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Chlorin e6 Functionalized Theranostic Multistage Nanovectors Transported by Stem Cells for Effective Photodynamic Therapy

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Abstract

Approaches to achieve site-specific and targeted delivery that provide an effective solution to reduce adverse, off target side effects are urgently needed for cancer therapy. Here, we utilized a Trojan-horse-like strategy to carry photosensitizer Chlorin e6 conjugated porous silicon multistage nanovectors with tumor homing mesenchymal stem cells for targeted photodynamic therapy and diagnosis. The inherent versatility of multistage nanovectors permitted the conjugation of photosensitizers to enable precise cell death induction (60%) upon photodynamic therapy, while simultaneously retaining the loading capacity to load various payloads, such as antitumor drugs and diagnostic nanoparticles. Furthermore, the mesenchymal stem cells that internalized the multistage nanovectors conserved their proliferation patterns and in vitro affinity to migrate and infiltrate breast cancer cells. In vivo administration of the mesenchymal stem cells carrying photosensitizer-conjugated multistage nanovectors in mice bearing a primary breast tumor confirmed their tropism toward cancer sites exhibiting similar targeting kinetics to control cells. In addition, this approach yielded in a > 70% decrease in local tumor cell viability after in vivo photodynamic therapy. In summary, these results show the proof-of-concept of how

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.7b05766.

SEM image to verify MSV pore size, FTIR spectra, Ce6 absorption analysis, SEM image of Ce6@MSV, MSV internalization by MSC, Ce6@MSV cytotoxicity, MSV migration in vitro, raw data of BLI signals before and after harvesting, and tumor cell viability of harvested tumors (PDF)

Movie S1, cell deformation after PDT (MPG)

The authors declare no competing financial interest.

photosensitizer conjugated multistage nanovectors transported by stem cells can target tumors and be used for effective site-specific cancer therapy while potentially minimizing potential negative side effects.

Graphical abstract

Keywords

mesenchymal stem cells; porous silicon; photodynamic therapy; cancer therapy; theranostics

INTRODUCTION

Cancer is the second leading cause of death in the world¹ and the leading cause of death in the US among adults between 40 and 79 years of age.² Traditional cancer treatment options include a combination of chemotherapy, surgery, radiation, and biologics.³ However, conventional chemotherapy and biologics lack the necessary features to successfully arrive at the cancer lesion, delivering less than 0.01% of the administered dose⁴ and yielding undesired side-effects to healthy tissue.⁵ This disconnect can be rooted in the presence of multiple biological barriers that exist to protect us from foreign pathogens and pose as obstacles that impede and obstruct the delivery of agents to the cancer site.⁶ Nanoparticles have emerged as powerful solutions to encapsulate and deliver biological agents but have predominantly fallen short in obtaining successful clinical translation, primarily due to their inability to adequately address these biological barriers (e.g., sequestration by phagocytic cells⁷ and dense extracellular matrix of tumors⁸). Hence, novel solutions are needed to circumvent these issues and impart functions on therapies that permit efficient negotiation of these biological barriers.

As an alternative to chemotherapy, photodynamic therapy (PDT) has emerged as a technique for cancer therapy which utilizes three components: photosensitizers (PS), light, and oxygen. Light excitation at a specific site triggers a photochemical reaction in PS resulting in the production of reactive oxygen species (ROS), which subsequently yields cell damage and death.⁹ PDT can provide accurate stimulus that triggers ROS production at a defined time and specific site resulting in a significant reduction of off-target effects on healthy tissues. However, direct in vivo use of PS can be problematic due to impaired cellular uptake¹⁰ or excessive aggregation, yielding reduced PDT activity.¹¹ A possible solution is to incorporate PS on the surface of biocompatible¹² and biodegradable^{13,14} porous silicon particles¹⁵ whose highly modifiable surface and variable pore volume permit the simultaneous delivery of various payloads (e.g., drugs¹⁶ or nanoparticles^{17,18}). We proposed that multistage nanovectors (MSV) composed of porous silicon could be used to sequentially address the biological barriers.¹⁹ MSV have previously been shown to effectively navigate blood flow and marginate toward the vascular wall,²⁰ however, targeting inflamed vasculature and

maintaining retention within tumors remains a challenge without modification of the particle surface, which can impede PS engraftment.²¹

