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Development and MPI tracking of novel hypoxia-targeted theranostic exosomes

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Abstract

Treating the hypoxic region of the tumor remains a significant challenge. The goals of this study are to develop an exosome platform that can target regions of tumor hypoxia and that can be monitored *in vivo* using magnetic particle imaging (MPI). Four types of exosomes (generated under hypoxic or normoxic conditions, and with or without exposure to X-ray radiation) were isolated from MDA-MB-231 human breast cancer cells. Exosomes were labeled by DiO, a fluorescent lipophilic tracer, to quantify their uptake by hypoxic cancer cells. Subsequently, the exosomes were modified to carry SPIO (superparamagnetic iron oxide) nanoparticles and Olaparib (PARP inhibitor). FACS and fluorescence microscopy showed that hypoxic cells preferentially take up exosomes released by hypoxic cells, compared with other exosome formulations. In addition, the distribution of SPIO-labeled exosomes was successively imaged *in vivo* using MPI. Finally, the therapeutic efficacy of Olaparib-loaded exosomes was demonstrated by increased apoptosis and slower tumor growth *in vivo*. Our novel theranostic platform could be used as an effective strategy to monitor exosomes *in vivo* and deliver therapeutics to hypoxic tumors.

Keywords

Cancer; Hypoxia; Exosomes; Drug delivery; Magnetic Particle Imaging (MPI)

1. Introduction

Tumor hypoxia is an important factor of resistance to therapy, particularly for radiotherapy and chemotherapy [1-3]. Because the tumor mass grows rapidly, there are regions of hypoxia

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Conflicts of interest.

The authors have declared that they have no competing interest.

in the tumor, where oxygen concentration is significantly lower than in healthy tissues. These hypoxic regions are associated with more aggressive cancer phenotypes and worse prognoses. Therefore, imaging and treating these hypoxic regions is an important clinical challenge for effective cancer therapy [4,5]. To address this problem, we investigate the feasibility of using exosomes to treat hypoxic tumor regions, focusing specifically on exosomes released *in vitro* by cancer cells exposed to hypoxia or ionizing radiation. We further investigate an imaging strategy for tracking these exosomes *in vivo* using magnetic particle imaging (MPI), a recently developing molecular imaging modality.

Exosomes are a type of cell-derived extracellular vesicles with diameter ranging from 30 to 200 nm [6,7]. These particles carry information for intercellular communication within the tumor microenvironment, including functional proteins, DNA, mRNA, and miRNA [8,9]. Recently, there has been significant interest in using exosomes for diagnostic and therapeutic applications [10-13]. In particular, exosomes are being investigated as nanoparticle carriers for therapeutics delivery [14-17]. Currently, the main methods to deliver therapeutics include liposomal, polymeric and other nanoparticles [18-22]. However, these nanoparticles have limitations due to issues such as immunogenicity, stability, and long-term safety. Exosomes could overcome these limitations due to their desirable features such as high stability under physiological conditions, ability to target specific tissues, non-immunogenic, non-toxicity, and high biocompatibility [23-25]. Therefore, exosomes are considered the ideal platform to deliver miRNA, drugs, and other molecules to specific cells. Until now, only few studies have investigated how environmental conditions such as hypoxia and exposure to ionizing radiation affect the release of exosomes from cancer cells [26-28]. Some of these studies have reported that these exogenous factors can directly impact the protein composition of exosomes [29,30]. However, it is not known whether these factors modulate the uptake of these exosomes by hypoxic cancer cells. Knowledge about how exosomes released from hypoxic or irradiated cells accumulate in hypoxic cells could help improve the efficacy of exosome-mediated drug delivery for cancer therapy.

Magnetic particle imaging (MPI) is an emerging non-invasive tomographic technique that directly images the distribution of superparamagnetic iron oxide (SPIO) nanoparticle *in vivo* [31,32]. This modality has significant advantages such as sub-micromolar sensitivity [33], high spatial resolution, and lack of ionizing radiation.

In this study, we propose to develop a novel theranostic exosome platform to image and deliver therapeutics to regions of tumor hypoxia. The specific goals are to quantify exosome uptake by hypoxic cancer cells for different types of exosomes, namely those released under conditions of hypoxia and/or radiation, and to evaluate, using MPI, the ability of these exosomes to deliver drugs to hypoxic cells in the tumor. The development of this exosome platform is innovative because no prior studies have explored *in vivo* imaging and simultaneous therapy of hypoxic cancer using exosomes. Quantification of exosome uptake in hypoxic cancer cells is also important to understand exosome-mediated cell-to-cell communication in the tumor microenvironment. In addition, this work is the first reported use of MPI for tracking exosomes *in vivo*.

