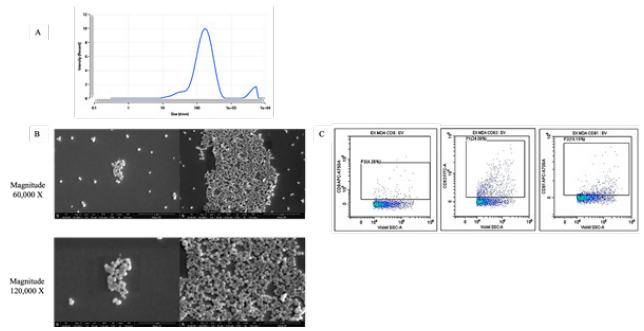

Fig. 1. Exosome identification using dynamic light scattering (A), scanning electron microscopy (B), and nanoflow cytometry analysis (C).

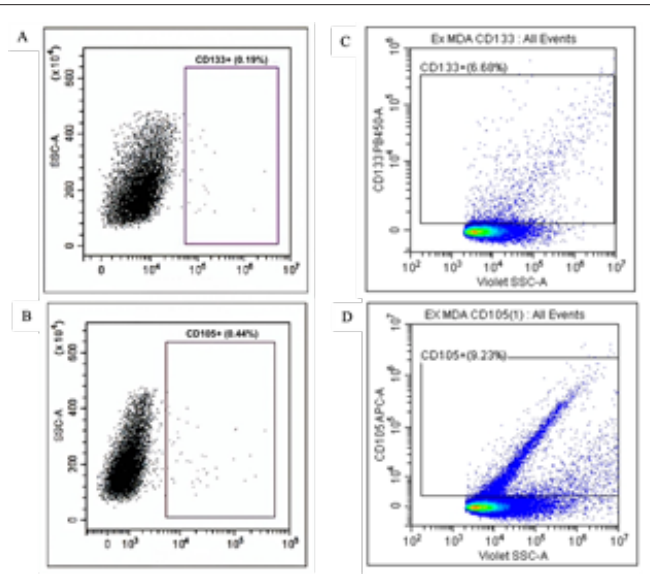

Fig. 2. CD133 and CD105 expression on MDA-MB-231 cells and MDA-MB-231 breast cancer cell-derived exosomes using nanoflow cytometry. (A-B) Surface expression of CD133-Brilliant Violet 421 and CD105-APC. (C-D) CD133-Brilliant Violet 421 and CD105-APC exosomes. The data are representative of three separate experiments.

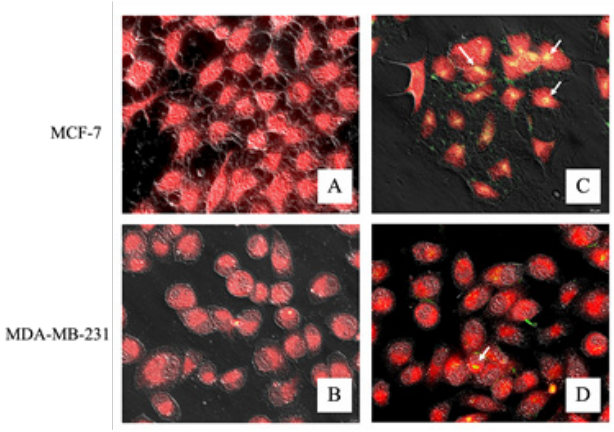

Fig. 3. Exosomes of MDA-MB-231 cells were taken up by breast cancer cells. Immunofluorescence staining of MDA-MB-231 cells (red) without (A-B) and with (C-D) exosomes (green).

3.4. MDA-MB-231 cell-derived CD133+ exosomes enhance cell signaling pathways related to cell survival and invasion

Our findings indicated that 41.64% of presorted exosomes were CD133+, and 90.11% of post-sorted exosomes were CD133+. Subsequently, the CD133+/CD105- exosomes were used for western blot analysis. Given that the MDA-MB-231 cell-derived exosomes were taken up by MCF-7 cells, we speculated whether this could affect the expression of the AKT/extracellular signal-regulated kinase (ERK)/cyclin D1 signaling pathway, promoting proliferation, invasion, and migration of MCF-7 cells. MDA-MB-231 cell-derived exosomes (CD133+) were used to stimulate MCF-7 cells at different exosome protein concentrations for 30 and 60 min. Low and high exosome protein concentrations substantially increased AKT phosphorylation (Figure 4). Low and high exosome protein concentrations led to ERK phosphorylation after 30 min of incubation. However, no significant difference was observed in cyclin D1 levels. A migration experiment was performed to assess the movement of MDA-MB-231 cells when cocultured with either CD133-negative or CD133-positive exosomes derived from MDA-MB-231 cells. The images illustrate cell migration over a 20-h duration. A visual examination indicated that MDA-MB-231 cells cocultured with CD133-positive exosomes had enhanced motility compared to that of cells cocultured with CD133-negative exosomes (Figure 5).

