

AWARD NUMBER: W81XWH-12-1-0478

TITLE: Targeting Prostate Cancer Stemlike Cells through Cell Surface-Expressed GRP78

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REPORT DATE: October 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE October 2014		2. REPORT TYPE Annual		3. DATES COVERED 30 Sept 2013– 29 Sept 2014	
4. TITLE AND SUBTITLE Targeting Prostate Cancer Stemlike Cells through Cell Surface-Expressed GRP78				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-12-1-0478	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Salvatore Pizzo, M.D., Ph.D; Dr. Robin E. Bachelder E-Mail: pizzo001@mc.duke.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke University Durham, N.C. 27705-4677				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT In our previous funding period, we showed that prostate cancer cells surviving short term chemotherapy in vitro express increased levels of cell surface GRP78. Based on our hypothesis that cancer stem-like cells express cell surface GRP78, in the current funding period, we tested if chemotherapy-enriched prostate cancer cells exhibit increased sphere forming ability compared to untreated cells. Results indicated that these chemoresidual cells do not show increased spherogenicity, a hallmark of cancer stem-like cells. Accordingly, we shifted our attention to sorting cell surface GRP78-positive and cell surface GRP78-negative prostate cancer cells and determining their relative sphere forming ability. In fact, we observed increased spherogenicity of cell surface GRP78-positive prostate cancer stem-like cells, thus addressing the hypothesis that cell surface GRP78 drives cancer stem-like cell behavior. We then tested effects of a GRP78 carboxy terminal(C-terminal) antibody on sphere forming ability of these sorted GRP78-positive cells. This GRP78 antibody abolished sphere formation by these cells. This data demonstrates efficacy of targeting cell surface GRP78 to reduce cancer stem-like cell behavior. In the previous grant cycle, we had a company generate new C-terminal GRP78 antibodies in mice. In the current cycle, we requested expansion/purification of two of the GRP78 antibodies that showed cell surface staining of prostate cancer cells. In the next grant funding cycle, we will compare the relative abilities of these new antibodies (and our previously studied C-terminal GRP78 antibody, C38) to inhibit sphere growth of GRP78-sorted prostate cancer cells.					
15. SUBJECT TERMS prostate cancer, cell surface GRP78, cancer stem cell, Snail-1					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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PROGRESS REPORT:

INTRODUCTION:

In our last progress report, we provided data indicating that short-term chemotherapy treatment of prostate cancer cells increases cell surface GRP78 expression. These studies resulted in our publication of one manuscript (Li et al., 2014), describing an in vitro model of prostate cancer dormancy/recurrence. In the current grant period, we tested the hypothesis that these chemo-residual prostate cancer cells with increased cell surface GRP78 expression are cancer stem cells. Data from this grant period indicate that these chemo-residual cells show decreased prostasphere forming ability compared to untreated cells, disproving our hypothesis. In light of this result, we next developed a sorting strategy for obtaining cell surface GRP78(+) and cell surface GRP78(-) prostate cancer cells (see Task 6 in the original SOW). Using this strategy, we tested the relative abilities of these two sorted populations to grow as self-renewing spheres to begin to address the hypothesis that cell surface GRP78 is expressed on prostate cancer stem-like cells. These studies were successful in showing that cell surface GRP78(+) tumor cells exhibited increased sphere forming ability compared to cell surface GRP78(-) tumor cells. We also showed that a GRP78 monoclonal antibody directed against the C-terminus of GRP78 prevents the growth of prostate cancer cells as self-renewing spheres. Finally, we have begun to characterize the binding properties of newly produced C-terminal GRP78 monoclonal antibodies generated using a GRP78 peptide immunization strategy. Collectively, these studies provide a foundation for testing relative efficacy of three C-terminal GRP78 monoclonal antibodies in eliminating prostate cancer stem-like cells (in year 3).

KEYWORDS: GRP78, prostate cancer stem-like cell, prostaspheres

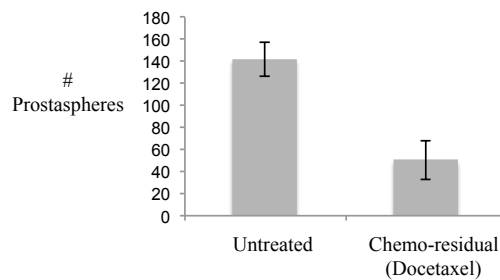
OVERALL PROJECT SUMMARY (Tasks refer to those outlined in approved Statement of Work):

Task 2:

OBJECTIVE: Investigate the relative expression of cell surface GRP78 in adherent prostate cancer cells and in prostate cancer stem-like cells.

RESULTS/DISCUSSION: In year 1, we determined that cell surface GRP78 expression levels were low in prostate cancer cells, making it difficult to sort cell surface GRP78(+) and cell surface GRP78 (-) prostate cancer cells (see Task 6). However, we showed that treatment of DU145 prostate cancer cells with chemotherapy in vitro resulted in the enrichment of a chemo-resistant population that expressed increased levels of cell surface GRP78 (compared to untreated cells). In the current year of funding, we tested whether these chemo-residual cells exhibit increased sphere forming ability compared to untreated cells. Data in Fig. 1 demonstrate that sphere forming ability of chemo-residual DU145 cells was not increased relative to that of untreated DU145 cells, indicating that chemotherapy does not enrich for prostate cancer stem-like cells. Accordingly, we attempted cell surface GRP78 sorting on untreated DU145 cells (see Task 6).

Figure 1: DU145 cells surviving short-term chemotherapy (docetaxel, 10 nM) do not exhibit increased sphere forming ability compared to untreated DU145 cells. DU145 cells were treated for 2 d with docetaxel (10 nM), after which chemotherapy was removed. Ten days after chemotherapy treatment, chemo-residual cells were harvested. Sphere forming ability of untreated and chemo-residual cells was then assessed. Prostatespheres were generated in serum-free RPMI 1640 (Gibco®, Life Technologies, USA) supplemented with B27, EGF (20 ng/ml) and FGF (20 ng/ml). Prostatesphere media included 1% methylcellulose to prevent sphere aggregation. Untreated and docetaxel-treated DU145 were harvested, counted and seeded in triplicate on ultra-low attachment 6-well plates (Corning Inc., Corning, NY, USA) at equal densities (DU145 = 10,000 cells/well; SUM159 = 25,000 cells/well). After 12 d of sphere growth, spheres >50 µm were counted on the GelCount™ Colony Counter (Oxford Optronix, Abington, UK). GelCount™ sphere settings were optimized for each cell line and were not changed between trials. Results represent three independent trials. Error bars represent the standard error of the



Task 6:

OBJECTIVE: Investigate the relative ability of cell surface GRP78(+) and cell surface GRP78(-) prostate cancer cells to grow as self renewing prostaspheres in vitro.

