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PRINCIPAL INVESTIGATOR: Robin E. Bachelder, Ph.D.

CONTRACTING ORGANIZATION: Duke UniversityÊÁÖ¸¦@æ¦ ÊÁ¢ÔÁÇ¦Ï€¦

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Targeting Nuclear FGF Receptor to Improve Chemotherapy Response in Triple-Negative Breast Cancer

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5b. GRANT NUMBER
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Dr. Robin E. Bachelder

E-Mail: robin.bachelder@duke.edu

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
During this funding period, we determined an optimal window of time (8 days post chemotherapy-treatment) in which to study chemo-resistance signaling in chemo-residual triple-negative tumor cells, establishing an in vitro model of triple-negative breast cancer dormancy/recurrence. This work resulted in a manuscript publication. Using this model, we investigated the effect of reducing FGF receptor 2 expression in TN tumor cells on their chemo-resistance. This data showed that FGFR2 does not drive chemoresistance in this breast cancer sub-type, disproving our original hypothesis. Using a Novartis FGFR inhibitor, we confirmed a role for FGFR family members in TN tumor resistance, and obtained new data implicating FGF receptor 3 in this function. These data provide a foundation for testing FGFR3 regulation of TN tumor cell chemo-resistance in the next grant period.

15. SUBJECT TERMS
triple-negative breast cancer, chemo-resistance, FGF receptor

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PROGRESS REPORT:

INTRODUCTION:

In this grant term, we have developed an in vitro model of triple-negative (TN) breast tumor dormancy/recurrence, resulting in one publication(*1*). Whereas our preliminary work studied signaling in chemoresidual TN tumor cells surviving 2 days chemotherapy treatment, our new results suggest that TN tumor cells continue to die up to 7 days after chemotherapy treatment. After 8 days, a small number of cells (representing 0.1% of the original tumor cell population) remain viable, and exist in a dormant state. Two weeks after chemotherapy removal, these dormant cells resume proliferation, establishing colonies. This model is highly relevant to this grant application because it indicates the importance of studying chemo-resistance pathways in TN tumor cells surviving chemotherapy on d8 of our dormancy/recurrence model.

Surprisingly, using this in vitro model of tumor dormancy/recurrence, we did not observe an effect of knocking down FGFR2 expression in TN tumor cells on their chemotherapy resistance. These findings disprove our original hypothesis that FGFR2 promotes TN breast cancer chemo-resistance. However, we did observe synergy between chemotherapy and a small molecule FGFR inhibitor in reducing recurrent TN colony formation. These results support our original hypothesis that FGFR inhibitors may synergize with chemotherapy to eliminate TN breast tumor cells.

During this grant period, we sought to determine which FGFR family members drive chemotherapy resistance in triple-negative breast tumor cells. Using a phospho tyrosine kinase receptor array, we observed high FGFR3 activity, but not high FGFR1 or FGFR4 activity, in SUM159 triple-negative tumor cells. Based on these discoveries in grant year 1, we have revised our original aims, focusing on activities for FGFR3 instead of FGFR2 in TN breast cancer chemoresistance. These revised aims are as follows:

REVISED AIMS:

Aim 1: Examine FGFR3 regulation of chemotherapy resistance in triple-negative (TN) breast tumor cells. *Hypothesis: Chemotherapy enriches for TN tumor cells expressing FGFR3. FGFR3 promotes chemo-resistance by driving AP-1-dependent Snail-1 transcription.*
1.1 Examine the ability of DNA-damaging (Adriamycin, Cyclophosphamide) and microtubule-targeting (Docetaxel) chemotherapies to enrich for TN tumor cells expressing FGFR3.
1.2 Investigate FGFR3 phosphorylation/kinase activity in TN breast tumor cells exposed to DNA-damaging and microtubule-targeting chemotherapies.
1.3 Using shRNAs, Snail-1 promoter constructs, and chromatin immunoprecipitation, test the hypothesis that FGFR3 drives AP-1-dependent Snail-1 transcription in chemotherapy-selected TN tumor cells.
1.4 Using shRNAs, investigate FGFR3/Snail-1 regulation of chemotherapy resistance (Adriamycin vs Cyclophosphamide vs Docetaxel) in TN breast tumor cells.
1.5 By immunohistochemistry, examine FGFR3 expression in tumor cells obtained from TN breast cancer patients pre- and post- neoadjuvant chemotherapy treatment [Adriamycin/Cyclophosphamide (AC) or Docetaxel/Cyclophosphamide (TC)].

