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Targeted Elimination of Breast Cancer Cells with Low Proteasome Activity is Sufficient for Tumor Regression

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Abstract

Breast cancers are thought to be organized hierarchically with a small number of breast cancer stem cells, able to regrow a tumor after sublethal treatment while their progeny lack this feature. Furthermore, breast cancer stem cells are highly resistant to conventional anti-cancer treatments. According to the cancer stem cell hypothesis, all cancer stem cells in a tumor have to be eliminated to achieve cancer cure. In this study we tested if targeted elimination of breast cancer stem cells leads to tumor regression.

Specific targeting of breast cancer stem cells was achieved via a unique imaging and targeting system that relies on their low proteasome activity. In our system breast cancer cells stably express a fluorescent fusion protein, thymidine kinase-ZsGreen-cODC, which is readily degraded after translation in cells with normal 26S proteasome activity. However, cells with low proteasome activity accumulate this fluorescent fusion protein, thus allowing for their identification, tracking and specific elimination.

Here, we show that the activity of the 26S proteasome was significantly down-regulated in MCF-7, T47D and MDA-MB-231 cultures enriched for breast cancer stem cells. Treatment with ganciclovir resulted in abrogation of sphere formation *in vitro*, and tumor regression *in vivo*, thus demonstrating that targeted elimination of breast cancer stem cells leads to loss of self-renewal *in vitro* and tumor regression *in vivo*. We conclude, that specific targeting of breast cancer stem cells could be a useful strategy to improve treatment outcome.

Keywords

Breast cancer stem cells; proteasome; xenografts; mammospheres; thymidine kinase

1. Introduction

Increasing experimental [1] and clinical evidence support [2] the idea that breast cancers are organized hierarchically with a small number of breast cancer stem cells (BCSCs) able to regrow a tumor after sublethal treatment, while their progeny lack this feature.

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Disclosures

The authors declare that they have no conflict of interest

Importantly, BCSC withstand classical chemotherapeutic agents and are relatively resistant to ionizing radiation [3, 4]. Resistance of BCSCs to conventional treatment modalities has been attributed to up-regulation of ABC transporter proteins, high expression levels of free-radical scavengers [3, 5], and altered DNA repair [3, 5, 6].

BCSCs can be prospectively identified based on surface marker expression [1], ALDH activity [2], or lack of proteasome function [7, 8]. We have used the latter to create an imaging system for CSCs in glioma and breast cancer [9]. It is based on constitutive expression of a fusion protein between a fluorescent protein, ZsGreen, and/or thymidine kinase (TK), and the C-terminal degron of murine ornithine decarboxylase (cODC). cODC is recognized by the 26S proteasome in an ubiquitin-independent fashion and directs the fusion protein to immediate 26S proteasomal degradation. In cells with low proteasome activity the fusion protein, TK-ZsGreen-cODC accumulates, and thus can be used to image (via ZsGreen) and/or target (via TK) these cells [10]. Importantly, in breast cancer, cells with low proteasome activity partially overlap with the population of CD24^{-low}/CD44^{high} population of cells in luminal breast cancers [7], as well as with the ALDH-1⁺ population [7], both markers that enrich for BCSCs [2].

In the present study, we used the above-described imaging and targeting system for BCSCs, to test the hypothesis that targeted elimination of BCSCs leads to tumor regression *in vivo*.

2. Material and Methods

2.1 Cell culture

Human MCF-7, T47D, and MDA-MB-231 breast cancer cell lines were purchased from American Type Culture Collection (Manassas, VA). MCF-7-ZsGreen-cODC, T47D-ZsGreen-cODC, and MDA-MB-231-ZsGreen-cODC, MCF-7-TK-ZsGreen-cODC, T47D-TK-ZsGreen-cODC, and MDA-MB-231-TK-ZsGreen-cODC were generated as described in Vlashi et al. [9]. All cells were cultured in log-growth phase in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) (supplemented with 10% fetal bovine serum and a penicillin/streptomycin cocktail) in a humidified incubator at 37°C with 5% CO₂.

2.2 Mammosphere Cultures

T47D and MDA-MB-231 cells were plated in mammosphere media (phenol-red free DMEM-F12, 10 ml/500ml B27 (Invitrogen), 5 µg/ml bovine insulin (Sigma), 4 g/ml heparin (Sigma), 20 ng/ml fibroblast growth factor 2 (bFGF, Sigma) and 20 ng/ml epidermal growth factor (EGF, Sigma)). To grow MCF7 mammospheres non-essential amino acids (NEAA, 100X) and sodium pyruvate (1mM) were added to the above mammosphere media. Cells were plated into 96-well ultra-low adhesion plates, at a density of 50 cells/well in 100 l of mammosphere media. The next day the cells were treated with increasing concentrations of ganciclovir (Sigma) as indicated. Growth factors, EGF and bFGF, and heparin were added every 3 days, and the cells were allowed to form spheres for ~ 20 days. The number of spheres formed per well was then counted. Three independent experiments were performed.