RESULTS/DISCUSSION: We implemented a sorting strategy for isolating cell surface GRP78(+) and cell surface GRP78 (-) prostate cancer cells (Task 6a; Fig. 2) with the goal of testing the relative abilities of these two populations to grow as self-renewing spheres, a property of cancer stem-like cells (Task 6b). As shown in Fig. 3, cell surface GRP78(+) DU145 cells were more efficient at sphere formation than cell surface GRP78(-) cells.

Figure 2: Strategy for sorting cell surface GRP78(+) and cell surface GRP78(-) DU145 prostate cancer cells. DU145 cells were harvested with 2 mM EDTA and stained with anti-Grp78-alexa fluor 488 (10 µg/10⁶ cells) and 7AAD to exclude dead cells. Cells were sorted into Grp78-positive (P6 gate) and Grp78-negative (P4 gate) populations.

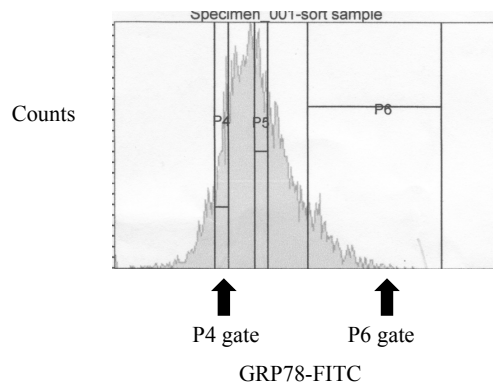
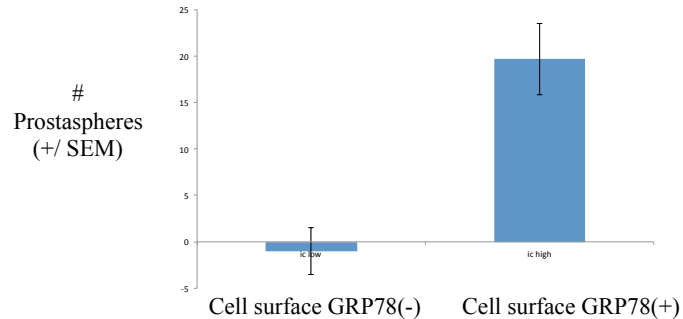


Figure 3: Testing relative sphere forming ability of cell surface GRP78(+) and cell surface GRP78(-) DU145 prostate cancer cells. Sorted populations from Fig. 2 were grown as prostaspheres according to the protocol described in Fig. 1. Prostasphere number from three wells (+/- SEM) was determined on d8.



Task 5:

OBJECTIVE: Determine the effect of incubating human prostate cancer stem-like cells with monoclonal antibodies specific for N-terminal and C-terminal domains of cell surface GRP78 on Akt/GSK-3/Snail-1 signaling.

RESULTS/DISCUSSION: We are delayed in performing the experiments because of our delay in addressing Task 6 (originally scheduled for year 1, moved to year 2; see above).

Task 7:

OBJECTIVE: Using Snail-1 shRNAs, assess the importance of Snail-1 for cell surface GRP78 regulation of the growth of self-renewing prostate cancer stem-like cells.

RESULTS/DISCUSSION: We are delayed in performing these experiments for the reasons described above (see Task 5).

Task 8:

OBJECTIVE: Determine the relative ability of cell surface GRP78(+) and cell surface GRP78 (-) prostate cancer cells to initiate tumor growth in a xenograft model.

RESULTS/DISCUSSION: We are delayed in performing these experiments for the reasons described above (see Task 5).

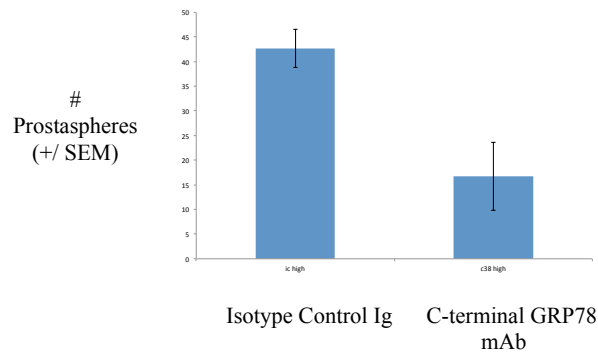
Task 9b (note that we initiated these studies in year 2 instead of in year 3, as proposed in original SOW):

OBJECTIVE: Determine the effect of incubating prostate cancer stem-like cells (derived from prostasphere cultures of 1-LN cells) with N- and C-terminal GRP78 monoclonal antibodies on their ability to grow as self-renewing cancer stem-like cells.

RESULTS/DISCUSSION: Based on our successful demonstration that cell surface GRP78(+) DU145 cells exhibit increased sphere forming ability compared to cell surface GRP78(-) cells (see Fig. 3), we investigated

the effect of a C-terminal GRP78 monoclonal antibody on sphere forming ability of cell surface GRP78(+) DU145 cells. As shown in Fig. 4, this C-terminal antibody prevented sphere growth of cell surface GRP78(+) tumor cells.

Figure 4: C-terminal GRP78 monoclonal antibody (C38) suppresses the ability of cell surface GRP78(+) DU145 tumor cells to grow as self-renewing prostaspheres. DU145 prostate cancer cells were sorted into cell surface GRP78(+) and cell surface GRP78(-) tumor cell populations as described in Fig. 2. Sorted populations were then cultured as prostaspheres (using the methods in Fig. 1) +/- C-terminal GRP78 monoclonal antibody C38 or an isotype control antibody (all antibodies at 5 $\mu\text{g/mL}$). Spheres were counted on d8, using the methods described in Fig. 1.



Supplementary Task 1:

OBJECTIVE: Purification of two additional monoclonal antibodies directed against the C-terminus of GRP78.

RESULTS/DISCUSSION: In July 2011, prior to the start date of this grant (September 2012), our lab attempted to produce C-terminal GRP78 monoclonal antibodies using a GRP78 C-terminal fragment immunization approach (see Progress Report, year 1). We pursued this effort with the goal of generating additional C-terminal GRP78 monoclonal antibodies with potential therapeutic efficacy in eliminating prostate cancer stem-like cells. In funding year 1, we identified six hybridoma supernatants that recognized cell surface GRP78 (See Progress Report, year 1). We selected two of the clones (415-579; 417-734) for expansion based on: 1) their ability to bind to cell surface GRP78 on human prostate cancer cells, and 2) their ability to induce cell death in DU145 prostate cancer cells (See Progress Report Year 1). In the current funding period, these clones were slowly adapted to growth in serum-free media, and then expanded to allow collection of 1.5L of spent media from each of the two lines. Purified antibody from the spent media of each clone was obtained by Protein A affinity purification. We are currently testing the binding of these C-terminal GRP78 antibodies to human prostate cancer cell lines (DU145, 1-LN) by flow cytometry, as well as their ability to suppress human prostasphere growth.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed a strategy for sorting cell surface GRP78(+) and cell surface GRP78(-) prostate cancer cells.
- Showed that cell surface GRP78(+) prostate cancer cells are more efficient at prostasphere formation than cell surface GRP78(-) prostate cancer cells.
- Demonstrated efficacy of a C-terminal GRP78 monoclonal antibody (C38) in targeting prostate cancer stem-like cells.
- Purified two new purified C-terminal GRP78 monoclonal antibodies to be tested in year 3 for their ability to target prostate cancer stem-like cells.