Aim 2: Perform pre-clinical studies of a novel combination therapy (chemotherapy + FGFR inhibitor) for triple-negative (TN) breast cancer. *Hypothesis: By suppressing FGFR3 activity, combination therapy (chemotherapy + FGFR inhibitor) is more effective than chemotherapy in eliminating TN tumor cells.*
2.1 Assess the impact of a selective FGFR inhibitor (Novartis; NVP-BGJ398) on FGFR3 phosphorylation/kinase activity and Snail-1 expression in chemotherapy-enriched TN tumor cells.
2.2 Investigate relative efficacy of combination therapy [chemotherapy + selective FGFR inhibitor (Novartis; NVP-BGJ398) versus chemotherapy alone in eliminating TN tumor cells *in vitro* and in an orthotopic mouse model.

KEYWORDS: chemotherapy resistance, triple-negative breast cancer, FGF receptor

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

Task 1:

OBJECTIVE: Examine the ability of DNA-damaging (Adriamcyin, Cyclophosphamide) and microtubule-targeting (Docetaxel) chemotherapies to select for triple-negative tumor cells expressing nuclear FGFR2.

RESULTS/DISCUSSION: During the first year, we developed an in vitro model of TN breast cancer dormancy/recurrence (Li et al., 2014; Fig. 1A). These studies indicate that TN breast tumor cells exposed for 2 days to chemotherapy continue to die for 7 days after initial chemotherapy treatment (Fig. 1B). In order to study a chemo-resistant TN tumor cell population, all tasks in our revised statement of work have been modified to study chemo-resistance signaling in TN breast tumor cells harvested 8 days after chemotherapy challenge.

Preliminary studies presented in our grant proposal demonstrated that tumor cells surviving 2 d chemotherapy treatment exhibited increased expression of nuclear-localized FGFR2. During this grant period, we studied FGFR2 expression in chemo-residual tumor cells harvested 8 days after chemotherapy treatment. In fact, at this time, we did not see increased FGFR2 expression compared to that observed in parental cells (Fig. 1C). This finding suggests that FGFR2 is NOT a determinant of TN breast cancer chemo-resistance.

Figure 1: Development of an in vitro model of TN tumor dormancy/recurrence. A. Schematic of experimental tumor dormancy/recurrence model. SUM159 TN tumor cells were treated short term (2d) with chemotherapy [docetaxel (100 nM) or adriamycin (1 µg/mL)] in vitro. After 8d, dormant tumor cells were observed. On d18, these tumor cells resumed growth, establishing "recurrent" colonies. **B.** SUM159 TN tumor cells were incubated with adriamycin (1 µg/mL) for 2 d, after which chemotherapy was removed. Kinetics of cell die-off were assessed by counting viable cells using trypan blue at the indicated times post-chemotherapy treatment. **C.** Nuclear protein extracts were obtained from parental SUM159 tumor cells, and chemotherapy-enriched dormant TN tumor cells (harvested on d8). Equivalent amounts of nuclear proteins were subjected to SDS-PAGE, and immunoblotted with FGFR2 or Lamin A antibodies, followed by IrDye-conjugated secondary antibody. Protein bands were detected by Odyssey infrared imaging. Note that FGFR2 is not enriched in chemo-residual TN tumor cells harvested on d8.
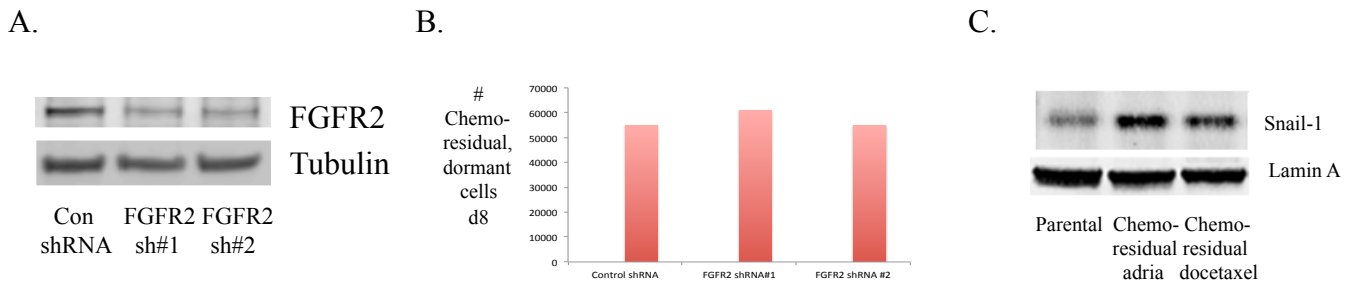