2. Materials and methods

2.1. Cell line and hypoxia/radiation exposure

The human breast cancer cell line MDA-MB-231 was cultured in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. Hypoxia was simulated by culturing cells with 200 μ M of Deferoxamine (DFO) (Sigma-Aldrich, St Louis, MO, USA) at 37°C and 5% CO₂ in a humidified environment for 24 hr. DFO, an iron chelator, has been found to up-regulate hypoxia-inducible factor 1 α (HIF-1 α) expression and increase expression of HIF-1 α target genes associated with hypoxia. Additionally, for radiation exposure, MDA-MB-231 cells were irradiated with 5 Gy in an X-RAD 320 irradiator (Precision X-Ray Inc., North Branford, CT, USA).

2.2. Exosome purification

The MDA-MB-231 cells (1×10^6 cells) were cultured in conditioned medium including 10% exosome-depleted FBS Media Supplement (System Bioscience, Mountain View, CA, USA) and 1% Penicillin-Streptomycin. After 48 h incubation, the culture medium was collected and exosomes were isolated using an ExoQuick™ (System Bioscience, Mountain View, CA, USA) exosome purification kit. Exosomes were quantified by protein concentrations using a BCA protein assay kit (Pierce, Rockford, IL, USA). The amount of four types of exosomes (generated under hypoxia and/or exposure to radiation) was compared according to protein concentration.

2.3. Transmission electron microscopy (TEM) and NanoSight

The exosomes were fixed with 2% glutaraldehyde (GAA) and deposited on 300 mesh carbon/formvar coated copper grids. The size of the exosome was analyzed by transmission electron microscopy (JEM-1400 series 120kV, JEOL USA Inc., Pleasanton, CA, USA) with a LaB6 emitter and a Gatan Orius 10.7 megapixel CCD camera. Size distribution of exosomes (100 mL; 10 ng/mL) was also analyzed by Nanoparticle Tracking Analyzer (NanoSight Ltd, Amesbury, UK).

2.4. Western blotting

Proteins were extracted from cells and purified exosomes with RIPA buffer containing 25 mM Tris HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS (Thermo scientific, Rockford, IL USA). Protein concentration was measured with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins (30 μ g) were loaded with 4%–12% gradient polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) and then blotted onto PVDF membranes (Millipore, Watford, UK). Primary antibodies were incubated overnight at 4°C on the membrane and included: anti-CD9, anti-CD63, and anti-Hsp70 (1:1000 dilution; System Bioscience, Mountain View, CA, USA). The secondary antibodies were incubated for 1 h at room temperature and included: anti-rabbit (1:2,000 dilution, System Bioscience, Mountain View, CA, USA) for CD9, CD63, and Hsp70. Immunoreactive bands were imaged via luminescence using an IVIS imaging system (XenogenCorp., Alameda, CA, USA) and detection reagents (Roche, Nutley, NJ, USA).

2.5. Fluorescence exosome imaging and flow cytometry

Lipophilic tracers such as DiO (Invitrogen, Carlsbad, CA, USA; green fluorescence; Ex: 484 nm, Em: 501 nm) and DiI (red fluorescence; Ex: 565 nm, Em: 594 nm) were prepared in stock solutions in dimethylsulfoxide (DMSO) for *in vitro* and *in vivo* studies. Exosomes under various conditions (hypoxic or radiation) were incubated with 1 μ M of DiO or DiI at 37°C for 15 min. The fluorescence-labeled exosomes (100 μ g/mL) were incubated with hypoxic MDA-MB-231 cells for 24 hr. The uptake of the fluorescent exosomes by hypoxic cells was visualized using an EVOS FL Cell Imaging System (Thermo fisher scientific, Santa Clara, CA, USA) equipped with DAPI, GFP, and Texas Red dichroic filter sets, a Plan Fluorite 4 $\times$ objective lens, and a 16-bit monochrome CCD camera. In addition, exosomes were imaged using a Leica DMI8 microscope (Leica Microsystems, Wetzlar, Germany) equipped with a FITC dichroic set, a HC PL Fluotar 10 $\times$ objective lens, and an 8-bit monochrome CCD camera. Fluorescence signals were quantified using the Image J software. The uptake of fluorescent exosomes by hypoxic cells was also quantified using a BD FACSAria Fusion sorter (BD bioscience, San Jose, CA, USA).

