Differential in vivo biodistribution of $^{131}$I-labeled exosomes from diverse cellular origins and its implication for theranostic application.

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Abstract

Exosomes are critical mediators of intercellular crosstalk and are regulator of the cellular/tumor microenvironment. Exosomes have great prospects for clinical application as a theranostic and prognostic probe. Nevertheless, the advancement of exosomes research has been thwarted by our limited knowledge of the most efficient isolation method and their in vivo trafficking. Here we have shown that a combination of two size-based methods using a 0.20 μm syringe filter and 100k centrifuge membrane filter followed by ultracentrifugation yields a greater number of uniform exosomes. We also demonstrated the visual representation and quantification of the differential in vivo distribution of radioisotope $^{131}$I-labeled exosomes from diverse cellular origins, e.g., tumor cells with or without treatments, myeloid-derived suppressor cells and endothelial progenitor cells. We also determined that the distribution was dependent on the exosomal protein/cytokine contents. The applied in vivo imaging modalities can be utilized to monitor disease progression, metastasis, and exosome-based targeted therapy.

Graphical Abstract

Exosomes are biological nano-particles, which have immense prospects for clinical application as a diagnostic marker, monitoring disease progression and potential therapeutic carrier. However,
this advancement has been impaired by lacking of optimized isolation method and inadequate techniques to track their in vivo distribution. Here we present a simple, rapid and high yielding exosome isolation technique utilizing a combination of size-based and ultracentrifugation method. We also demonstrated the exact visualization and quantification of radioisotope-labeled exosomes from different cell types with or without treatment, in the primary solid tumors and metastatic area, which are dependent on the protein/cytokine contents of the exosomes.

Keywords
Exosomes; isolation; in vivo imaging; radioisotope labeling; MDSC exosomes; EPC exosomes

Background
Exosomes are highly heterogeneous lipid bilayered membranous vesicles (30–150 nm) of endosomal origin produced by all cell types and are released into biological fluids or cell culture medium. The initial concept of exosomes as “garbage bag” has been changed drastically as exosomes are now thought to be an integral part of intercellular communication. At any given time point, exosomes can contain all the known bioactive constituents of a cell, including proteins, lipids and nucleic acids. In addition to their physiological functions such as maintenance of cellular-stemness, immunity, tissue homeostasis, protein clearance, and signaling; exosomes contribute to the pathophysiology of several diseases. Tumor cell-derived exosomes (TDEs) are capable of altering the tumor microenvironment (TME) by stimulating the secretion of growth factors and cytokines from TME-associated cells. Thus TDEs play an imperative role in epithelial to mesenchymal transition (EMT), immune escape, tumorigenesis, tumor growth, angiogenesis, invasion, cancer stemness, and tumor drug resistance. TDEs arbitrate tumor-associated immune suppression and trigger pre-metastatic niche formation. Exosomes have immense prospects for clinical application as a diagnostic marker and for monitoring treatment response and disease progression. Recently, an exosome-based therapeutic delivery system has drawn tremendous attention owing to the distinctions between exosomes and synthetic nano-carriers. Nevertheless, few unsettled queries still affect the possible application of exosomes, e.g., the most efficient and reproducible approach for large-scale production of quality exosomes in a short time, the loading efficiency of therapeutic agents and the precise detection of exosome biodistribution.

Despite the intense research for understanding the biological and pathophysiological functions of exosomes, only a few studies have scrutinized exosome biodistribution. A breakthrough to investigate their in vivo distribution and track exosomes is immensely desired for safe and effective clinical application. Yet, to track their whereabouts, only a few effective methods are reported; these have mostly adopted fluorescent and bioluminescence imaging, either through labeling them with lipophilic membrane dye or manipulating them to exhibit a membrane reporter. Contrarily, the utilization of nuclear medicine imaging techniques, such as single-photon emission computed tomography (SPECT) or positron emission tomography (PET), that are non-invasive, can be combined with anatomical imaging, such as computed tomography (CT) or magnetic resonance imaging (MRI) can be
barely found in published articles. These nuclear imaging techniques have indisputable advantages over fluorescent and bioluminescence imaging, owing to their excellent sensitivity for the deeper tissues and quantitative measurement potential of the clinical grade labeling radioisotopes ($^{99m}$Tc, $^{131}$I, $^{111}$In-oxine).

Here, we propose a simpler and quicker isolation technique by a combination of size based and ultracentrifugation methods. We also demonstrate the visual representation and \textit{in vivo} distribution of exosomes isolated from tumor cells with or without treatment, and from myeloid-derived suppressor cells (MDSCs) and endothelial progenitor cells (EPCs) in metastatic breast cancer animal models by SPECT/CT. We also show that the differential biodistribution is related to the protein/cytokine contents of the collected exosomes.

\textbf{Methods}

Description of cell lines, nanoparticle tracking analysis, flow-cytometry, isolation of MDSCs and EPCs, protein quantification, thin layer paper chromatography, quantitative analysis of radioactivity, \textit{ex vivo} gamma activity, and statistical analysis are described in Supplementary Material.

\textbf{Ethics statement}

All the experiments were performed according to the National Institutes of Health (NIH) guidelines and regulations. The Institutional Animal Care and Use Committee (IACUC) of Augusta University (protocol \#2014–0625) approved all the experimental procedures. All animals were kept under regular barrier conditions at room temperature with exposure to light for 12 hours and dark for 12 hours. Food and water were offered \textit{ad libitum}. All efforts were made to ameliorate the suffering of animals. CO2 with a secondary method was used to euthanize animals for tissue collection.