CONCLUSION: We developed a sorting strategy for purifying cell surface GRP78(+) and cell surface GRP78(-) prostate cancer cells. We also showed that cell surface GRP78(+) prostate cancer cells exhibit increased ability to grow as self-renewing prostaspheres [compared to cell surface GRP78(-) prostate cancer cells]. Finally, we showed efficacy of targeting cell surface GRP78 with a C-terminal GRP78 monoclonal antibody in preventing prostasphere growth. These accomplishments in year 2 provide a foundation for studying therapeutic efficacy of C-terminal GRP78 monoclonal antibodies in a mouse model of prostate cancer (year 3).

PUBLICATIONS, ABSTRACT, AND PRESENTATIONS:

Peer-reviewed scientific journals:

1. S. Li, M. Kennedy, S. Payne, K. Kennedy, V. L. Seewaldt, S. V. Pizzo, R. E. Bachelder, Model of tumor dormancy/recurrence after short-term chemotherapy. *PLoS One* **9**, e98021 (2014)10.1371/journal.pone.0098021).

Presentations:

- 2013 Duke University School of Medicine, Basic Science Research Day. “Size matters: Targeting giant tumor cells to prevent recurrence”
- 2013 Duke University Medical Center, Department of Pathology Grand Rounds. “Targeting chemotherapy-enriched dormant tumor cells to prevent recurrence”

INVENTIONS, PATENTS, AND LICENSES: Nothing to report

REPORTABLE OUTCOMES:

1. Development of an in vitro model of TN breast cancer dormancy/recurrence

OTHER ACHIEVEMENTS: Nothing to report

REFERENCES:

1. S. Li, M. Kennedy, S. Payne, K. Kennedy, V. L. Seewaldt, S. V. Pizzo, R. E. Bachelder, Model of tumor dormancy/recurrence after short-term chemotherapy. *PLoS One* **9**, e98021 (2014)10.1371/journal.pone.0098021).

APPENDICES:

Published manuscript:

1. S. Li, M. Kennedy, S. Payne, K. Kennedy, V. L. Seewaldt, S. V. Pizzo, R. E. Bachelder, Model of tumor dormancy/recurrence after short-term chemotherapy. *PLoS One* **9**, e98021 (2014)10.1371/journal.pone.0098021).



Model of Tumor Dormancy/Recurrence after Short-Term Chemotherapy

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Abstract

Although many tumors regress in response to neoadjuvant chemotherapy, residual tumor cells are detected in most cancer patients post-treatment. These residual tumor cells are thought to remain dormant for years before resuming growth, resulting in tumor recurrence. Considering that recurrent tumors are most often responsible for patient mortality, there exists an urgent need to study signaling pathways that drive tumor dormancy/recurrence. We have developed an *in vitro* model of tumor dormancy/recurrence. Short-term exposure of tumor cells (breast or prostate) to chemotherapy at clinically relevant doses enriches for a dormant tumor cell population. Several days after removing chemotherapy, dormant tumor cells regain proliferative ability and establish colonies, resembling tumor recurrence. Tumor cells from “recurrent” colonies exhibit increased chemotherapy resistance, similar to the therapy resistance of recurrent tumors in cancer patients. Previous studies using long-term chemotherapy selection models identified acquired mutations that drive tumor resistance. In contrast, our short term chemotherapy exposure model enriches for a slow-cycling, dormant, chemo-resistant tumor cell sub-population that can resume growth after drug removal. Studying unique signaling pathways in dormant tumor cells enriched by short-term chemotherapy treatment is expected to identify novel therapeutic targets for preventing tumor recurrence.

Citation: Li S, Kennedy M, Payne S, Kennedy K, Seewaldt VL, et al. (2014) Model of Tumor Dormancy/Recurrence after Short-Term Chemotherapy. PLoS ONE 9(5): e98021. doi:10.1371/journal.pone.0098021

Editor: Irina U. Agoulnik, Florida International University, United States of America

Received: December 20, 2013; **Accepted:** April 28, 2014; **Published:** May 20, 2014

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Funding: National Cancer Institute R21 CA141223, www.cancer.gov; National Cancer Institute R01 CA155664, www.cancer.gov; Department of Defense Congressionally Directed Medical Research W81XWH-12-1-0478, cdmp.army.mil/; Department of Defense Congressionally Directed Medical Research W81XWH-13-1-0404, cdmp.army.mil/. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Despite the apparent efficacy of chemotherapy in “shrinking” primary tumors, chemotherapy-resistant tumor cells are thought to contribute to future tumor recurrence, the leading cause of patient mortality [1]. The identification of proteins that confer chemotherapy resistance has historically relied on studies of signaling pathways supported by tumor cells subjected to long-term, high dose drug selection [2,3]. These long-term selection models select for mutations/epigenetic modifications that result in acquired expression/activity of proteins involved in therapy resistance. The clinical relevance of these long term selection models remains controversial [4].

Other models propose that tumors are heterogeneous, consisting of therapy-sensitive and therapy-resistant tumor cell subpopulations [5,6,7,8,9,10]. According to these models, following chemotherapy treatment, chemo-resistant tumor cells exist in a dormant (sleeping) state for many years before resuming growth, resulting in tumor recurrence. Methods are needed to enrich for dormant tumor cells, allowing for studies of their unique signaling properties. Such studies will be critical to defining logical therapeutic targets for preventing tumor recurrence.

Using short term chemotherapy treatment to enrich for drug-resistant tumor cells, we have developed an *in vitro* model of tumor recurrence. In this model, short-term exposure of breast and

prostate tumor cells to clinically-relevant chemotherapy classes/doses enriches for a population of slow-cycling (dormant) tumor cells. Chemotherapy-enriched dormant tumor cells resume proliferation approximately ten days after chemotherapy withdrawal, forming colonies resembling a tumor recurrence. Colonies emanating from chemotherapy-enriched dormant cells exhibit increased resistance to the original chemotherapy insult, similar to recurrent tumors in cancer patients. Contrasting with evolution models of therapy resistance, the existence of drug-resistant tumor cell subpopulations in the original tumor suggests that we can effectively eliminate tumor recurrence by implementing combination therapies [chemotherapy (targeting proliferative cells)+therapy targeting drug-resistant tumor cells].

