Disruption of Circulating Extracellular Vesicles as a Novel Therapeutic Strategy against Cancer Metastasis

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Metastasis is the main cause of cancer mortality for many types of cancer; however, difficulties remain in effectively preventing metastasis. It has been recently and widely reported that cancer-derived extracellular vesicles (EVs) contribute to cancer metastasis. Thus, therapeutic strategies targeting cancer-derived EVs hold great promise because of the possibility of EVs driving the cancer microenvironment toward metastasis. Here, we provide a novel strategy for therapeutic antibody treatment to target cancer-derived EVs and inhibit the metastasis of breast cancer in a mouse model, establishing a rationale for further clinical investigation. Treatment with human-specific anti-CD9 or anti-CD63 antibodies significantly decreased metastasis to the lungs, lymph nodes, and thoracic cavity, although no obvious effects on primary xenograft tumor growths were observed. In in vitro and in vivo experiments, the EVs incubated with the targeted antibodies were preferentially internalized by macrophages, suggesting that antibody-tagged cancer-derived EVs would be eliminated by macrophages. Our results suggested that therapeutic antibody administration effectively suppresses EV-triggered metastasis in cancer and that the removal of EVs could be a novel strategy for cancer therapy.

INTRODUCTION

Metastasis is the main cause of human cancer mortality,1 and preventing metastasis is a critical issue in improving cancer survival rates. To suppress tumor metastasis, many researchers have been investigating the mechanisms of tumor metastasis and trying to identify metastatic suppressor genes. Therapies that specifically target metastatic suppressors are expected to suppress metastasis at a toxicity lower than that of conventional chemotherapies.2 There are a number of metastatic suppressor candidates, such as intercellular communication factors (e.g., NM23-H1/H2,3,4 KISS1,5 and RhoGD12), cell surface proteins and receptors (e.g., KAI1 [CD82],7) and transcription factors (e.g., LSD18) (reviewed in Smith and Theodorescu2 and Hurst and Welch9). In addition, drugs that recover the expression of these metastatic suppressor genes have been developed, such as medroxyprogesterone acetate, which targets NM23,10,11 and atrasentan, which is an endothelin receptor antagonist regulated by RhoGD12.12 However, the most of these have been shown to be ineffective in clinical trials,13,14 and no metastatic suppressor drugs are yet available for use in clinical treatments. Therefore, new strategies have been desired to prevent metastasis.

Recent studies have revealed a novel biomolecule, the exosome, which supports cancer metastasis.15 Exosomes are a subset of extracellular vesicles (EVs), which are lipid bilayer vesicles secreted from cells.16 Exosomes have a diameter of 30–200 nm, and they transfer miRNA, mRNA, DNA, and proteins to other cells, thereby mediating intercellular communications.17 Exosomes released from cancer cells have been revealed to support cancer metastasis at multiple steps,18 including angiogenesis at the primary tumor,19 immune reaction modulation,20,21 modification of the microenvironment,22 preparation of metastasis-supportive microenvironments (pre-metastatic niche),23,24 and even the determination of organ specificity in metastasis.25 In addition, we have previously shown that inhibiting exosome secretion via nSMase2 knockdown in cancer cells clearly decreases tumor metastasis in a xenograft breast cancer model, without affecting primary tumor size.19 These results illustrated the importance of exosomes in the different stages of metastatic cancer. Therefore, studies aiming to achieve cancer-derived exosome depletion from the circulation could be promising for the development of novel therapeutics that would mitigate tumor metastasis.26,27

In this study, we demonstrated the therapeutic concept of suppressing cancer metastasis via the depletion of cancer-derived EVs. Antibodies to human-specific CD9 and CD63, which are considered to localize at the surface of most exosomes,26 were utilized to inhibit...
were observed by electron microscopy (Figure 1A). A NanoSight procedure.

The vesicular structures of the EVs isolated from the highly metastatic human breast cancer cell line MDA-MB-231-luc-D3H2LN were measured using a NanoSight nanoparticle tracking system. (C) Immunoelectron microscopy images of MDA-MB-231-luc-D3H2LN EVs labeled by anti-human CD9 and anti-human CD63 antibodies. Scale bar, 100 nm. (D) Human-specific recognitions of anti-human CD9 and anti-human CD63 antibodies. The EVs and cell lysates from a human breast cancer cell line (MDA-MB-231-luc-D3H2LN; depicted as MM231) and a mouse breast cancer cell line (4T1; depicted as 4T1) were analyzed by western blot using anti-human CD9, anti-mouse CD9, anti-human CD63, and anti-mouse CD63 antibodies. Hsp70 was used as the loading control.