Cell-based therapies have recently spurred the development of novel approaches relying on innate advantages of cells to actively respond to stimuli and direct migration, bestowing them with attributes that surpass those exhibited by biologics and targeted nanoparticles.²² Mesenchymal stem cells (MSC) have emerged as a source that can be easily isolated from various locations, manipulated ex vivo for various applications, and exhibit directed migration to inflamed tissues (e.g., tumors).²³ Previous studies have leveraged on this attractive migration phenomenon to deliver nanoparticles²⁴ or prodrug converting enzymes.²⁵ However, these approaches are susceptible to exocytosis of nanoparticles, require elaborate genetic modifications to achieve an effect, and lack the versatility to effortlessly adjust the therapeutic payload.²⁶ With this in mind, we chose to develop a Trojan horse-like strategy to deliver 1 μ m theranostic MSV, surface functionalized with PS, as a new platform for PDT-based cancer therapy and imaging (Figure 1). Herein, we demonstrated precise cell death and decreased tumor cell viability upon PDT and conserved MSC migration to tumor both in vitro and in vivo. To the best of our knowledge, this is the first study utilizing PS conjugated MSV, showing in vivo success of MSC to induce localized cell death.

RESULTS AND DISCUSSION

MSV have been extensively characterized by our group²⁷ and, for this study, we used uniformly discoidal 1000 $\times$ 400 nm MSV (Figure 2a) with 30–60 nm pores (Figure S1) to maximize internalization, while avoiding exocytosis.²⁸ Zeta potential measurements confirmed successful NH₂ grafting on the surface of MSV yielding a change of 70 mV, whereas surface conjugation with Chlorin e6 (Ce6, Ce6@MSV) slightly decreased the zeta potential by 9 mV (Figure 2b). Fourier transform infrared spectroscopy (FTIR) spectra corroborated the zeta potential measurements and displayed the emergence of –NH and –CH₂ bending peaks at 1560 and 1490 cm^{–1} after amine modification, respectively (Figure S2). Similar to previous reports,²⁹ peaks at 1630 and 1560 cm^{–1} were detected after Ce6 conjugation corresponding to the carboxyl group³⁰ and C–C³¹ vibrations in the pyrrole rings of the Ce6 molecule, respectively. In addition, Ce6@MSV exhibited the characteristic absorption and fluorescence peaks of Ce6 (Figure 2c & d) and demonstrated ~7% (w/w) of Ce6 on the surface (Figure S3).

Scanning electron microscopy (SEM) images of Ce6@MSV revealed that the pores of MSV remained accessible after conjugation (Figure S4), though covalent conjugation of molecules to porous silicon particles may have an effect on their loading capacity.³² To evaluate if Ce6@MSV preserved their ability to load and retain nanoparticles, we examined the simultaneously loading of therapeutic (doxorubicin (DOX) micelles) and diagnostic (quantum dots, QD) payloads into the pores of MSV. As expected, Ce6@MSV displayed similar loading capacity for both payloads compared to bare MSV (Figure 2e), with fluorescent microscopy revealing successful conjugation of Ce6.³³ Hence, Ce6@MSV conserved the unique loading properties of MSV demonstrating its potential as a powerful theranostic nanovectors for therapy³⁴ and imaging³⁵ after conjugation with PS.

The success of our approach relies on Ce6@MSV provoking minimal impact on the viability and biological function of MSC. As such, the internalization of Ce6@MSV was evaluated using fluorescence microscopy relying on the intrinsic fluorescence of Ce6 to detect Ce6@MSV (Figure 3a). These images exhibit successful internalization of Ce6@MSV (red) by MSC (green) and bright field images (Figure S5) suggest Ce6@MSV accumulation in the peri-nuclear region, similar to previous results with bare MSV.³⁶ The viability and proliferation of MSC following Ce6@MSV internalization was assessed using MTT and alamarBlue assays, respectively. At 24 h, minimal differences were detected in the viability of unloaded MSC (control) to MSC+Ce6@MSV exposed at 50, 100, and 200 MSV:MSC ratios (Figure S6). The proliferation of MSC was assessed for 72 h and revealed MSC +Ce6@MSV paralleled control MSC growth rates (Figure 3b). These assays function based on active mitochondrial enzymatic activity³⁷ and verified that the introduction of Ce6@MSV at various ratios failed to induce any significant change in the normal metabolic activity of MSC, similar to previous results with MSV.³⁶

