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Elimination of epithelial-like and mesenchymal-like breast cancer stem cells to inhibit metastasis following nanoparticle-mediated photothermal therapy

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Abstract

Increasing evidence suggesting breast cancer stem cells (BCSCs) drive metastasis and evade traditional therapies underscores a critical need to exploit the untapped potential of nanotechnology to develop innovative therapies that will significantly improve patient survival. Photothermal therapy (PTT) to induce localized hyperthermia is one of few nanoparticle-based treatments to enter clinical trials in human cancer patients, and has recently gained attention for its ability to induce a systemic immune response targeting distal cancer cells in mouse models. Here, we first conduct classic cancer stem cell (CSC) assays, both *in vitro* and in immune-compromised mice, to demonstrate that PTT mediated by highly crystallized iron oxide nanoparticles effectively eliminates BCSCs in translational models of triple negative breast cancer. PTT *in vitro* preferentially targets epithelial-like ALDH + BCSCs, followed by mesenchymal-like CD44+/CD24– BCSCs, compared to bulk cancer cells. PTT inhibits BCSC self-renewal through reduction of mammosphere formation in primary and secondary generations. Secondary implantation in NOD/SCID mice reveals the ability of PTT to impede BCSC-driven tumor formation. Next, we explore the translational potential of PTT using metastatic and immune-competent mouse models. PTT to inhibit BCSCs significantly reduces metastasis to the lung and lymph nodes. In immune-competent BALB/c mice, PTT effectively eliminates ALDH + BCSCs. These results suggest the feasibility of incorporating PTT into standard clinical treatments such as surgery to enhance BCSC destruction and inhibit metastasis, and the potential of such combination therapy to improve long-term survival in patients with metastatic breast cancer.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2016.06.045>.

Keywords

Photothermal therapy; Breast cancer stem cells; Iron oxide nanoparticles; Metastasis inhibition; Triple negative breast cancer

1. Introduction

Cancer stem cells pose what is arguably the greatest challenge in developing therapies that will truly cure cancer patients. First discovered in 1997 in acute myeloid leukemia by Dick and colleagues [1], soon evidence for a small population of stem-like cells with the ability to drive tumor formation began to arise in solid cancers [2–6]. For more than a decade Wicha and others have conducted extensive studies to characterize BCSC populations identified by either the Aldefluor enzymatic assay (ALDH+) or the surface receptor phenotype CD44+/CD24– [2,7–11]. In an effort to better understand the role of BCSCs in tumor growth, dissemination, and metastasis formation, recent genetic profiling by the Wicha group revealed the two BCSC populations are distinct and yet plastically transition from one form to the other as signaled by the tumor microenvironment [12]. The authors report that the ALDH+ BCSCs exist in a mesenchymal-to-epithelial (MET) state and are primarily proliferative and concentrated at the tumor interior. In contrast, the CD44+/CD24– BCSCs exist in an epithelial-to-mesenchymal (EMT) state and are primarily quiescent and concentrated at the tumor invasive edge. Their findings suggest that ALDH+ MET BCSCs and EMT CD44+/CD24– BCSCs may have distinct roles in driving tumor growth and metastasis.

In light of this recent discovery it remains pertinent that future breast cancer therapies not only account for the BCSCs in addition to the differentiated cancer cells [13], but also distinguish the two distinct BCSC populations. However, for most patients it is unlikely the BCSCs will be localized to the primary tumor at the time of treatment. Up to 30% of Stage I breast cancer patients already have micrometastases detectable from bone marrow biopsies [14]. With metastasis being the primary cause of death in breast cancer patients [15,16], to be most effective future therapies must perform two essential roles: act locally against all populations of cancer cells to prevent future metastasis, and act systemically against all populations of cancer cells in existing metastases. However the resistance of CSCs to traditional therapy such as chemotherapy or radiation, which when used alone can enrich the CSC population [17–19], remains a significant challenge. Therapeutic regimens exploiting advances in nanoparticle development offer a tremendous potential to enhance treatment efficacy against CSCs.

Photothermal therapy (PTT) is one of the few examples of nanoparticle-mediated treatments to enter clinical trials in human cancer patients [20,21]. Gold-based nanoparticles and carbon nanotubes have been the predominant subjects of laboratory investigation to produce heat targeted to kill cancer cells in response to near infrared (NIR) light [21–34]. Similarly, in the presence of an alternating magnetic field, iron oxide nanoparticles have been explored for cancer hyperthermia [21,35–37]. Such therapy offers several advantages to cancer patients. External control of localized hyperthermia translates to reduced off-target toxicity

compared to other intravenously injected therapies (e.g., chemotherapy). Biologics and many small molecule drugs rely on inhibition of a particular molecular pathway, which limits the patient population who will respond to such therapy and can even lead to development of resistant cancer cells that have learned to rely on alternative survival pathways [9,38]. In contrast, nanoparticle-mediated PTT offers broader applicability, including difficult-to-treat subtypes such as triple negative breast cancer which lacks estrogen, progesterone, and Her2 receptors exploited for targeted therapy. Development of imagable, targeted nanoparticles may even allow for localized PTT at sites of macrometastases. Perhaps most promising are the initial findings that nanoparticle-mediated hyperthermia induces a systemic cancer-specific immune response in which cytotoxic T-cells are trained to recognize and inhibit distal cancer cells [36], a response that may be strengthened by coupling with immunotherapy [32,39], suggesting the potential to inhibit already formed micro- and macrometastases in human patients.

Extensive research into the effects of photothermal therapy on CSCs will become critical as basic research and clinical studies suggest its feasibility in human patients with local and metastatic cancers. PTT was first realized into a commercial therapy applicable to cancer patients through AuroLase Therapy, which uses silica-cored gold nanoshells. Clinical trials began in patients with head and neck cancer and more recently expanded to patients with primary and/or metastatic lung tumors. In *Science Translational Medicine* Atkinson et al. conducted an elaborate study using patient-derived xenografts, revealing that mild hyperthermia (42 °C) via AuroLase therapy can sensitize MET ALDH+ BCSCs to ionizing radiation (IR). They found PTT impairs their ability to repair double-stranded breaks in DNA caused by IR, which was suggested to be the result of changes in heat shock protein expression caused by PTT [18]. However, it remains unclear whether differences exist in the sensitivities of MET and EMT BCSCs to PTT alone.