2.6. Exosome drug loading

To load the exosomes with a therapeutic drug, 100 μ M of Olaparib was gently mixed with 100 μ g of exosomes in 200 mL of electroporation buffer. Electroporation was performed at 150 μ F and 350 V in electroporation cuvettes using a Gene Pulser Xcell Electroporatoion System (Bio-Rad, Hercules, CA, USA). Subsequently, the mixture was incubated at 37 °C for 30 min to allow the exosome membrane to recover. After loading Olaparib, the exosomes were purified again using exoQuick and free Olaparib was removed by PBS washing.

2.7. Cell viability assay

MDA-MB-231 cells (2.5×10^3 cells/well) were grown in 96-well plates with 200 μ M DFO to induce HIF-1 α expression and simulate hypoxic conditions. To evaluate the cytotoxicity of Olaparib-loaded exosomes in these hypoxic cells, cell viability assays were performed 48 hr after treatment with Olaparib (100 μ M) or Olaparib-loaded exosomes (0, 20, 40, 80, 160, 320 μ g/mL). The cell plate was incubated with Cell Counting Kit-8 (CCK-8) solution (Sigma-Aldrich, St Louis, MO, USA), and after 2 hr, the mean value of the OD was measured at 450 nm. All measurements were corrected by subtracting a blank reading and by normalizing the values to the control to estimate % viability.

2.8. Apoptosis assay

MDA-MB-231 cells (2.5×10^4 cells/well) were grown with 200 μ M DFO in 24-well plates. Olaparib-loaded exosomes (100 μ g/mL) or Olaparib (100 μ M) were administered to the hypoxic cells and incubated at 37 °C for 48 hr. After removing the medium, the cells were washed three times using PBS. Apoptosis and necrosis were detected using an Apoptosis Detection Kit (ab176749, Abcam, Cambridge, England). Fluorescence of cells was examined using an EVOS FL Cell Imaging System (Thermo fisher scientific, Santa Clara, CA, USA) and a Leica DMI8 microscope (Leica Microsystems, Wetzlar, Germany). Fluorescence was also measured and quantified using a BD FACSAria Fusion sorter (BD bioscience, San Jose, CA, USA).

2.9. Nanoparticle (SPIO)-labeled exosomes for *in vivo* MPI

For SPIO labeling of exosomes, MDA-MB-231 cells (1×10^6 cells/plate) were incubated with 200 $\mu\text{g/mL}$ SPIO nanoparticles (VivoTrax, Magnetic Insight, Inc. Alameda, CA, USA) for 24 hr. The cells were then washed three times with PBS to remove free SPIO from the medium. After another 48 hr incubation in exosome-depleted medium, the cultured medium was collected and exosomes were isolated using ExoQuick™ (System Bioscience, Mountain View, CA, USA).

2.10. Serum stability assay

Exosomes were isolated from cell medium treated with DFO (200 μM), SPIO (200 $\mu\text{g/mL}$), and Olaparib (100 μM) to confirm the stability of exosomes under physiological condition. Exosomes (100 μg) were incubated with 10% of exosome-depleted FBS (System Bioscience, Mountain View, CA, USA) at 37 °C for 24 hr. At 4 hr and 24 hr after incubation, the size of exosomes was measured using a Nanoparticle Tracking Analyzer (NanoSight Ltd, Amesbury, UK).

2.11. Tumor xenografts in nude mice

All procedures involving animals were approved by the Administrative Panel on Laboratory Animal Care of Stanford University. MDA-MB-231 cells (1×10^6 cells) were transplanted subcutaneously into the right flanks of 8-week-old female BALB/c nu/nu mice weighing about 20 g on average. Mouse tumors were allowed to grow to a tumor size of 10 mm in diameter for *in vivo* studies.