Materials and Methods

Cell Culture/Reagents

SUM159 cells were obtained from Duke Cell Culture Facility and maintained in Ham's F-12 medium containing 5% heat-inactivated FBS, 5 µg/ml insulin, and 1 µg/ml hydrocortisone. DU145 prostate cancer cells were obtained from the Duke Cell Culture Facility and maintained in RPMI 1640 containing 10% heat-inactivated FBS.

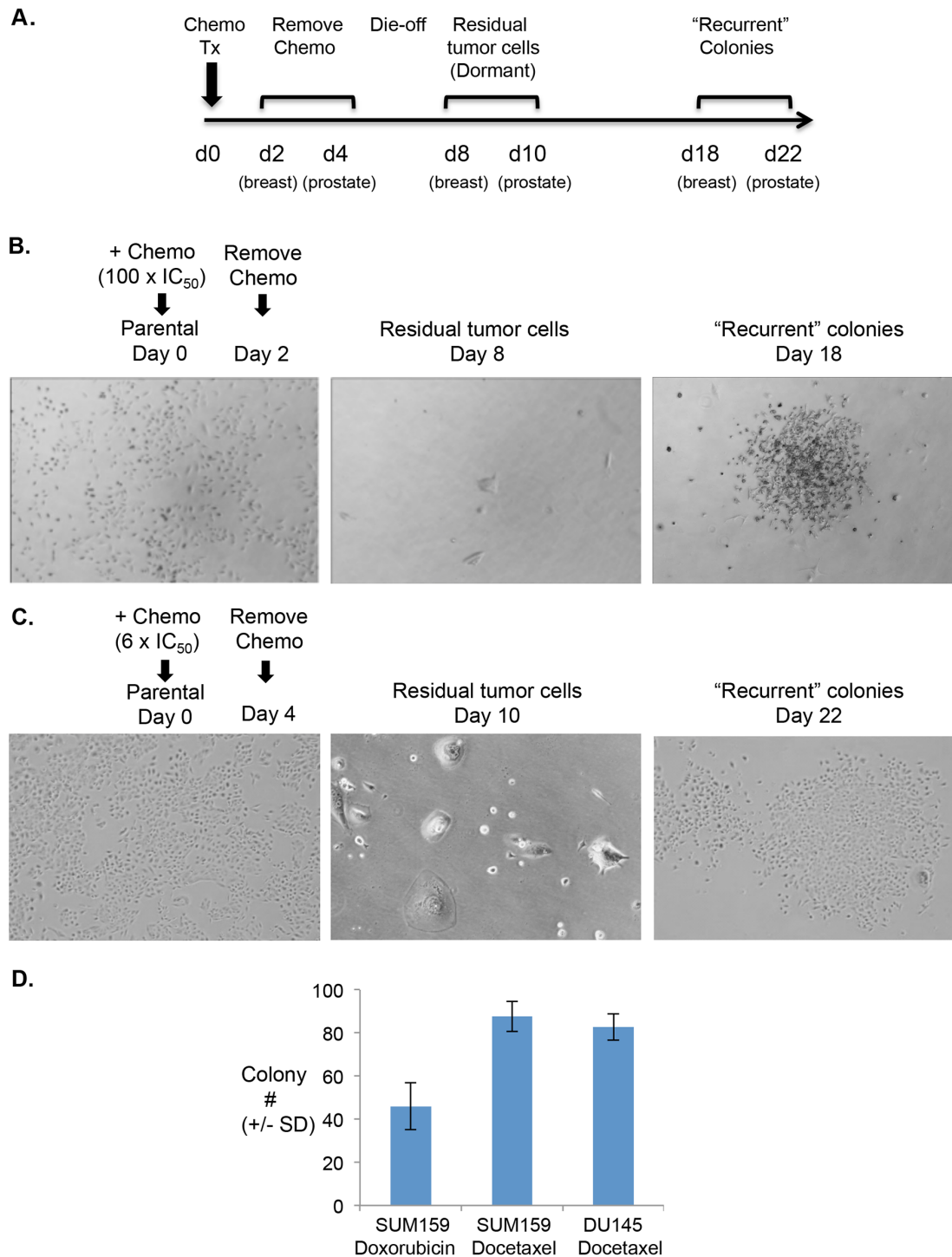


Figure 1. *In vitro* model of tumor dormancy/recurrence after short-term chemotherapy treatment. **A.** Schematic of experimental tumor dormancy/recurrence model. Breast (SUM159) or prostate (DU145) tumor cells were treated short term (breast 2 d; prostate 4 d) with chemotherapy *in vitro*. After 8 d (breast) or 10 d (prostate), dormant tumor cells (breast d8; prostate d10) were observed. Over time (breast d18; prostate d22), these dormant tumor cells resumed growth, establishing "recurrent" colonies. **B.** SUM159 breast tumor cells (Parental, left panel; 4X) were incubated with Docetaxel (100 nM; 100 fold IC₅₀) for 2 d, after which chemotherapy was removed and fresh culture medium added. Residual tumor cells were imaged on d8 after treatment (Residual tumor cells, middle panel; 4X). Colonies evolving from residual tumor cells were imaged on d18 ("Recurrent" colonies, right panel; 4X). Similar results were obtained using SUM159 cells incubated with Doxorubicin (Dox) for 2 d (1 µg/ml; 100 fold IC₅₀; data not shown). **C.** DU145 prostate cancer cells (Parental, left panel; 4X) were incubated with Docetaxel (10 nM) for 4 d, after which chemotherapy was removed and fresh culture medium added. Residual tumor cells were imaged on d10 after treatment (Residual tumor cells, middle panel; 10X). Colonies were imaged on d22 ("Recurrent" colonies, right panel; 4X). **D.** SUM159 were incubated with Doxorubicin or Docetaxel as in "B". Recurrent colonies were counted using crystal violet on d18. Likewise, DU145 cells were incubated with Docetaxel as in C. Recurrent colonies were counted using crystal violet on d22. Results are representative of at least three independent trials.
doi:10.1371/journal.pone.0098021.g001

Time Course- Cell Death Following Acute Chemotherapy Treatment

SUM159 were incubated with doxorubicin (1 μM) for 2 d, after which chemotherapy was removed, and new media added. Photographs were taken using an Olympus inverted microscope with a Canon EOS Rebel T4I. Final magnifications were 4X and 10X. Viable cell number was determined by performing trypan blue stains on cells harvested at 6 h, d1, d2, d3, and d7 post-chemotherapy treatment. Alternatively, DU145 tumor cells were incubated with docetaxel (10 nM). Chemotherapy was removed after 4 d. Viable cell number was determined as above for chemotherapy-treated SUM159 cells.

Time Course- Regrowth of Chemo-residual Tumor Cells

Six days after chemotherapy removal, SUM159 cells were harvested with trypsin-EDTA, and replated in 96 well plates (1000 cells/well). Tumor cell proliferation was assessed on a daily basis by measuring thymidine uptake. For the DU145 model, DU145 cells were harvested with accutase six days after chemotherapy removal, and replated in 96 well plates (1000 cells/well). Tumor cell proliferation was assessed on a daily basis by measuring thymidine uptake.