Previous studies examining the effects of nanoparticle-mediated hyperthermia alone on CSCs have been conducted using models that are more difficult to interpret whether the results would translate to human cancers: using an Ecadherin knockdown model to examine CSCs separately rather than grown in the presence of differentiated cancer cells [40], exposing cells to nanoparticles *in vitro* before injecting them into mice for *in vivo* hyperthermia [26], or evaluating tumor growth on cells that received nanoparticle-mediated hyperthermia *in vitro* before implantation into mice [41]. There is a need for rigorous analysis using functional assays to evaluate treatment effect on stem cell properties such as self-renewal and tumor initiating frequency of treated tumors. Further investigation, particularly using translational *in vivo* models, is required to better characterize the effect of photothermal therapy on BCSCs and, consequently, to inhibit cancer metastasis to ultimately improve survival.

Photothermal therapy may be a prime candidate for inclusion in cancer therapy that is effective in the two essential roles to eliminate both MET and EMT BCSCs as well as differentiated cancer cells, both locally and systemically. Here we sought to establish the efficacy of PTT in this first role, laying the foundation for future investigations into its systemic potential. We conduct a rigorous analysis of the local effects of PTT, providing rationale for incorporating it into standard breast cancer therapy – to inhibit BCSCs at the

primary tumor site to prevent future metastasis. As such, we sought to discern the sensitivities of both MET and EMT BCSCs to various clinically relevant PTT conditions. Our lab has previously demonstrated the ability to treat mice bearing orthotopic tumors derived from human breast cancer cells via photothermal therapy mediated by biodegradable highly crystallized iron oxide nanoparticles (HCIONPs). In contrast to most PTT mediators, the HCIONPs produced by our lab are capable of magnetic resonance imaging (MRI) and efficient intratumoral accumulation following tail vein injection [42]. Here we conduct translational studies to evaluate the effect of PTT via HCIONPs on BCSCs in order to inhibit the spread of metastasis from the primary tumor site. Our work focuses on triple negative breast cancer (TNBC), which is generally more aggressive and metastatic than other subtypes. We reveal *in vitro* that PTT eliminates MET BCSCs with the greatest sensitivity and identify conditions in which EMT BCSCs are also eliminated preferentially to differentiated cancer cells. For the first time, we show that PTT inhibits breast cancer stem cell self-renewal *in vitro*. Through secondary implantation in immune-compromised NOD/SCID mice we demonstrate the ability of *in vivo* PTT to inhibit BCSCs in triple negative breast tumors. Furthermore, we show *in vivo* that employing PTT to inhibit BCSCs before standard surgical treatment can reduce metastasis formation in TNBC. Finally, PTT effectively eliminates ALDH+ 4T1 cells *in vitro* and in xenograft tumors in immune-competent BALB/c mice to reduce lung metastasis.

2. Materials and methods

2.1. Preparation of HCIONPs

Polymer-coated HCIONPs were prepared as previously described by our lab. Briefly, HCIONPs (~15 nm diameter) were synthesized in organic solvent by thermal decomposition [42]. Single core nanocrystals were rendered soluble in aqueous solution after coating with diblock copolymer poly(ethylene oxide)-b-poly(γ -methacryloxypropyl trimethoxysilane) (PEO-b-P γ MPS), which is synthesized by reversible addition fragmentation chain transfer (RAFT) polymerization, and purifying by a magnetic separator (Frantz laboratory) [42,43]. The purified, polymer-coated HCIONPs were viewed by transmission electron microscopy (TEM) (JEOL JEM-1400Plus 120 kV). Hydrodynamic size and zeta potential were determined by dynamic light scattering (DLS) on a Zetasizer Nano S90 (Malvern). The HCIONP iron concentration was determined using o-phenanthroline as previously described [42]. HCIONPs were diluted in phosphate buffered saline (PBS) for *in vitro* and *in vivo* studies.

2.2. Photothermal effect of HCIONPs in vitro

For all *in vitro* studies, HCIONPs were suspended in cell culture medium (0.1 mg Fe/mL, 200 μ L/well) on a 96-well plate resting on a hot plate set to 37 °C. Solutions were heated by a NIR laser (885 nm, spot size 5 $\times$ 8 mm², MDL-III-885, OPTO Engine LLC, Midvale, UT) at various powers for 10–20 min and the temperature recorded using an infrared camera (FLIR Systems, i7, Boston, MA). For SUM159 studies, laser powers were identified to produce heating curves achieving final temperatures of 42, 45, and 48 °C (0.34, 0.42, and 0.49 W, respectively).

2.3. Cell culture

SUM159 and MCF7 human breast cancer cells and 4T1 murine breast cancer cells were grown as adherent monolayers incubated at 37 °C with 5% CO₂. For SUM159 cell culture, Ham's F12 medium was supplemented with 5% fetal bovine serum, 1% antibiotic-antimycotic, 5 µg/mL insulin, 1 µg/mL hydrocortisone, and 4 µg/mL gentamicin. Eagle's Minimum Essential Medium (EMEM) was supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic, and 10 µg/mL insulin for MCF7 culture. 4T1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic.

2.4. Alamar blue cell viability studies

SUM159 cells were seeded at 1000 cells/well in 200 µL on a 96-well plate and were treated the following day. After returning to the incubator for 72 h, 20 µL of alamarBlue reagent (AbD Serotec, Raleigh, NC) were added to each well and the fluorescence was measured 4 h later (540/25 nm excitation, 590/20 nm emission, 570 nm mirror) using a BioTek microplate reader (Synergy 2).

2.5. Flow cytometry

Given the 5 × 8 mm² laser spot size, suspensions of cells (500,000/well) were treated in a 96-well plate. After PTT the cells were collected, added to 25 cm² flasks, and allowed to recover overnight. The following day cells were collected and counted using Trypan Blue. For SUM159 studies, cells were dividing into separate tubes to stain for ALDH⁺ CSCs and CD44⁺/CD24[−]/EpCAM⁺ CSCs. The Aldefluor Assay (StemCell Technologies, Durham, NC) was conducted according to the manufacturer's instructions. Antibodies used were CD44-APC, CD24-FITC, EpCAM-PE-Cy7 (BD), which incubated for 30 min on ice with cells in HBSS + 2% FBS. DAPI was used for cell viability. For analysis of *in vivo* PTT in 4T1 tumors, mice were humanely euthanized two days after PTT (Day 4). 4T1 tumors were harvested and digested into single cells using a gentleMACS Octo Dissociator (MACS Miltenyi Biotec, San Diego, CA) according to the manufacturer's protocol, before staining with Aldefluor reagent and DAPI. All samples were run on a FACSCanto II (BD) to collect 10,000 live cells, and analyzed using Flowing Software 2.5.1 (Perttu Terho, Turko Center for Biotechnology, University of Turko, Finland).