2.12. *In vivo* MPI imaging

MPI images of *in vitro* cells and *in vivo* mice were acquired using the projection field-free line (FFL) method on the Momentum MPI scanner (Magnetic Insight, Inc. Alameda, CA, USA). Nanoparticle (SPIO)-labeled exosomes (100 μg) were intravenously injected in mice. One hour after injection, the mice were scanned using the MPI system to evaluate exosome uptake in brain, heart, lungs, liver, spleen, kidneys, and intestine, *in vivo* and *ex-vivo*. Mice were translated along the z-axis using the motorized stage of the scanner. The field of view (FOV) was 6 cm $\times$ 8 cm, and the imaging acquisition time 10 seconds per projection (total time ~40 min including image reconstruction). CT images were also acquired using a MicroCT scanner (TriFoil Imaging CT120, Northridge, CA, USA). VivoQuant software was used for 2D and 3D co-registration of the MPI images with CT scans.

2.13. Tumor growth measurements

In tumor xenograft mice, tumor growth was monitored for 3 weeks following intratumoral treatment with PBS (control), Olaparib-loaded exosomes (100 $\mu\text{g/mL}$), and Olaparib (100 μM) every 2 days. The size of tumors was measured with calipers every 3 days and calculated using the Response Evaluation Criteria in Solid Tumors (RECIST).

2.14. *Ex vivo* studies

Ex vivo studies were performed to confirm morphologic changes by Hematoxylin and Eosin staining (H&E), exosome uptake by fluorescence microscopy, and the level of apoptosis by

cleaved caspase 3 staining in tumor tissues. The distribution of the fluorescent exosomes was imaged in tumor tissues using an EVOS FL Cell Imaging System (Thermo fisher scientific, Santa Clara, CA, USA) and cleaved caspase 3 staining was imaged by Zeiss Axioimager A2 (Carl Zeiss microscopy, NY, USA)

2.15. Statistical analysis

All data are presented by mean $\pm$ standard deviation. P-values < 0.05 were considered to be statistically significant and were obtained using Student's unpaired t-test.

3. Results

3.1. Characterization of exosome release by irradiated and hypoxic cancer cells

We first quantified exosome release by hypoxic and irradiated cancer cells. Due to the effects of X-ray radiation and hypoxia, cancer cells displayed morphological changes on microscopy imaging as well as lower number of cells compared to control cells (Fig. 1A & Supplementary Fig. 1A). Exosome release after 48 hr was quantified by measuring protein amount after exosome isolation (Fig. 1B). Radiation and hypoxia both contributed to increase the amount of exosomes released, and the highest release was observed for the combination of the two conditions (about 6 fold higher than control). These results are even more significant considering that fewer cells are viable after radiation and hypoxia. Subsequently, NanoSight Track Analysis found the distribution of exosome size to be almost same for these four different types of exosomes, ranging from 30 to 200 nm diameter (Fig. 1C). In addition, TEM imaging confirmed the presence of microvesicles within the expected size range for exosomes (~100 nm; Supplementary Fig. 1B). Finally, western blot confirmed that purified exosomes displayed specific markers such CD9, CD63, and Hsp70 compared to cells (Supplementary Fig. 1C).

3.2. Quantification of exosome uptake by hypoxic cancer cells using fluorescence (DiO) labeling

Fluorescence microscopy was used to measure and quantify the uptake by hypoxic cancer cells of the four different types of fluorescent exosomes: control exosomes (Con Exo), irradiated exosomes (Rad Exo), hypoxic exosomes (DFO Exo), and irradiated hypoxic exosomes (DR Exo). Green fluorescence, corresponding to DiO-labeled exosomes, was higher in cells treated with exosomes than in control cells (Fig. 2A). Nuclear staining is shown in blue. Quantification of fluorescence images showed that the uptake of exosome by hypoxic cells was greater when these exosomes were produced following hypoxia or irradiation. The highest uptake was achieved for hypoxic exosomes (DFO Exo). Similar to fluorescence microscopy, FACS data showed that cells treated with hypoxic exosomes had the highest green fluorescence signals (about 97% of cells positive) compare to cells treated with the other exosome preparations (Fig. 2B). These results indicate that hypoxic cells have a preference for exosomes produced by other hypoxic cells. The difference in uptake for hypoxic and irradiated exosomes (DFO Exo and Rad Exo) was not deemed significant. In addition, these exosomes had no significant effect on proliferation of hypoxic cells (CCK-8 assay, Supplementary Fig. 2) or apoptosis (apoptosis assay, Fig. 2C).