Figure 1. Specific Recognition of Anti-CD9 and Anti-CD63 Antibodies on the Surfaces of Cancer-Derived EVs

(A) A representative image of EVs isolated from MDA-MB-231-luc-D3H2LN cells under transmission electron microscopy. Scale bar, 100 nm. (B) The particle size distributions and concentrations of EVs from MDA-MB-231-luc-D3H2LN cells were measured using a NanoSight nanoparticle tracking system. (C) Immunoelectron microscopy images of MDA-MB-231-luc-D3H2LN EVs labeled by anti-human CD9 and anti-human CD63 antibodies. Scale bar, 100 nm. (D) Human-specific recognitions of anti-human CD9 and anti-human CD63 antibodies. The EVs and cell lysates from a human breast cancer cell line (MDA-MB-231-luc-D3H2LN; depicted as MM231) and a mouse breast cancer cell line (4T1-luc; depicted as 4T1) were analyzed by western blot using anti-human CD9, anti-mouse CD9, anti-human CD63, and anti-mouse CD63 antibodies. Hsp70 was used as the loading control.

Anti-CD9 and Anti-CD63 Antibodies Stimulated the Removal of Cancer-Derived EVs by Macrophages

Next, we assayed whether the anti-CD9 or anti-CD63 antibodies affected the behavior of the EVs. Previous studies have demonstrated that exogenous EVs are removed by macrophages in vitro and in vivo.31,32 Because macrophages are among the phagocytic cells, phagocytosis is stimulated when antibodies are attached to antigens by the opsonization effect. Therefore, we examined whether the anti-CD9 and anti-CD63 antibody treatment enhanced the EV clearance by stimulating the uptake by macrophages and whether the EV uptake occurred via phagocytosis. EVs from MDA-MB-231-luc-D3H2LN cells were labeled with the green fluorescent dye PKH67, which labels the lipid bilayer membrane. To examine the mechanism of the EV uptake by the macrophages, we used two inhibitors, i.e., cytochalasin D, which inhibits both phagocytosis and macropinocytosis, and 5-(N-ethyl-N-isopropyl) amiloride (EIPA), which is a macropinocytosis inhibitor. We found that 5 μM cytochalasin D was sufficient to inhibit the phagocytosis of zymosan by macrophages, and 25 μM EIPA partially inhibited the macropinocytosis of dextran red. The mouse macrophage cell line RAW264.7 was incubated with the inhibitors for 30 min prior to incubation with the EVs. Cytochalasin D inhibited the uptake of the EVs (Figure 2A, left panel), whereas EIPA stimulated the uptake of EVs compared with the control groups (Figure 2A, right panel). These results suggested that the EVs were not internalized via macropinocytosis and that the majority of the internalization of EVs by macrophages may have been due to phagocytosis. However, cytochalasin D has recently been reported to affect other endocytosis mechanisms,33,34 such as inhibiting actin fiber formation.35,36 Therefore, we were unable to define whether the EVs were taken up by phagocytosis. When the lysosomes were labeled with LysoTracker red, the EVs were likely to co-localize...
with the lysosomes (Figures 2B, S1A, and S1B). Therefore, the EVs that were internalized by the macrophages were likely to be degraded by the lysosomes. Next, we observed the effects of the antibodies on the macrophage uptake of the EVs. The labeled EVs were incubated with or without antibodies and were then added to a culture of RAW264.7 cells. The EVs without antibodies and those with the control IgG were internalized by the macrophages to some extent, but the incubation of the EVs with the anti-CD9 and anti-CD63 antibodies further stimulated uptake (Figures 2B, 2C, and S1A–S1C). The non-internalized EVs remaining in the culture medium were significantly decreased when they were incubated with anti-CD9 and anti-CD63 antibodies compared with the control IgG (Figure 2D). These results suggested that the EV-recognizing anti-CD9 and anti-CD63 antibodies were able to stimulate the removal of the cancer-derived EVs by the macrophages. The antibodies continued to stimulate the uptake of EVs in the presence of cytochalasin D (Figures 2B, S1A, and S1B). However, with cytochalasin D, the EVs exhibited a fluctuated distribution and a lower co-localization with the lysosomes (Figure 2B). We hypothesized that the antibodies were able to stimulate the recognition of the EVs by the macrophages, but the intracellular transportation of the EVs to the lysosome was inhibited by cytochalasin D.