2.6. Mammosphere assay

MCF7 mammosphere culture was conducted in Mammary Epithelial Basal Medium (MEBM) supplemented with 1% antibiotic-antimycotic, 5 µg/mL insulin, 1 µg/mL hydrocortisone, and 4 g/mL gentamicin, 0.02 µg/mL epidermal growth factor (EGF), 0.02 µg/mL basic fibroblast growth factor (bFGF), B27 (Invitrogen), 1:25,000,000 β-mercaptoethanol [44]. For PTT, given the 5 × 8 mm² laser spot size, suspensions of MCF7 cells (100,000/well) were heated in an ultralow adhesion 96-well plate. To achieve final temperatures of 42, 45, and 48 °C on the ultralow plate required laser powers, designated P1-P3, of 0.37, 0.52, and 0.66 W, respectively. After PTT the cells were collected and added to an ultralow adhesion 6-well plate at a density of 6000 cells in 2 mL mammosphere medium per well. Primary spheres were allowed to form for seven days and then were

counted and imaged using an inverted microscope (Nikon TE 2000S). The medium from each well was then passed through a 40 μ M strainer to collect the mammospheres, which were dissociated into single cells. The single cells were counted and re-plated on a fresh ultralow adhesion 6-well plate (Non-Treated, 42 °C PTT, and 45 °C PTT treatment groups) or 96-well plate (48 °C PTT treatment group). Secondary mammospheres were allowed to form for seven days before counting and imaging.

2.7. Mouse models for PTT

All mouse studies were conducted in accordance with a standard animal protocol approved by the University of Michigan University Committee on the Use and Care of Animals. Five week old NOD/SCID (non-obese diabetic/severe combined immunodeficient) immune-compromised or BALB/c immune-competent mice were obtained from Harlan laboratories. Primary xenograft tumors were formed in the no 4 inguinal mammary pad by orthotopic injection of 0.5×10^6 SUM159 cells or 0.5×10^4 4T1 cells suspended in matrigel. Upon the detection of palpable tumors, tumor volumes were calculated according to the following equation: volume = $0.5 \times \text{length} \times (\text{width})^2$. Mice were divided into treatment groups (n = 5) once tumor volumes reached $\sim 100 \text{ mm}^3$ (NOD/SCID SUM159 tumors) or $\sim 55 \text{ mm}^3$ (BALB/c 4T1 tumors). PTT for all *in vivo* studies was conducted as previously described [42]. Briefly, mice were intravenously injected with HClONPs by tail vein (20 mg Fe per kg mouse body weight). PTT was conducted under anesthesia 24 or 48 h later. Tumor surface temperatures were continuously monitored using an infrared camera (FLIR Systems, i7, Boston, MA).

2.8. Secondary implantation studies

Primary NOD/SCID mice were subjected to ten minute PTT using laser powers of 1.0 W or 0.5 W (2.5 W/cm² or 1.25 W/cm² irradiance, respectively). Mice were humanely euthanized 72 h after PTT, and tumors were harvested and digested using a gentleMACS Octo Dissociator (MACS Miltenyi Biotec, San Diego, CA) according to the manufacturer's protocol. Single tumor cells were counted, stained with DAPI to isolate viable cells and H2K[d]-APC to discriminate mouse cells, and sorted on a FACS Aria III (BD). DAPI-/H2K[d]-cells were pooled from each tumor per treatment group and injected double-sided into the no 4 mammary fat pads of secondary NOD/SCID mice (five weeks, n = 4) in limiting dilutions (5,000, 500, 50 cells). Secondary mice in each treatment group were sacrificed together according to total tumor burden per mouse.

2.9. Metastasis studies

For detection of lymph node metastasis in NOD/SCID mice, Luciferase-expressing SUM159 cells [9] were used for xenograft tumor formation in the no 4 inguinal mammary pad as described above. Mice were randomly divided into treatment groups (n = 8) once tumor volumes reached $\sim 75 \text{ mm}^3$. Mice were imaged using an IVIS 200 and Living Image software (PerkinElmer, Waltham, MA) one day following PTT (0.5 W). Ten days following PTT (tumors <1 cm diameter), tumor resections were performed using a high temperature cauterizer (Bovie Change-A-Tip, Clearwater, FL). Mice were monitored weekly by IVIS for metastasis formation following removal of surgical wound clips, and sacrificed at six weeks post-PTT due to tumor burden. Results of IVIS bioluminescence images were confirmed via

necropsy and Pan-cytokeratin (AE1/AE3) staining of metastatic tissues following fixation in formalin and embedding in paraffin. For detection of lung metastasis in BALB/c mice, 1.0×10^6 4T1 cells suspended in 100 μ L PBS were injected subcutaneously into the flank. Mice were randomly divided into treatment groups ($n = 5$) once tumor volumes reached $\sim 50 \text{ mm}^3$. Mice were sacrificed 18 days following PTT (0.5 W, 10 min) at 24 and 48 h post-HCIONP injection. Lungs were harvested and stained with India ink to quantify metastatic nodules.

2.10. Statistical analysis

All data are represented as mean $\pm$ standard deviation. The significance values of data sets with more than two groups were calculated using Minitab software via one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc comparisons to the non-treated control, and proportions were compared via the Chi-square test [45]. Statistical analysis of limiting dilution transplantation data was conducted using Extreme Limiting Dilution Analysis (ELDA) software as described by Hu and Smith [18,46]. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. HCIONPs enable selective hyperthermia of cell culture medium under near-infrared laser light

Our lab previously demonstrated the ability to use biodegradable, highly crystallized iron oxide nanoparticles (HCIONPs) coated with antifouling polymer for photothermal therapy *in vivo* [42]. The intravenously injected HCIONPs effectively accumulate in breast cancer tumors via the enhanced permeation and retention (EPR) effect [42,47,48], and their highly crystallized structure enables effective tumor heating upon stimulation by near infrared (NIR) light. Representative size and zeta potential measurements reveal HCIONPs less than 20 nm in diameter with a slightly negative charge (-14.5 mV), as shown in Fig. 1A and B, obtained through transmission electron microscopy (TEM) and dynamic light scattering (DLS).