\textbf{Exosome isolation}

Exosomes were isolated from the culture supernatants of 4T1 and AT3 tumor cell lines. Briefly, $5 \times 10^6$ tumor cells were plated in $175 \text{cm}^2$ flasks and grown overnight with 10% FBS complete media in normoxia (20% oxygen). The media was removed and replenished by exosome-free complete media. Exosomes were depleted from the complete media by ultracentrifugation for 70 minutes at 100,000x g using an ultracentrifuge (Beckman Coulter) and SW28 swinging-bucket rotor. The cells were then treated with control (DMSO), colony stimulating factor-1 receptor (CSF1R) antagonist (GW2580, 1μM) and 20-HETE synthase inhibitor (HET0016, 100μM) in hypoxia (1% oxygen) for 48 hours. The cell culture supernatant was centrifuged at 700x g for 15 minutes to get rid of cell debris. We employed five different methods as follows – 1) ultracentrifugation only by initial step with 10,000x g for 30 minutes followed by two steps with 100,000x g for 70 minutes each, 2) size-based method by passing through 0.20 μm syringe filter (Coming, USA) followed by centrifugation with 100k membrane tube (Pall Corporation, USA) at 3200x g for 30 minutes, 3) combination of two steps of size-based method by passing through 0.20 μm syringe filter and centrifugation with 100k membrane tube at 3200x g for 30 minutes followed by a single step of ultracentrifugation at 100,000x g for 70 minutes, 4) combination of one step size-
based method by passing through 0.20 μm and single ultracentrifugation at 100,000x g for 70 minutes and 5) commercially available density gradient separation by total exosome isolation reagent (Invitrogen™, USA). The reagent was added to the culture supernatant sample and incubated overnight at 4°C. The precipitated exosomes were recovered by centrifugation at 10,000x g for 60 minutes.

Tumor model
Both 4T1 and AT3 cells expressing the luciferase gene were orthotopically implanted in syngeneic BALB/c and C57BL/6 mice, respectively (Jackson Laboratory, USA). All the mice were between 5-6 weeks of age and weighed 18–20g. Animals were anesthetized using a mixture of Xylazine (20mg/Kg) and Ketamine (100 mg/Kg). Either 50,000 4T1 cells or 100,000 AT3 cells in 50μL matrigel (Corning, USA) were injected into the right mammary fat pad.

Radiolabeling of exosomes using Iodine-131 (131I)
Isolated TDEs were labeled by Pierce™ Iodination Beads (Thermo Scientific™). In short, 4–5 iodination beads were cleaned with sterile normal saline and allowed to air dry. The beads were added directly to 5 mCi of 131I solution (Cardinal Health, Inc.) and then incubated at room temperature. After 5 minutes, exosomes resuspended in PBS were added to the reaction tube and incubated at room temperature for 30 minutes. To stop the iodination reaction, the beads were taken out from the reaction tube. To get rid of free 131I, the labeled exosomes were washed and centrifuged with extra PBS using a 100k membrane tube at 3200x g for 15 minutes.

In vivo SPECT/CT imaging
After the intravenous injection of 350±50 μCi of 131I-labeled exosomes in 100μL into the tail vein, all animals underwent SPECT-CT scanning. During the whole procedure, the animals were anesthetized and maintained using a combination of 1.5% isoflurane and 1 L/min medical oxygen flow, and their body was immobilized in an imaging chamber to restrain movements. Whole body CT followed by SPECT-imaging was acquired by a nanoScan 4-headed micro-SPECT-CT scanner (Mediso, USA). The image acquisitions were commenced 3 hours after the injection of 131I-labeled exosomes. The reconstructed image size was 205×205×205μm.

Protein array
Exosomal proteins were evaluated for the expression profiles of 44 factors in duplicate by mouse cytokine antibody array (RayBiotech, Inc.). 500μg of protein sample was loaded onto the membrane and the chemiluminescent reaction was detected using LAS-3000 imaging machine (Fuji Film, Japan). All signals emitted from the membrane were normalized to the average of 6 positive control spots of the corresponding membrane using ImageJ software.
Results

Optimizing the exosome isolation method.

To optimize the exosome isolation method, we collected exosomes from 4T1 cell culture supernatant using five different techniques- 1) ultracentrifugation (UC) only, 2) size based only (0.20 μm and 100k membrane), 3) combination of size based (0.20 μm and 100k membrane) and UC, 4) combination of single size based (0.20 μm) and UC and 5) density gradient separation. Among the different methods employed, the density gradient separation technique (#5) yielded the most concentrated exosomes with $4 \times 10^{10}$ particles/mL, while (#1) UC alone and its (#4) combination with size based (0.20 μm) separation yielded the lowest concentration of $1.6 \times 10^{10}$ particles/mL and $1 \times 10^{10}$ particles/mL, respectively (Figure 1A). The combination of size based (0.20 μm and 100k) separation followed by UC (#3) yielded a concentration of $3 \times 10^{10}$ particles/mL. However, after the isolation process, there was visible sedimentation of co-isolated impurities and polymer or reagent along with the exosomes isolated by density gradient separation. The mean diameter of exosomes isolated by (#1) UC alone was 131.86±13.42 nm, (#3) combination of size based (0.20 μm and 100k membrane) and UC was 119.8±4.95 nm and (#5) density gradient method was 122.93±18.06 nm (Figure 1B). The size distribution curve of density gradient separation showed a wider base with a thick tail extending towards the smaller size (Figure 1C). There was no significant difference in common exosomal markers, CD9 and CD63 between the samples by flow-cytometry (Figure 1D). We also quantified total number of exosomes after each step of the proposed method (#3) (Figure 1E) that showed 19% of the exosomes from culture supernatant were recovered after the final step. There was no significant difference in exosomes size after each step (Figure 1F). Flow-cytometric analysis showed no significant changes in CD9 and CD63 on the surface of the exosomes (Figure 1G). Transmission electron microscopy (TEM) images for the exosomes isolated by method #3 showed normal morphology of exosomes without any distortion (Figure 1H).