3.3. Olaparib-loaded exosomes for cancer drug delivery

Following these results, exosomes released by hypoxic cells (DFO Exo) were selected to deliver therapeutic drugs because of their higher targeting of hypoxic cells. As a next step, exosomes were loaded with Olaparib for cancer therapy. Brightfield microscopy images showed fewer cells in the field of view 48 hr after treatment with Olaparib-loaded exosomes or free Olaparib (Supplementary Fig. 3A). This was verified by performing a CCK-8 assay: cells exposed to Olaparib-loaded exosomes had lower viability (about 50%) compared to control cells (Supplementary Fig. 3B). As a positive control, cells were also treated with Olaparib and showed about 85% lower viability. Next, apoptosis and necrosis were imaged by fluorescence microscopy. Cells treated with Olaparib-loaded exosomes and free Olaparib both displayed increased apoptosis (Fig. 3A). According to FACS analysis, cells treated with Olaparib-loaded hypoxic exosomes had 71% apoptotic cells while those treated with free Olaparib had about 68%, while untreated cells had only 4% apoptotic cells (Fig. 3B). Interestingly, Olaparib-loaded hypoxic exosomes (Olaparib-loaded DFO Exo) triggered higher apoptosis (about 71%) than Olaparib-loaded control exosomes (Olaparib-loaded Con Exo; about 42%). These results confirm our hypothesis that exosomes released by hypoxic cells can more efficiently target other hypoxic cancer cells for cancer therapy and thus induce apoptosis more effectively. Finally, using a CCK-8 assay, we verified that Olaparib-loaded exosomes could decrease the viability of hypoxic cells in a dose-dependent manner, with a decrease of 70% observed for the highest dose (Fig. 3C). These results showed that Olaparib-loaded exosomes have significant therapeutic effects on apoptosis and viability.

3.4. Nanoparticle (SPIO)-labeled exosomes for *in vivo* MPI

For *in vivo* applications, exosomes were modified by labeling them with SPIO nanoparticles for MPI imaging. Cells and exosomes labeled with SPIO have a visibly distinct color *in vitro* (Supplementary Fig. 4A). MPI images also showed that cells and exosomes transfected with SPIO have strong MPI signal (Supplementary Fig. 4B). The MPI signal increased linearly as a function of the amount of exosomes (Supplementary Fig. 4C). Using TEM, we verified that SPIO nanoparticles were successively encapsulated in transfected exosomes (Fig. 4A). In addition, there was no significant difference in exosome size before and after transfection of SPIO (Fig. 4B). Furthermore, a serum stability assay was performed to show that there was no significant change in the size distribution of exosomes at 4 hr and 24 hr (Supplementary Fig. 5A). There were also no significant differences in the average diameter of the different types of exosomes used in this study (Supplementary Fig. 5B). Finally, to validate the feasibility of imaging exosomes *in vivo*, SPIO-labeled exosomes were injected intravenously in a healthy mouse, and MPI/CT imaging was performed (Fig. 4C). *In vivo* and *ex vivo* images showed that exosomes accumulated primarily in the liver.

3.5. Therapeutic effect of Olaparib-loaded exosomes in mouse tumor models

To confirm the therapeutic efficacy of drug delivery using these exosomes *in vivo*, mice bearing MDA-MB-231 xenografts received intratumoral injections of PBS (vehicle), Olaparib-loaded exosomes, or Olaparib every two days. Using MPI imaging, we verified the successful administration of the exosomes into the tumor (Figure 5A). Two weeks after this treatment, we observed a significant delay in the growth of the tumors treated with Olaparib-

loaded exosomes and Olaparib, compared to the control, indicating the therapeutic efficacy of Olaparib-loaded exosomes. The tumor tissue was then characterized by histology. H&E staining did not highlight any differences between control tumors and tumors treated with Olaparib-loaded exosomes (Figure 5B). Furthermore, confocal microscopy imaging allowed us to confirm the uptake of Olaparib-loaded exosome within the tumor tissues, owing to the red fluorescence of DiI-labeled exosomes. To confirm apoptosis, cleaved caspase 3 staining was performed and showed increased apoptosis in tumors treated with Olaparib-loaded exosomes.