Here we compared heating curves on 96-well plates containing cell culture medium spiked with HCIONPs or PBS (Laser Only control). We hypothesized that conducting PTT at increasing laser powers for our *in vitro* studies would allow us to determine whether a difference exists in the sensitivities of BCSCs and differentiated cancer cells. As shown in Fig. 1C, laser powers less than 0.5 W, designated P1, P2, and P3, are sufficient to trigger hyperthermia up to 42, 45, and 48 $^{\circ}\text{C}$ after 10 min of laser irradiation in medium containing HCIONPs. In contrast, the same laser powers result in minimal temperature increases in control media without HCIONPs.

3.2. PTT by HCIONPs inhibits cell viability of triple negative breast cancer cells

The majority of our studies were conducted using the human SUM159 breast cancer cell line, which is triple negative and has quantifiable populations of both ALDH⁺ and CD44⁺/CD24⁻/EpCAM⁺ BCSCs [49]. For our first *in vitro* study we investigated the effect of HCIONP-mediated photothermal therapy (10 min) at P1, P2, and P3 laser powers on SUM159 cell viability after 72 h, as shown in Fig. 1D. Control treatments tested include

Laser Only (P1, P2, P3), HCIONPs Only, and Non-Treated. While cell viabilities after Laser Only and HCIONPs Only control treatments are not statistically different from the Non-Treated group, a dose-dependent decrease is observed for PTT-treated cells irradiated at increasing laser powers in the presence of HCIONPs. Significant decreases in viability are observed after irradiation at power levels P1 and P2, but some cells are able to survive and proliferate within 72 h. This capacity is abrogated by PTT at P3, for which the relative number of viable cells is 4.7% of the Non-Treated control.

3.3. MET ALDH+ BCSCs are preferentially sensitive to PTT

Next we performed flow cytometry on PTT-treated SUM159 cells to examine the relative sensitivities of the ALDH+ epithelial-like (MET) BCSCs and CD44+/CD24-/EpCAM+ mesenchymal-like (EMT) BCSCs to each other and the bulk cancer cells. Cells were counted and bulk viability determined 24 h following PTT, prior to quantification of each BCSC population by flow cytometry. As shown in Fig. 2A, a dose-dependent decrease in the ALDH+ MET BCSC percentage occurs for 10 min PTT at increasing laser powers. PTT significantly decreases the percentage of ALDH+ BCSCs using the P2 laser power while the P3 power is required to significantly decrease cell viability (Fig. 2C), suggesting an increased sensitivity compared to bulk cancer cells. The significant decrease in CD44+/CD24-/EpCAM+ EMT BCSC absolute number, but not percentage, at the P3 laser power (Fig. 2B) suggests that the sensitivity of EMT CSCs is similar to the bulk cancer cells after 10 min PTT. Control treatments with P3 Laser Only and HCIONPs Only do not affect either BCSC population (Fig. S1). Representative flow cytometry plots of PTT-treated cells are shown in Fig. S1.

An enhancement of BCSC sensitivity to PTT relative to bulk cancer cells is more apparent after 20 min irradiation using the same laser powers. Hyperthermia at the P1 laser power reveals that a significant decrease in the percentage of ALDH+ MET CSCs (Fig. 2D) occurs without a significant decrease in the overall cell viability (Fig. 2F). Increasing the irradiation time to 20 min also enhances the sensitivity of EMT CSCs, for which a significant difference in percentage and absolute number is observed after P2 PTT compared to the Non-Treated group (Fig. 2E).

3.4. Mammosphere formation reveals PTT inhibits BCSC self-renewal

We next employed the mammosphere assay, the classic *in vitro* functional BCSC assay, to evaluate the effect of HCIONP-mediated PTT on BCSC self-renewal. Treatment is performed in non-adherent conditions in medium lacking serum, and stem cells that survive form three-dimensional mammospheres. These primary spheres are then passaged and replated, without further treatment, to form secondary mammospheres. A reduction in secondary mammosphere formation suggests inhibition of CSC self-renewal.

Here, for the first time, the effect of PTT on BCSC self-renewal is studied based on the formation of secondary mammospheres. Seven days following PTT (10 min) of single MCF7 breast cancer cells, primary mammospheres were quantified and imaged. As shown in Fig. 3A, PTT treatment with both HCIONPs and laser irradiation results in a significant decrease in primary mammosphere formation, which occurs in a dose-dependent manner

with increasing laser power. Furthermore, increases in laser power reduce mammosphere size in addition to mammosphere number, as shown in Fig. 3B. Control treatments with P3 Laser Only and HClONPs Only do not affect mammosphere formation or size (Fig. 3E and F). Following counting and imaging, the primary mammospheres were collected, dissociated into single cells, and re-plated to quantify CSC self-renewal to form secondary mammospheres over seven days. As shown in Fig. 3C and D, secondary spheres are significantly reduced in number and size in the P1 PTT treatment group. Most secondary spheres from the P2 PTT treatment group just met the size threshold for counting (45 μ M). In the P3 PTT treatment group, of the few primary spheres large enough to be collected and re-plated as single cells their ability to form secondary spheres is completely abrogated.

Although the MCF7 breast cancer cell line is one of the most widely used for the mammosphere assay due to an acceptable sphere-forming frequency of non-treated cells, inhibition of CSC self-renewal in TNBC cells is highly desired. Therefore, we further evaluated the effect of PTT on mammosphere formation using the triple negative SUM149 cell line. Our data revealed a similar trend in inhibition of primary mammosphere formation and size reduction following PTT (Figs. S2A and S2B). It is worth noting that primary mammosphere inhibition at the highest laser power (P3) left insufficient quantities to passage and evaluate the effect on secondary mammosphere formation. At the P1 and P2 laser powers, similar decreases in secondary mammosphere formation were observed (Figs. S2C and S2D); however, our data suggested that BCSC inhibition may be very sensitive to slight differences in the temperature profiles generated by PTT.

3.5. PTT delays BCSC-driven tumor formation after secondary implantation

The functional assay for inhibition of CSCs *in vivo* is secondary implantation, which assesses the degree to which treatment affects the CSCs' ability to self-renew and differentiate to drive tumor formation in immunodeficient mice. Treated tumors are removed from primary mice and isolated as single cells for implantation at limiting dilutions into non-treated secondary mice, to calculate a tumor initiating cell frequency [2,7,9]. Here we performed PTT in mice bearing orthotopic SUM159 tumors as previously described by our lab [42] using immunodeficient NOD/SCID mice. Tumors of primary mice were subjected to 1.0 W and 0.5 W PTT (10 min), resulting in final tumor temperatures of 60 ± 5 °C and 42 ± 2 °C, respectively. Mice were sacrificed 72 h after PTT to remove tumors for double-sided secondary implantation at dilutions of 50, 500, and 5000 cells per injection per treatment group.