Binding efficiency and serum stability of $^{131}$I-labeled exosomes

For confirming the binding efficacy of $^{131}$I to the exosomes and serum stability, we implemented thin layer paper chromatography (TLPC) [16]. Most of the free $^{131}$I alone moved from the spotted point in the bottom to the top half (Figure 2A). Contrarily, $^{131}$I bound to the exosomes remained at the bottom and barely moved to the top half, indicating $^{131}$I bound to the exosomes and very little dissociation of the $^{131}$I from the exosomes (Figure 2B). We appraised the labeling stability of $^{131}$I-labeled exosomes in serum by incubating $^{131}$I labeled exosomes with 20% FBS for 4 and 24 hours in 37° C followed by TLPC similar to the previous experiment. Identical to the $^{131}$I bound exosomes, $^{131}$I from serum-challenged $^{131}$I labeled exosomes hardly moved to the top, implying the labeling of exosomes with $^{131}$I was stable in serum even after 24 hours (Figure 2C).

Detection and quantification of radioisotope $^{131}$I-labeled tumor cell-derived exosomes in the primary tumor and metastatic site.

$^{131}$I labeled-TDEs in 100 μL solution was injected into tumor-bearing (tumor exo) and tumor-free (tumor-exo – no tumor) animals via tail vein. After 3, 24 and 48 hrs of administration, the animals were scanned by SPECT/CT, and the reconstructed images were
analyzed by ImageJ. We also injected free $^{131}$I in tumor-bearing mice (free I-131) to
determine the uptake of free $^{131}$I to the tumors. We observed an ample amount of radioactive
intensity after 3 hrs at the primary tumor site and metastatic site (lung) in the animals that
received $^{131}$I-labeled TDEs (Figure 2D). There was almost no radioactivity in the tumor
area, and negligible radioactivity in the lungs of the group injected with free $^{131}$I. The group
of animals without any tumor and injected with radiolabeled TDEs showed almost no
radioactivity in the mammary fat pad but plenty of radioactivity in the lungs (Figure 2E and
2F). We observed notable levels of radioactivity in thyroid and stomach. Radioactivity was
also high in the bladder for renal clearance.

**Distribution of MDSCs, EPCs and HEK293-derived exosomes in the primary and metastatic
site.**

Next, we wanted to see the biodistribution of exosomes derived from other cell types that
play a crucial role in tumor progression and metastasis. As a non-cancerous, non-specific
cell line, we used human embryonic kidney 293 (HEK293) cells. MDSCs were collected
from the spleen of tumor-bearing mice with more than 99% purity, and EPCs were isolated
from the bone marrow of normal mice with more than 85% purity (Figure 3A). TDEs (tumor
exo) were collected from the cell culture supernatant. The mean diameter of isolated
exosomes from HEK293 cells (HEK293 exo), MDSCs (MDSC exo) and EPCs (EPC exo)
was 97.6 nm, 131.1 nm, and 140.1 nm, respectively (Figure 3B and 3C). Three hours after
injecting the $^{131}$I-labeled exosomes intravenously into tumor-bearing animals, exosomes
from all the groups accumulated in the primary breast tumor and metastatic sites in the lungs
except for the HEK293 exo (Figure 3D). Interestingly, exosomes from the EPCs were
abundantly located in the primary tumor site, and an ample amount of exosomes from the
MDSCs were visualized more in the metastatic site-lungs than any other groups (Figure 3E and
3F).

**Ex-vivo gamma activity measurement of a different organ.**

After the final scan, we euthanized the animals and measured the weight and emitted gamma
activity of individual harvested organs. While most of the organs as a whole showed
negligible radioactivity, only the tumor and liver retained a significant load of radioactivity
in all groups of animal (Supplemental Figure S1A). Interestingly, lungs from the mice
injected with MDSC-exo showed considerably higher level of radioactivity than the other
groups. For mice injected with the EPC-exo, the primary tumor showed a notably higher
level of gamma activity than the other organs. We also calculated the radioactivity per
milligram of individual organs which showed similar changes of radioactivity as of the
whole organ. The non-tumor bearing group of mice that were injected with $^{131}$I-labeled
tumor exosomes showed gamma activity mostly in the liver (Supplemental Figure S1B).

**Cytokine array of MDSCs and EPCs-derived exosomal protein content**

To understand the variation in biodistribution of exosomes, we analyzed exosomal protein
content from MDSCs and EPCs compared with those of TDEs using a membrane-based
cytokine array. Angiogenic factors such as endoglin, Intercellular adhesion molecule-1
(ICAM-1), vascular endothelial growth factor receptor-2 (VEGFR2) and Platelet factor-4
(PF-4) were increased in both MDSC exo and EPC exo (Figure 4). Epidermal growth factor (EGF) was higher in both the MDSC and EPC exo than the tumor exo. Among the invasion factors, E-cadherin and E-selectin levels were increased in both MDSC and EPC exo. Notably, matrix metalloproteinase-2 (MMP-2) was increased in MDSC exo compared to tumor and EPC exo. Factors for myeloid activation and function such as granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF) and monocyte chemoattractant protein-1 (MCP-1/CCL2) were over-expressed in both MDSC and EPC exo than the tumor exo. In addition, MDSC exo also had higher expression of macrophage-inflammatory protein-1-alpha (MIP-1α/CCL3) and stromal cell-derived factor-1 (SDF-1α). T-cell function modulating factors such as interleukin (IL)-2, IL-7, L-selectin, and thymic stromal lymphopoietin (TSLP) were increased in MDSC and EPC exo. From the immunomodulatory cytokines, IL-2, IL-4, keratinocyte-derived chemokine (CXCL1/KC), C-X-C motif chemokine-5 (CXCL5/LIX), chemokine (C-C motif) ligand-5 (CCL5/RANTES) and tumor necrosis factor-α (TNF-α) were significantly high in both MDSC and EPC exo than the control exo. Furthermore, there was an obvious increase in IL-13 and IL-1 receptor antagonist (IL-1ra) in MDSC exo.