2.3 Proteasome Function Assays

Chymotryptic, tryptic, and caspase-like proteasome activities were measured as described previously [11] with a few minor modifications. Cells were washed with PBS, and pelleted by centrifugation. Glass beads and homogenization buffer (50 mM Tris/HCl, 50 mM MgCl₂, 250 mM sucrose, 5 mM ATP) were added at 2x the volume of the cell pellet and tubes were vortexed for 1 minute. Beads and cell debris were removed by centrifugation at 4°C. Protein concentration was determined with a Micro BCA protocol (Pierce, Rockford, IL) with BSA (Sigma) as standard. To measure 26S proteasome activity, 100 µg of protein from crude

cellular extracts of each sample was diluted with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM MgCl₂, 2 mM ATP) to a final volume of 1 mL in quadruplicates. The fluorogenic proteasome substrates Suc-LLVY-AMC (chymotryptic substrate, Biomol, Plymouth Meeting, PA), Z-ARR-AMC (tryptic substrate, Calbiochem, San Diego, CA), and Z-LLE-AMC (caspase-like substrate, Biomol), were dissolved in DMSO and added to a final concentration of 80 μ M in 1% DMSO. Proteolytic activities were continuously monitored by measuring the release of the fluorescent group, 7-amido-4-methylcoumarin (AMC), in a fluorescence plate reader (Spectramax M5, Molecular Devices, Sunnyvale, CA) at 37° C, at excitation and emission wavelengths of 380 and 460 nm, respectively.

2.4 Animals

Nude (nu/nu), 6–8-week-old female mice, originally obtained from The Jackson Laboratories (Bar Harbor, ME) were re-derived, bred and maintained in a pathogen-free environment in the American Association of Laboratory Animal Care-accredited Animal Facilities of Department of Radiation Oncology, University of California (Los Angeles, CA) in accordance to all local and national guidelines for the care of animals.

2.5 Tumor xenotransplantation

T47D-TK-ZsGreen-cODC and MDA-MB-231-TK-ZsGreen-cODC cells were generated as described previously [9] and injected subcutaneously into the thighs of 6-week old female Nu/Nu mice (10⁶ cells per inoculum) within Matrigel (BD Biosciences). Each mouse was inoculated with cells expressing ZsGreen-cODC in one thigh, and cells expressing TK-ZsGreen-cODC on the opposite thigh. When tumors reached a diameter of approximately 5 mm on average, animals were imaged using the Maestro imaging system. Animals were then treated with 5 daily i.p. injections of 50 mg/kg ganciclovir after which imaging was repeated. For MDA-MB-231-TK-ZsGreen-cODC an extra group of animals was included, for which treatment with ganciclovir started the same day as tumor inoculation. Tumor growth was followed for 4–6 weeks, and animals were sacrificed when the combined tumor burden reached the maximum size allowed by the approved animal protocol.

2.6 Statistics

Results shown are represented as means $\pm$ standard error mean (SEM). Unless indicated otherwise result from at least 3 biologically independent repeats are shown. A p-value of <0.05 in Student's t-test was considered statistical significant.

3. Results

3.1. 26S proteasome activity is down-regulated in mammospheres

Monolayer cultures of MCF7-ZsGreen-cODC, T47D-ZsGreen-cODC, and MDA-MB-231-ZsGreen-cODC, stably expressing the fusion protein, ZsGreen-cODC, contain a very small population of ZsGreen-cODC-positive cells (Fig. 1A, top panels), indicating that the majority of the cells in monolayer cultures has normal proteasome activity. However, when these cells were grown in suspension in serum-free conditions, which promote the growth of cancer stem cells, the ZsGreen-cODC-positive population of cells was highly enriched (Fig. 1A, bottom panels). Quantification of the percentage of ZsGreen-cODC-positive cells by flow cytometry showed a significant increase in ZsGreen-cODC-positive cells in sphere cultures for all three cell lines analyzed (MCF-7-ZsGreen-cODC: 0.17 ± 0.042 % (n=12) to 1.78 ± 0.49 % (n=11), $p=0.008$; T47D-ZsGreen-cODC: 0.275 ± 0.1 % (n=8) to 2.14 ± 0.56 % (n=11), $p=0.03$; MDA-MB-231-ZsGreen-cODC: 0.3 ± 0.065 % (n=6) to 7.56 ± 1.6 (n=4), $p=0.02$) (Fig. 1B).