Secondary tumor growth curves up to 50 days post-implantation of 5000 and 500 cells are shown in Fig. 4A and B, respectively. This data and the images in Fig. 4C clearly show that both 1.0 W and 0.5 W PTT delay secondary tumor formation and result in smaller tumor sizes compared to secondary mice who received cells from Non-Treated mice. As shown in Fig. 4D, extreme limiting dilution analysis [46] of secondary tumor formation for all three dilutions reveals a significant difference among treatment groups at Day 40 ($p < 0.01$) and Day 50 ($p < 0.001$). However, optimization of PTT conditions (e.g., increasing irradiation time) may be required to enhance the effect on ultimate tumor formation and tumor initiating cell frequency.

3.6. PTT reduces metastasis formation in axillary lymph nodes

Advancement to a metastatic disease is the primary cause of death in breast cancer patients, at which point treatment becomes palliative [15,51,52]. As such, following a thorough demonstration of HCIONP-mediated PTT efficacy in classic BCSCs studies *in vitro* and in immune-compromised mice, we conducted initial studies in both metastatic and immune-competent mouse models to suggest the translational potential of PTT in human patients. Based on our observations that SUM159 cells can metastasize to axillary lymph nodes, in our next study we sought to demonstrate that hyperthermia by PTT can be used to perform the previously described first essential role of cancer therapy – to act locally against all populations of cancer cells to prevent future metastasis. Wanting to incorporate PTT into a clinically relevant model with minimized off-target toxicity, we hypothesized that inhibition of BCSCs by PTT would prevent metastasis from spreading from the primary tumor when applied before surgery. To concentrate on the effects against metastasis strictly caused by PTT-mediated hyperthermia, immune-compromised NOD/SCID mice were inoculated with Luciferase-transfected SUM159 cells and monitored via an *in vivo* imaging system (IVIS) to track primary tumor and metastatic development after treatment. Mice received either PTT (0.5 W, 10 min) ten days prior to surgical tumor resection (PTT + SR), or surgery without PTT (SR Only). As denoted in the bioluminescence imaging scheme in Fig. 5A, mice in both treatment groups (n = 8) were imaged immediately following PTT (Fig. 5B) and weekly following removal of surgical wound clips. Despite our best efforts to remove all tumor tissue during surgery, initial imaging following surgery revealed virtually all tumors growing back in both groups (Fig. S3). Metastasis formation was tracked by IVIS for six weeks following PTT and confirmed via necropsy and staining for AE-1/AE-3 to detect the presence of human SUM159 cells (Fig. S4) [53]. As shown in Fig. 5B–D, PTT before surgery significantly reduces the proportion of mice that ultimately develop metastases ($p < 0.05$). The incidence of lymph node metastasis is 25% in mice that received PTT versus 62.5%, with the majority of these mice containing additional metastatic growths (1/2 in PTT + SR, 4/5 in SR Only).

3.7. PTT inhibits MET ALDH+ BCSCs and lung metastasis in immune-competent mice

For our final study we sought to demonstrate HCIONP-mediated PTT efficacy in a translational immune-competent metastatic breast cancer model to lay a foundation for future studies of PTT to induce a systemic anticancer immune response. We selected the 4T1 mouse cell line for *in vitro* and *in vivo* studies, as it has been used as a model for stage IV metastatic triple negative breast cancer and has a quantifiable ALDH+ BCSC population [54–56]. We first evaluated the ability of PTT to eliminate ALDH+ BCSCs *in vitro* by flow cytometry. As before, 4T1 cells were subjected to PTT (20 min) in a 96-well plate and compared to Laser Only and HCIONP Only control treatments. Using slightly larger HCIONPs (18 nm vs 15 nm) resulted in more efficient heating curves. As shown in Fig. S5A medium containing cells exposed to HCIONPs prior to 0.24 W laser treatment reaches a temperature of ~46.5 °C within 10 min, at which point the temperature has essentially plateaued (~47 °C at 20 min). In contrast, a minimal temperature increase from ~31 to ~35 °C is observed in the medium containing cells in the Laser Only treatment group. As before, 24 h following PTT cells were counted and bulk viability determined prior to BCSC quantification by flow cytometry. While Laser Only and HCIONP Only control treatments

do not significantly affect 4T1 cell viability, the relative viability of PTT-treated cells drops to 26% ($p < 0.001$) of the Non-Treated control (Fig. 6A). As shown in Fig. 6B only in the PTT-treated group does the ALDH⁺ cell population significantly decrease ($p < 0.001$), 3-fold from ~2% in the Non-Treated control. The treatment effect is more apparent considering the absolute BCSC number (Fig. 6B), accounting for the dramatic decrease in overall cell survival.

The 4T1 cell line is syngeneic in BALB/c immune-competent mice and spontaneously metastasizes to the lungs and other organs [57,58]. To analyze HCIONP-mediated PTT inhibition of ALDH⁺ BCSCs *in vivo*, orthotopic 4T1 tumors were grown in the inguinal mammary fat pad of BALB/c mice. Mice with 4T1 tumors (~55 mm³) were administered HCIONPs by tail vein (20 mg Fe/kg body weight) 24 h before 20 min laser application at a power of 0.52 W. As shown in Fig. S5B, this resulted in a rapid increase in tumor surface temperature to ~55 °C within five minutes. Mice were sacrificed 48 h after PTT and tumors were digested for Aldefluor staining of single cells. Analysis by flow cytometry in Fig. 6C reveals an average population of ALDH⁺ cells in the Non-Treated group of ~1.6%, consistent with our *in vitro* results. As expected, PTT significantly decreased the ALDH⁺ cells 3-fold to ~0.5% ($p < 0.001$). Finally, we investigated whether PTT can reduce lung metastasis in immune-competent mice bearing 4T1 tumors. Mice were treated with 10 min PTT (0.5 W) at 24 and 48 h post-HCIONP injection and sacrificed 18 days after PTT. The lungs were harvested and stained with India ink to quantify metastatic nodules (Fig. S5). As shown in Fig. 6D, PTT significantly reduced the average number of lung metastases to 13 nodules compared to 42 nodules in the Non-Treated mice. These results in combination with recent efforts to develop CSC vaccines specifically targeting ALDH⁺ BCSCs [59–61] highlight the importance of future studies to investigate the potential of PTT of primary tumors to trigger T-cell inhibition of not only differentiated cancer cells at distal sites [32,36,39] but also BCSCs present there.