Evolution of “Recurrent” Colonies

SUM159 dormant cells were harvested 5–6 d after chemotherapy removal with trypsin-EDTA, and re-plated in 6-well plates (10^3 cells/well). Media was changed every 3–4 d. Recurrent colonies (d18–d22) were stained with crystal violet and colonies containing >50 cells were counted. DU145 dormant cells were harvested with accutase 6 d after chemotherapy removal and replated in 6-well plates (2.5×10^3 cells/well). Media was changed every 5–6 d. Recurrent colonies were stained with crystal violet on d22 and counted using the GelCount.

Western Blots

Cells were harvested using trypsin-EDTA, washed with PBS, incubated in RIPA buffer on ice for 20 min, and then subjected to high speed centrifugation to obtain total cellular protein in the soluble fraction. For nuclear protein extraction, harvested cells were first incubated in cytosolic lysis buffer (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl_2 , 0.5% NP40, and proteinase inhibitors) on ice for 20 min, centrifuged, and the supernatants were collected as cytosolic protein lysates. The residual pellets were washed with cytosolic lysis buffer once, and then incubated in nuclear lysis buffer (50 mM TRIS, 1% SDS, and proteinase inhibitors) plus Benzonase (Sigma, St. Louis, MO) on ice for 20 min. The supernatants after centrifugation were collected as nuclear protein extracts. Protein concentrations were determined by BCA assay. Equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with the following primary antibodies, followed by the appropriate species IRDye-conjugated secondary antibody (Invitrogen): p21 (Cell Signaling), GAPDH (GenScript), Actin (Sigma). Proteins were detected using Odyssey infrared imaging system (LI-COR, Lincoln, NE).

Thymidine Uptake

Cells were plated in 96-well plates (2×10^3 cells/well). After overnight incubation, cells were incubated with 0.5 μCi /well [Methyl- ^3H]-Thymidine (Perkin Elmer) for 4–6 h before harvesting onto glass-fiber filters. [^3H]-Thymidine incorporation was measured as counts per minute (CPM) using a Tri-Carb 2100TR time-resolved liquid scintillation counter (Perkin Elmer).

Alamar Blue

Cells were plated in 96-well black, clear bottom plates (2×10^3 cells/well) in 100 μl complete medium. After 6 h, 10 μl /well alamarBlue (Life Technologies) reagent was added and, after 3 h, fluorescence was measured using a Cytation3 plate reader (BioTek).

PKH Labeling Study

SUM159 and DU145 cells were labeled using the PKH26 Red Fluorescent Cell Linker Kit (Sigma) according to the manufacturer's instructions. The labeled SUM159 cells were treated with doxorubicin (1 $\mu\text{g}/\text{ml}$) to generate chemotherapy enriched dormant cells, as described above. Likewise, PKH26-labelled DU145 were treated with docetaxel (10 nM) to generate chemotherapy-enriched dormant cells, as described above. Labelled cells were detected using the Guava EasyCyte Plus flow cytometer (Millipore).

Measuring Chemotherapy Sensitivity of Recurrent Tumor Cells

SUM159 and DU145 “recurrent” colonies (as described above) were re-plated in T75 tissue culture flasks and grown as a monolayer. Parental tumor cells and recurrent tumor cells were plated in 96-well plates (2×10^3 cells/well). After overnight incubation, cells were incubated with media only, doxorubicin, or docetaxel at the indicated concentrations for 2 d. [Methyl- ^3H]-Thymidine was added (0.5 $\mu\text{Ci}/\text{well}$) 6 h before harvesting onto glass-fiber filters. [^3H]-Thymidine incorporation was measured as described above. Data were reported as fold change relative to cells cultured in media alone.

Results

Several studies indicate that drug-resistant, slow-cycling tumor cells are represented at low frequency in human tumors, and are therapy resistant [5,6]. The contribution of these cells to tumor recurrence following chemotherapy treatment is not known. We investigated the hypothesis that short-term exposure of tumor cells to chemotherapy enriches for a slow-cycling, chemo-resistant tumor cell sub-population that can, over time, resume growth, thus resembling tumor recurrence. To test this hypothesis, we exposed human breast (SUM159) and prostate (DU145) tumor cells to acute chemotherapy treatment (Fig. 1A). SUM159 breast tumor cells were exposed to Docetaxel (100 nM; 100-fold IC_{50}) or Doxorubicin (1 $\mu\text{g}/\text{mL}$; 100-fold IC_{50}). DU145 prostate tumor cells were exposed to Docetaxel (10 nM; 6-fold IC_{50}). Chemotherapy was removed on d2 for SUM159 cells and on d4 for DU145 cells, and fresh culture medium was added. After 8 days (SUM159) or 10 days (DU145), the majority of tumor cells were dead. However, we noted that a small number of residual tumor cells remained (Fig. 1B and 1C). These residual tumor cells appeared to be non-proliferative, as indicated by the fact that their numbers did not increase for several days (data not shown). Approximately 10 d after chemotherapy removal, these residual tumor cells resumed proliferation (Fig. 3C) and eventually formed colonies, resembling a tumor recurrence (Fig. 1B–1D).

Tumor dormancy has been defined as a condition in which residual cancer cells stop dividing [11]. It is thought that these cells remain dormant for a prolonged period before receiving signals (intrinsic or extrinsic) that cause them to resume growth and establish recurrent tumors. Fitting this definition of dormancy, both breast tumor cells and prostate tumor cells surviving short term chemotherapy in our model represented a sub-population of cells that did not take up appreciable thymidine (Fig. 2A), but were