**Distribution of exosomes from treated tumor cells.**

To explore further, we wanted to investigate whether exosomes collected from tumor cells treated with drugs that decrease cancer growth and metastasis, would distribute differently in vivo. Previously, our laboratory showed that HET0016 and GW2580 treatment decreases lung metastasis of breast tumor-bearing mice [17, 18], and HET0016 decreases neovascularization and tumor growth with increased survival in a glioblastoma model [17, 18]. GW2580 is a selective small molecule kinase inhibitor of CSF1R, and HET0016 is a highly selective inhibitor of CYP4A in the arachidonic acid pathway that has been shown to decrease recruitment of tumor-infiltrating myeloid cells and decrease tumor-associated macrophages (TAMs) polarization towards M2 macrophages [19]. There were no significant differences in exosome size and marker (CD9 and CD63) after GW2580 and HET0016 treatment of cells (GW2580 exo and HET0016 exo, respectively) (Figure 5A, 5B and 5C). After labeling with 131I, the exosomes were injected into tumor-bearing mice, and in vivo imaging was acquired by SPECT/CT after 3 hours, 24 hours and 48 hours. There was increased radioactivity at the primary tumor site in all the groups (Figure 5D). Although there was an increased localization of the HET0016 exo in the primary tumor, it was not statistically significant (Figure 5E). However, there was an ascertainable decline of radioactivity in the lungs of GW2580 exo, and HET0016 exo groups compared to that of control exo groups (Figure 5F).

**Distribution in other organs and clearance**

In addition to the tumor and lungs, we also measured the radioactivity and clearance over time in other organs (Supplemental Figure S2). After 3 hours, the highest percentage of radioactivity was observed in the urinary bladder area, which subsequently cleared. A considerable amount of radioactivity was observed in the stomach, which was scarcely detectable in subsequent scanning. Among the organs, the liver, lungs, and tumor retained a perceptible amount of radioactivity over time. Furthermore, the thyroid gland in all the
animal groups showed no substantial change of radioactivity in all three scans (3, 24 and 48 hrs), suggesting no dissociation of the $^{131}$I from the exosomes that would eventually increase the free $^{131}$I uptake by the thyroid.

**Cytokine array of tumor-derived exosomal protein content after treatment.**

Finally, because of the differential biodistribution of exosomes isolated from tumor cells with or without treatment, we wanted to see if the treatment of 4T1 tumor cells with GW2580 or HET0016 affects the protein contents of exosomes. We extracted the exosomal proteins from untreated 4T1 cells (control), GW2580-treated cells, and HET0016-treated cells. We performed cytokine array analysis to evaluate the differences of 44 cancer-related factors for these three samples. Among the angiogenesis factors, VEGFR2, ICAM-1, bFGF were significantly reduced in exosomes from the treated cells compared to control cells (Figure 6). Among the chemotactic factors, the level of KC and macropage-derived chemokine (MDC) was significantly downregulated in GW2580 exo and HET0016 exo, respectively. Among the immune-modulatory cytokines, IL-6, TNF-$\alpha$, IL-12, IL-10, and IL-13 were decreased, and IL-4 was increased in the treated samples. The level of the invasive factors E-cadherin, Eotaxin, E-selectin and tissue inhibitors of metalloproteinases-2 (TIMP-2) was significantly reduced in exosomes from GW2580-treated cells. Interestingly, both GW2580 and HET0016 treatment of tumor cells increased the level of Fas ligand (FasL/CD95L) in the exosomes.

**Discussion**

To expedite the research and clinical application of exosomes, it is fundamental to be able to more explicitly and more effectively isolate exosomes from a wide spectrum of impurities. Until now, five isolation techniques have been developed by exploiting a particular trait, such as the size, density and surface markers of exosomes [20]. Here, we compared five different isolation techniques and implemented a modified reproducible protocol of isolating quality exosomes more efficiently for downstream experiments in a shorter period of time. The UC only method takes approximately 4-5hrs to isolate exosomes from the culture supernatant, and the density gradient-based technique needs a longer incubation time (overnight). Although size-based methods (alone or in combination) need the shortest period of time, it is not possible to pellet the exosomes from the media and subsequent wash. Our optimized method using a combination of size-based technique along with a UC method requires less than 2 hrs. We demonstrated that it yields a greater number of exosomes with uniform size, and almost 19% recovery of culture supernatant exosomes without changing the common exosome markers. Using a 0.20 $\mu$m syringe filter allows passing of vesicle size <200 nm, ensuring the exclusion of other larger extracellular vesicle types. Following 100k membrane centrifugation ensures removing most of the protein impurities that could be co-isolated with the exosomes by other methods. Although the density gradient separation techniques yielded more exosomes, the size distribution curve was not uniform, and there were visible impurities along with isolated exosomes. We did not compare the immune-affinity capture-based isolation method as it separates only the specific subgroup of exosomes that possesses the antigen of interest.
A few articles have reported the tumor targeting and metastatic tumor behavior of exogenously administered exosomes. While most of them adopted either fluorescence imaging [21–25] or bioluminescence imaging [26], only one article reported limited tumor accumulation of the injected exosomes by nuclear imaging [24]. However, the authors did not demonstrate visualization in tumor and metastatic sites in vivo. Fluorescence imaging shows inferior tissue penetration, auto-fluorescence of biological tissues, low resolution and often requires euthanasia. Bioluminescence has low sensitivity for the deeper organs with the necessity of injecting toxic substrates to generate optical signals, which is incompatible for sequential imaging [27]. We administered 131I-labeled TDEs and showed significant accumulation of exosomes in the primary tumor in tumor-bearing mice compared to the free 131I injected group and in the mammary fat pad (MFP) of non-tumor bearing mice. It is noteworthy to mention that there was almost no accumulation of TDEs in normal MFP, but they accumulated even in the normal lungs, implying the propensity of the breast TDEs for the future metastatic sites. The thyroid and stomach showed significant radioactivity due to the presence of a sodium-iodide symporter (NIS), which is an intrinsic plasma membrane glycoprotein that actively arbitrates iodide transport into the thyroid follicular cells [28] and several extra-thyroidal tissues, e.g., salivary glands, gastric mucosa and lactating mammary glands [28, 29]. However, the NIS expression level is lower in extra-thyroidal tissues than in thyroid tissues, and longstanding retention of iodide does not occur in these tissues [30], which is why the initial high level of radioactivity in the stomach was almost undetectable in subsequent scanning.