**Primary Tumors Were Not Affected by Anti-CD9 or Anti-CD63 Antibodies**

We prepared a mouse xenograft model of metastatic breast cancer and evaluated the effects of the anti-CD9 and anti-CD63 antibodies on primary and metastatic tumors. The experimental scheme is illustrated in Figure 3A. MDA-MB-231-luc-D3H2LN cells mixed with 50% extracellular matrix (ECM) gel were implanted orthotopically to the right inguinal fat pad of each mouse. At 6, 9, and 12 days after implantation, anti-CD9 or anti-CD63 antibodies dissolved in PBS (--) were injected intravenously at an amount of 45 μg/mouse. For the controls, an equal amount of control IgG protein or PBS (--) was injected. The tumor size was evaluated using in vivo imaging of luciferase-expressing cancer cell bioluminescence by injecting luciferin intraperitoneally. After 35 days, the tumor tissues were resected from the mice, and the estimated tumor volumes were calculated from the images. During the treatments, no significant reduction of mouse weight was observed in the mice that received either the anti-CD9 or the anti-CD63 antibody treatment, suggesting that the antibodies did not cause adverse effects in the mice (Figure 3B). There were no statistically significant differences in the primary tumor sizes among the treatments. In vivo imaging results (Figure 3C) or tumor volume measurements (Figure 3D). According to a previous report,19 cancer-derived EVs stimulate vascular formation in primary tumors, which results in increased metastasis. We assessed the vascular formation of the primary tumors using anti-CD31 antibody immunohistochemistry to visualize blood vessels, and we analyzed whether the antibody treatments decreased angiogenesis in the primary tumors. The density levels of the blood vessels in the tumor tissue that received the anti-CD9 antibody treatment were the same as those in the control.
Body exhibited almost the same growth rate as the cells incubated using an MTS assay. The cells incubated with the anti-CD9 antibodies on the metastatic characteristics of cancer cells in vitro. We next analyzed the effects of the anti-CD9 and anti-CD63 antibodies on cancer cell proliferation, invasion, and EV secretion. Effects of Antibodies on Cancer Cell Proliferation, Invasion, and EV Secretion

We next analyzed the effects of the anti-CD9 and anti-CD63 antibodies on the metastatic characteristics of cancer cells in vitro. Cell proliferation in the presence of these antibodies was evaluated using an MTS assay. The cells incubated with the anti-CD9 antibody exhibited almost the same growth rate as the cells incubated with control IgG and PBS (−), whereas the cells that were incubated with anti-CD63 exhibited increased cell proliferation (Figures S3A and S3B). Although the anti-CD63 antibody seemed to be capable of promoting cell proliferation in vitro, the effect was subtle in vivo because the tumor volume remained unchanged (Figures 3C and 3D). Next, cellular invasive abilities were evaluated via invasion assays with anti-CD9 or anti-CD63 antibody treatment. The cells that were incubated with anti-CD9 and those incubated with anti-CD63 antibody did not exhibit any differences in invasion ability (Figure S3C).

We also examined whether the antibodies inhibited EV secretion in vitro. As illustrated in Figures S3D and S3E, there were no significant differences in the particle numbers or protein amounts of the EVs from the cells that were treated with the anti-CD9 or the anti-CD63 antibodies and those from the control cells. Collectively, the antibody treatments did not have significant phenotypic effects on the cancer cells in vitro or in vivo. Suppression of Metastasis by the Anti-CD9 and Anti-CD63 Antibody Treatments

We detected metastatic cells using cancer cell bioluminescence in vivo and ex vivo 35 days after implantation. The orthotopically implanted cancer cells metastasized to the axillary lymph nodes, thoracic cavity, and the lungs (Figures 4A, 4B, S4A, and S4B). At the axillary lymph nodes, the anti-CD63 antibody treatment significantly suppressed metastasis (Figures 4A and S4A). Anti-CD9 antibody treatment did not significantly suppress metastasis, but did reduce the frequency of the highest level of metastasis (Figures 4A and S4A). In the thoracic cavity, the antibody treatment elicited no statistically significant suppression of metastasis (Figure 4A). However, the anti-CD63 antibody treatment resulted in a low frequency of metastasis, and the anti-CD9 antibody treatment slightly decreased the high level of metastasis (Figure S4A). In the lungs, the mice treated with the antibodies exhibited lower degrees of metastasis; specifically, the anti-CD63 antibody treatment dramatically suppressed metastasis (Figures 4B and S4B).

To accurately assess the lung metastasis rate, lung tissues were isolated, and bioluminescence was measured using an in vivo imaging system (Figure 4B). Importantly, the anti-CD9 and anti-CD63 antibody treatments significantly decreased the bioluminescence signals from the lungs of the mice. Furthermore, we performed immunohistochemistry with sections of the lungs to observe the disseminated tumor cells. The metastatic foci of the human breast cancer cells metastasized to the axillary lymph nodes, thoracic cavity, and lungs (Figures 4A, 4B, S4A, and S4B). At the axillary lymph nodes, the anti-CD63 antibody treatment significantly suppressed metastasis (Figures 4A and S4A). Anti-CD9 antibody treatment did not significantly suppress metastasis, but did reduce the frequency of the highest level of metastasis (Figures 4A and S4A). In the thoracic cavity, the antibody treatment elicited no statistically significant suppression of metastasis (Figure 4A). However, the anti-CD63 antibody treatment resulted in a low frequency of metastasis, and the anti-CD9 antibody treatment slightly decreased the high level of metastasis (Figure S4A). In the lungs, the mice treated with the antibodies exhibited lower degrees of metastasis; specifically, the anti-CD63 antibody treatment dramatically suppressed metastasis (Figures 4B and S4B).