A.

B.

C.
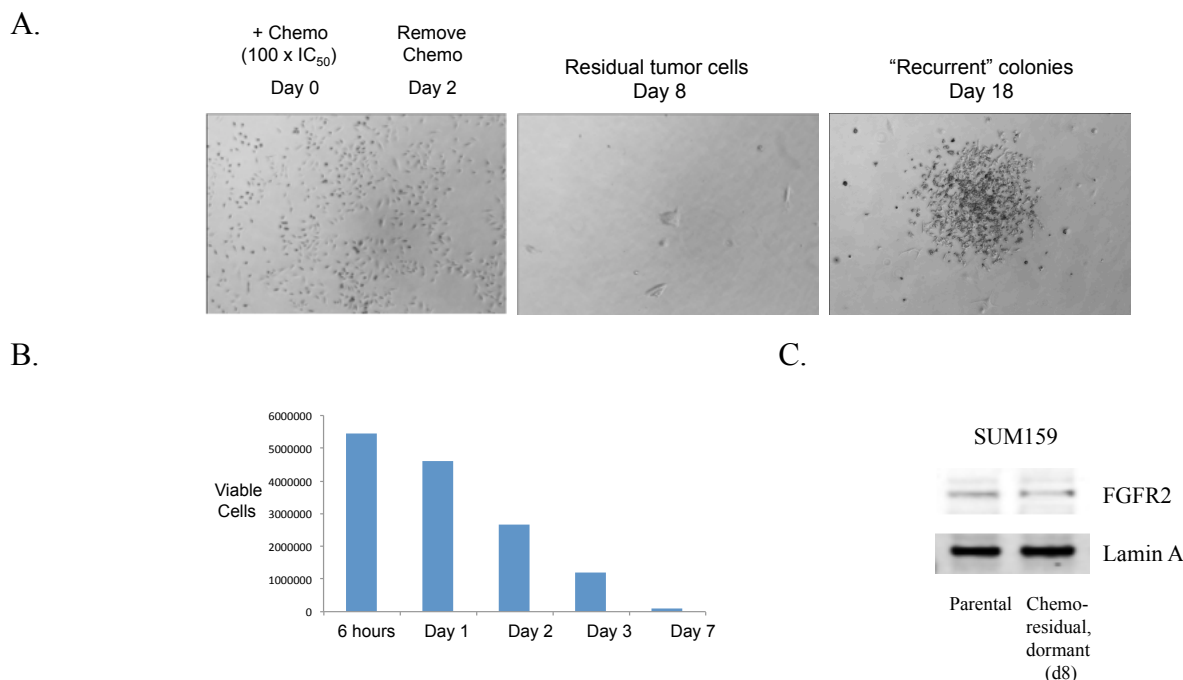
Task 3:

OBJECTIVE: Using shRNAs, investigate nuclear FGFR2/Snail-1 regulation of chemotherapy resistance (Adriamcyin vs Cyclophosphamide vs Docetaxel) in triple-negative breast tumor cells.