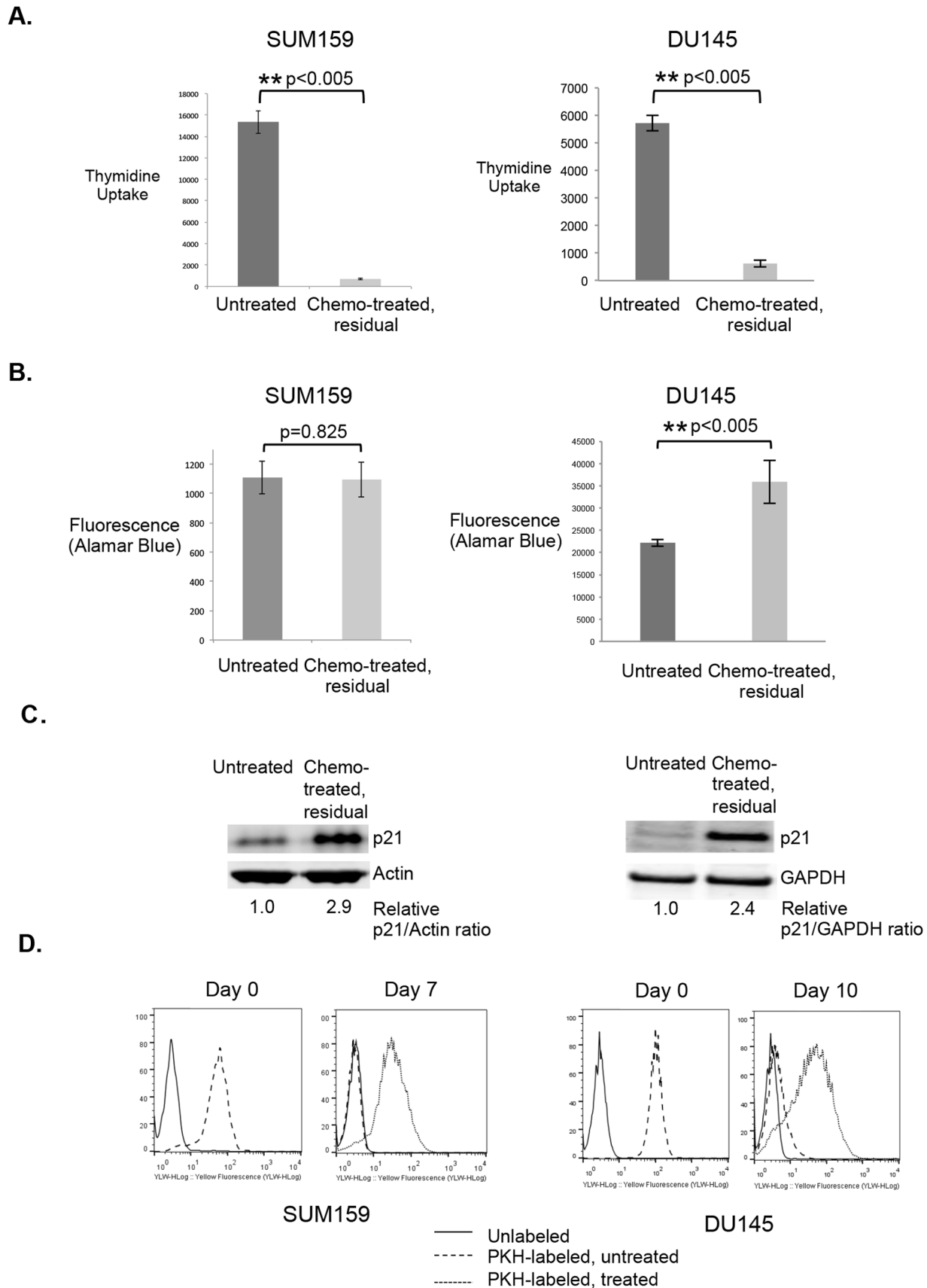
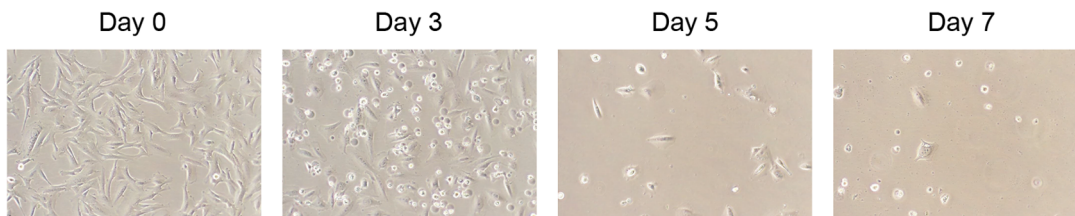


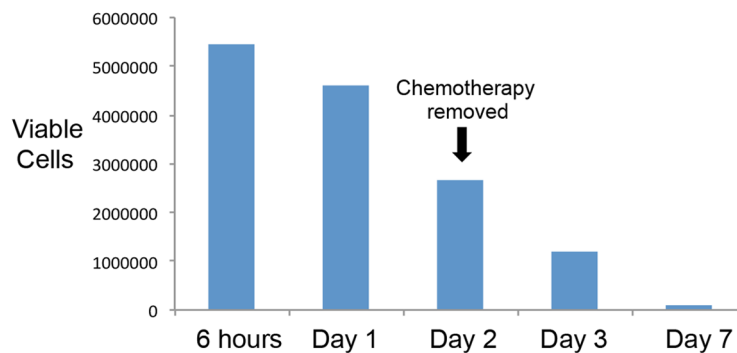
Figure 2. Chemotherapy enriches for dormant tumor cells. **A and B.** SUM159 breast and DU145 prostate cancer cells were exposed to acute Doxorubicin or Docetaxel treatment, respectively (as described in Fig. 1). Residual tumor cells surviving short-term chemotherapy treatment were

harvested on d8 (breast) or d10 (prostate), and seeded at 2000 cells/well in triplicate wells of a 96 well plate. Proliferation was determined by thymidine incorporation (+/-SD). Cell viability was assessed by alamar blue (fluorescence +/- SD) (B). Statistical significance for (A) and (B) was determined using a two-tailed student's t-test, with $p < 0.05$ being considered significant. $p \leq 0.05$ (*); $p \leq 0.005$ (**). C. Total cellular protein was extracted from parental and residual, chemo-resistant tumor cells, and equivalent amounts were immunoblotted with p21 antibody, followed by IrDye-conjugated secondary antibody. Protein loading was assessed using Actin or GAPDH antibodies. Protein bands were detected by infrared imaging. Protein bands were quantified using Image J software (NIH), and the relative ratio of p21 to loading control is shown for each lane. Similar results were obtained in 3 independent trials. D. SUM159 or DU145 tumor cells were stained with the label-retaining dye PKH26, and labeling efficiency was assessed by flow cytometry on Day 0. PKH26-labelled SUM159 cells were either left untreated (- - -) or incubated for 2 d with Doxorubicin (1 $\mu\text{g}/\text{ml}$; ---). PKH26-labelled DU145 cells were either left untreated (- - -) or incubated for 4 d with Docetaxel (10 nM; ---). The % label-retaining cells was determined on d7 (SUM159) or d10 (DU145) after treatment. Note that at the time of harvest, the majority of untreated cells (proliferative) had lost the dye, whereas slow-cycling dormant cells enriched by chemotherapy had retained the dye.
doi:10.1371/journal.pone.0098021.g002

A.



B.



C.