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Organ Distribution of the EVs Treated with the Antibodies in Animals

We monitored the in vivo distributions of the cancer-derived EVs in the mouse breast cancer model and determined whether the anti-CD9 or anti-CD63 treatments affected their distributions. We first quantified the cancer-derived EVs in the mouse blood. Anti-CD9, anti-CD63, or control IgG antibody was injected intravenously into mice harboring orthotopic MDA-MB-231-luc-D3H2LN tumors. After 24 hr, whole blood was collected, and the total EVs were concentrated by ultracentrifugation from the filtrated sera. The cancer-derived EVs were detected with an Exoscreen system™ using anti-human CD9 and anti-human CD63 antibodies. Although the signals from the cancer-derived EVs were low, we were able to detect cancer-derived EVs in the mouse sera (Figure S5). The anti-CD63 antibody treatment reduced the signal from the cancer-derived EVs, whereas the anti-CD9 antibody treatment did not significantly reduce the EVs in our experiment. These results suggest that at least the anti-CD63 antibody reduced the amount of EV in the mouse blood.

Next, we observed the organ distribution of the cancer-derived EVs. The EVs collected from the human breast cancer cell line MDA-MB-231-luc-D3H2LN were labeled with a near-infrared fluorescent dye DiR and were then incubated with anti-CD9, anti-CD63, or control IgG antibodies. Five micrograms of the EVs were intravenously injected into each mouse. At 24 hr after injection, the mice were sacrificed, and the fluorescence intensities were measured to determine the distributions of the EVs in each organ using the in vivo imaging system. The fluorescence intensities were lower in the organs of the mice that were treated with the EVs with anti-CD9 or anti-CD63 antibodies compared with the mice that were treated with the EVs without antibodies (Figure 5, the left panel). The control IgG antibody did not reduce the signal of the DiR-EVs. Although the signals from the lungs were not significantly reduced (Figure S6A), the fluorescent signals were specifically reduced in the spleens and livers (Figures S6B and S6C). These results suggest that the anti-CD9 and anti-CD63 antibodies reduced the accumulation of EVs in the mouse organs.

Stimulation of the Clearance of Cancer-Derived EVs by the Anti-CD9 and Anti-CD63 Antibodies Was Macrophage Dependent

Finally, we analyzed whether these stimulations of EV clearance were macrophage dependent in vivo. Mouse innate macrophages were depleted via the injection of clodronate liposome into the tail veins. Macrophage depletion was confirmed via immunohistochemistry for the tissue macrophages in the lungs, liver, and spleen using the anti-F4/80 antibody. The clodronate liposomes removed the majority of the macrophages from the lungs, spleen, and liver (Figure S7). After 5 days of clodronate treatment, the DiR-stained EVs reacted with anti-CD9, anti-CD63, or control IgG antibodies were intravenously injected, and the remaining EVs in the organs were evaluated via ex vivo imaging after 24 hr. The total EVs remaining in the entire organs were elevated via macrophage depletion treatment (Figure 5, right panel). After removing the macrophages, the EVs that were incubated with the anti-CD63 antibody exhibited signals comparable to those of the control groups. The anti-CD9 antibody elicited a weak reduction in the EVs, but the reduction rate was much lower than that of the mouse without clodronate treatment. These results indicated that the effect of the antibodies in the promotion of the removal of
EVs was macrophage dependent. The injection of control liposomes without clodronate only slightly decreased the DiR signals from the organs, but this decrease was not significant. In the lungs, the antibodies did not significantly alter the amounts of the EVs (Figure S6A). In the spleen, the clodronate treatment significantly reduced the signals from the EVs to the background level (Figure S6B). These results indicated that in the spleen, the injected EVs were primarily retained by tissue-resident macrophages. In the liver, the clodronate treatment eliminated the stimulation of the macrophage uptake of the anti-CD9 and anti-CD63 antibodies (Figure S6C).

**DISCUSSION**

We demonstrated that treatment with antibodies that recognize cancer-derived EVs was able to suppress cancer metastasis. The antibodies were intended to accelerate the removal of cancer-derived EVs by macrophages before the EVs could promote metastasis.

Several studies have demonstrated that cancer-derived EVs contribute to cancer progression, including metastasis; thus, the strategy of eliminating cancer-derived EVs has been considered to be a promising method to suppress cancer malignancy. However, this idea has not been fully experimentally supported. Our results demonstrated that the concept of the inhibition of the function of cancer-derived EVs using antibodies is effective in the prevention of cancer metastasis, which is applicable in terms of therapeutic antibodies in clinical situations. This concept will contribute to the development of novel cancer treatment strategies for the prevention of metastasis. Because the suppression of cancer metastasis critically leads to longer survival and alleviates pain, this treatment would be beneficial for patients.