4. Discussion

Here we conduct translational studies to rigorously analyze the ability of PTT via highly crystallized iron oxide nanoparticles to inhibit two breast cancer stem cell populations (ALDH⁺, CD44⁺/CD24[−]/EpCAM⁺) in TNBC *in vitro* and *in vivo*, to inhibit metastasis from primary tumors when used alone or in combination with surgery. The use of HCIONPs for PTT via NIR laser light is beneficial for clinical translation for multiple reasons. Compared to the AuroLase gold nanoshells that are 150 nm in diameter, the smaller size of our stealthy HCIONPs facilitates efficient tumor accumulation after intravenous injection due to the EPR effect [62,63]. However, all nanoparticles will undergo significant uptake in off-target organs due to the reticuloendothelial system (RES) or mononuclear phagocyte system (MPS) [63–65], suggesting another advantage of the biodegradable HCIONPs [42] over biocompatible gold-based nanoparticles [18,66,67]. While iron oxide nanoparticles (IONPs) have been more often investigated for cancer hyperthermia by employing an alternating magnetic field, the translation of this approach to humans is limited. The inability to localize this therapy to the tumor tissue generally requires the particles be intratumorally injected [36,37] to avoid off-target heating in other organs where IONPs accumulate after intravenous injection.

Here we show that HCIONPs enable selective heating of cell culture medium in response to near infrared (885 nm) laser irradiation, which can be tuned to precisely control PTT temperatures to kill cancer cells. A dose-dependent inhibition of SUM159 cell viability is observed with increasing PTT laser power (P1 – P3) 72 h after treatment. PTT at P3 reduces the viability to 4.7% of the Non-Treated control, suggesting that HCIONP-mediated hyperthermia for only 10 min can eliminate proliferation of triple negative breast cancer cells *in vitro*. This is not surprising considering the findings by Burke et al., who compared carbon nanotube-mediated PTT and conventional hyperthermia by water bath. Investigating two settings for rate of temperature increase (ROTI) by water bath on a suspension of breast cancer stem-like cells (HMLER^{shEcaderin}), they showed that hyperthermia induced by NIR-responsive nanoparticles enhances cytotoxicity compared to water bath-mediated hyperthermia at equivalent exposure times and temperatures [40]. While rapid heat transfer from PTT causes the overall solution temperature to become mildly hyperthermic [68], the cytotoxic effect will be amplified by the presence of HCIONPs in close proximity to the cells as the heat transfer is significantly greater at the nanoparticle surface [69].

Next we sought to evaluate the effect of HCIONP-mediated PTT on the distinct epithelial-like and mesenchymal-like BCSCs [12]. Decreases in the percentages of SUM159 ALDH+ MET BCSCs after 10 and 20 min PTT at increasing laser powers (P1-P3) reveals an increased sensitivity of this highly proliferative BCSC population to HCIONP-mediated hyperthermia compared to the bulk cell line. While Atkinson et al. demonstrated that gold nanoshell-mediated PTT and ionizing radiation together reduce the percentage of ALDH+ BCSCs in patient-derived xenografts [18], this is the first report that PTT alone can reduce ALDH+ CSCs in human breast cancer cells. When we examined the CD44+/CD24–/ EpCAM+ EMT BCSCs, we found their number decreases in proportion to bulk cancer cells after 10 min PTT. A significant decrease in EMT BCSC percentage is observed at the P2 laser power after 20 min PTT; this suggests preferential elimination of both BCSC populations is possible within breast tumors with optimization of PTT conditions, especially considering recent findings that EMT BCSCs are more localized at the invasive edge [12] where the nanoparticles will easily accumulate within the tumor tissue. Hyperthermia has been known to trigger several cellular and molecular changes, including impairment of protein and DNA/RNA synthesis, protein denaturation and aggregation, inhibition of repair enzymes, and induction of heat shock protein synthesis [30,70]. Due to their highly proliferative nature, as determined by elevated ki-67 expression [7,12], it is conceivable that the enhanced sensitivity of the ALDH+ BCSCs to hyperthermia is due to damaged proteins necessary to meet high metabolic demands and to coordinate DNA replication and repair. Further investigations into the response of heat shock proteins within each breast cancer cell subtype may also provide insight into differences in sensitivity to nanoparticle-mediated hyperthermia [18].

Previous studies of nanoparticle-mediated hyperthermia have yet to examine the treatment's effect on CSC self-renewal by evaluating mammosphere formation beyond the primary generation. In the assay adapted by Dontu and colleagues [71,72], single cells are plated at clonal densities in non-adherent conditions in medium containing growth factors but lacking serum. The result is massive anoikis. The surviving cells grow in three-dimensional colonies, termed mammospheres, that are enriched in stem cells. First studied using human

mammary epithelial cells, the cells from these colonies are able to differentiate into all three types of mammary epithelial lineages and able to self-renew to form mammospheres after serial passaging. To suggest inhibition of BCSC self-renewal, treatment applied to the primary generation of sphere-forming cells must result in a decrease in mammosphere formation in subsequent generations.

By conducting PTT at increasing laser powers, we show that PTT reduces both the number and size of primary mammospheres formed using both MCF7 and SUM149 breast cancer cell lines. This observation is most apparent after heating at P3, which after seven days resulted in few primary mammospheres large enough to collect and passage for the second generation. For the first time we demonstrate the ability of PTT to inhibit secondary mammosphere formation. Consistent with the proliferation study, P3 PTT treatment is the most drastic; it completely abrogates self-renewal to form secondary spheres.

