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The Resistance of Breast Cancer Stem Cells to Conventional Hyperthermia and their Sensitivity to Nanoparticle-mediated Photothermal Therapy

Andrew R. Burke¹, Ravi N. Singh¹, David L. Carroll², James C. S. Wood^{1,6}, Ralph D'Agostino Jr.^{3,6}, P.M. Ajayan⁴, Frank M. Torti^{1,6}, and Suzy V. Torti^{5,6,*}

¹Department of Cancer Biology, Wake Forest School of Medicine, Winston Salem, NC 27157

²Department of Physics, Wake Forest University, Winston Salem, NC 27109

³Department of Biostatistical Sciences, Wake Forest School of Medicine, Winston Salem, NC 27157

⁴Department of Mechanical Engineering and Materials Science, Rice University, 6100 Main Street, Houston, Texas 77006

⁵Department of Biochemistry, Wake Forest School of Medicine, Winston Salem, NC 27157

⁶Comprehensive Cancer Center of Wake Forest School of Medicine, Winston Salem, NC 27157

Abstract

Breast tumors contain a small population of tumor initiating stem-like cells, termed breast cancer stem cells (BCSCs). These cells, which are refractory to chemotherapy and radiotherapy, are thought to persist following treatment and drive tumor recurrence. We examined whether BCSCs are similarly resistant to hyperthermic therapy, and whether nanoparticles could be used to overcome this resistance. Using a model of triple-negative breast cancer stem cells, we show that BCSCs are markedly resistant to traditional hyperthermia and become enriched in the surviving cell population following treatment. In contrast, BCSCs are sensitive to nanotube-mediated thermal treatment and lose their long-term proliferative capacity after nanotube-mediated thermal therapy. Moreover, use of this therapy in vivo promotes complete tumor regression and long-term survival of mice bearing cancer stem cell-driven breast tumors. Mechanistically, nanotube thermal therapy promotes rapid membrane permeabilization and necrosis of BCSCs. These data suggest that nanotube-mediated thermal treatment can simultaneously eliminate both the differentiated cells that constitute the bulk of a tumor and the BCSCs that drive tumor growth and recurrence.

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*To whom correspondence should be addressed: Department of Biochemistry, Wake Forest University School of Medicine, Medical Center Blvd, Winston Salem, NC 27157, Phone: 336-716-9357, Fax: 336-716-0255, storti@wakehealth.edu.

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1. Introduction

Many malignancies [1–8], including breast cancer [9, 10], are thought to be sustained by a small, slow-cycling population of transformed stem-like cells that enable key aspects of disease progression including expansion of the primary tumor [11] and generation of tumor metastasis [10, 12]. In breast cancer, these cells, variously termed cancer stem cells (CSCs) or tumor-initiating cells (TICs), are distinguished by characteristic markers, such as the cell surface antigens CD44^{high}/CD24^{low} and ALDH1 enzymatic activity [10]. CSCs are inherently refractory to standard treatment modalities such as chemotherapy [13, 14] and radiotherapy [6, 15, 16]. The current inability to ablate this critical subpopulation is thought to account for disease recurrence. Accordingly, new treatment strategies that can effectively eliminate both the CSCs and their more differentiated daughter cells that constitute the bulk of the tumor will be necessary to achieve durable treatment remissions in breast cancer patients following therapy.

Among breast cancers, those with a “triple negative” phenotype (estrogen receptor, progesterone receptor, and HER-2 negative) are the most difficult to treat. These cancers lack the targets against which current clinical therapies are directed [17, 18] and are enriched in CD44^{high}/CD24^{low} stem-like cells [19].

Heat-based cancer treatments represent a promising approach for the clinical management of treatment-resistant cancers, including breast cancer. These therapies involve the elevation of malignant tissues to supraphysiologic temperatures [20–25]. In addition to direct toxic effects on tumor cells, thermal treatments may enhance the efficacy of both radiotherapy and some chemotherapeutics [26–28]. Despite these notable benefits, widespread clinical adoption of hyperthermic therapy has been limited by toxicities resulting from diffuse heating of non-tumor tissues and the relative invasiveness of thermal ablative instrumentation [29]. To address these limitations, rapid, minimally invasive, and highly localized, nanotechnology-based thermal tumor ablation therapies are being developed (reviewed in [30]) with a variety of nanomaterials, including single walled carbon nanotubes [31], multiwalled carbon nanotubes [32], graphene [33], gold nanorods [34] and shells [35, 36].

Carbon nanotubes (CNTs) display several properties that make them promising candidates for minimally invasive thermal therapy of cancer. These include efficient antenna behavior (strong absorbance of electromagnetic radiation) and thermal conductance [37–39]. In this application, CNTs are localized to tumors and stimulated with tissue-transparent [40] near infrared radiation (NIR) or radiofrequency (RF) energy to generate localized heat [31, 41–43]. We previously demonstrated that the treatment of tumors with the combination of multiwalled carbon nanotubes (MWCNTs) and NIR results in rapid tumor regression and long-term survival in a mouse model [32]. CNT-mediated thermal therapy addresses several limitations inherent in contemporary clinical methodologies. First, the heating is confined to the intended lesion, greatly diminishing off-target toxicities. Second, each nanoparticle generates heat in response to NIR or RF stimulation, creating a more uniform temperature distribution throughout the tumor mass. Third, NIR-stimulated nanoparticles are compatible with concurrent MRI temperature mapping techniques, allowing confirmation of the treated tissue volume following therapy [32, 35]. Finally, the procedure is minimally-invasive, potentially expanding the type and location of tumors that can be treated by this method.