Targeting EVs has several advantages compared with targeting tumor suppressor genes for the prevention of metastasis. First, the strategy of targeting EVs is widely applicable to tumors in different organs and of different subtypes because EVs are secreted from nearly all cells, including a variety of cancer cells. By contrast, tumor suppressors have a rather narrow applicability because the mechanism of cancer metastasis seems to depend on the cancer type. Some metastatic suppressors even elicit pre-metastatic results, e.g., NM23 in neuroblastomas and KISS1 in hepatocellular carcinomas. Additionally, because EVs promote cancer metastasis at multiple steps, the blocking of EVs will be effective against multiple processes. EVs are easily targeted by therapeutics reagents because they are secreted outside of the cells, whereas some tumor suppressors are located inside of the cells and are difficult to target using antibodies. These advantages increase the feasibility of the clinical application of EVs for metastasis suppression.

The key to this strategy is antibody specificity. In this experiment, we used human-specific antibodies to recognize EVs from human cancer cells in mice. The anti-human CD9 and anti-human CD63 antibodies in this experiment were not suitable for direct use in humans as antibody drugs because CD9 and CD63 are ubiquitous in the human body, and antibody treatment would cause severe side effects. Rather, this strategy can be clinically applied via the use of antibodies that target specific proteins on the surfaces of cancer-derived EVs. Molecules that are specific to the surfaces of cancer-derived EVs have been surveyed as cancer biomarkers and include CD147 in colon cancer and CD24 and EpCAM in ovarian cancer. Recently, EVs from several cancer cells have been found to display integrin β1, whereas normal cells did not; thus, this is a possible therapeutic target. Additionally, not only the surface proteins but also sugar chains, lipids, and other surface structures can potentially be targeted by antibodies. The targeting of these cancer-specific molecules on the surfaces of EVs via antibodies will enable the inhibition of EV function via the stimulation of the removal of cancer-derived EVs by opsonization. For clinical development, studies that are generally required for antibody drugs, such as humanization, in vivo distribution, and dose determinations, also apply in this case. However, cancer antigens often exist on the surfaces of non-cancer-derived EVs and normal cells. This low specificity will damage organs and tissues when the antibodies are administered. To overcome this difficulty, cancer-derived EVs may be removed through a dialysis column, such as the Hemopuri. The Hemopuri is applied to remove the Her2 exosomes from blood to enhance the therapeutic effects of Herceptin. By attaching antibodies targeting cancer-derived EVs to the column, the cancer-derived EVs can be removed without injecting a large amount of antibodies into the human body.

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**Figure 5. Macrophage-Dependent Stimulation of EV Clearance by Anti-CD9 and Anti-CD63 Antibodies**

The localizations of the EVs with anti-CD9 or anti-CD63 antibodies in the whole organs were evaluated using the DiR-stained EVs. The results from mice without treatment (macrophage +, the left panel) and with depletion of the macrophages with clodronate liposomes (macrophage −, the right panel) are shown. Representative images are shown in the lower panels. The DiR-stained EVs were incubated with antibodies and intravenously injected into mice via the tail vein. PBS without EVs stained by DiR was injected for the controls. The relative amounts of EV were evaluated according to the radiant efficiency in the areas of the organs at 24 hr after injection. The graph represents the mean ± SD. Macrophage +: EVs only n = 11, CD9 n = 9, CD63 n = 9, IgG n = 5. Macrophage −: EVs only n = 6, CD9 n = 4, CD63 n = 4, IgG n = 4. **p < 0.001, ANOVA with Dunnett’s test.”
Our in vitro and in vivo studies demonstrated that the cancer-derived EVs that were recognized by the antibodies were likely to be removed by macrophages (Figures 2 and 3). Previous reports have demonstrated that macrophages remove exogenous EVs in vitro31 and in vivo.32 The liver and spleen are organs in which externally injected EVs are likely to accumulate,33,34 and the resident macrophages of the liver and spleen have been suggested to uptake EVs.32 Accelerated EV removal via antibodies occurred in our mouse experiments. We demonstrated that the anti-CD9 and anti-CD63 antibodies reduced the amount of EVs that accumulated in the organs, particularly in the liver and spleen (Figures 5, S6B, and S6G). After the depletion of macrophages, the clearance of EVs was not stimulated by anti-CD9 and anti-CD63 antibodies (Figures 5 and S6A–S6C), which indicates that the macrophages were responsible for the stimulated EV uptake by antibodies. We also demonstrated that anti-CD63 treatment reduced the circulating cancer-derived EVs in mouse blood (Figure S5). Based on these results, the EVs that were recognized by the anti-CD9 and anti-CD63 antibodies were preferentially eliminated by the macrophages.