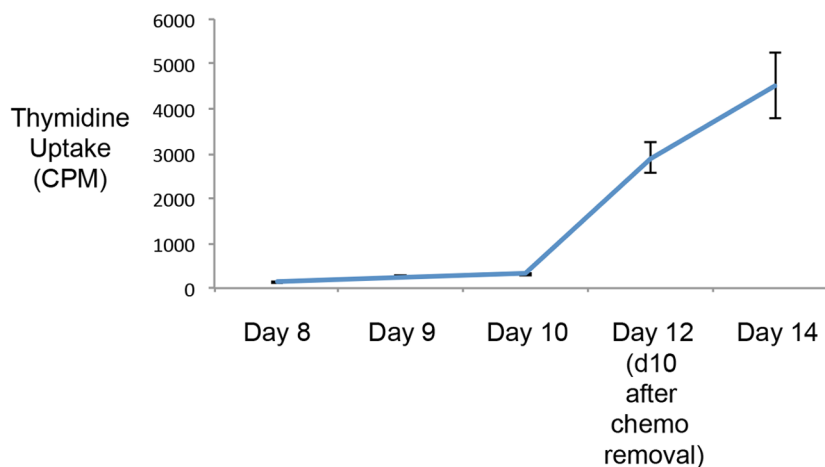


Figure 3. Kinetics of “recurrent” colony growth. SUM159 tumor cells were incubated with Doxorubicin (2d) as indicated in Fig. 1. **A and B.** Kinetics of cell die-off were assessed by imaging representative fields (**A**) as well as by counting viable cells using trypan blue (**B**) at the indicated times post-chemotherapy treatment. **C.** Proliferative status of residual tumor cells was measured over time by performing thymidine incorporation assays on cells (2000 cells/well) harvested at the indicated times post-chemotherapy treatment.
doi:10.1371/journal.pone.0098021.g003

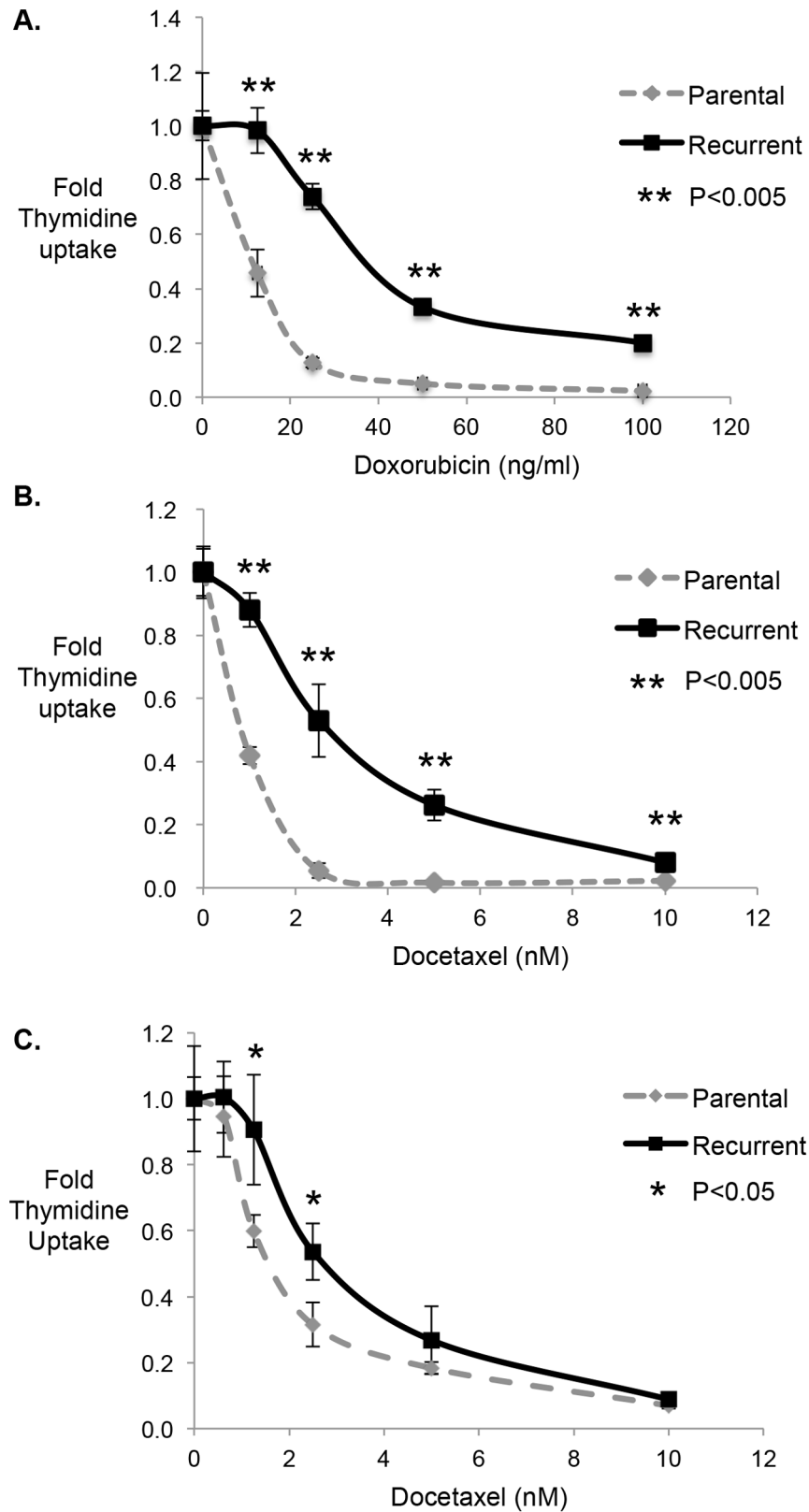


Figure 4. Tumor cells from recurrent colonies are more resistant to chemotherapy than parental tumor cells. A and B. SUM159 breast tumor cells were incubated with Doxorubicin (A) or Docetaxel (B) as in Fig. 1. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting in the evolution of “recurrent” colonies. Tumor cells from recurrent colonies, as well as parental tumor cells, were re-challenged with the indicated concentrations of Doxorubicin (A) or Docetaxel (B). Chemo-sensitivity was assessed by thymidine incorporation. Data for each point are expressed as fold change relative to cells cultured in media only. $n=4$, error bars represent S.D., * $p<0.05$, ** $p<0.005$. **C.** DU145 prostate tumor cells were incubated with Docetaxel as in Fig. 1. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting

in the evolution of “recurrent” colonies. Tumor cells from recurrent colonies and parental tumor cells were re-challenged with the indicated concentrations of Docetaxel. Chemo-sensitivity was assessed by thymidine incorporation, as in A and B.
doi:10.1371/journal.pone.0098021.g004

metabolically active, as indicated using an alamar blue assay (Fig. 2B). Notably, chemo-residual DU145 prostate cancer cells exhibited increased alamar blue positivity compared to parental DU145 cells, suggesting that these enriched cells may have elevated metabolism. Chemo-residual tumor cells also expressed increased levels of p21 (Fig. 2C), a cell cycle arrest protein. Contrasting with parental tumor cells, chemotherapy-enriched tumor cells were slow-cycling, as indicated by their retention of the lipophilic dye PKH26 (Fig. 2D).