In this manuscript, we explored whether nanotube-mediated thermal therapy could be used to effectively ablate breast cancer stem cells *in vitro* and *in vivo*.

2. Materials and Methods

2.1. Cell culture

HMLER^{shEcadherin} breast cancer stem cells and control HMLER^{shControl} cells as well as non-tumorigenic HMLE^{shEcadherin} and HMLE^{shControl} breast cells were generously provided by Dr. Robert Weinberg (MIT) [44]. Cells were cultured in a 1:1 mixture of MEGM and DMEM supplemented with 10% FBS, insulin and hydrocortisone. SUM 159 (Asterand) cells were maintained in F12 + 5% FBS, insulin and hydrocortisone and DMEM/F12 + 10% FBS with 1% antibiotic mixture, respectively. All cells were cultured in humidified incubators maintained at 37°C with 5% CO₂.

For microscopy studies, cells were grown in 6-well plates and imaged using an Olympus IX70 inverted microscope. All image adjustments (cropping and resizing only) were performed using the Image J software package.

2.2. MWCNT suspensions

Amide-functionalized (PD15L1–5-NH₂, lot number: 60809) multiwalled carbon nanotubes (MWCNTs-NH₂) were purchased from NanoLab (Waltham, MA). MWCNTs were suspended in sterile saline with 1% (wt/wt) DSPE-PEG 5000 (Avanti Polar Lipids) through probe tip sonication (Branson). All preparations were autoclaved prior to use. MWCNT suspensions were endotoxin free (<10 EU/ml). Mean length was 591 ± 464 nm and mean diameter was 29 ± 11 nm. Additional physico-chemical characterization of this material is provided in Burke et al [45].

2.3. MTT-based viability

Cells were dispensed in 96 well plates. Wells were washed with 200 µL PBS and overlaid with 200 µL fresh media prior to initiating the assay. 50 µL of MTT reagent (5 mg/mL thiazolyl blue tetrazolium bromide in PBS) (Sigma) was added to each well and incubated at 37°C for 1–2 hrs. 3 wells that did not contain cells were similarly treated and used as blanks. After incubation, media was aspirated and formazan crystals were solubilized in 200 µL DMSO. pH was adjusted by adding 25 µL Sorensen glycine buffer (pH 10.5) per well and then well contents were mixed for 10 min on a titer plate shaker. Absorbance at 560 nm was determined using a plate reader (Molecular Devices). Absorbance at 490 nm for each treatment group were averaged then normalized to the indicated control conditions.

2.4. MTS-based viability

Sample wells in 96-well plate format containing 100 µL media were incubated with 20 µL mixed MTS + PMS reagent (Cell Titer 96 AQueous Non-radioactive Cell Proliferation Assay, Promega) according to the manufacturer's instructions. Samples were incubated at 37°C for 2–4 hrs. OD₄₉₀ values were determined use a plate reader (Molecular Devices). OD₄₉₀ values for each treatment group were averaged then normalized to the indicated control conditions.

2.5. HMLER sensitivity to cytotoxic agents

HMLER^{shControl} and HMLER^{shEcadherin} cells were plated in quintuplicate at 5,000 cells per well in a 96 well plate and allowed to adhere overnight. Paclitaxel and salinomycin were purchased from Sigma. The HSP90 inhibitor 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) was purchased from In vivo Gen. All drugs were dissolved in DMSO. Compounds were serially diluted to the indicated concentrations in normal growth media and cells were overlaid with 200 µL per well. Plates were incubated for 3 days at 37°C prior to being assessed by MTT (described above).

2.6. MWCNT heating model

MWCNTs-NH₂ were suspended in PBS + 2% FBS (FACS buffer) at a final concentration of 50 µg/mL. 500 µL aliquots of suspension were placed into wells of a 48 well cell culture plate. Wells were exposed to a 1064 nm near-infrared continuous wave fiber laser (IPG Photonics: YLR-20-1064-LP) set at 3 W for 5–45 seconds. Pre and post-exposure temperature measurements were determined by thermocouple. Change in temperature was plotted as a function of laser exposure time and a linear regression was performed to develop the heating model described by the equation $T = 0.627 t + 1.6971$.

2.7. MWCNT-mediated thermal therapy

In experiments where cells were heated by the combination of MWCNTs and laser, cell suspensions were mixed with MWCNTs to generate a final nanotube concentration of 50 µg/mL in 500 µL total sample volume. Baseline temperature measurements of each sample were then acquired by thermocouple and used to determine the change in temperature necessary to reach the desired final temperature. This value was substituted for “T” in the heating model equation and “t” was solved for to determine the laser exposure time (in seconds) necessary to heat the cell sample to the desired final temperature. As controls, samples that received MWCNTs but no laser exposure (termed “MWCNT alone”) or samples that received laser exposure but no MWCNTs (termed “laser alone”) were also generated. Following laser exposure the actual final temperature of each sample was measured by thermocouple to validate the accuracy of the model.

2.8. Water bath-mediated hyperthermia

Cells were resuspended in normal growth media at 250,000 cells in 600 µL. Triplicate samples were prepared for each cell type. Samples were then placed in a circulating water bath set between 43–53°C. At the indicated timepoints, 100 µL volumes were withdrawn from each sample and plated in a 96-well plate. After the final timepoint 100 µL of fresh media was added to each well and cells were allowed to recover overnight at 37°C. Wells were then prepared for MTT or MTS-based viability analysis as described above.