Following the evaluation of the impact of Ce6@MSV on the health of MSC, their ability to migrate and infiltrate cancer cells was assessed. MSC and cancer cells were seeded within a culture insert to separate each cell line by 500 μm (Figure S7a). Once the insert was removed, both control MSC and MSC+MSV began crawling and spreading toward the cancer cells. At 24 h, both groups successfully migrated to the edge of the cancer cells. Upon arriving at this border, control MSC and MSC+MSV continued to infiltrate the cancer cell population such that by 120 h, no discernible boundary could be observed between MSC and the cancer cells (Figure S7a). Confocal microscopy at 24 and 120 h (Figure S7b) confirmed that MSC arrived at the border and infiltrated the cancer cell cultures while successfully carrying MSV. Thus, the incorporation of MSV into MSC did not impact the viability, proliferation, or migration potential of MSC toward cancer cells.

The capacity for Ce6@MSV to generate ROS was evaluated using a ROS-specific detection assay that induces fluorescent activation upon oxidation. Following PDT, both control MSC and MSC+MSV failed to induce any increase in ROS signals (Figure 3c, d), whereas MSC +Ce6@MSV demonstrated a significant increase in local ROS signal, specifically within the laser's radius (Figure 3c, white circle). Furthermore, similar to previous results,³⁸ we witnessed a bubble-like formation after PDT, followed by rapid cell deformation (Movie S1). To evaluate if ROS generation by Ce6@MSV is successful at triggering cell death, we used the LIVE/DEAD assay to concurrently stain both viable and dying MSC. LIVE/DEAD assay was selected to adequately address the precise cell killing ability of Ce6@MSV with PDT. Moreover, the utilization of relatively small stimulation area would not have induced sufficient effect for e.g. MTT assay without multiple stimulations or prolonged time that could have caused cells to die naturally in the absence of a suitable atmosphere. After excitation, control MSC and MSC+MSV confirmed our previous ROS generation results and displayed minimal MSC death within the laser's radius, with the majority of MSC remaining viable (Figure 3e). Interestingly, MSC+Ce6@MSV exhibited prominent cellular death within the laser's radius, whereas MSC outside of the laser's range remained viable (Figure 3e). Quantification revealed that, following PDT, nearly 60% of MSC+Ce6@MSV exhibited damaged membranes and were possibly undergoing rapid necrosis while MSC+MSV demonstrated only 3% cellular death, likely a result of the unspecific heat absorption of

porous silicon (Figure 3f).³⁹ Hence, Ce6@MSV can serve as an effective nanovector to generate ROS and induce localized cellular death in vitro. In addition, when combined with the tumor homing features of MSC, PDT with MSC+Ce6@MSV represents a promising tool for effective, site-specific cancer therapy.

Orthotopic 4T1 murine mammary tumors were established in the mammary fat pad of female BALB/c mice to evaluate the capacity of MSC to migrate toward tumors in vivo. This murine breast tumor model was selected to provide a favorable, immunocompetent syngeneic environment to preserve critical factors (e.g., cell-to-cell communication, signal transduction, etc.) that govern the homing of MSC to tumors. The migration of MSC to tumors was monitored longitudinally for 72 h with fluorescence and bioluminescent imaging (BLI) to locate labeled MSC and tumor cells, respectively (Figure 4a). To quantify this distribution from 24 to 72 h, we drew regions of interest over the thoracic, abdominal, and tumor regions. This analysis revealed that both groups exhibited similar kinetics for all three areas of interest, with the thoracic and abdominal cavities showing 20 and 30% decreases at 72 h, respectively (Figure 4b). Concurrently, the tumor region demonstrated a gradual 20% increase from 24 to 72 h for both groups over this span (Figure 4b). Similar to our results, previous investigations established that MSC successfully vacated the lungs of homeostatic (i.e., normal) mice by 24 h^{40,41} and showed increased homing and incorporation within tumors over 60 days.⁴⁰ Hence, this apparent increased accumulation observed at the tumor site could be attributed to MSC leaving the thoracic and abdominal areas to either be cleared or home to tumors.