In order to assess the relative differences in proteasome activity in BCSCs versus differentiated breast cancer cells more quantitatively, we seeded T47D and MDA-MB-231 cells in serum free media to allow mammosphere formation, enriched in BCSCs, and compared the activity of the 26S proteasome in mammospheres and the corresponding, more differentiated monolayer cultures. In both cell lines, culture conditions that allow for mammosphere formation selected for cells in which all three cleavage activities of the 26S proteasome were significantly down-regulated. (T47D spheres: chymotryptic: $48.2 \pm 10\%$, $n=3$, $p=0.011$; tryptic: $50.3 \pm 11.4\%$, $n=3$, $p=0.025$; caspase-like: $32 \pm 16.6\%$, $n=3$, $p=0.028$; MDA-MB-231 spheres: chymotryptic: 58.4 ± 26.8 , $n=3$, $p=0.0981$; tryptic: 63.3 ± 14.8 , $n=3$, $p=0.0461$; caspase-like: $53.6 \pm 21.7\%$, $n=3$, $p=0.05$; Fig. 2A and B, low proteasome activities for MCF-7 mammospheres were reported in the Supplementary Material of [9]).

3.2 Elimination of BCSCs with low proteasome activity leads to tumor regression

To further explore the role of breast cancer cells with low proteasome activity for the maintenance of mammary tumors, we engineered three breast cancer cell lines, MCF-7, T47-D, and MBA-MB-231 to stably express a fusion protein of thymidine kinase (TK), ZsGreen, and the C-terminal decon of murine ornithine decarboxylase (cODC).

First, we tested if cells with low proteasome activity in breast cancer are the self-renewing cell population responsible for mammosphere propagation, *in vitro*. All three cell lines expressing either the TK-ZsGreen-cODC or the ZsGreen-cODC vector were seeded at clonal densities into low adhesion plates to initiate the formation of mammospheres. Treatment with ganciclovir prevented the formation of mammospheres in cell lines expressing the TK-ZsGreen-cODC vector, in a dose-dependent manner, and resulted in a few surviving cells with high proteasome activity, while the sphere forming capacity in the cell lines expressing the ZsGreen-cODC vector remained unaffected (Fig. 3A-C).

Next, we used xenotransplantation models to analyze the importance of breast cancer cells with low proteasome activity in initiating and propagating tumors *in vivo*. Using TK-ZsGreen-cODC-expressing T47D cells, we found that 5 consecutive i.p. injections of ganciclovir (i.p., 50mg/kg) resulted in a significant reduction of the fluorescent signal, indicating the efficient *in vivo* elimination of cells with low proteasome activity in established tumors (Fig. 4A and B). More importantly, elimination of these cells was sufficient for subsequent tumor regression (Fig. 4C, dashed line), further supporting the relevance of this cell population for tumor maintenance. In contrast, xenografts of T47D-ZsGreen-cODC cells continued to grow after ganciclovir (GC) treatment (Fig. 4C, solid line). We confirmed these results also using the MDA-MB-231-TK-ZsGreen-cODC, and MDA-MB-231-ZsGreen-cODC tumor model (Fig. 4D, blue growth curves and 4E). We set up these *in vivo* experiments by inoculating mice with ZsGreen-cODC expressing tumor cells on the right thigh, and TK-ZsGreen-cODC tumor cells on the other thigh on the same mouse, in order to account for any variability between mice during tumor growth and treatment with ganciclovir. Therefore, once the ZsGreen-cODC tumors reached a size that required sacrificing of the mice, we could no longer follow the growth of TK-ZsGreen-cODC tumors, which at the time point of sacrificing the animal were significantly smaller (Fig. 4E). However, in a parallel experiment we inoculated mice with MDA-TK-ZsGreen-cODC tumors and started ganciclovir treatment right after tumor inoculation, in order to investigate the effects of eliminating the cell population with low proteasome activity on tumor formation. The latter experiment demonstrated that eliminating cells with low proteasome activity significantly delayed tumor formation (by approximately 40 days), but did not completely abrogate it.

4. Discussion

Since the first description of the prospective identification of BCSCs [1], the cancer stem cell hypothesis, which proposes a model of hierarchical organization of breast cancers [12] has gained wide acceptance, even though a more recent report indicates the possibility that breast cancer cells may exhibit plasticity of their phenotype under certain conditions [7]. The clinical importance of therapy-resistant BCSCs is supported by data in which patient outcome was correlated to the pre-treatment number of BCSCs in their tumors [2, 13, 14].

To formally investigate the role of BCSCs for tumor formation and maintenance, we utilized the lack of 26S proteasome activity in BCSCs to accumulate the product of a suicide gene, thymidine kinase, while the high proteasome activity in non-CSCs results in immediate degradation after its translation [9]. We have previously shown that in breast cancer, the cell population with low proteasome activity overlaps with the BCSC population as defined by surface markers validated by other laboratories [7]. Here, we demonstrate, in a quantitative manner that cell cultures enriched in BCSCs have low proteasome activity compared to more differentiated cell cultures. Using this system we were able to specifically target, and eliminate *in vivo* BCSCs accumulating the TK-ZsGreen-cODC fusion protein, via ganciclovir treatment.