MDSCs are a heterogeneous population of immature myeloid cells that are directly implicated in the escalation of tumor metastases by participating in the EMT, tumor cell invasion, promoting angiogenesis and formation of pre-metastatic niche [31, 32]. EPCs are progenitor cells with the ability to differentiate into endothelial cells and play a pivotal role in tumor growth and metastasis by tumor neovascularization, promoting the transition from micro to macro-metastasis. Even though the critical roles of MDSCs and EPCs in tumor progression and metastasis have been studied extensively, the similar roles of exosomes derived from these cells are yet to be explored. Only a few articles are available on exosomes derived from MDSCs [14, 33–36] and EPCs [37–39], but they are not directly related to their contribution to cancer progression and metastasis. Interestingly, we observed the highest retention of MDSC-derived exosomes in lungs of breast tumor-bearing animals than any other groups, highlighting their metastatic properties.

Furthermore, we noticed a maximal load of accumulation of EPC-derived exosomes in the primary tumor than in any other groups, which could be due to their neovascularization effects in the TME. Ex vivo measurements of radioactivity from individual organs by gamma counter also correspond to the in vivo analysis. To substantiate prior findings, we investigated the level of tumor-associated cytokines in exosomal protein content from MDSCs and EPCs compared to the tumor cells. There was a significant increase of endoglin, ICAM-1, VEGFR2 and PF-4 level in the MDSC exo and EPC exo than the tumor exo. Endoglin [40], VEGFR2 [41], ICAM-1 [42] and bFGF [43] are established factors that promote neovascularization. Myeloid activation and functionally relevant cytokines such as GM-CSF, G-CSF, M-CSF, and MCP-1 were over-expressed in exosomal content from both MDSCs and EPCs.
Furthermore, MDSC exo contained a higher level of MIP-1α and SDF-1α. GM-CSF and G-CSF potently induce the expansion of MDSCs, leading to enhanced metastatic growth\cite{44}. GM-CSF also promotes tumor proliferation and invasion while M-CSF instigates tumor invasion\cite{45}. TSLP and IL-7 were over-expressed in MDSC and EPC exo, these cytokines drive the differentiation of Th2 cells followed by secretion of IL-13 and IL-4, leading to the recruitment and activation of MDSCs and TAMs, which promote metastasis by secreting tumor cell migration-stimulating factors such as SDF-1α, IL-6, MMP – 2, LIX, KC, RANTES and TNF-α\cite{46, 47}. All these cytokines were significantly increased in MDSC-derived exosomes than EPC exo or TDEs, suggesting their prospective role in tumor proliferation, angiogenesis, invasion, and metastasis. While we used non-cancerous cell (HEK293)-derived exosomes, we did not see any retention of exosomes in the primary tumor or metastatic site, indicating that only the exosomes from tumor cells or TME-associated cells have the propensity to be distributed in tumor or metastatic areas.

We explored the possibility that chemotherapeutics or cell-targeted therapies may change the behavior and contents of the TDEs. We isolated exosomes from tumor cells with GW2580 or HET0016 treatment or without treatment (control). We observed more accumulation of treated exosomes in the tumor area, albeit it was not statistically significant. We also observed a significant reduction of radioactivity in lungs of the animal groups injected with GW2580 exo and HET0016 exo compared to the group injected with control exo, elucidating the diminution of the metastatic competency of the exosomes after treating the parent cells with the therapeutics. For validating this finding, we profiled 44 cancer-related factors in the exosomal contents by cytokine array. Distinguishable cancer-promoting factors that declined following the treatment of cancer cells compared to the control are VEGFR2, ICAM-1, bFGF, KC, TNF-α, IL-6, IL-12, IL-10, and IL-13. KC promotes the growth of the primary tumor and formation of a pre-metastatic niche in a different tumor model\cite{48, 49}. Elevated levels of IL-6 are associated with aggressive tumor growth, poor response to therapies, poor prognosis and shorter survival\cite{50} It should be noted that the FasL level was elevated in the exosomes from treated cancer cells. Up-regulation of FasL often occurs following chemotherapy, from which the tumor cells attain apoptosis resistance\cite{51, 52}.

In summary, we favorably demonstrated a simple, rapid, high-yielding exosome isolation technique utilizing a combination of size based and ultracentrifugation methods. As per our knowledge, we are the first group to demonstrate the exact visualization and quantification of radioisotope-labeled exosomes from different cell types, with or without drug treatment, in primary solid tumors and metastatic area, which are dependent on the protein/cytokine contents of the exosomes. Our imaging technique and quantification of exosomes could be applied for potential metastatic site prediction, monitoring tumor progression and targeting efficacy of exosome-based therapy, thus unlocking a theranostic potential for these exosomes.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
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Abbreviations:

- bFGF: basic fibroblast growth factor
- CSF1R: colony stimulating factor 1 receptor
- CT: computed tomography
- CTLA4: cytotoxic T-lymphocyte-associated protein 4
- EGF: epidermal growth factor
- EMT: epithelial to mesenchymal transition
- EVs: extracellular vesicles
- EPCs: endothelial progenitor cells
- FasL: Fas ligand
- G-CSF: granulocyte-colony stimulating factor
- GM-CSF: granulocyte-macrophage colony-stimulating factor
- HGF: hepatocyte growth factor
- HSP: heat shock protein
- ICAM-1: intercellular adhesion molecule 1
- IFN-gamma: interferon gamma
- IL-1beta: interleukin-1 beta
- IL-1ra: interleukin-1 receptor antagonist
- IL-2: interleukin-2
- IL-4: interleukin-4
- IL-6: interleukin-6
- IL-7: interleukin-7
- IL-10: interleukin-10
- IL-12: interleukin-12
- IL-13: interleukin-13
IL – 17  interleukin-17
KC  keratinocyte-derived chemokine
LIX  lipopolysaccharide-induced CXC chemokine
M-CSF  macrophage colony-stimulating factor
MCP-1  monocyte chemoattractant protein 1
MDC  macrophage-derived chemokine
MDSCs  myeloid derived suppressor cells
MFP  mammary fat pad
MIP-1α  macrophage-inflammatory protein-1 alpha
MMP-2  matrix metalloproteinase-2
MRI  magnetic resonance imaging
NIS  sodium iodide symporter
NTA  nanoparticle tracking analysis
PET  positron emission tomography
PF-4  platelet factor 4
RANTES  regulated on activation, normal T cell expressed and secreted
ROIs  regions of interest
SDF-1α  stromal cell-derived factor-1
SEM  standard error of the mean
SPECT  single-photon emission computed tomography
SCF  stem cell factor
TAMs  tumor-associated macrophages
TEM  transmission electron microscopy
TIMP 2  tissue inhibitors of metalloproteinases 2
TLPC  thin layer paper chromatography
TME  tumor microenvironment
TNF-α  tumor necrosis factor-α
TSLP  thymic stromal lymphopoietin
UC ultracentrifugation
VEGF-A vascular endothelial growth factor A
VEGFR2 vascular endothelial growth factor receptor 2

References:
1. Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, et al. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. Proceedings of the National Academy of Sciences of the United States of America. 2016;113(8):E968–E77. [PubMed: 26858453]
2. Kowal J, Tkach M, Théry C. Biogenesis and secretion of exosomes. Current Opinion in Cell Biology. 2014;29:116–25. [PubMed: 24959705]
3. Alipoor SD, Mortaz E, Garsen J, Movassaghi M, Mirmoadehi M, Adcock IM. Exosomes and Exosomal miRNA in Respiratory Diseases. %J Mediators of Inflammation. 2016;2016:11.
4. Balaj L, Lessard R, Dai L, Cho Y-J, Pomeroy SL, Breakefield XO, et al. Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. Nature communications. 2016;2:180-.
5. Kalluri R. The biology and function of exosomes in cancer. The Journal of Clinical Investigation. 2016;126(4):1208–15. [PubMed: 27035812]
6. Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Research. 2014;24:766. [PubMed: 24710597]
7. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nature Cell Biology. 2007;9:654. [PubMed: 17486113]
8. Bobrie A, Colombo M, Raposo G, Théry C. Exosome Secretion: Molecular Mechanisms and Roles in Immune Responses. 2011;12(12):1659–68.
9. Mittelbrunn M, Gutiérrez-Vázquez C, Villarroela-Beltri C, González S, Sánchez-Cabo F, González MA, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nature Communications. 2011;2:282.
10. Johnstone RM, Adam M, Hammond JR, Orr L, Turvide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J Biol Chem. 1987;262(19):9412–20. [PubMed: 3597417]
11. Cossetti C, Iaci N, Mercer Tim R, Leonardi T, Alpi E, Drago D, et al. Extracellular Vesicles from Neural Stem Cells Transfer IFN-γ via Ifngr1 to Activate Stat1 Signaling in Target Cells. Molecular Cell. 2014;56(2):193–204. [PubMed: 25242146]
12. Gangoda L, Boukouris S, Liem M, Kalra H, Mathivanan S. Extracellular vesicles including exosomes are mediators of signal transduction: are they protective or pathogenic? Proteomics. 2015;15(2-3):260–71. [PubMed: 25307053]
13. Syn N, Wang L, Sethi G, Thiery J-P, Goh B-C. Exosome-Mediated Metastasis: From Epithelial–Mesenchymal Transition to Escape from Immunosurveillance. Trends in Pharmacological Sciences. 2016;37(7):606–17. [PubMed: 27157716]
14. Zhang X, Yuan X, Shi H, Wu L, Qian H, Xu W. Exosomes in cancer: small particle, big player. Journal of Hematology & Oncology. 2015;8(1):33. [PubMed: 26156517]
15. Yu S, Liu C, Su K, Wang J, Liu Y, Zhang L, et al. Tumor Exosomes Inhibit Differentiation of Bone Marrow Dendritic Cells. The Journal of Immunology. 2007;178(11):6867–75. [PubMed: 17513735]
16. Arbab AS, Koizumi K, Araki T. A revised method of labeling mouse IgG with yttrium-90. Yamanashi Ika Daigaku Zasshi. 1996;10(4):121–4.
17. Angara K, Rashid MH, Shankar A, Ara R, Iskander A, Borin TF, et al. Vascular mimicry in glioblastoma following anti-angiogenic and anti-20-HETE therapies. Histol Histopathol. 2017;32(9):917–28. [PubMed: 27990624]
18. Jain M, Gamage N-DH, Alsulami M, Shankar A, Achyut BR, Angara K, et al. Intravenous Formulation of HET0016 Decreased Human Glioblastoma Growth and Implicated Survival Benefit in Rat Xenograft Models. Scientific Reports. 2017;7:41809. [PubMed: 28139732]
19. Chen XW, Yu TJ, Zhang J, Li Y, Chen HL, Yang GF, et al. CYP4A in tumor-associated macrophages promotes pre-metastatic niche formation and metastasis. Oncogene. 2017;36(35):5045–57. [PubMed: 28481877]
20. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in Exosome Isolation Techniques. Theranostics. 2017;7(3):789–804. [PubMed: 28255367]
21. Bellavia D, Raimondo S, Calabrese G, Forte S, Cristaldi M, Patinella A, et al. Interleukin 3-receptor targeted exosomes inhibit in vitro and in vivo Chronic Myelogenous Leukemia cell growth. Theranostics. 2017;7(5):1333–45. [PubMed: 28435469]
22. Hoshino A, Costa-Silva B, Shen T-L, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour exosome integrins determine organotropic metastasis. Nature. 2015;527:329. [PubMed: 26524530]
23. Ohno S-i, Takamash M, Sudo K, Ueda S, Ishikawa A, Matsuyama N, et al. Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells. Molecular therapy : the journal of the American Society of Gene Therapy. 2013;21(1):185–91. [PubMed: 23032975]
24. Smyth T, Kullberg M, Malik N, Smith-Jones P, Graner MW, Anchorodouqy TJ. Biodistribution and delivery efficiency of unmodified tumor-derived exosomes. Journal of Controlled Release. 2015;199:145–55. [PubMed: 25525319]
25. Watson DC, Bayik D, Srivatsan A, Bergamaschi C, Valentin A, Niu G, et al. Efficient production and enhanced tumor delivery of engineered extracellular vesicles. Biomaterials. 2016;105:195–205. [PubMed: 27522254]
26. Lai CP, Mardini O, Ericsson M, Prabhakar S, Maguire CA, Chen JW, et al. Dynamic Biodistribution of Extracellular Vesicles in Vivo Using a Multimodal Imaging Reporter. ACS Nano. 2014;8(1):483–94. [PubMed: 24383518]
27. Gangadaran P, Hong CM, Ahn B-C. An Update on in Vivo Imaging of Extracellular Vesicles as Drug Delivery Vehicles. Frontiers in Pharmacology. 2018;9(169).
28. Ahn B-C. Sodium iodide symporter for nuclear molecular imaging and gene therapy: from bedside to bench and back. Theranostics. 2012;2(4):392–402. [PubMed: 2259935]
29. Kogai T, Taki K, Brent GA. Enhancement of sodium/iodide symporter expression in thyroid and breast cancer. 2006;13(3):797.
30. Baril P, Martin-Duque P, Vassaux G. Visualization of gene expression in the live subject using the Na/I symporter as a reporter gene: applications in biotherapy. British journal of pharmacology. 2010;159(4):761–71. [PubMed: 19814733]
31. Condamine T, Ramachandran I, Youn J-I, Gabrilovich DI. Regulation of Tumor Metastasis by Myeloid-Derived Suppressor Cells. 2015;66(1):97–110.
32. Solito S, Pinton L, Mandruzzato S. In Brief: Myeloid-derived suppressor cells in cancer. The Journal of pathology. 2017;242(1):7–9. [PubMed: 28097660]
33. Burke M, Choksawangkarn W, Edwards N, Ostrand-Rosenberg S, Fenselau C. Exosomes from Myeloid-Derived Suppressor Cells Carry Biologically Active Proteins. Journal of Proteome Research. 2014;13(2):836–43. [PubMed: 24295599]
34. Deng Z, Rong Y, Teng Y, Zhuang X, Samykuttay A, Mu J, et al. Exosomes miR-126a released from MDSC induced by DOX treatment promotes lung metastasis. Oncogene. 2016;36:639. [PubMed: 27345402]
35. Wang Y, Tian J, Tang X, Rui K, Tian X, Ma J, et al. Exosomes released by granulocytic myeloid-derived suppressor cells attenuate DSS-induced colitis in mice. Oncotarget. 2016;7(13):15356–68. [PubMed: 26885611]
36. Zöller M Janus-Faced Myeloid-Derived Suppressor Cell Exosomes for the Good and the Bad in Cancer and Autoimmune Disease. 2018;9(137).
37. Han C, Sun X, Liu L, Jiang H, Shen Y, Xu X, et al. Exosomes and Their Therapeutic Potentials of Stem Cells %J Stem Cells International. 2016;2016:11.
38. Wu X, Liu Z, Hu L, Gu W, Zhu L. Exosomes derived from endothelial progenitor cells ameliorate acute lung injury by transferring miR-126. Exp Cell Res. 2018;370(1):13–23. [PubMed: 29883714]