For the experiment described in Supplemental Figure 5, HMLER^{shControl} and HMLER^{shEcadherin} cells were pretreated for 1 hr at 37°C with 125 ng/mL 17-DMAG. Cells were then washed, suspended in normal growth media and treated with water bath hyperthermia at 47°C as described above.

2.9. Slow ROTI water bath hyperthermia

Cells were resuspended in normal growth media at 250,000 cells in 500 µL. Triplicate samples were prepared for each cell type using microcentrifuge tubes. Samples were then placed in a circulating water bath set between 43–53°C. Tubes were removed from the water bath 3 minutes after immersion and 100 µL volumes were withdrawn from each sample and plated in a 96-well plate. 100 µL of fresh media was added to each well and plates were allowed to recover overnight at 37°C. Wells were then prepared for MTT or MTS-based viability analysis as described above.

2.10. Rapid ROTI water bath hyperthermia

10 mL of normal growth media in 15 mL conical tubes were preheated to between 43–53°C by immersion in a temperature controlled, circulating water bath. 250,000 cells were suspended in 100 µl normal growth media and rapidly injected into the preheated media. Cells were maintained at the preset temperatures for 30 seconds followed by cooling on ice. Samples were centrifuged and cell pellets resuspended in 500 µL growth media. 100 µL volumes were withdrawn from each sample and plated in a 96-well plate. 100 µL of fresh

media was added to each well and plates were allowed to recover overnight at 37°C. Wells were then prepared for MTT or MTS-based viability analysis as described above.

2.11. Rate of temperature increase (ROTI) calculations

ROTI values for each heating method were determined by the formula: $\Delta T/\Delta s$; with units of °C per second(s).

For MWCNT-mediated thermal therapy the ROTI was given as the slope of the linear equation $T = 0.627 t + 1.6971$.

For “slow ROTI” water bath hyperthermia the initial temperature of each suspension was 23°C and the time required for each sample to reach the preset water bath temperature following immersion was empirically determined to be ~180 seconds. Thus for $T_{\text{final}} = 43^\circ$, 45° , 47° , 49° , 51° or 53°C the ROTI values ranged from 0.111–0.167°C/s.

For “Rapid ROTI” water bath hyperthermia the cell suspensions were calculated to reach the prevailing water bath temperature within ~5 milliseconds based on literature values describing the timescale of heat transfer in cells and tissues [46, 47]. Therefore, given an initial temperature of 23°C and $T_{\text{final}} = 43^\circ$, 45° , 47° , 49° , 51° or 53°C , the ROTI values ranged from 4,000–6,000°C/s.

2.12. Mammosphere formation

The method was adapted from Dontu *et al.* [48] with modifications. HMLER^{shControl} and HMLER^{shEcadherin} cells were suspended at 5,000 cells/mL in complete MammoCult media (Stem Cell Technologies) and plated in triplicate wells of a 6-well ultra-low attachment culture plate (Corning). Cells were incubated at 37°C for 7–10 days. Wells were imaged by inverted microscope and mammosphere diameters were determined using the Image J software package. At least 50 cells or cell clusters (mammospheres) were counted per condition. Single cells had diameters ranging ≈ 15 –25 μm . Mammosphere data are graphed as box plots describing the median diameters along with the 25th and 75th percentiles. Outlier values are indicated by filled circles.

In the experiment described in Figure 5 HMLER^{shEcadherin} cells were suspended at 100,000 cells in 100 μL normal growth media. Cell suspensions were heated by water bath at the indicated temperatures as described in *Materials and Methods: Rapid ROTI water bath hyperthermia*. After treatment, cells were washed and diluted in MammoCult to 5,000 cells/mL. Duplicate wells were plated for each treatment temperature. Suspensions of HMLER^{shEcadherin} cells were also treated with MWCNTs and laser to reach the indicated final temperatures as described in *Materials and Methods: MWCNT-mediated thermal therapy*. Immediately after heat treatment cell suspensions were centrifuged and washed twice with PBS to remove nanotubes. Cells were then resuspended at 5,000 cells/mL in MammoCult and plated at 2 wells per treatment group as described previously.

2.13. Animal Handling

All animal studies were performed in compliance with the institutional guidelines on animal use and welfare (Animal Care and Use Committee of Wake Forest University Health Sciences) under an approved protocol. Female *nu/nu* athymic mice were obtained from Charles River Laboratories (5–8 wks old). Mice were housed 5 per cage in standard plastic cages, provided food and water ad libitum, and maintained on a 12-h light/dark cycle.

2.14. Tumor Regression and Survival Study

For the experiment described in Figure 6, a donor tumor was prepared by subcutaneous injection of an athymic female mouse with 2×10^6 HMLER^{shEcadherin} cells suspended in 100 μ L of 1:1 Matrigel (BD Biosciences) and PBS. When the tumor reached a diameter $\sim 1,000$ mm³ it was resected and minced under sterile conditions into 30 mm³ fragments. Fragments were surgically implanted into the flanks of 50 athymic female mice and allowed to grow to ~ 150 mm³; 7–10 days.