RESULTS/DISCUSSION: As proposed in our original statement of work, we produced triple-negative breast cancer cells stably expressing either of two FGFR2 shRNAs or a control shRNA. We confirmed reduced FGFR2 expression in FGFR2 shRNA transfectants by western blotting (Fig. 2A). These transfectants were exposed to Adriamycin or Docetaxel for 2 d, after which chemotherapy was removed. Chemo-residual dormant tumor cells were counted on d8 after chemotherapy treatment (Fig. 2B). Note that reducing FGFR2 expression did not impact the number of chemo-residual cells on d8. FGFR2 shRNA-expressing cells also did not exhibit reduced "recurrent" colony formation compared to control shRNA cells (data not shown). These results disprove our original hypothesis that FGFR2 drives TN breast cancer chemotherapy resistance. Similar results were obtained using docetaxel. Trends were confirmed in three independent trials.

We also investigated whether Snail-1, a transcription factor that we hypothesized to be downstream of FGFR2 signaling, is upregulated in chemo-residual TN tumor cells harvested d8 after chemotherapy treatment. As shown in Fig. 2C, nuclear Snail-1 expression levels were significantly increased in chemo-residual dormant TN tumor cells harvested 8d after adriamycin or docetaxel treatment.

Figure 2: FGFR2 shRNA does not reduce number of chemo-residual dormant tumor cells evolving after chemotherapy treatment: A. SUM159 cells were transfected stably with either a control shRNA or either of two distinct FGFR2 shRNAs (Sigma). Stable transfectants were selected in puromycin (5 µg/mL). Equivalent amounts of total cellular protein extracts from these transfectants were immunoblotted with FGFR2 antibody (R&D Systems; MAB4862) or tubulin antibody (Sigma) as a loading control, followed by IRdye secondary antibody. Proteins were detected using Odyssey infrared imaging. **B.** shRNA-expressing cells (2×10^6) were treated with adriamycin (1 µg/mL) for 2 d, after which chemotherapy was removed and new culture medium was added back. After 8d, chemo-residual dormant TN tumor cells were counted. No differences were observed in the number of recurrent colonies counted on d18 (data not shown). **C.** SUM159 cells were treated for 2 d with adriamycin (adria) or docetaxel as in Fig. 1. Nuclear protein extracts were obtained from chemo-residual tumor cells on d8. Equivalent amounts were immunoblotted with Snail-1 (Cell Signaling Technology) or Lamin-A (Sigma) antibodies, followed by IrDye secondary antibody. Protein bands were detected by Odyssey infrared imaging.

A.

FGFR2
Tubulin

Con shRNA   FGFR2 sh#1   FGFR2 sh#2

B.

Chemo-residual, dormant cells d8

Control shRNA   FGFR2 shRNA#1   FGFR2 shRNA #2

C.

Snail-1
Lamin A

Parental   Chemo-residual adria   Chemo-residual docetaxel

Task 7:

OBJECTIVE: Investigate relative efficacy of combination therapy [chemotherapy + selective FGFR inhibitor (Novartis; NVP-BGJ398)] versus chemotherapy alone in eliminating TN tumor cells *in vitro*.

RESULTS/DISCUSSION: Having disproven our hypothesis that FGFR2 drives triple-negative breast tumor chemo-resistance, we realized the immediate need to determine if other FGF receptor family members promote TN breast tumor cell chemotherapy resistance. Accordingly, we tested efficacy of FGFR inhibitors (TKI258, BGJ398; Novartis) in increasing TN breast cancer chemo-sensitivity. As shown in Fig 3A, using our in vitro model of tumor dormancy/recurrence, a Novartis FGFR inhibitor (TKI258) significantly reduced the number of "recurrent" colonies evolving from chemo-residual tumor cells (post-adriamycin treatment). We could not make any conclusions regarding efficacy of a second Novartis FGFR inhibitor (BGJ398) in this model because DMSO (control treatment) prevented recurrent colony growth (Fig. 3A). In future studies, we will solubilize BGJ398 in a less toxic solvent and assess its activity in our model. In addition, we will study the effect of these FGFR inhibitors on our docetaxel-generated dormancy/recurrence model (see revised SOW).