39. Zhang J, Chen C, Hu B, Niu X, Liu X, Zhang G, et al. Exosomes Derived from Human Endothelial Progenitor Cells Accelerate Cutaneous Wound Healing by Promoting Angiogenesis Through Erk1/2 Signaling. International journal of biological sciences. 2016;12(12):1472–87. [PubMed: 27994512]

40. FARSHAD NASSIRI MDC, SCHEITHAUER BERNDW, ROTONDO FABIO, FAZIO ALESSANDRA, YOUSEF GEORGEM, SYRO LUISV, KOVACS KALMAN and LLOYD RICARDOV. Endoglin (CD105): A Review of its Role in Angiogenesis and Tumor Diagnosis, Progression and Therapy. Anticancer Research. 2011;31(6):2283–90. [PubMed: 21737653]

41. Shibuya M Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. Genes & cancer. 2011;2(12):1097–105. [PubMed: 22866201]

42. Kevil CG, Orr AW, Langston W, Mickett K, Murphy-Ullrich J, Patel RP, et al. Intercellular Adhesion Molecule-1 (ICAM-1) Regulates Endothelial Cell Motility through a Nitric Oxide-dependent Pathway. Journal of Biological Chemistry. 2004;279(18):19230–8. [PubMed: 14985356]

43. Murakami M, Nguyen LT, Zhang ZW, Moodie KL, Carmeliet P, Stan RV, et al. The FGF system has a key role in regulating vascular integrity. The Journal of Clinical Investigation. 2008;118(10):3355–66. [PubMed: 18776942]

44. Ouzounova M, Lee E, Piranioglou R, El Andaloussi A, Kolhe R, Demirci MF, et al. Monocytic and granulocytic myeloid derived suppressor cells differentially regulate spatiotemporal tumour plasticity during metastatic cascade. Nature Communications. 2017;8:14979.

45. Üemura Y, Kobayashi M, Nakata H, Kubota T, Bandobashi K, Saito T, Taguchi H. Effects of GM-CSF and M-CSF on tumor progression of lung cancer: Roles of MEK1/ERK and AKT/PKB pathways. International Journal of Molecular Medicine. 2006;18(2):365–73. [PubMed: 16820947]

46. Allavena P, Sica A, Solinas G, Porta C, Mantovani A. The inflammatory micro-environment in tumor progression: The role of tumor-associated macrophages. Critical Reviews in Oncology/Hematology. 2008;66(1):1–9. [PubMed: 17913510]

47. Esquivel-Velázquez M, Ostoa-Saloma P, Palacios-Arreola MI, Nava-Castro J, Morales-Montor J. The role of cytokines in breast cancer development and progression. Journal of interferon & cytokine research: the official journal of the International Society for Interferon and Cytokine Research. 2015;35(1):1–16.