The opsonic effects of EV-recognizing antibodies may stimulate a vaccination effect against cancer-derived EVs because macrophages have antigen-representing abilities. After activation by stimuli, such as interferon gamma, macrophages express MHC class II proteins45–47 and present antigens on their surfaces. Therefore, it is expected that if antibodies stimulate the uptake of cancer-derived EVs by opsonization, antigen presentation by macrophages will be stimulated. The represented antigens will activate T cells to attack both cancer cells and cancer-derived EVs. Although the mice used in this study were SCID mice and did not have either T cells or B cells, the activation of T cells can occur in immune-competent mice and humans.

However, the removal of EVs by macrophages may be a part of the dysfunctional mechanism of cancer-derived EVs, and there is still room for the consideration of other mechanisms. We also assessed the possibility that the anti-CD9 and anti-CD63 antibodies inhibited the uptake of the cancer-derived EVs. The EVs isolated from the MDA-MB-231-luc-D3H2LN cells labeled with PKH67 were incubated with a series of concentrations of anti-CD9 antibodies, anti-CD63 antibodies, and control IgG and then supplemented with human umbilical vein endothelial cells (HUVECs). Confocal fluorescent microscopy images revealed that the labeled EVs had been incorporated into the cells and had accumulated around the nuclei (Figure S8A). Fluorescence microscopic observations of the EV uptake revealed that the CD9 antibodies influenced EV uptake by the HUVECs in a dose-dependent manner, whereas the CD63 antibodies only slightly inhibited uptake (Figure S8B). Our finding that the anti-CD9 antibody inhibited the uptake of EVs by HUVECs is consistent with a previous report that stated that an antibody against CD9 inhibited the uptake of EVs by dendritic cells.48 Therefore, it is also possible that the EVs with the anti-CD9 antibodies may have been inhibited in terms of uptake into the mouse organs. Indeed, the anti-CD9 antibody still slightly reduced the amount of EVs accumulated in the organs, especially the liver, even in macrophage-depleted conditions (Figures 5 and S6C). This result implies that the anti-CD9 antibody reduced amount of EVs in the organs by both stimulating the removal of EVs by macrophages and inhibiting the uptake of EVs.

Additionally, on the basis of these experiments, we cannot exclude the possibility that the antibodies attached to circulating tumor cells (CTCs) and stimulated the removal of the CTCs by phagocytosis. We assessed the effects of anti-CD9 and anti-CD63 antibodies on breast cancer cell colonization in the lungs. Following the injection of breast cancer cells treated with antibodies into the mice, the cancer cells that were treated with anti-CD9 and anti-CD63 antibodies colonized the lungs at the same levels as the cells that were incubated with the control IgG, which suggests that the antibody treatments did not affect cell attachment to or proliferation from a small population in the lungs.

In summary, we suggest a new treatment strategy for suppressing cancer metastasis via the inhibition of the pro-metastatic functions of cancer-derived EVs using antibodies. This strategy can be applied to prevent the metastasis of a wide variety of tumors by selecting antibodies that are specific to cancer-derived EVs.

**MATERIALS AND METHODS**

**Reagents**

The anti-human CD9 antibody (clone 12A12) and anti-human CD63 antibody (clone 8A12) dissolved in PBS (--) were developed and kindly given by Shionogi & Co. The anti-human CD9 antibody (clone 12A12, SHI-EXO-M01) and anti-human CD63 antibody (clone 8A12, SHI-EXO-M02) were purchased from Cosmo Bio for the in vitro experiments. The LEAF-Purified Mouse IgG2b, κ Isotype Ctrl (clone MPC-11) was purchased from BioLegend and used as the control antibody. Mouse IgG2b, kappa monoclonal [MPC-11]-isotype control (low endotoxin, azide free) (clone ab18457, Abcam), was also used as a control for the observations of the in vivo clearance of the DiR-stained EVs. In the western blot analyses of the human CD9 and human CD63, ECL anti-mouse IgG, HRP-linked whole antibody (from sheep) (GE Healthcare), was used as secondary antibody. The mouse CD9 and CD63 were detected with anti-CD9 antibody (clone ab82390, Abcam) and anti-CD63 antibody (clone R5G2, MBL), followed by ECL anti-rat IgG, HRP-linked whole antibody (from goat) (GE Healthcare). For the detection of Hsp70, purified mouse anti-Hsp70 (clone 7/Hsp70, BD Biosciences) was used. For the immunohistochemistry analysis, the vessels in the primary tumors were stained with an anti-CD31 antibody (clone ab28364, Abcam), followed by an Alexa Fluor 594-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Thermo Fisher Scientific). The metastatic cancer cells in the mouse lungs were detected with an anti-human vimentin antibody (clone SP20, Vector Laboratories), followed by ImmPRESS reagent, anti-rabbit IgG (Vector Laboratories). The macrophages in the liver, spleen, and lungs were detected using an anti-F4/80 antibody (CI:A3-1, Bio-Rad), followed by ImmPRESS anti-rat IgG (Vector Laboratories). For the inhibition
of macrophage uptake in vitro, cytochalasin D (Sigma-Aldrich) and EIPA (Sigma-Aldrich) were used. To label the lysosomes, LysoTracker Red (Thermo Scientific) was added before the observations.