4. Discussion

The use of exosomes for diagnostic and therapeutic applications has received considerable attention [34-36]. Because of their unique characteristics such as high bioavailability, low toxicity, high targeting capabilities, and excellent membrane permeability, exosomes are ideal carriers to deliver miRNA, proteins, drugs, and other molecules [25,37-38]. Our study is innovative because no prior study has explored the use of hypoxia to enhance the ability of exosomes to target hypoxic cancer cells. This work is also the first to use MPI for imaging the distribution of exosomes *in vivo*. This study could also be useful to investigate the biological role of exosomes in the tumor microenvironment, which could open new perspectives for anti-cancer treatments.

Previous reports have demonstrated that exosomes from irradiated cells can trigger apoptosis in unirradiated cells, an effect known as the bystander effect [39,40], and that hypoxic exosomes can upregulate angiogenesis [41,42]. However, our study further shows that exosome uptake by hypoxic cancer cells can be modulated by exposing the cells producing the exosomes to hypoxia and/or ionizing radiation. Here, exosomes were isolated after exposing cancer cells to four different conditions: control, X-ray irradiation, hypoxia, and combination of X-ray irradiation and hypoxia. Not only did the amount of exosomes increase due to hypoxia and ionizing radiation, these exosomes were also taken up more avidly by hypoxic cancer cells. In particular, the highest exosome uptake was measured for exosomes released by hypoxic cells (DFO Exo). However, it is still not clear what specific proteins on the surface of exosomes are responsible for these changes. Elucidating the role of different surface proteins in exosome uptake could help better understand exosome-mediated cell-to-cell signaling in the tumor microenvironment. The molecular profile of membrane protein from hypoxic or irradiated exosomes, and its relation to cellular uptake, could also help explain the mechanism of exosome uptake in specific organs.

Many groups around the world have imaged the distribution of exosomes *in vivo* in mouse models by fluorescence imaging, bioluminescence imaging, and radioisotope imaging [43]. Lipophilic tracers such as DiR or fluorescence dyes such as Cy7 have been used for fluorescence imaging [15,16]. Other groups have used reporter genes to specifically tag exosomes, developing GFP/RFP tagged vector using highly expressed exosomal proteins such as CD9/CD63 or combining Gaussia luciferase with exosome membrane proteins [44,45]. In addition, radioisotope imaging of exosomes was demonstrated *in vivo* [46]. However, these imaging methods have limited use for clinical application due to low tissue penetration, low sensitivity, low resolution, and/or use of ionizing radiation. To overcome

these limitations, we developed a novel method for non-invasive tracking of exosomes *in vivo* using MPI. One strength of MPI lies in the use of SPIO nanoparticles for contrast. These particles are extremely safe for human use, they provide long-lasting contrast, and they have been used for numerous MRI applications. The use of SPIO nanoparticles with MPI enables the acquisition of *in vivo* images with greater contrast, sensitivity, and quantitation than MRI. Compared to radioisotope methods, MPI tracers are non-ionizing, FDA-approved, and available clinically [47,48]. To the best of our knowledge, our study is the first to image exosomes using MPI.

Furthermore, we showed that exosomes successfully delivered Olaparib drug to breast cancer cells *in vitro* and *in vivo*. Olaparib, an inhibitor of the enzyme poly ADP ribose polymerase (PARP) involved in DNA repair, is an FDA-approved targeted therapy for cancer [49,50]. This drug is indicated against BRCA1 or BRCA2-mutated cancers, such as those of the breast, prostate, and ovaries. In addition, Olaparib can be effective as radiosensitizer drug due to its inhibiting effect on DNA repair. Preclinical studies have shown enhanced radiosensitivity with use of Olaparib in cancer cells [51-53]. Because hypoxic tumor regions are resistant to therapy, particularly radiotherapy, delivery of Olaparib to hypoxic cancer regions could be a potent therapeutic strategy for radioresistant hypoxic cancer cells. This new exosome platform can potentially deliver other anti-cancer drug and radiosensitizers to various tumors.