48. Wang D, Sun H, Wei J, Cen B, DuBois RN. CXCL1 Is Critical for Premetastatic Niche Formation and Metastasis in Colorectal Cancer. Cancer Research. 2017;77(13):3655–65. [PubMed: 28455419]

49. Yuan M, Zhu H, Xu J, Zheng Y, Cao X, Liu Q. Tumor-Derived CXCL1 Promotes Lung Cancer Growth via Recruitment of Tumor-Associated Neutrophils. Journal of immunology research. 2016;2016:6530410-. [PubMed: 27446967]

50. Kumari N, Dwarakanath BS, Das A, Bhatt AN. Role of interleukin-6 in cancer progression and therapeutic resistance. Tumor Biology. 2016;37(9):11553–72. [PubMed: 27260630]

51. Hannun YA. Apoptosis and the Dilemma of Cancer Chemotherapy. Blood. 1997;89(6):1845–53. [PubMed: 9058703]

52. Igney FH, Krammer PH. Tumor counterattack: fact or fiction? Cancer Immunology, Immunotherapy. 2005;54(11):1127–36. [PubMed: 15889255]
Figure 1.
(A) NTA analysis showing exosome concentration per mL and (B and C) size distribution of exosomes isolated using five different methods as described in the text. (D) Flow-cytometric analysis of common exosome markers (CD9 and CD63). (E and F) NTA analysis showing total number, recovery and size of exosomes isolated by each step of method #3 (combination of size based and UC methods). (G) Flow-cytometric analysis of common exosome markers in each step of method #3. (H) Transmission electron microscopy image for exosomes isolated by method #3. Scale bar depicts 100 nm.
Figure 2.
Binding of $^{131}$I to exosomes and serum stability (of binding) were confirmed by thin layer paper chromatography (TLPC). (A) A major proportion of the free $^{131}$I moved from the spotted point in the bottom of the TLPC paper to the top half, confirming the efficacy of the eluent. (B) Binding of $^{131}$I to exosomes was validated as shown by a much lower percentage of $^{131}$I (free, dissociated) that moved to the top to the plate, compared to the amount remaining in the bottom part, which represented the $^{131}$I labeled exosomes. (C) Serum stability of $^{131}$I bound exosomes was very high as only a very small amount of free $^{131}$I disengaged from the bound exosomes to move to the top half. (D) Reconstructed and co-registered in vivo SPECT/CT images (coronal view) at 3 hrs, from the animals injected with $^{131}$I-labeled tumor exosomes. (E and F) Free $^{131}$I and exosome distribution in the tumors and lungs. Quantitative data are expressed in mean ± SEM. *$P<.05$, ***$P<.001$. $n = 3$. 
Figure 3.

(A) Flow-cytometric analysis of isolated MDSCs, showing that more than 99% of cells are positive for CD11b+ and Gr1 (left panel), and EPCs showing more than 85% positive for CD117 (right panel). (B and C) NTA showing size distribution curve and mean size of the exosomes from tumor cells (tumor exo), HEK293 cells (HEK293 exo), myeloid-derived suppressor cells (MDSC exo) and endothelial progenitor cells (EPC exo). (D) Reconstructed and co-registered in vivo SPECT/CT images (coronal view) of the abovementioned \textsuperscript{131}I labeled exosomes after 3 hrs of intravenous injection in tumor-bearing mice. (E) Quantification of radioactivity in tumor showed significant aggregation of EPC exo in contrast to control exo and MDSC exo. (F) Quantification of radioactivity in the lungs showed the highest percentage of radioactivity in mice injected with MDSC exo. There was no retention of non-specific HEK293 exo in either tumor or lungs. Quantitative data are expressed in mean ± SEM. *P<.05, ***P<.001. n = 3.
Figure 4.

*In vitro* quantification of the level of cytokines in the protein samples, collected from the exosomes of AT3 tumor cells, MDSCs, and EPCs. Exosomal proteins from MDSCs and EPCs showed a significant increase in some pro-angiogenic, immune modulatory, myeloid activation and function, and T cell-related factors compared to the exosomal proteins from tumor cells. Quantitative data are expressed in mean ± SEM. *P*<.05, **P**<.01, ***P***<.001, ****P***<.0001. n = 4.
Figure 5.

(A and B) NTA analysis showing no significant differences in the size distribution of exosomes isolated from tumor cells (control exo), GW2580-treated tumor cells (GW2580-exo), and HET0016-treated tumor cells (HET0016-exo). (C) Flow-cytometric analysis of exosomal markers CD9 and CD63 for the abovementioned treatment conditions. (D) Reconstructed and co-registered in vivo SPECT/CT images (coronal view) of the animals injected with the abovementioned 

\[ ^{131} \text{I} \]

labeled exosomes after 3 hrs. (E) Quantification of radioactivity in tumor showed insignificant higher aggregation in mice injected with \[ ^{131} \text{I} \]-labeled GW2580 exo and HET0016 exo compared to control exo. (F) Quantification of radioactivity in lungs showed significant reduction of exosomes localization in GW 2580 exo and HET0016 exo than the control exo injected groups. Quantitative data are expressed in mean ± SEM. *\( P<.05 \), **\( P<.01 \), ***\( P<.001 \), ****\( P<.0001 \), n = 3.
Figure 6.
*In vitro* quantification of the level of cytokines in protein samples, collected from the exosomes of 4T1 tumor cells without any treatment and with GW2580 and HET0016 treatment for 48 hrs. Exosomal proteins from treated cells showed a significant decrease of some pro-angiogenic, immune modulatory, and chemotactic factors. Quantitative data are expressed in mean ± SEM. *P < .05, **P < .01, ***P < .001, ****P < .0001. n = 4.