4. Discussion

Here, DLS, SEM, nanoflow cytometry, and western blotting were performed to characterize the exosomes from a breast cancer cell line. Our analysis showed that exosomes from MDA-MB-231 cells contained tetraspanins including CD9, CD63, and CD81 [22]. These exosomes were approximately 40–140 nm in radius and exhibited a uniformly round shape. These exosome characteristics are comparable to those reported in other studies, suggesting successful exosome isolation [23,24]. We subsequently examined the expression of CD133 and CD105 in MDA-MB-231 cells and exosomes [16,20,25,26]. The cancer cells exhibited low expression of these markers, whereas the exosomes exhibited elevated expression.

Previous studies have described CD 133 and CD105 low-phenotype MDA-MB 231 cells [27,28]. The findings of our study revealed that MDA-MB 231 cells with low CD 133 and CD105 levels produced considerable amounts of CD133+ and CD105+ exosomes. This suggests that they may contribute to the invasive properties of cancer cells.

Breast cancer cells exposed to exosomes exhibited diffused fluorescence, and no discernible difference was noted in the exosome uptake efficiency between MDA-MB 231 and MCF-7 cells. However, further studies are required to clarify uptake efficiency, which could be influenced by the origin of exosomes and recipient cells [29].

We categorized exosomes into four distinct types: CD133+/CD105-, CD133+/CD105+, CD133-/CD105+, and CD133-/CD105- to clarify the effect of exosome-derived MDA-MB 231 cells on MCF-7 cells. The sorting procedure was restricted to yielding only CD133+/CD105- and CD133-/CD105- exosomes. CD133+/CD105-exosome induced the expression of AKT and ERK phosphorylation in MCF-7 cells, suggesting that CD133 is involved in the activation of the mitogen-activated protein kinase (MAPK)/ERK pathway [30]. The relatively high cellular migration with CD133+ exosomes suggests that CD133 plays a crucial role in cancer metastasis and transport of oncoproteins to recipient cells [31,32].

5. Conclusion

To the best of our knowledge, this is the first study to assess the functions of highly expressed CD133 and CD105 cell-derived exosomes in the MAPK/ERK pathway [30, 33]. Exosomes produced by tumors help in cancer advancement by exerting an effect on signal-transducing proteins that control cell proliferation and migration. Furthermore, exosomes stimulate the invasiveness of recipient cells and promote tumor dissemination. Therefore, understanding how exosomes aid these effects is crucial for developing innovative cancer treatments. Suppression of exosome production or their interactions with recipient

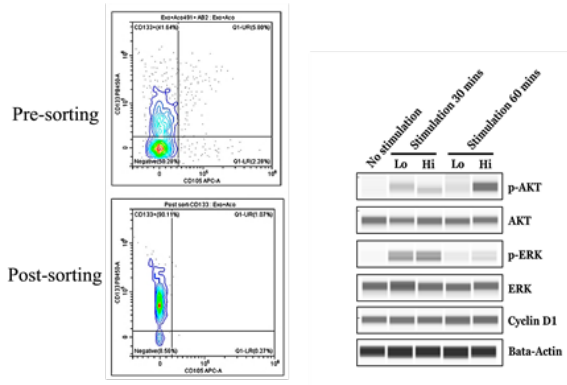

Fig. 4. Expression of phosphorylated and total AKT, ERK, and cyclin D1 in MCF-7 cells. Lo and Hi represent low and high exosome protein concentrations (1 and 5 µg/L, respectively).

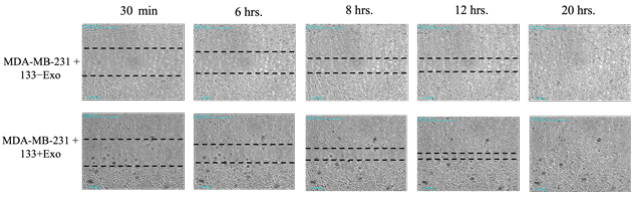

Fig. 5. Migration assay of MDA-MB-231 cells cocultured with MDA-MB-231 cell-derived exosomes (CD133-) and (CD133+).

cells can prevent cancer progression and spread. Further investigations into the intricate functions of exosomes in cancer are required.

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Author contributions
Songjang W, Jiraviriyakul A: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents and materials; Wrote the paper. Nensat C: Analyzed and interpreted the data; Wrote the paper. Adulyaritthikul P, Boonkoom T, Seetasang S, Promchai S: Performed the experiments. All authors contributed to manuscript editing and approved the final version.

Conflict of interest
The authors declare that they hold no competing interests.

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