Mice were then randomized into 3 control (Untreated, Laser Only and CNT Only) and one experimental group (CNT + Laser) with 10 animals per group. Mice in the “Laser Only” group received an intratumoral injection of 50 μ L saline with 1% DSPE-PEG. Mice in the “CNT Only” and “CNT + Laser” groups received intratumoral injections of 100 μ g MWCNTs-NH₂ suspended in sterile saline with 1% DSPE-PEG (See *Materials*). Following injection, mice in the “Laser Only” and “CNT + Laser” groups had their tumors irradiated with a 3 W/cm² 1,064 nm continuous wave NIR laser (IPG Photonics) for 30 seconds.

After treatment, changes in tumor volume for all mice were monitored every three days by digital caliper measurements. Mice were removed from the study (considered “dead”) when their tumor volumes exceeded 1,000 mm³ or were deemed moribund by veterinary consult.

2.15. Immunohistochemistry

HMLER^{shControl} and HMLER^{shEcadherin} cells were washed in PBS, resuspended in normal growth media and pelleted by centrifugation. The supernatant from each sample was aspirated and the cell pellets flash-frozen in liquid nitrogen. Pellets were harvested and formalin fixed into a paraffin embedded block. Four μ m sections were then cut and processed for ER, PR and Her-2 as performed in breast cancer tissue using a Leica Bond 3 system for ER and PR and the Dako system for Her-2. Cellular antigens were retrieved using epitope retrieval solutions obtained from the kits. The primary antibodies for ER, PR were from Leica and dilutions were 1:200 and 1:100 respectively. The Her-2 antibody was obtained from the Dako kit with no dilution. The incubations times at room temperature were 15 minutes for ER and PR and 30 minutes for Her-2. The DAB expression of specific antigens was developed using the Leica micro polymer detection reagents for ER and PR while the Her-2 expression was obtained by using the Dako polymer detection reagent and cells were counter stained with H&E. The analysis for ER and PR was defined as intense brown staining in the nucleus and HER-2 was defined based on the DAKO assay definition. All assays contained a positive control for each marker.

2.16. Antibodies

Antibodies for immunoblotting were purchased from the following vendors: H-Ras (C-20, Santa Cruz Biotechnology), E-Cadherin, HSP27 (G31), HSP70 (D69) and HSP90 (all from Cell Signaling Technologies). Antibodies for flow cytometry were as follows: PerCP-Cy5.5 mouse anti-human CD44 (C26), PerCP-Cy5.5 mouse IgG2b κ isotype (both from BD Pharmingen) and APC mouse anti-human CD24 (ML5), APC mouse IgG2a κ isotype (both from Biolegend). The apoptotic/necrotic cell detection kit was purchased from BD Biosciences with PE Annexin V and 7-AAD viability dye (559763).

2.17. Flow cytometry

HMLER^{shControl} and HMLER^{shEcadherin} cells were washed and resuspended at 1×10^6 cells in 100 μ L FACS buffer. Cells were labeled at 4°C for 30 minutes with APC CD24, PerCP-Cy5.5 CD44, both or the appropriate isotypes (see *Antibodies*) at the manufacturer’s recommended concentrations. Samples were then washed once with cold PBS and fixed

with 1× formaldehyde in PBS. Cells were analyzed on a FACS Aria (Becton Dickinson) or an Accuri C6. Data was exported and graphed using FCS Express (DeNovo Software).

For the experiment described in Figure 2, HMLER^{shControl} cells were pretreated with water bath hyperthermia at 43, 45 and 47°C for 75, 30 and 5 minutes, respectively and then replated and allowed to recover at 37°C for 24 hours. Plates were then washed to remove dead cells and samples were prepared for flow cytometric analysis as described above.

For the experiment described in Figure 3, HMLER^{shControl} cells were pretreated with MWCNT-mediated hyperthermia at 43, 45 and 47°C as described in *Materials and Methods: MWCNT-mediated hyperthermia* and then replated and allowed to recover at 37°C for 24 hours. Plates were then washed to remove dead cells and samples were prepared for flow cytometric analysis as described above.

For the detection of apoptotic and necrotic cells following heat treatment, cells were treated as previously described then washed with PBS and resuspended in Annexin V binding buffer. One sample from each treatment condition was not resuspended but was replated at 37°C for analysis at the 24 hr timepoint. For all other timepoints samples were withdrawn, labeled with reagents according to the manufacturer's instructions and analyzed by FACS.

2.18. Immunoblotting

Lysates were prepared from HMLER^{shControl} and HMLER^{shEcadherin} cell cultures that were in log-phase growth using standard protocols. 50 µg total protein from each lysate was loaded per lane on 10% polyacrylamide gels. Proteins were electrophoresed and transferred to nitrocellulose. Membranes were blocked with tris-buffered saline + 2% BSA (TBS-B) and were probed with antibodies diluted to the manufacturer's recommended concentrations in TBS-B. Appropriate HRP-conjugated secondary antibodies were diluted 1:10,000 in TBS-B and membranes were developed using enhanced chemiluminescence (Pierce Pico). Membranes were digitally imaged using an LAS 3000 (Fuji). Densitometry was performed in Image J. Membranes were stripped using Restore stripping buffer (Sigma) according to the manufacturers recommendations and re-probed as described above.

2.19. Statistics

All analyses were performed with SPSS software (SPSS). In instances of multiple comparisons, ANOVA was performed with post-hoc testing for the desired pair-wise comparisons. In instances of individual pair-wise comparisons the Student's t test was performed. For the Kaplan–Meier plot nonparametric survival analysis models were fit to compare groups. Log-rank tests were used to determine differences between groups.