**Cell Lines and Cultures**
A highly metastatic human breast cancer cell line, MDA-MB-231-luc-D3H2LN, was obtained from Xenogen. A mouse breast cancer cell line, 4T1-luc, was previously described.19 MDA-MB-231-luc-D3H2LN and 4T1-luc cells were grown in RPMI 1640 (GIBCO from Thermo Fisher Scientific) supplemented with 10% (v/v) heat-inactivated FBS (GIBCO), 1% (v/v) antibiotic-antimycotic (GIBCO). Mouse macrophage RAW264.7 cells, which were purchased from DS Pharma Biomedical, were cultured in DMEM supplemented with 10% heat-inactivated FBS (GIBCO), 1% antibiotic-antimycotic (GIBCO), and 1% L-glutamine (GIBCO). HUVECs were purchased from Lonza and cultured in EGM-2 BulletKit (Lonza). All cells were cultured in a 5% CO2, humidified atmosphere at 37°C.

**Preparation of EVs**
After being washed with PBS (–), MDA-MB-231-luc-D3H2LN or 4T1-luc cells were cultured in Advanced RPMI 1640 medium (GIBCO) supplemented with 1% antibiotic-antimycotic and 1% GlutaMAX (GIBCO) for 2 days to collect the spent medium. The cells were supplemented with fresh medium and incubated for 2 days to collect the additional spent medium. The spent medium was centrifuged at 2,000 × g for 10 min at 4°C, and the supernatant was filtered with a 0.22-μm filter unit (Millipore). The filtered medium was ultracentrifuged at 35,000 rpm for 70 min at 4°C using SW41 Ti rotor (Beckman Coulter). The pellet was washed with PBS (–) once and resuspended in the remaining PBS (–). The protein concentration of EVs was measured using a Qubit Protein Assay kit (Molecular Probes from Thermo Fisher Scientific). The particle number of EVs was measured by NanoSight LM10 with NTA2.3 Analytical software (NanoSight).

For the preparation of fluorescent-labeled EVs, a PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma-Aldrich) or DiR (Caliper Life Sciences) was used. Purified EVs were incubated with 2 μM PKH67 in PBS for 5 min or 20 μM DiR in PBS for 15 min at room temperature without light. The EVs were washed with PBS three times on a Vivacon 500 (100,000 MWCO) filter (Sartorius Stedim) to remove unincorporated dye. The stained EVs were resuspended in PBS (–) and used for assays. The same volume of PBS (–) without EVs was treated in the same way for the controls.

**Electron Microscopy**
The EVs purified from MDA-MB-231-luc-D3H2LN cells were observed using phase contrast electron microscopy, as described in Kosaka et al.19 For the immunoelectron microscopy, EVs were allowed to adsorb to form var-coated nickel grids at room temperature. Thereafter, the grids were incubated with primary antibody (the anti-human anti-CD9 or anti-CD63) in 1% BSA-PBS for 2 hr at room temperature; then, the grids were washed with 1% BSA-PBS. The grids were subsequently incubated with secondary antibody conjugated to 10-nm gold particles (goat anti-mouse IgG polyclonal antibody, British BioCell International) for anti-CD63 antibody and 5-nm gold particles (goat anti-mouse IgG polyclonal antibody, British BioCell International) for anti-CD9 antibody for 90 min at room temperature. After being washed with PBS, the grids were stained with 2% phosphor tungstic acid solution (pH 7.0) for 15 s. The grids were observed by a transmission electron microscope (JEM-1400Plus; JEOL) at an acceleration voltage of 80 kV.

**Immunoblot Analysis**
MDA-MB-231-luc-D3H2LN and 4T1-luc cells were dissolved in M-PER Mammalian Protein Extraction Reagent (Pierce from Thermo Fisher Scientific) to prepare cell lysates. The protein concentration was determined by a micro BCA assay. The cell lysates or EV suspensions were denatured in 4× sample buffer solution without 2-ME (Wako). The signals were detected using ImmunoStar LD (Wako).