Removal of treated primary tumor cells for secondary implantation at limiting dilutions into immune-compromised mice has become the gold standard for *in vivo* efficacy of treatment on CSCs [73,74]. Here we conduct the first study of secondary implantation from mice solely treated with nanoparticle-mediated hyperthermia, using NOD/SCID mice bearing SUM159 tumors subjected to 10 min PTT. While significant decreases are observed in secondary tumor volumes and tumor formation rates at 40 and 50 days post-implantation of PTT-treated primary tumor cells, ultimate secondary tumor formation rates may have been affected by a very small population of residual progenitor or cancer stem cells. While PTT conditions may need to be fine-tuned (e.g., increase laser irradiation time) to obtain optimal results, it is clear the ability of BCSCs to drive tumor formation is hindered following PTT. Interestingly, in contrast to our previous study in nude mice [42], with all other variables the same except tumor size at the time of treatment (100 vs 40 mm³), 1.0 W PTT was unable to reduce tumor growth in primary NOD/SCID mice (data not shown). Despite repeating treatment in NOD/SCID mice with smaller tumors, 1.0 W PTT was unable to prevent tumor growth. These contrasting effects in secondary versus primary mice are consistent with observations made for primary tumor treatments that specifically target CSCs [75]. Taken together, this *in vivo* data suggests two important features of PTT treatment: 1) consistent with our *in vitro* data, BCSCs are more sensitive to PTT *in vivo* than differentiated cancer cells comprising the bulk of the primary tumor, and 2) PTT alone is not sufficient to eliminate the bulk of differentiated cancer cells in mice with highly compromised immune systems.

Several factors beyond application time and temperature may play a role in the degree of response observed after PTT treatment of primary tumors. For instance, the ability of the photo-responsive nanoparticles to extravasate deep into tumor tissue will affect the heating distribution by the laser light. Likewise, in terms of clinical translation, the depth of laser light penetration will dictate the efficiency to heat various types of tumors depending on their location within the human body. In our current study, the variable that differed from our previous study, in which significant tumor reduction was observed following PTT in nude mice, was the immune functionality of the mice. Our data combined suggest that the immune function of the mice plays a role in the ability to shrink primary tumors following PTT. Others have recently reported that in immune-competent mice proinflammatory

cytokines and chemokines released by dying cells after nanoparticle-mediated hyperthermia trigger a tumor-specific immune response [36,39], suggesting the importance of mouse model selection given the purpose of the intended study. While future studies of photothermal cancer therapy alone may be more efficacious in immunocompetent mice, and in fact may be enhanced if coupled with immunotherapy [32,39], immune-compromised NOD/SCID mice make it possible to discern the effect strictly from hyperthermia on both stem-like and differentiated cancer cells.

The advancement to a metastatic disease, which eventually occurs for many women diagnosed with early stage breast cancer, is the primary cause of death in women with breast cancer [15,51]. While patients diagnosed with metastatic breast cancer are currently considered incurable, palliative treatment options exist [15,51,52]. For breast cancer patients with less advanced disease, first-line therapy generally includes surgery and depends on factors such as the type of breast cancer, stage, and grade; preceding or following surgery, patients may also receive radiation, chemotherapy, hormone/endocrine therapy, or biologics (Herceptin). Given the evidence suggesting that CSCs are the culprit responsible for metastasis and are resistant to and possibly enriched by chemotherapy and radiation [17–19,76], there is a critical need to develop first-line breast cancer therapy that is effective in two roles: eliminating all types of cancer cells, including CSCs, at the primary tumor site to prevent future metastases, and eliminating all types of cancer cells at existing metastatic sites. While extensive research is still required, initial reports that nanoparticle-mediated PTT can inhibit CSCs and induce a systemic cancer-specific immune response [32,36,39] suggest it may be a prime candidate to meet both components of this critical unmet need.

Given our demonstration of PTT inhibition of MET and EMT BCSCs, we sought to establish the potential of PTT of primary tumors to inhibit subsequent metastasis formation. To facilitate translation for human clinical trials, PTT was performed in combination with surgery which, like PTT, is localized and minimizes off-target toxicity. Metastasis development was examined in NOD/SCID mice containing Luciferase-transfected SUM159 tumor xenografts treated with PTT ten days before surgical resection. Weekly IVIS imaging up to six weeks following treatment revealed that PTT before surgery significantly reduces the percentage of mice with BCSC-driven macrometastases. This study reveals that hyperthermia by PTT can be used to perform the previously described first essential role of cancer therapy – to act locally against all populations of cancer cells to prevent development of future metastasis. However, up to 10% of patients have Stage IV or metastatic breast cancer at the time of diagnosis, and it is estimated that metastatic recurrence accounts for 20–30% of breast cancer cases [77]. Therefore, to have the greatest clinical impact, new treatments must be effective in the second essential role of cancer therapy – to act systemically against all populations of cancer cells in existing metastases.

Other recent works have begun to examine the mechanism by which PTT affects tumor growth and metastasis by evoking an immune response, without focusing on the potential role of CSC inhibition. Initial findings reveal that nanoparticle-mediated hyperthermia induces a systemic cancer-specific immune response in which cytotoxic T-cells are trained to recognize and inhibit distal cancer cells [36], a response that may be strengthened by coupling with immunotherapy [32,39]. The data collectively show that PTT at the primary

tumor site triggers a release of inflammatory cytokines and chemokines, such as those seen in burn patients, that attract antigen presenting cells (APCs) such as dendritic cells (DCs) to the tumor site. This results in activated DCs in tumor draining lymph nodes that present to CD8⁺ cytotoxic T-cells, which are drawn to the tumor site by the released chemokines. Together, the results of these studies and recent studies to develop CSC-specific vaccines [59–61] highlight the importance to conduct future studies to investigate the potential of PTT as a platform to induce death of differentiated cancer cells and CSCs in primary tumors, and to subsequently trigger a T-cell-mediated immune response to selectively eliminate both these populations present at metastatic sites.

For our final study, we sought to establish conditions in which localized PTT mediated by biodegradable iron oxide nanoparticles effectively eliminates BCSCs in an immune-competent mouse model of metastatic breast cancer, laying the groundwork for future studies to demonstrate that local PTT inhibition of BCSCs will evoke a cancer/CSC-specific immune response targeting those cells that have migrated to distal sites. We selected the 4T1 mouse cell line for *in vitro* and *in vivo* studies, as it has been used as a model for stage IV metastatic triple negative breast cancer and has a quantifiable ALDH⁺ BCSC population [54–56]. Both bulk cancer cells and the ALDH⁺ BCSC population were successfully inhibited following 20 min heating to 46 °C via HClONP-mediated PTT. In BALB/c mice bearing 4T1 tumors, ALDH⁺ BCSCs were significantly inhibited 48 h after PTT heating to ~55 °C. With little research to validate CD44⁺/CD24[–] as a marker for 4T1 (mesenchymal-like) BCSCs [78–81], the ability to analyze the effect of PTT on both ALDH⁺ epithelial-like BCSCs and CD44⁺/CD24[–]mesenchymal-like BCSCs would be ideal for future studies. Finally, we showed that PTT of 4T1 tumors significantly reduces BCSC-driven formation of lung metastasis 20 days post-PTT compared to Non-Treated mice.