3. Results

We obtained breast cancer stem cells (HMLER^{shEcadherin}) and bulk (non-stem) breast cancer cells (HMLER^{shControl}) from the laboratory of Dr. Robert Weinberg [44] and confirmed that these cells exhibit the anticipated phenotypes. Specifically, the stem cell population exhibited a mesenchymal morphology; a ~20-fold increase in cells displaying the CD44^{high}/CD24^{low} antigen profile characteristic of tumor initiating breast cancer cells [9]; the ability to propagate as floating sphereoids (termed “mammospheres” or “tumorspheres”) in non-adherent conditions; and a ~10-fold increase in resistance to the chemotherapeutic drug paclitaxel (Supplemental Figures 1–3). Further, we found that these cells exhibit properties of a triple negative breast cancer cells, lacking expression of estrogen receptors, progesterone receptors and HER-2 (Supplemental Figure 4). Collectively, these data confirm previous findings using this cell system indicating their functional identity to BCSCs

isolated from established cell lines or patient samples [44]. Furthermore, we identify these cells as a new model of stem cell-driven, triple-negative breast cancer.

3.1. Response of breast cancer stem cells to traditional hyperthermia

We next determined the sensitivities of breast cancer stem and bulk (non-stem) breast cancer cells to hyperthermia. For this study, stem (BCSCs) and bulk breast cancer cells were heated to a defined temperature (between 43–49°C) in a circulating water bath and changes in viability over time were determined. As shown in Figure 1, BCSCs were significantly more resistant to the effects of hyperthermia than bulk breast cancer cells across the entire temperature range. For example, at a treatment temperature of 47°C, viability (expressed as a fraction relative to untreated cells) of the bulk breast cancer cells following 10, 15, 30 or 60 minutes of treatment was reduced to 0.33, 0.23, 0.11 and 0.05, respectively. In contrast, hyperthermia under identical conditions reduced the viability of BCSCs to 0.81, 0.76, 0.49 and 0.28. Thus the thermal resistance of stem cells compared to bulk breast cancer cells at 47° ranged from 2.7 fold at 10' to 5.6 fold at 60'. These differences were statistically significant ($p \leq 0.01$).

Mechanisms of thermoresistance are multifactorial; however, in several cell types, overexpression of members of the heat shock protein (HSP) family has been implicated in resistance to heat-based treatments [49]. To determine if HSPs were involved in the observed differences in sensitivity to hyperthermia between BCSCs and bulk breast cancer cells, expression of HSP27, HSP70 and HSP90 was analyzed by western blotting. HSP90 was overexpressed ~4.5-fold in BCSCs relative to bulk breast cancer cells. No difference in the basal expression levels of HSP27 or HSP70 was observed (Figure 1e). Consistent with a role for HSP90 in thermal resistance, treatment with the HSP90 inhibitor 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) partially sensitized BCSCs but not bulk breast cancer cells to hyperthermia (Supplemental Figure 5).

3.2. Enrichment of BCSCs following conventional hyperthermia

Because our findings indicated that BCSCs were more resistant to hyperthermia than their bulk breast cancer counterparts, we tested whether BCSCs in a mixed cell population would preferentially survive hyperthermia and become enriched in the remaining cell fraction following treatment. We treated bulk breast cancer cells (which contain a small endogenous stem cell component, see Supplemental Figure 1c) at 43°, 45° and 47°C for 75, 30 and 5 minutes, respectively. These time and temperature combinations were chosen to result in partial cell death so that we could assess the phenotype of surviving cells. Viable cells were analyzed by flow cytometry to determine the relative percentage of CD44^{high}/CD24^{low} cells. As shown in Figure 2, hyperthermia significantly enriched the stem cell fraction under all treatment conditions ($P < 0.001$). Specifically, the stem cell fraction increased from $15.7 \pm 1.7\%$ before treatment to $25.3 \pm 0.7\%$ (43°C), $29.2 \pm 1.8\%$ (45°C) and $29.2 \pm 0.9\%$ (49°C) following treatment, indicating enrichment of the stem cell fraction of the surviving population by 1.6 – 1.9 fold ($p < 0.001$). Furthermore, stem cell enrichment increased as a function of total cell death. These data confirm that breast cancer stem cells preferentially survive hyperthermia and persist following treatment.

3.3. Sensitivity of BCSCs to MWCNT-mediated thermal therapy

We hypothesized that the ability of nanoparticles to generate intense, sub-cellularly localized heat [39, 50] may differ from hyperthermia delivered by conventional means [51], and may overcome the resistance of BCSCs to traditional hyperthermic treatment. To test this hypothesis, we assessed the response of BCSCs and bulk breast cancer cells to nanoparticle-mediated hyperthermia at the same temperatures we used previously when heating cells in a water bath (Figure 1). To achieve the desired final temperatures, we

established experimental conditions under which the change in temperature of a suspension of multiwalled carbon nanotubes was controlled by laser exposure time ($R^2 = 0.997$) (Figure 3a; see *Materials and Methods*)

Next, cells were mixed with MWCNTs immediately prior to treatment then exposed to NIR laser light for lengths of time calculated to generate the indicated final temperatures. Final temperatures were verified by thermocouple in parallel samples. Immediately following laser irradiation, cells were washed extensively to remove MWCNTs then replated and allowed to recover overnight. Viability was assessed by MTT.