We next sought to determine the time after chemotherapy removal that dormant tumor cells resumed growth after chemotherapy removal. The number of viable breast tumor cells decreased for five days after chemotherapy removal, as demonstrated in Fig. 3A and B. However, residual tumor cells did not resume proliferation until approximately 10 days after chemotherapy removal, as assessed by thymidine uptake (Fig. 3C). Similar kinetics of growth were observed using the DU145/docetaxel prostate cancer model (data not shown).

Recurrent tumors are frequently detected in cancer patients many years after initial chemotherapy treatment, and these tumors are chemo-refractory. Similar to recurrent tumors in patients, recurrent tumor cells evolving in our model from chemotherapy-enriched dormant cells exhibited increased chemotherapy resistance (Fig. 4). Increased therapy resistance was observed in both recurrent breast tumor cells (Fig. 4A and B) and in recurrent prostate tumor cells (Fig. 4C). Notably, resistant recurrent breast tumor colonies were observed independent of the class of chemotherapy treatment (taxane vs anthracycline) (Fig. 4A and 4B).

Discussion

Our results demonstrate that dormant, chemo-resistant tumor cells can be enriched from human breast and prostate tumor cell lines by short-term chemotherapy treatment. DNA-damaging (Doxorubicin) and microtubule-modifying (Docetaxel) chemotherapies, representing standard treatment regimens for breast and prostate cancer patients respectively, enriched for these dormant cells at clinically relevant doses [12,13], indicating broad relevance to patient treatment (Fig. 1).

The current study focused on the ability of these dormant tumor cells to resume growth upon chemotherapy withdrawal, resembling the process of tumor recurrence. Notably, “recurrent” tumor cells evolving after chemotherapy withdrawal were more resistant to subsequent chemotherapy challenge than parental tumor cells. The therapy resistance of recurrent tumor cells in our model

resembles therapy resistance of recurrent tumors in cancer patients [4].

The resistant phenotype of “recurrent” tumor cells evolving from our chemotherapy-enriched dormant cells contrasts with the reversibly-resistant phenotype of tumor cells subjected to long-term drug selection [6,14]. To date, we have observed continued resistance of our “recurrent” breast tumor lines for 50 days after chemotherapy withdrawal (representing approximately 40 doubling times for these cells; data not shown). The irreversible resistance of these drug resistant tumor cells has important implications for patient treatment. Specifically, the existence of irreversible drug resistant phenotypes in the original tumor argues against models suggesting that recurrent tumors arising in patients after a gap in treatment (“drug holiday”) may benefit from retreatment with the same therapy [4]. Studies are ongoing to determine if “recurrent” tumor cells from our *in vitro* model remain chemo-refractory for months after therapy withdrawal.

We are currently defining resistance mechanisms (DNA repair, drug efflux) of recurrent tumor cells evolving from our short term chemotherapy enrichment model. Notably, recurrent colonies exhibiting increased chemotherapy resistance relative to parental tumor cells were obtained regardless of the chemotherapy class studied [DNA-damaging (Doxorubicin) or microtubule-modifying (Taxane)]. This finding raises the important possibility that chemo-resistant tumor cells may be cross-resistant to multiple chemotherapy classes, a topic of current investigation.

Our *in vitro* model of tumor dormancy/recurrence is important because it enriches for a dormant tumor cell population that is normally under-represented in the parental tumor cell line. Current studies in the lab are focused on identifying novel signaling pathways that drive tumor dormancy/recurrence using this short-term chemotherapy enrichment strategy. These studies have the potential to identify: 1) logical therapeutic targets in chemo-resistant, dormant tumor cell populations, and 2) biomarkers that predict recurrence-free survival.

Acknowledgments

We thank Drs. Rupa Ray, Gustaaf de Ridder, and George Cianciolo for their useful comments on these studies.

Author Contributions

Conceived and designed the experiments: SL MK SP KK REB. Performed the experiments: SL MK SP KK. Analyzed the data: SL MK SP KK VLS SVP REB. Wrote the paper: SL KK REB.

References

- Weiss RB, Woolf SH, Demakos E, Holland JF, Berry DA, et al. (2003) Natural history of more than 20 years of node-positive primary breast carcinoma treated with cyclophosphamide, methotrexate, and fluorouracil-based adjuvant chemotherapy: a study by the Cancer and Leukemia Group B. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology* 21: 1825–1835.
- Calcagno AM, Salcido CD, Gillet JP, Wu CP, Fostel JM, et al. (2010) Prolonged drug selection of breast cancer cells and enrichment of cancer stem cell characteristics. *Journal of the National Cancer Institute* 102: 1637–1652.
- Puhr M, Hoefler J, Schafer G, Erb HH, Oh SJ, et al. (2012) Epithelial-to-mesenchymal transition leads to docetaxel resistance in prostate cancer and is mediated by reduced expression of miR-200c and miR-205. *The American journal of pathology* 181: 2188–2201.
- Kuczynski EA, Sargent DJ, Grothey A, Kerbel RS (2013) Drug rechallenge and treatment beyond progression—implications for drug resistance. *Nature reviews Clinical oncology* 10: 571–587.
- Moore N, Houghton J, Lyle S (2012) Slow-cycling therapy-resistant cancer cells. *Stem cells and development* 21: 1822–1830.
- Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, et al. (2010) A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* 141: 69–80.
- Fillmore CM, Kuperwasser C (2008) Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast cancer research: BCR* 10: R25.
- Iliopoulos D, Hirsch HA, Wang G, Struhl K (2011) Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via IL6 secretion. *Proceedings of the National Academy of Sciences of the United States of America* 108: 1397–1402.

9. Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, et al. (2008) Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *Journal of the National Cancer Institute* 100: 672–679.
10. Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, et al. (2007) Molecular definition of breast tumor heterogeneity. *Cancer cell* 11: 259–273.
11. Lin WC, Rajbhandari N, Wagner KU (2014) Cancer Cell Dormancy in Novel Mouse Models for Reversible Pancreatic Cancer: A Lingering Challenge in the Development of Targeted Therapies. *Cancer research* 74: 2138–2143.
12. Brunsvig PF, Andersen A, Aamdal S, Kristensen V, Olsen H (2007) Pharmacokinetic analysis of two different docetaxel dose levels in patients with non-small cell lung cancer treated with docetaxel as monotherapy or with concurrent radiotherapy. *BMC cancer* 7: 197.
13. Liu J, Zheng H, Tang M, Ryu YC, Wang X (2008) A therapeutic dose of doxorubicin activates ubiquitin-proteasome system-mediated proteolysis by acting on both the ubiquitination apparatus and proteasome. *American journal of physiology Heart and circulatory physiology* 295: H2541–2550.
14. Williams KP, Allensworth JL, Ingram SM, Smith GR, Aldrich AJ, et al. (2013) Quantitative high-throughput efficacy profiling of approved oncology drugs in inflammatory breast cancer models of acquired drug resistance and re-sensitization. *Cancer letters* 337: 77–89.