Considering the results presented here and the findings by Atkinson and colleagues, incorporating nanoparticle-mediated PTT into routine breast cancer therapy such as surgery may improve long-term patient outcomes by avoiding sparing or enriching the CSC population. The preliminary data presented here lays the groundwork for future studies in immune-competent mice to demonstrate the potential to combine PTT with clinically used therapies to inhibit not only BCSCs located at the primary tumor site but those at distal sites by evoking a cancer/CSC-specific immune response. Successful development of such translational nanomedicine combining PTT with clinically used treatments such as surgery to kill distal cancer stem cells would have potential to improve survival for not only breast cancer patients with incurable metastasis but patients with other metastatic cancers as well.

5. Conclusions

In this study we evaluate the ability of photothermal therapy via highly crystallized iron oxide nanoparticles to inhibit breast cancer stem cells to inhibit metastasis following treatment of the primary tumor. Here we conduct rigorous, translational studies to evaluate the effect of PTT via HClONPs on BCSCs, including both MET and EMT BCSC populations, in triple negative breast cancer *in vitro* and *in vivo*, and apply PTT alone or in combination with surgical resection to inhibit lymph node and lung metastasis. We reveal *in vitro* that PTT eliminates MET BCSCs with the greatest sensitivity and identify conditions

in which EMT BCSCs are also eliminated preferentially to differentiated cancer cells. For the first time, we show that PTT inhibits breast cancer stem cell self-renewal *in vitro*. Through secondary implantation we demonstrate that PTT impedes BCSC-driven tumor formation. Lastly, using metastatic and immune-competent mouse models we explore the translational potential of PTT in human breast cancer patients. We show that PTT before surgery to inhibit BCSCs significantly reduces metastasis development in TNBC. Additionally, we reveal effective elimination of ALDH+ BCSCs and lung metastasis by PTT in an immune-competent metastatic mouse model of TNBC. Taken together, these results suggest the potential to combine nanoparticle-mediated photothermal therapy with current clinical treatments such as surgery to enhance CSC destruction and improve long-term survival in breast cancer patients, and also provide a viable treatment option for those with currently incurable metastatic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

(A) TEM image of polymer-coated HCIONPs. (B) DLS characterization of HCIONP hydrodynamic size and zeta potential. (C) Ten minute heating curves of SUM159 medium subjected to PTT (Laser + HCIONPs), compared to Laser Only controls. Laser powers tested were 0.34, 0.42, 0.49 W, designated P1, P2, P3, respectively. Solutions were spiked with HCIONPs (0.1 mg Fe/mL) for PTT or PBS for Laser Only control treatment. (D) SUM159 cell viability determined by alamarBlue 72 h after PTT (10 min) at P1, P2, and P3 laser powers, compared to HCIONP Only, Laser Only (P1-P3), and Non-Treated controls. Addition of alamarBlue Reagent results in a colorimetric change that is proportional to the number of metabolically active cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ via Dunnett's post-hoc comparison to Non-Treated control.

Fig. 2. SUM159 MET-like ALDH+ BCSC percentage and absolute number 24 h after 10 min (A) and 20 min (D) PTT. EMT-like CD44+/CD24-/EpCAM+ BCSC percentage and absolute number 24 h after 10 min (B) and 20 min (E) PTT. Cell viability 24 h after 10 min (C) and 20 min (F) PTT. It should be noted that after 20 min PTT at P3, the number of viable cells was insufficient for analysis by flow cytometry. For cell viabilities and percentages of BCSCs, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ via Dunnett's post-hoc comparison to Non-

Treated control. For absolute numbers of BCSCs, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ via Dunnett's post-hoc comparison to Non-Treated control.

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Fig. 3.

(A) MCF7 primary mammosphere formation seven days after PTT (10 min) at P1, P2, and P3 laser powers, grown at a density of 6000 cells/well. (B) Representative images of primary mammosphere size seven days after PTT. (C) Secondary mammosphere formation 14 days after primary PTT, normalized by number of cells plated per well. (D) Representative images of secondary mammosphere size 14 days after primary PTT. Primary spheres were collected on Day 7, digested to single cells and plated for secondary mammosphere formation. (E) Primary mammosphere formation seven days after HClONP Only and Laser

Only (P3 power, 10 min) control treatment. (F) Representative images of primary mammosphere size seven days after control treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ via Dunnett's post-hoc comparison to Non-Treated control.

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Fig. 4. Tumor growth curves for secondary mice ($n = 4$) receiving dual injections of 5000 (A) and 500 (B) treated SUM159 tumor cells from primary mice. (C) Representative images of secondary mice at Day 40 after receiving 5000 treated cells and at Day 49 after receiving 500 treated cells. (D) Tumor formation in secondary mice after receiving injections of 5,000, 500, or 50 treated SUM159 tumor cells from primary mice. Primary mice were administered HClONPs (20 mg Fe/kg body weight) intravenously 48 h prior to laser application. Secondary mice were sacrificed based on total tumor burden per mouse. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ via Extreme Limiting Dilution Analysis software as described by Hu and Smith [18,46].

Fig. 5. (A) Schematic of bioluminescence imaging of SUM159 tumor-bearing NOD/SCID mice, relative to treatment. † denotes bioluminescence imaging. ‡ denotes removal of surgical wound clips. (B) IVIS bioluminescence images of mice one day and six weeks following PTT on Day 0. To image metastases with higher sensitivity, the lower portions of all mice were covered to mask the tumor signal. (C) Percentage of mice with metastasis detectable via bioluminescence imaging over time after treatment. (D) Summary of axillary lymph node (ALN) and other metastases detected in treated mice during necropsy. * $p < 0.05$ by Chi-square test of proportions.

Fig. 6.

(A) 4T1 cell viability 24 h after 20 min PTT compared to HCIONP Only and Laser Only controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ via Dunnett's post-hoc comparison to Non-Treated control. (B) 4T1 ALDH+ BCSC percentage and absolute number 24 h after 20 min PTT. For percentages of BCSCs, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ via Dunnett's post-hoc comparison to Non-Treated control. For absolute numbers of BCSCs, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ via Dunnett's post-hoc comparison to Non-Treated control. (C) ALDH+ BCSC percentage in 4T1 tumor cells isolated from BALB/c mice 2 d after 20 min PTT. (D) Number of lung metastases formed in BALB/c mice 20 d after two treatments of 10 min PTT to 4T1 tumors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ via student's t-test.