As shown in Figure 3b, the combination of MWCNTs and laser (but none of the control conditions) lead to significant, temperature dependent decreases in cell viability. Critically, the stem and bulk breast cancer cells were equally sensitive to nanotube-mediated thermal therapy (NMTT). This contrasts with the treatment resistance of stem cells to water bath hyperthermia (Figure 1), indicating that hyperthermic therapy mediated by nanotube-generated heat may overcome the inherent resistance of breast cancer stem cells to thermal therapy.

To confirm that both stem and bulk breast cancer cells had equivalent sensitivity to NMTT, the surviving fraction was analyzed by flow cytometry. Bulk breast cancer cells were treated with a combination of MWCNTs + laser to achieve final temperatures of 43°, 45°, 47° or 49°C. Cells were allowed to recover for 24 hours and then analyzed by flow cytometry. Unlike water bath hyperthermia (Figure 2), NMTT did not lead to an increase in the BCSC fraction ($p=0.177$) (Figure 3c). In fact, at the higher temperature (49°), the surviving stem cell fraction was decreased relative to bulk breast cancer cells. Similar results were obtained using an independent breast cancer stem cell model system, SUM159 cells [10]: the stem cell fraction in this breast cancer cell line was also resistant to water bath-mediated hyperthermia but not MWCNT-mediated hyperthermia (not shown). This finding demonstrates that in a population including both stem and bulk breast cancer cells (such as seen in human tumors), both are equally sensitive to MWCNT-mediated thermal treatments.

3.4. Effect of rapid temperature changes on BCSC survival

Water bath-mediated hyperthermia produced a rate of temperature increase (ROTI) calculated to be 0.1–0.2°C/second (see *Materials and Methods*) while the ROTI achieved during NMTT was 0.6°C/second, a difference of 3–6 fold. We next investigated whether BCSC killing was nanoparticle-specific or a general response of cells experiencing rapid temperature change. The water bath procedure was modified to produce an extremely rapid temperature change (See *Materials and Methods: Rapid Water Bath Hyperthermia*), enhancing the rate of temperature increase to a calculated value of 5,000°C/second. BCSCs were then treated to reach 43–53°C by either the “slow” (0.1–0.2°C/second) or “rapid” (5,000°C/second) water bath hyperthermia method. As shown in Figure 4a, a rapid ROTI did not enhance cell death *per se*. Indeed, the transient heat increase significantly promoted the survival and proliferation of BCSCs. Moreover, NMTT was significantly more cytotoxic to BCSCs than either hyperthermic treatment (Figure 4a). For example, in cells treated to 43–49°C, rapid ROTI hyperthermia *increased* cell counts at 24 hours post-treatment to 128.8, 146.9, 136.2 and 110.8% of Untreated, respectively (Figure 4a). In contrast, NMTT at the same time and treatment temperatures *decreased* cell counts to 87.6, 77.7, 64.8 and 59.4% of untreated ($p \leq 0.0005$) (Figure 4a). Overall, these data demonstrate that the enhanced sensitivity of breast cancer stem cells to NMTT is not due to a rapid ROTI.

3.5. Membrane permeabilization and necrosis following MWCNT-mediated thermal therapy

One hypothesis for the equivalent sensitivity of breast cancer stem and bulk breast cancer cells to nanotube-mediated thermal therapy is that the intense, nano-scale heat generated by NIR-stimulation of MWCNTs causes critical membrane damage to cells adjacent to nanotubes and consequent necrotic cell death. To test this, we treated stem and bulk breast cancer cells by either rapid ROTI water bath hyperthermia or NMTT and measured Annexin V labeling (a marker of apoptosis) and 7-AAD permeability (an indicator of plasma membrane integrity) at timepoints ranging from 30 minutes to 24 hours post treatment.

Consistent with our previous findings (Figure 4a) rapid ROTI water bath hyperthermia did not lead to robust cell death. Specifically, 7-AAD positivity increased for stem and bulk breast cancer cells, respectively, from 2.1% and 4.6% pre-treatment to 14.7% and 12.8% at 24 hours post-treatment (Figure 4c). In contrast, 7-AAD positivity in both cell types treated with NMTT reached 76.3% and 79.4%, respectively, over the same time period (Figure 4c). Moreover, cell death occurred more rapidly following NMTT than water bath hyperthermia. For example, with BCSCs, maximal 7-AAD positivity was observed 4 hrs post NMTT (84.5%) while the maximum (14.7%) was not reached until 24 hours following water bath hyperthermia (Figure 4b). Additionally, sustained increases in apoptotic cells (labeled with Annexin V and not 7-AAD) were not seen over the 24 hour course of the study, indicating that necrosis was the predominant form of cell death observed in both stem and bulk breast cancer cells following NMTT. Taken together, these data demonstrate that MWCNT-mediated thermal treatments promote rapid, necrotic cell death in breast cancer cells that are resistant to standard hyperthermia.

3.6. Proliferation of BCSCs following nanotube-mediated thermal therapy

We tested whether NMTT diminishes the long-term proliferative ability of breast cancer stem cells using mammosphere formation, a rigorous assay of stem cell self-renewal and proliferation. In this method, individual cells must survive and divide to form floating clonal colonies (~10 rounds of replication must occur for a single cell to form a mammosphere 200 μm in diameter). BCSCs were heat treated to 43°, 45°, 47° or 49°C by either rapid ROTI water bath hyperthermia or NMTT then replated as single-cell suspensions in ultra-low attachment conditions to track mammosphere formation over 7 days.