After 72 h, mice were sacrificed and organs were harvested to analyze the biodistribution of MSC. Confirming our longitudinal assessment, control MSC and MSC+Ce6@MSV showed similar biodistribution with no significant differences observed between the two groups in the liver, spleen, lung, kidney, heart, or tumor (Figure 4c). After 72 h, the liver and spleen exhibited a substantial accumulation whereas minimal signal was observed in the kidneys and heart, similar to previous studies.⁴⁰ The lung and tumor exhibited similar accumulation signals and demonstrated the ability of MSC+Ce6@MSV to conserve the capacity of MSC to actively target tumors. Furthermore, if examined mice at further time points (6–14 days), we believe even greater MSC tumor accumulation could be observed.^{40,42} Transporting nanoparticles within MSC provide yet another level of possible protection by avoiding the exposure of potentially cytotoxic payloads to healthy tissues as MSC could retain these payloads after accumulating within filtering organs and are cleared before releasing their payloads. Hence, MSC can serve as effective carriers that retain their homing to tumors after incorporation with theranostic MSV, permitting their potential use for cancer therapy or imaging.

For in vivo PDT, mice with established 4T1 tumors were separated into two groups and treated with direct intratumoral injections of either control MSC or MSC+Ce6@MSV. Intratumoral injections are widely utilized for PDT in vivo validation^{43–45} and further enabled evaluation for pinpoint accurate stimulation and strictly bound cell death in vivo on target. For these studies, we chose to administer 1×10^5 MSC to represent a reasonable percentage of MSC capable of accumulating in tumors based on previous studies.⁴² Furthermore, we chose to study the intratumoral route, as opposed to intravascular route, due

to the absence of high power laser.^{43,44} PDT was achieved by employing a microscope suitable for intravital microscopy (IVM) with a laser similar to what was utilized to obtain the in vitro results. After administration, the locations of labeled MSC or MSC+Ce6@MSV were imaged using IVM and photoactivated for 15 min at each injection site. The PDT time was increased from in vitro setup to achieve satisfactory radiation after penetrating through several cell layers to stimulate the MSV efficiently. Evaluation of site-specific PDT was done by assessing fluorescence and BLI using an IVIS Spectrum to locate injection spots and to assess the viability of cancer cells, respectively. Photographic images from each group demonstrated that tumors treated with MSC+Ce6@MSV displayed a possible local increase in edema compared to control MSC treatment (Figure 4d, yellow arrows). Overlaying the regions around the injection sites (fluorescence, Figure 4d) on BLI images (Figure 4d) revealed negligible differences between the site of injection and the surrounding area for control MSC whereas tumors treated with Ce6@MSV exhibited a local decrease of BLI signal at the injection site compared to the surrounding tumor areas. Quantification of BLI signals at the injection sites, normalized to the surrounding area of the injection site per tumor and treatment (Figure S8), confirmed these observations demonstrating a 60% reduction in tumor cell viability for MSC+Ce6@MSC compared to the 13% increase in viability for control MSC (Figure 4e). In addition, when normalized to the viability (i.e., BLI signal) of the overall tumor, MSC+Ce6@MSV treated tumors continued to exhibit greater than 85% difference in viability compared to tumors treated with control MSC (Figure 4f). Moreover, employing the same analysis on harvested tumors revealed similar significance between the treatments (Figure S9).