One limitation of this study is that exosomes were administered intratumorally rather than systemically. Although we found high accumulation of hypoxic exosomes *in vitro* in hypoxic cancer cells, intravenously injected hypoxic exosomes (100 µg) did not yield a detectable MPI signal in the tumor (Supplementary Fig. 6). According on the reported sensitivity of MPI (5 ng per voxel, equivalent to ~10 ng/mL), MPI should detect an injected dose of 100 µg of exosomes, assuming 1% ID/g uptake in the tumor and 5% w/w Fe labeling [31-33]. *In vitro*, we were able to visualize 2.5 µg of exosomes. The lack of detectable MPI signal in the tumor suggests that accumulation of exosomes is too low for MPI to detect. Other groups have also shown similarly low uptake of exosomes *in vivo* in tumors [54,55]. It is therefore necessary to develop additional strategies for targeting *in vivo* hypoxic tumor regions with exosomes [56,57]. For example, hypoxia-targeting peptides and antibodies could be used to bioengineer the exosomes and enhance their ability to target hypoxic cells *in vivo* [58,59]. Furthermore, exosomes released from different types of cells, such as cancer cells, immune cells, or stem cells, could be more effective in targeting hypoxic cancer *in vivo* [16,60]. Additionally, these *in vivo* studies could be helpful to understand the role of exosomes as vaccine for cancer immunotherapy [61-63].

5. Conclusions

In this study, the uptake of exosomes from cells conditioned using X-ray irradiation and/or hypoxia was successfully quantified in hypoxic cancer cells, demonstrating the enhanced ability of exosomes released by hypoxic cancer cells to target hypoxic cancer cells. Furthermore, these “hypoxic” exosomes were successfully modified for MPI imaging and *in vivo* drug delivery. This new theranostic platform using hypoxic exosomes could be useful for delivering anti-cancer drug and radiosensitizers to various kinds of hypoxic cancer, while

imaging the efficacy of the treatment using MPI. However, we also found that exosomes from MDA-MB-231 cells administered intravenously in mice could not effectively target regions of tumor hypoxia. To increase the accumulation of exosomes in hypoxic cells *in vivo*, it may be necessary to develop a targeting method to increase overall exosome accumulation in the tumor. In future studies, we are planning to bioengineer exosomes using hypoxia-targeting agents or use exosomes released from different types of cells, which could help enhance hypoxic targeting *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Characterization of exosomes in radiation and hypoxia condition

(A) Four different types of exosomes (generated under hypoxic or normoxic conditions, and with or without exposure to radiation) were isolated from human breast cancer MDA-MB-231 cells. (B) Protein assay shows that radiation and hypoxia increased the release of exosomes from cancer cells; the highest amount was achieved for the combination of the two conditions. (C) NanoSight shows that the hydrodynamic size of the exosomes was distributed in the range 30~200 nm. * P value <0.05.

Fig. 2. Quantification of exosome uptake by hypoxic cancer cells using fluorescence (DiO) labeling

(A) Uptake of fluorescence (DiO)-labeled exosomes in hypoxic cancer cells was quantified by fluorescence microscopy. The highest uptake was for exosomes released from hypoxic cells (DFO Exo). (B) FACS also shows the same pattern as fluorescence microscopy. (C) Apoptosis assay showed that exosomes have no effect on apoptosis.

Fig. 3. Drug (Olaparib)-loaded exosomes to delivery therapeutic drug

(A) Apoptosis assay shows induction of apoptosis in hypoxic cells after 48 hr of treatment. (B) FACS analysis demonstrates that increased apoptosis was increased in cells treated with Olaparib-loaded exosomes or Olaparib, with the highest signals for cells treated with DFO Exo. (C) CCK assay shows that the number of viable cells to decrease in dose-dependent manners with exosome concentration.

Fig. 4. Nanoparticle (SPIO)-labeled exosomes for *in vivo* MPI

(A) TEM images show successful encapsulation of SPIO nanoparticles in exosomes. (B) NanoSight nanoparticle size distribution shows no significant difference in exosome size after SPIO labeling. (C) MPI and MicroCT images of exosomes show distribution in healthy mice *in vivo* and *ex vivo*.

Fig. 5. Therapeutic effect of Olaparib-loaded exosomes in mouse tumor models

(A) To evaluate the therapeutic effects of Olaparib-loaded exosomes in tumor xenograft mice, tumor size was measured after intratumoral injection of PBS, olaparib-loaded exosomes, and olaparib. In comparison with the control group, the mice treated olaparib-loaded exosomes exhibits slower tumor growth, confirming the therapeutic effects of exosomes. (B) *Ex vivo* tissues analyses showing, from left to right, tumor histology, uptake of fluorescence exosomes, and tumor apoptosis. * P value <0.05.