Rapid ROTI water bath hyperthermia led to significantly increased mammosphere size. Specifically, by day 7, median mammosphere size increased from 203.8 μm in the untreated condition to 250 μm , 281.6 μm , 250.8 μm and 236.3 μm in the 43°, 45°, 47° and 49°C water bath hyperthermia groups, respectively ($P=0.00028$) (Figure 5a). These findings are consistent with data shown in Figure 4a indicating that rapid water bath heat treatment promotes increased cell proliferation as early as 24 hours following treatment. In contrast, NMTT completely abrogated the mammosphere-forming ability of breast cancer stem cells (Fig. 5b).

3.7. Effect of nanotube-mediated thermal therapy on tumor-bearing mice

To determine the efficacy of NMTT *in vivo*, athymic mice were implanted subcutaneously with breast cancer stem cells and randomized into 4 groups ($n=10$) consisting of an untreated control, a cohort injected intratumorally with vehicle followed by laser exposure (Laser Only), a cohort injected intratumorally with 100 μg MWCNTs (CNT Only) and a cohort injected with 100 μg MWCNTs followed by laser exposure (CNT+Laser). Laser treatment consisted of irradiating the tumors with a 1,064 nm NIR laser at 3W/cm² for 30 seconds. Survival and tumor size were monitored and mice were removed from the study when their tumor burden reached 1,000 mm³ or were deemed moribund by veterinary consult. As shown in Figure 6, control group animals displayed marked tumor burdens

(Untreated = $1125.7 \pm 146.7 \text{ mm}^3$; Laser Only = $1020.9 \pm 209.1 \text{ mm}^3$ and CNT Only = $1113.8 \pm 205.9 \text{ mm}^3$; mean $\pm$ standard error) and significant mortality (Untreated = 11% alive, Laser Only = 22% alive and MWCNT Only = 20% alive) 45 days post treatment. In contrast, the combination of MWCNTs and laser exposure led to complete tumor regression (CNT + Laser = $0 \pm 0 \text{ mm}^3$) and significantly enhanced overall survival (100%) relative to the control groups ($p < 0.05$). Thus, NMTT is an effective therapy for stem cell driven breast cancer *in vivo*.

4. Discussion

In this article we characterize the response of human breast cancer stem cells and bulk breast cancer cells to heat treatment. We demonstrate that BCSCs are resistant to classic hyperthermia across a range of temperatures, and that these heat treatments do not diminish the long-term proliferative capacity of these cells. In contrast, carbon nanotube-mediated thermal treatments are lethal to both stem and bulk breast cancer cells. Furthermore, breast cancer stem cells that survive following NMTT do not retain long-term proliferative capabilities. Accordingly, tumors derived from BCSCs are sensitive to NMTT (Figure 6).

Our observations also demonstrate that nanotube-mediated thermal therapy is fundamentally distinct from classical hyperthermia in that the biological effect (cell death) is not simply a function of treatment time and temperature. When we controlled for both rate of temperature increase (ROTI) and final temperature, nanotube-mediated thermal treatments were significantly more cytotoxic to breast cancer cells overall and BCSCs in particular than the equivalent traditional hyperthermic treatment (Figure 4a). Mechanistically, nanotube-mediated cell death triggered rapid necrosis (Figure 4), which distinguishes it from the apoptotic cell death induced by classical hyperthermia [52]. Induction of necrotic death may be therapeutically advantageous, since mechanisms of resistance to apoptotic cell death are bypassed. For example, we observed that HSP90 is elevated in breast cancer stem cells (Figure 1); although this conferred at least partial protection to classical hyperthermic cell death, it did not protect breast cancer stem cells from nanotube-mediated hyperthermic death (Figure 4). Although further studies will be required to elucidate the details of the interaction between NIR-stimulated nanotubes and the cancer cell surface that leads to cell death, it is likely that the high surface temperature of NIR-stimulated MWCNTs [50] irreversibly permeabilizes cell membranes [53, 54]. This would account for the rapid loss of membrane integrity observed exclusively in NMTT-treated cells (Figure 4c). Nanotube-mediated thermal therapy may represent a promising option for breast cancer treatment that targets both the bulk breast tumor and the breast cancer stem cell.

5. Conclusions

Our results demonstrate that breast cancer stem cells are highly resistant to conventional thermal treatments. This resistance can be overcome through the use of nanoparticle-based photothermal therapies, which promote necrotic cell death. Nanotube-mediated hyperthermia may serve as a simple therapy that simultaneously eliminates both the stem cells and bulk cancer cells that constitute a breast tumor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Breast cancer stem cells are resistant to hyperthermic cell death and over-express HSP90

a–d, Relative viability of cells 24 hours after heat treatment. Cells were heated by water bath at 43°C (**a**), 45°C (**b**), 47°C (**c**) or 49°C (**d**) for 0–60 minutes. MTT absorbance values were normalized to the untreated condition (“0” minutes heat shock). Graphed are means ($n=4$) $\pm$ s.d. P-values correspond to the indicated pair-wise comparisons and were determined by post-hoc testing following an ANOVA that determined overall significance. (**e**) Expression levels of heat shock proteins (HSPs) 27, 70 and 90 were determined by Western blot. E-cadherin levels were used to confirm status of cell lines. β -actin served as a loading control. HSP90 is overexpressed ~4.5-fold in BCSCs relative to bulk breast cancer HMLER cells.