Photoactivation of MSC+Ce6@MSV provided effective PDT, permitting precise in vivo cancer cell killing. For clinical applications, this delivery system can leverage on the intrinsic fluorescent nature of Ce6@MSV to allow clinicians to dictate where to apply PDT and permit a more effective treatment. Although similar stem-cell-mediated delivery platforms for PS have been employed in vitro³⁸ and in vivo,⁴⁶ we developed a system that achieves favorable cell killing at a fraction of the power in minimal activation time.⁴³ Compared to earlier publications,^{43,44} 20-times less powerful laser was utilized in our study to obtain significant therapeutic effect. Thus, future experiments with higher laser power could yield more effective results and be applied to test the efficacy after systemic administration. In addition, the Ce6 absorption band at longer wavelength (Figure 2c) (650–680 nm) enables utilizing laser in this range to increase available treatment depth and additionally improve the potential therapeutic efficacy. Furthermore, the singlet oxygen produced by PDT alone may not be sufficient to produce long-term impact as it is estimated to diffuse less than 300 nm before converting back to its unreactive state.⁴⁷ Thus, the addition of theranostic MSV loaded with powerful therapeutics could be used to simultaneously provide PDT and chemotherapy to increase the therapeutic benefit. Furthermore, the transport of these MSV by MSC can further enhance the diffusion of nanoparticles and therapeutics to deeply penetrate the tumor matrix because of the crawling and extravasation features of MSC. In addition to triggering cell death, the versatility of the MSV platform could be used in combination with MSC to yield beneficial outcomes to provide aid for tissue regeneration applications.⁴⁸

CONCLUSIONS

The versatility of MSV allows the development of powerful theranostic agents, permitting access to its surface for conjugation of photosensitizers (i.e., Ce6) and loading of smaller nanoparticles within its porous matrix. Here, we show a proof-of-concept where we leverage on the intrinsic tropism of MSC to tumors for transportation of Ce6@MSV. The addition of PS to MSV permits accurate site-specific therapy, providing a potent solution for achieving effective cancer therapy by maximizing tumor response and minimizing toxic side effects. MSC containing Ce6@MSV demonstrated in vitro and in vivo tumor infiltration and homing and, upon excitation with PDT, induced effective cancer cell death. Furthermore, the negligible toxicity of Ce6@MSV incorporated within responsive MSC could be strategically employed to promote regenerative properties, thus providing a powerful tool for not only cancer therapy but various other biomedical applications.

MATERIALS AND METHODS

Materials

N-Hydroxysuccinimide (NHS), dimethyl sulfoxide (DMSO), 3-(aminopropyl)triethoxysilane (APTES), 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA), bovin serum albumin (BSA), doxorubicin (DOX), Tris-HCl, and 2-propanol (IPA) were purchased from Sigma-Aldrich. 2-(*N*-morpholino)ethanesulfonic acid (MES), DiO, DiD and DiR cell-labeling solutions, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC), trypsin, LIVE/DEAD assay, alamarBlue, Qdot 525 ITK carboxyl quantum dots (QD), and SlowFade Gold antifade reagent were purchased from Thermo Fisher Scientific. Chlorin e6 (Ce6) was purchased from Frontier Scientific. Stem cell medium and supply kits were purchased from Cell Biologics. Phosphate buffered saline (PBS) and Hanks balanced salt solution (HBSS) were obtained from HyClone. D-Luciferin was obtained from Gold Biotechnology. 1,2-Distearoyl-phosphatidylethanolamine-polyethylene glycol amino-200 was purchased from Avanti Polar Lipids. 4T1 cancer cells coexpressing firefly luciferase and TdTomato were purchased from PerkinElmer.

MSV Fabrication

Discoidal MSV (1 $\mu\text{m} \times 400 \text{ nm}$) were manufactured by electrochemical etching, consequent photolithographic patterning and oxidized with H₂O₂ as described earlier.²⁷ MSV was amine-modified with APTES (8% v/v) for 2 h in a thermomixer (35 °C, 1400 rpm) to produce amine-grafted MSV (NH₂-MSV), whereas Ce6 was activated with EDC (0.1 M) and NHS (0.3 M) in DMSO for 4 h. NH₂-MSV were dispersed in MES (0.2 M, pH 5), added to equal volume of Ce6 in DMSO, and agitated for 18 h at room temperature (RT, 23 °C). Following overnight agitation, unconjugated Ce6 was washed from Ce6@MSV with IPA until supernatant became transparent.