Our results clearly show that the specific elimination of BCSCs with low proteasome activity is sufficient to prevent self-renewal *in vitro*. Most importantly, elimination of TK-ZsGreen-cODC-positive cells *in vivo* the same day of tumor inoculation (by ganciclovir treatments) significantly delayed tumor formation (Fig. 4D, red growth curve). However, TK-ZsGreen-cODC cell inoculations treated with ganciclovir beginning the day of tumor inoculation eventually began to form tumors. This could be explained by the possibility that the ganciclovir treatments (dose and duration) we used in these studies may have been insufficient for a complete elimination of cells with low proteasome activity on the day of tumor inoculation, perhaps due to poor vascularization of sub-cutaneous inoculation sites. Alternatively, we do not exclude the possibility that there might be another subpopulation of breast tumor cells that eventually gave rise to the tumors. Nonetheless, these experiments clearly demonstrated the importance of breast cancer cells with low proteasome activity on tumor initiation (Fig. 4D, red growth curve).

Of particular importance was the finding that when breast tumor xenografts were allowed to first establish a tumor *in vivo*, the elimination of BCSCs with low proteasome activity is necessary and sufficient to halt tumor growth and cause subsequent tumor regression *in vivo* (Fig. 4D, blue growth curves). Previous studies by other laboratories have only indirectly tested this hypothesis, by analyzing the effect of treatments, not specific for BCSCs, on tumor regression. To our best knowledge, the approach presented here is the first to directly and specifically eliminate BCSCs from an established tumor, and allow for subsequent study of the consequences.

We are aware of the limitations of our approach, as ganciclovir treatment has a certain bystander effect that may have also led to the death of non-BCSCs. However, given the low frequency and uneven distribution of BCSCs within a tumor it is very unlikely that the effect on non-BCSCs would explain the complete regression of the tumors.

We conclude that our data supports targeting of BCSCs as a promising strategy to overcome treatment resistance of breast cancer.

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Figure 1.

(A) MCF7-ZsGreen-cODC, T47D-ZsGreen-cODC, or MDA-MB-231-ZsGreen-cODC cells were cultured as monolayer or mammospheres. Mammospheres of all three cell lines were enriched for ZsGreen-cODC⁺ cells with low proteasome activity. (B) Flow cytometry analysis of the percentage of ZsGreen-cODC⁺ cells in monolayer and spheres in MCF7-ZsGreen-cODC, T47D-ZsGreen-cODC, and MDA-MB-231-ZsGreen-cODC.

Figure 2.
(A) Cleavage activities of the 26S proteasome in cell free crude extract of T47D or (B) MDA-MB-231 cells. When cells are grown under serum free conditions to enrich for BCSCs all three activities of the 26S proteasome are down-regulated.

Figure 3.

Mammosphere formation of (A) MCF-7-ZsGreen-cODC, MCF-7-TK-ZsGreen-cODC, (B) T47D-ZsGreen-cODC, T47D-TK-ZsGreen-cODC, (C) MDA-MB-231-ZsGreen-cODC, MDA-MB-231-TK-ZsGreen-cODC in the presence of ganciclovir. Elimination of cells with low proteasome activity prevented mammosphere formation.

Figure 4.

Cells with low proteasome activity can be imaged via the ZsGreen-cODC accumulation in T47D-TK-ZsGreen-cODC tumors (A, white arrow), and five consecutive injections of ganciclovir eliminated all cells with low proteasome activity (B), which was sufficient to cause tumor regression in xenografts of T47D-TK-ZsGreen-cODC cells (C). Vertical dotted lines: days of ganciclovir treatment. (D) Elimination of cells with low proteasome activity leads to delay in tumor initiation (D, blue and red squares), and regression of established tumors (D, red circles). The mice used to generate the blue tumor growth curves were implanted with 1×10^6 MDA-MB-231-ZsGreen-cODC on the left thigh ($n=7$), and MDA-MB-231-TK-ZsGreen-cODC ($n=6$) on the right thigh and treated with ganciclovir when the tumor diameters had reached an average of 5 mm (blue arrows). The mice used to generate the red tumor growth curve were implanted with 1×10^6 MDA-MB-231-TK-ZsGreen-cODC on both thighs ($n=6$) and treated with ganciclovir the same day as tumor inoculation (red arrows). (E) Representative images of mice implanted with MDA-MB-231-ZsGreen-cODC on the left thigh, and MDA-MB-231-TK-ZsGreen-cODC on the right thigh and treated with ganciclovir (for 5 consecutive days) when tumor diameters had reached an average of 5 mm. The MDA-MB-231-TK-ZsGreen-cODC tumor growth was significantly delayed.