Figure 2. Sub-lethal hyperthermia enriches for the BCSC phenotype in bulk breast cancer cells
 Changes in the CD44^{high}/CD24^{low} stem cell fraction of bulk breast cancer cells 24 hours after water bath heat shock at 43°, 45° or 47°C as determined by flow cytometry. These treatments killed 67.7, 71.3 and 82% of the starting cells by the time of analysis. **a**, Representative density dot plots show significant enrichment of CD44^{high}/CD24^{low} stem cells (indicated by black arrows) in the fraction of cells surviving heat treatment. **b**, Quantification of 3 experiments showing a significant enrichment of the CD44^{high}/CD24^{low} stem cell phenotype in viable cells 24 hours after treatment. Graphed are mean percent changes ($n=3 \pm$ s.d.) in the CD44^{high}/CD24^{low} cell fraction normalized to the Untreated condition (which is set as 1.0, i.e. “0 percent change”). Dashed lines indicate the 95% C.I.

for the Untreated condition. All heat treatments led to significant increases in the stem cell fraction ($p < 0.0001$) relative to Untreated. Significance was determined by ANOVA with post-hoc testing.

Figure 3. Stem and bulk breast cancer cells are equally sensitive to MWCNT-mediated thermal therapy

Breast cancer stem cells (BCSCs) and bulk breast cancer cells were rapidly heat treated to specific temperatures by the combination of MWCNTs and 3W NIR laser irradiation. NIR exposure ranged from 5–46 seconds. **a**, Change in temperature of a 50 μ g/mL MWCNT suspension is linearly related ($R^2 = 0.997$) to seconds of 3W NIR laser exposure. **b**, Relative viability of breast cancer cells 24 hours after MWCNT-mediated thermal therapy was determined by MTT. Graphed are means ($n=4$) $\pm$ s.d. normalized to the “Untreated” conditions. “CNT Only” describes samples that were mixed with MWCNTs but were not laser treated. “Laser Only” describes samples that were laser treated but did not contain

MWCNTs. The indicated seconds of laser exposure in the “Laser Only” group correspond to the laser exposure times used in the “CNT + Laser” group. “CNT + Laser” describes samples that were heat shocked to the indicated final temperatures by the combination of 50 $\mu\text{g/mL}$ MWCNTs and 3W laser radiation. P-values indicate significant differences for both cell types relative to the “Untreated” conditions. Statistical significance was determined by ANOVA and post-hoc testing. c, Bulk breast cancer cells were heat treated to the indicated final temperatures by nanotube-mediated thermal therapy. Changes in the $\text{CD44}^{\text{high}}/\text{CD24}^{\text{low}}$ stem cell fraction were determined by flow cytometry. Quantification of 3 experiments showing no enrichment of the stem cell phenotype in viable cells 24 hours after treatment. “Laser Only” indicates samples that were laser treated but did not contain MWCNTs. “CNT Only” indicates samples that were mixed with 50 $\mu\text{g/mL}$ MWCNTs but were not laser treated. Graphed are mean percent changes ($n=3 \pm \text{s.d.}$) in the $\text{CD44}^{\text{high}}/\text{CD24}^{\text{low}}$ cell fraction normalized to the “Untreated” condition (defined as “0 percent change”). Dashed lines indicate the 95% C.I. for the “Untreated” condition. Significance was determined by ANOVA with post-hoc testing.

Figure 4. Nanotube-mediated thermal therapy is more cytotoxic than water bath hyperthermia at equivalent exposure times and treatment temperatures

a, Relative viability of BCSCs cells following slow (“Slow ROTI”) or rapid (“Rapid ROTI”) induction of hyperthermia by water bath treatment or nanotube-mediated thermal treatments. Viability was assessed 24 hours after treatment. Graphed are mean ($n=4$) $\pm$ s.d. for each treatment group normalized to the appropriate Untreated condition. P-values correspond to the statistical differences between the NMTT treatment and ROTI treatments at a given temperature. Overall significance was determined by ANOVA with post-hoc tests for the pair-wise comparisons. (ROTI = rate of temperature increase; see Materials and Methods).

b, Representative dot plots of BCSCs showing 7-AAD uptake and Annexin V labeling as a

function of time post heat treatment. **c**, Quantification of total cell death (as determined by 7-AAD uptake) in both stem and bulk breast cancer cells following rapid ROTI water bath hyperthermia or NMTT to 53°C. Graph is representative of two independent experiments. Solid colors indicate cell type; pattern indicates form of heat treatment.

Figure 5. Nanotube-mediated thermal therapy but not water bath hyperthermia abrogates mammosphere-forming ability of BCSCs

BCSCs were heat shocked to 43–49°C by either rapid ROTI water bath hyperthermia (a) or NMTT (b) and then allowed to form mammospheres for 10 days. Graphed are median mammosphere diameters for each treatment group along with 25th and 75th percentiles. Outlier values are indicated by filled circles. At least 40 mammospheres were measured per treatment group. **b**, Inset provides detail on median mammosphere sizes formed after NMTT. Statistical differences were determined by ANOVA. ** $P < 0.0001$ relative to Untreated.

Figure 6. Long-term survival of mice bearing stem cell driven breast tumors following nanotube-mediated thermal therapy

Cohorts of mice (n=10 per group) bearing tumors derived from the breast cancer stem cell line were treated with NMTT (CNT + Laser) or control conditions (Untreated, CNT Only or Laser Only). Changes in tumor volume and overall survival were tracked for 45 days following treatment. Kaplan-Meier plot details the significant survival advantage of mice treated with NMTT relative to all other control groups ($p < 0.05$).