Characterization

Scanning electron microscopy (SEM) was used for pore size and morphological studies. Briefly, 1×10^6 MSV suspended in IPA were spotted onto acetone-washed SEM stubs and

allowed to dry overnight. Visualization was performed under a high vacuum at 20.00 kV with a spot size of 5.0. Images were all collected using an FEI Quanta 400 FEG ESEM apparatus and a Hitachi S-5500 In-lens FE-SEM. Zetasizer Nano-ZS (Malvern Instruments Ltd.) was utilized to measure the surface charge (zeta potential). Photosensitizer Ce6 conjugation was confirmed with transmission Fourier transform infrared spectroscopy (FTIR, Thermo Scientific Nicolet 6700) and ultraviolet–visible spectroscopy (UV–vis, Synergy H4, BioTek).

Loading Payloads into MSV Pore Structure

Ten million MSV or Ce6@MSV were dispersed in Tris-HCl buffer (10 μL , pH 7.3). QD (5 μL , 8 μM) and Doxorubicin (5 μL , DOX) micelles prepared using a previously published protocol⁴⁹ were added and sonicated to ensure homogeneous dispersion. MSV were spun down and washed with Tris-HCl buffer after 30 min incubation at RT to remove unloaded QDs and DOX micelles. DOX-QD-MSV (10 μL) and DOX-QD-Ce6@MSV (10 μL) were dried on microscope slide and mounted with SlowFade antifade reagent and imaged using confocal microscopy (Nikon A1+).

MSV Internalization into MSC

Mouse MSC derived from the bone marrow of BALB/c mice were utilized as a carrier platform for Ce6@MSV. MSC were grown in stem cell media supplemented with 10% of fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin (P/S), and 1% nonessential amino acids. MSC were maintained at 37 °C, 95% humidity, and 5% CO₂. The culture media was changed every 2 days and MSC were split after reaching 80–90% confluence.

Sixty thousand MSC/cm² were seeded into wells of a 4-well chambered coverglass (Nunc Lab-Tek II). After cell adherence, the growth media was replaced with media containing 200 Ce6@MSV/cell and incubated overnight (>16 h). Cells were washed three times with PBS to remove free MSV. The internalization was confirmed with confocal microscopy after labeling the MSC with green fluorescent DiO-dye. Loaded cells were denoted as MSC +Ce6@MSV.

Cytotoxicity

Ce6@MSV cytotoxicity was evaluated using an MTT and alamarBlue cell viability assay. MSC were seeded into wells of a 96-well plate (6×10^4 MSC/cm² for MTT and 1×10^4 MSC/cm² for alamarBlue) and incubated with Ce6@MSV (0, 50, 100, and 200 MSV:MSC) for 24 h and subsequently washed with PBS to remove free MSV. alamarBlue was diluted to 10% working solution with cell culture media and incubated with MSC+Ce6@MSV for 3 h. Reacted alamarBlue was collected for analysis, cells washed with PBS x3 and fresh media was replaced. Cell proliferation was analyzed with UV–vis at 24, 48, and 72 h after particle internalization. MTT was dissolved in PBS to yield 12 mM solution and diluted to 10% working solution with cell growth media. After 3 h incubation with MTT solution, the media was replaced with DMSO, and subsequently analyzed with UV–vis.

ROS Production

ROS generation was evaluated with H₂DCFDA. MSC+Ce6@MSV (6×10^4 MSC/cm², 200 MSV:MSC) were incubated with H₂DCFDA (20 μ M) in cell culture media at 37 °C for 1 h. Noninternalized dye was washed away with PBS $\times$ 3 and fresh media was replaced prior to the PDT. The MSC+Ce6@MSV were exited with 405 nm laser (100 mW) for 3 min and subsequently imaged with confocal microscopy.

