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# Exosomes derived from breast cancer lung metastasis subpopulations promote tumor self-seeding

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ABSTRACT

Lung metastasis is a primary obstacle in the clinical treatment of metastatic breast cancer. Most patients with lung metastasis eventually die of recurrence. Recurrence may be related to self-seeding, which occurs when circulating tumor cells re-seed into the tumors they originated from (metastasis or carcinoma *in situ*). Tumor-derived exosomes have been intensively revealed to promote the progression of various cancers. However, whether tumor-derived exosomes play roles in tumor self-seeding has not yet been identified. By establishing a self-seeding nude mouse model, we found that exosomes derived from MDA231-LM2 cells (subpopulations of breast cancer lung metastasis) potentiate the growth of MDA-MB-231 xenografts. More importantly, laser confocal microscopy and flow cytometry results identified that MDA231-LM2-secreted exosomes promote the seeding of MDA231-LM2 cells into MDA-MB-231 xenografts. These findings suggest MDA231-LM2-secreted exosomes as a promising target to treat breast cancer lung metastasis.



## 1. Introduction

Breast cancer (BrC) is the commonest carcinoma and the leading cause of cancer-related mortality among women worldwide [1]. Metastasis to distant organs is one of the central hallmarks of a malignant tumor; it overwhelmingly contributes to poor prognosis and death of patients with BrC [2]. Locoregional relapse (recurrence in the breast, chest wall, or regional lymph nodes) was found to be associated with concomitant distant metastasis in approximately 30% of cases [3]. Organotropic metastasis occurs frequently among various cancers [4], and metastatic BrC has been widely reported to most often metastasize to the lungs [5]. Furthermore, an estimated 60%–70% of patients with lung metastasis eventually die as a result of relapse [6]. However, successful treatment remains a clinical challenge for metastatic BrC owing to the unclear recognition of the mechanism that drives relapse.

The complete cascade of primary tumor cells metastasizing to distant organs was conventionally viewed as a unidirectional process [7]. Dramatically, a subpopulation of hyperaggressive circulating tumor cells (CTCs, either derived from primary tumors or metastatic colonies) was shown to reinfiltrate their original tumor via a process termed “tumor self-seeding” [8,9]. Self-seeded tumor cells in animal models of BrC, melanoma, and osteosarcoma have recently been observed to rescue and potentiate the progression of primary tumors by accelerating tumor growth, angiogenesis, and stromal recruitment [8]. Mechanistically, Kim et al. [8] demonstrated that the primary tumor-derived cytokines IL-6 and IL-8 attract CTCs, whereas MMP1 and FSCN1 mediate CTC infiltration into mammary tumors. Additionally, using adenocarcinoma models, Eleonora et al. [10] demonstrated that chromogranin A suppresses tumor self-seeding by inhibiting the transendothelial migration of cancer cells. In addition, Zhang et al. [11] revealed that IL-6 suppression reduced CTC seeding in primary tumors in an osteosarcoma nude mouse model. Although MDA231-LM2 cells (lung metastasis subpopulations derived from MDA-MB-231 cells) were identified as donor tumor cells in a self-seeding model of BrC

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[8], how these cells infiltrate recipient tumors requires further elucidation.

As the major component of extracellular vesicles, exosomes are derived from the luminal membranes of multivesicular bodies. With a diameter of approximately 30–150 nm, exosomes have a lipid bilayer and a saucer-like shape [12]. Recently, exosomes have been widely studied as cell-to-cell communicators because of their ability to carry and deliver various types of signaling molecules, including lipids, proteins, and nucleic acids [13–15]. Strikingly, tumor-derived exosomes have been intensively identified to regulate tumor progression in various cancers. In BrC, exosomes have been proved to regulate metastasis, stem cell stimulation, apoptosis, immune suppression, and drug resistance [7,16,17]. Moreover, breast tumor-released exosomes have been demonstrated to regulate organotropic metastasis via delivering integrins [4]. However, further evidence is required to reveal the role of exosomes in tumor self-seeding.

Given the above uncertainties, we sought to determine the role of BrC cell-secreted exosomes in tumor self-seeding. We constructed self-seeding models via the subcutaneous injection of BrC cells. We next isolated and identified exosomes derived from MDA-MB-231 and MDA231-LM2 cells. The exosomes were introduced into the self-seeding models via tail vein injections. The seeding of donor tumor cells in recipient tumors was determined using laser confocal microscopy and flow cytometry. Our results identified a novel role of exosomes derived from subpopulations of BrC lung metastatic cells in promoting tumor self-seeding.