**In Vitro Macrophage Uptake Assay**
Approximately 2 × 10^4 RAW264.7 cells were plated on black-walled 96-well plates in 100 μL of medium, and the plates were incubated overnight to allow for cell attachment. One microgram, based on the amount of protein, of PKH67-labeled EVs was incubated with 1 μg of anti-CD9 antibody or control IgG at room temperature for 1 hr. The EVs were diluted into RPMI 1640 medium without FBS. After removing the spent medium, 3 μg/100 μL of EVs with antibodies or only EVs were added to the cells, which were then incubated at 37°C for 15 hr. The EVs that remained in the supernatant were quantified using a Synergy H4 plate reader (BioTek) at excitation/emission wavelengths of 480/502 nm. The nuclei of the cells were labeled with 1 μg/mL Hoechst 33342 (Dojindo) in PBS for 20 min at 37°C. Next, the cells were fixed with 4% paraformaldehyde phosphate buffer solution (Wako) for 30 min. The plates were subjected to microscopic observations using a BZ-X710 (Keyence) with a 20× objective lens. The incorporated EVs were evaluated according to the fluorescence intensities of the images using ImageJ software. Cytochalasin D was used as a phagocytosis and macropinocytosis inhibitor, and EIPA was used as a macropinocytosis inhibitor. The RAW264.7 cells were treated with cytochalasin D or EIPA for 30 min, and then 5 μg of pKH67-stained EVs suspended in FluorBrite DMEM media (GIBCO) with Prolong Live Antifade Reagent (Molecular Probes) were added to the cells cultured in eight-well chambered coverglasses. For the non-treated control, the same volume of DMSO (lower than 0.5%) was added. To label the lysosomes, LysoTracker Red was added at the final concentration of 1:10,000 before the observations. The cells were observed with a Leica TCS SP5 confocal microscopy (Leica Microsystems).

**Mouse Studies**
The animal experiments in this study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. The immunodeficiency mouse C.B-17/Icr-scid/scid models were purchased from CLEA Japan or Charles River Laboratories, Japan. Female mice aged 5–6 weeks
were used for tumor implantation. A MDA-MB-231-luc-D3H2LN cell suspension in PBS (−) was mixed with an equal volume of an ECM gel from Engelbreth-Holm-Swarm murine sarcoma (Sigma, E1270), and after the anesthetization of the mice with isoflurane gas, a 2×10^6 cells/50 µL solution was orthotopically implanted into the right inguinal fat pads. Six days after implantation, the mice were treated with anti-human CD9 (12A12), anti-human CD63 (8A12), or the isotype control IgG2b (MPC-11, BioLegend), or PBS (−). The antibodies were intravenously injected at 45 µg in 100 µL of PBS into each mouse via the lateral tail vein in a single injection. The tumor volumes were calculated using the standard formula 0.5 × L × W^2, in which L is the longest diameter and W is the shortest diameter.49–51

In Vivo Imaging of Primary Tumors and Metastatic Cells

Beetle luciferin, potassium salt (Promega) was dissolved in PBS (−) to a final concentration of 15 mg/mL and then filtered using a 0.22-µm filter. The luciferin solution was injected at 200 µL per 20 g of mouse weight into each mouse intraperitoneally. After 10 min, bioluminescence was imaged using an IVIS Lumina II (PerkinElmer). Tumor volume was quantified by photon radius using Living Image software, version 4.3.1 (Caliper Life Sciences). The metastatic levels at the primary tumor tissues dissected from the mice were fixed in 4% paraformaldehyde phosphate buffer solution (Wako) and embedded in paraffin. Each section was deparaffinized and rehydrated, and then the antigens were activated by heating at 95°C for 45 min in an ImmunoSaver solution (Nissin EM). After blocking with BSA, the microvessels in the sections were stained with anti-CD31 antibody (1:50 dilution) at 4°C overnight, followed by staining with anti-rabbit secondary antibody with Alexa Fluor 594 (1:500 dilution) at room temperature for 30 min using Can Get Signal Immunoreaction Enhancer Solution (Toyobo). The sections were mounted with Vecta Shield mounting medium with DAPI (Vector Laboratories). The tumor cells were detected via incubation with anti-human vimentin antibody (1:100 dilution) at 4°C overnight, followed by incubation with ImmPRESS anti-rabbit IgG (Vector Laboratories), DAB staining was achieved using a Metal-Enhanced DAB Substrate Kit (Thermo Fisher Scientific), hematoxylin staining, and dehydridation. The slides were subjected to microscopic observations using a BZ-X710 microscope (Keyence) with a 10× objective lens.

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Observation of EV Distribution in Organs

The DiR-stained EVs were injected with the same protein amounts of anti-CD9 (12A12), anti-CD63 (8A12), and isotype control IgG2b (MPC-11, Abcam) antibodies for 1 hr at room temperature. Five micrograms, based on the amount of protein, of the DiR-stained EVs without antibodies and with antibodies, were intravenously injected into the tail veins of 6-week-old mice. At 24 hr after injection, the mice were sacrificed, and the organs were isolated to measure the radiant efficiency from the organs using an IVIS Spectrum system (PerkinElmer). The relative intensities of the organs were calculated by normalizing the radiant efficiency of the region of interest to that of the organs from mice injected with EVs without antibodies. For macrophage depletion, small-size clodronate liposomes (Xygieia Bioscience) were intravenously injected into the tail veins of 5-week-old female SCID mice at 100 µL/mouse. After 5 days, the DiR-stained EVs with or without antibodies were injected.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures and can be found with this article online at http://dx.doi.org/10.1016/j.ymthe.2016.10.009.

AUTHOR CONTRIBUTIONS


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