In Vitro Apoptosis Evaluation for PDT

LIVE/DEAD assay based on green-fluorescent calcein-AM and red-fluorescent ethidium homodimer-1 was used to discriminate live and dead cells respectively to evaluate effectiveness of PDT in vitro. Briefly, calcein-AM (5 μ L) and ethidium homodimer-1 (20 μ L) were added to 0.2% BSA containing HBSS (10 mL) to yield working solution for LIVE/DEAD staining. MSC were seeded into wells of a 4-well plate (6×10^4 MSC/cm²), allowed to adhere and incubated with Ce6@MSV (200 MSV:MSC) for 24 h. MSC+Ce6@MSV were stimulated with 405 laser (100 mW) for 3 min, incubated with LIVE/DEAD assay solution (0.5 mL) for 30 min in RT, and subsequently imaged with confocal microscopy.

In Vitro Migration

Ten thousand MSC and MSC+MSV (at 1:200 MSV per cell) were seeded into one side of an Ibidi culture inserts within a Ibidi 35 mm high μ -Dish. Separated by 500 μ m, 3×10^4 MDA-MB-231 cells were seeded on the other side. MSC and MDA-MB-231 cells were transduced by Dr. Brian Rabinovich at The University of Texas M.D. Anderson Cancer Center in the Department of Pediatrics with mCherry and green fluorescent protein, respectfully. After allowing the cells to adhere, inserts were removed and left at 37 °C and 5% CO₂ for 120 h, removing dishes at 18, 24, 48, and 120 h for pictures taken with a Nikon TS100 equipped with a DS-Fi1. At 24 and 120 h, inserts were analyzed with a Nikon A1 confocal to image the cellular border and infiltration of MSC into cancer cells.

Animal Care

Animal studies were performed in accordance with the guidelines of the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals based on approved protocols by The Houston Methodist Research Institute's Institutional Animal Care and Use Committee. Female BALB/c mice (4–8 weeks old) were purchased from Charles Rivers Laboratories and maintained as previously described.¹²

In Vivo Migration

Red-fluorescent 4T1-Luc breast cancer cells were grown in RPMI 1640 cell media supplemented with 10% of FBS, 1% L-glutamine and 1% P/S. The tumors were implemented to mammary fat pads of BALB/c by subcutaneous injection of 4T1-Luc cancer cells (5×10^4) and grown for 10–14 days to reach appropriate size. MSC+Ce6@MSV were dyed with DiR before IV injection (1×10^6) ($n = 3$). DiR labeled empty MSC (1×10^6) were used as a control group ($n = 3$). IVIS imaging was performed 24, 48, and 72 h after stem cell administration to determine MSC location. Luciferin (1 mg/mice/ time point) was injected intraperitoneally (IP) prior to each time point to verify tumor locations. The mice were

sacrificed after 72 h and the organs were collected to further analyze MSC biodistribution with IVIS.

In Vivo Phototherapy

4T1-Luc breast cancers were grown and implanted in mice as described in in vivo Migration-section. All the tumors received at least three intratumoral injections of either MSC +Ce6@MSV (1×10^5) ($n = 3$) or DiD-labeled MSC (1×10^5) ($n = 3$). Each injection site was located with IVM and subsequently photo-activated for 15 min with 405 nm laser (100 mW). Following photoactivation, mice were injected with luciferin (IP, 1 mg/mice), sacrificed, and imaged with IVIS to collect fluorescence (DiD/Ce6) and BLI (tumor viability) of mice to locate injection sites and analyze the corresponding tumor viability at those points. Three mice per group were imaged before the tumor was harvested (i.e., Before Harvesting), and five mice per group were imaged after tumors were harvested (i.e., After Harvesting). Fluorescence and BLI images were analyzed using Living Image software. Quantification of signals at the injection sites of MSC and MSC+Ce6@MSV was done by first identifying the injection sites using the fluorescence signal and transferring these ROIs to the BLI image of tumor cell viability. In addition to the ROIs for injection spots, two additional ROIs were drawn to represent the area surrounding the injection site (solid red line, in Figure 4D) and the area that encompasses the entire tumor (dashed white line, in Figure 4D). The average radiance from these ROIs were exported to Microsoft Excel and the injection spots identified on each tumor were normalized to either the average radiance of the signal found surrounding the injection or the overall tumor. Data is represented as percentage above or below the average radiance of the signal in the tumor. For the analysis shown in Figure 4 (i.e., before harvesting), we inspected 9–10 injection spots on 3 tumors per group. For the analysis shown in the FigureS9 (i.e., after harvesting), we inspected 8–10 injection spots on 5 tumors.