## 2. Materials and methods

Abbreviations

| | |
|---|---|
| BrC | breast cancer |
| CTCs | circulating tumor cells |
| IL | interleukin |
| MMP1 | matrix metallopeptidase 1 |
| FSCN1 | fascin actin-bundling protein 1 |
| FBS | fetal bovine serum |
| CM | conditioned medium |
| eGFP | enhanced green fluorescent protein |
| MOI | multiplicity of infection |
| PEG | polyethylene glycol |
| PBS | phosphate buffered saline |
| TEM | transmission electron microscopy |
| RT | room temperature |
| NTA | nanoparticle tracking analysis |
| sCMOS | scientific complementary metal-oxide semiconductor |
| SDS | sodium dodecyl sulfate |
| PVDF | polyvinylidene difluoride |
| BSA | bovine serum albumin |
| TBS-T | Tris buffered saline containing 0.1% Tween 20 |
| Alix | apoptosis-linked gene 2-interacting protein X |
| HSC70 | heat-shock cognate 70 |

### 2.1. Cell lines and culture

The human BrC cell lines BT-549, MDA-MB-231, and T-47D were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), and MDA231-LM2 was purchased from the BeNa Culture Collection (BNCC; Beijing, China). BT-549, MDA-MB-231, and T-47D cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (FBS; PAN-Biotech, Germany), whereas MDA231-LM2 cells were cultured in Leibovitz's L15 Medium (Thermo Fisher Scientific) supplemented with 10% FBS and 1% penicillin/streptomycin in a 5% $CO_2$ (100% air for MDA231-LM2) humidified incubator at 37 °C. To prepare the conditioned medium (CM), the cells were cultured in DMEM or L15 medium containing 10% exosome-free FBS for 48 h.

### 2.2. Construction of stable expression cell lines

We generated MDA231-LM2 and BT-549 cell lines that displayed stable expression of mCherry (MDA231-LM2-mCherry and BT-549-mCherry) and MDA-MB-231 and T-47D cell lines that displayed stable expression of enhanced green fluorescent protein (eGFP; MDA-MB-231-eGFP and T-47D-eGFP) by infection with lentiviral vectors (Obio, Shanghai, China; Hanbio, Shanghai, China). Lentiviral infection was performed following the manufacturer's instructions. Briefly, the cells were seeded in a 6-well plate at a density of 50% and cultured overnight for attaching. The next day, the cells were infected with lentiviral vectors (MOI = 30) for 24 h. To establish stably expressing cell lines, the infected cells were treated with 1 μg/ml puromycin (Thermo Fisher Scientific) for two weeks. The expressions of mCherry and eGFP in the above-mentioned cell lines were evaluated using an inverted fluorescent microscope (Nikon, Japan).

### 2.3. Exosome isolation

We isolated and purified exosomes using a method that combined polyethylene glycol (PEG)-based enrichment and ultracentrifugation as reported previously [18]. Briefly, a 2 × stock solution of PEG6000 (Sigma, MO, USA) was prepared. The CM was centrifuged at 500 × g for 5 min followed by 2000 × g for 30 min at 4 °C to remove cellular debris and large apoptotic bodies. Subsequently, the CM was filtered through a 0.22-μm filter (Merck Millipore, MA, USA) to remove microvesicles. Subsequently, an equal volume of prepared 2 × PEG solution was added to the CM and the contents were then incubated with rocking for 12 h at 4 °C. The next day, the samples were centrifuged at 3220 × g for 1 h at 4 °C and resuspended with particle-free phosphate buffered saline (PBS). The exosomes were washed to remove contaminated protein and PEG by ultracentrifugation at 110,000 × g for 70 min at 4 °C using an Optima XPN-100 ultracentrifuge (Beckman Coulter, NJ, USA). Finally, the exosomes were resuspended in particle-free PBS for immediate use or were stored at −80 °C.

### 2.4. Transmission electron microscopy (TEM)

After dilution in particle-free PBS, the exosomes were placed onto copper grids to be absorbed for 3 min at room temperature (RT), followed by negative staining with 3% phosphotungstic acid solution (pH 7.0) for 3 min at RT. The excess solution was removed, and the copper grids were placed onto a filter paper and were dried for 20 min at RT. The size and morphology of the exosomes were observed and recorded using a 120 kV transmission electron microscope (JEM-1400, JEOL, Japan) with a magnification of 20 × K.

### 2.5. Nanoparticle tracking analysis (NTA)

Nanoparticle parameters were identified using the NanoSight NS3000 system (NanoSight Technology, Malvern, UK). The exosomes were diluted to match 20 and 100 objects per frame and were then gently injected into the laser chamber. Each sample was equipped with a blue (488 nm) laser and a scientific complementary metal-oxide semiconductor (sCMOS) camera and was captured for 60 s. The detection threshold was maintained at 7 to ensure accurate and consistent detection of small particles. Finally, the

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