Statistical Significance

Statistics were obtained using GraphPad Prism software. Statistics for cytotoxicity, ROS production, cell viability (LIVE/DEAD) and in vivo ear migration were defined using a One-Way ANOVA followed by Dunnett's post-test to correct for multiple comparisons. Statistics for MSC biodistribution analysis was determined using a Two-way ANOVA with a Sidak post-test to correct for multiple comparisons. All graphs show values represented with the mean $\pm$ s.e.m. For all cases, the statistical significance is denoted by probabilities set of **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic illustration of PDT with Trojan-horse-like approach. (A) Chlorin e6 is conjugated to MSV to prepare theranostic Ce6@MSV. Ce6@MSV are (B) internalized within MSC and (C) transported with MSC to cancer to (D) induce reactive oxygen species after photodynamic therapy for (E) localized cell death for cancer therapy.

Figure 2.

Assembly of Ce6@MSV. (a) Discoidal 1000 × 400 nm MSV, scale bar 500 nm. (b) ζ -potential of modified MSV $\pm$ s.e.m. ($n = 3$). (c) Absorption and (d) fluorescence spectrum comparing MSV, Ce6@MSV and pure Ce6. Vertical adjustment was done for clarity. (e) Fluorescent imaging of MSV and Ce6@MSV (purple) simultaneously loaded with DOX micelles (red) and QD (green). From left: bright field, TRITC, FITC, Cy5, and merged images. Scale bars, 500 nm.

Figure 3.

Ce6@MSV internalization, cytotoxicity, and PDT with MSC. (a) Confocal imaging of MSC, MSC+MSV, and MSC+Ce6@MSV demonstrating the internalization and characteristic Ce6 fluorescence. From left: MSC (green), Ce6@MSV (red), and merged images. Scale bar, 100 μm . (b) MSC proliferation up to 72 h after Ce6@MSV internalization with various concentrations, values represented as mean $\pm$ s.e.m. ($n = 8$). (c) ROS production in MSC, MSC+MSV and MSC+Ce6@MSV after PDT. Scale bar, 200 μm . (d) Quantification of ROS analysis in the excitation area from MSC, MSC+MSV, and MSC+Ce6@MSV before and after PDT, values represented as mean $\pm$ s.e.m. ($n = 3$). (e) MSC viability after PDT with MSC, MSC+MSV, and MSC+Ce6@MSV determined with LIVE/DEAD assay. LIVE cells are green, DEAD cells are red. Scale bar, 200 μm . The PDT was done with 100 mW, 405 nm laser for 3 min. (f) Analysis on the percentage of LIVE and DEAD MSC after PDT within excitation area comparing MSC, MSC+MSV, and MSC+Ce6@MSV, values represented as mean $\pm$ s.e.m. ($n = 3$). * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$.

Figure 4.

In vivo cancer homing and PDT. (a) Longitudinal NIR fluorescence imaging at 0, 24, 48, and 72 h comparing the accumulation of DIR labeled MSC and MSC+Ce6@MSV. (b) Quantification and subsequent normalization of the MSC accumulation within the thoracic, abdominal, and tumor regions. (c) Analysis of organ biodistribution of MSC and MSC +Ce6@MSV allowing for 72 h for migration. All data represented as mean $\pm$ s.e.m ($n = 3$). (d) Photographic images of tumors (outlined in dashed white line), with yellow arrows indicating areas displaying edema. Fluorescent images of tumors identifying the injection spots of MSC or MSC+Ce6@MSV (white circles). BLI images of luciferase in tumors

showing the injection spots (white circles) and identifying the area surrounding the injection (solid red line) and entire tumor (dashed white line). (e, f) Quantification of cancer cell viability evaluated (e) from the area surrounding the injection and (f) from the whole tumor after PDT with 100 mW, 405 nm laser for 15 min. All data represented with mean $\pm$ s.e.m. ($n = 3$). *** $P < 0.001$; **** $P < 0.0001$.