Figure 3: FGFR inhibitor (Novartis) reduces "recurrent" colony growth in in vitro recurrence model. **A.** SUM159 tumor cells were incubated with adriamycin (1 µg/mL) +/- either of two FGFR inhibitors (Novartis; TKI258 or BGJ398) at a concentration of 50 nM for two days. Media alone served as a control for TKI258, while DMSO served as a control for BGJ398). After chemotherapy removal, new media containing FGFR inhibitor in the absence of chemo was added every 4 days. Number of "recurrent" colonies was counted on the indicated days. Note that TKI258 significantly reduces recurrent colony formation. No conclusions could be made regarding efficacy of BGJ398 in reducing recurrent colony formation because this compound was resuspended in DMSO, which proved toxic on its own in the in vitro recurrence model. **B.** SUM159 tumor cells were incubated with docetaxel (100 nM) for 2 days, after which chemotherapy was removed. Recurrent colonies were counted on d18. Results indicate mean number recurrent colonies from triplicate wells (+/- SD).

A.
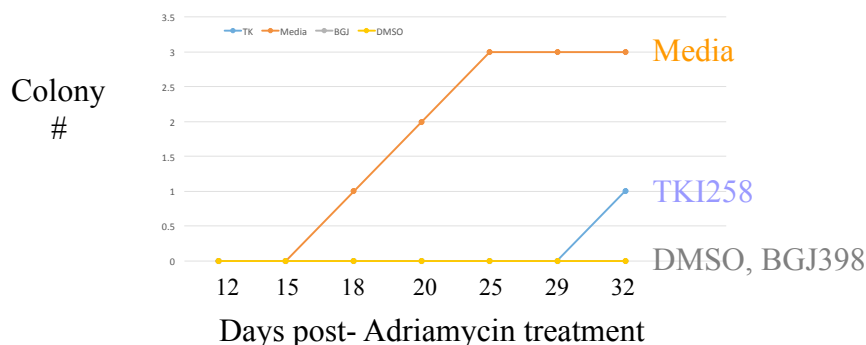
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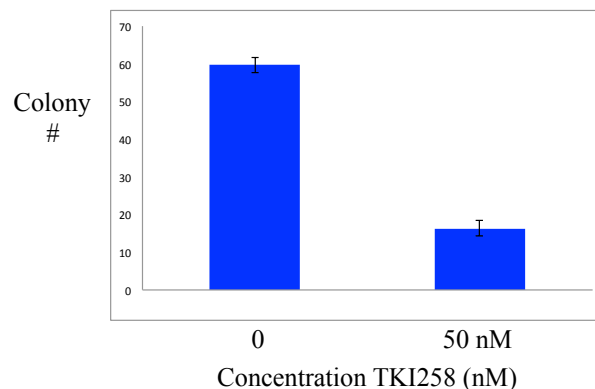
Task 2:

OBJECTIVE: Investigate nuclear FGFR2 phosphorylation/kinase activity in triple-negative breast tumor cells exposed to DNA-damaging and microtubule-targeting chemotherapies.

RESULTS/DISCUSSION: Results from task 7 (above) suggest that an FGF receptor other than FGFR2 drives TN breast tumor cell chemo-resistance. To explore which FGFR is relevant, we investigated the levels of phosphorylated FGF receptors in SUM159 TN tumor cells. As shown in Fig. 4A, levels of phosphorylated FGFR3 were significantly elevated in these tumor cells compared to levels of other phosphorylated FGF receptors. Notably, in a screen of 28 receptor tyrosine kinases, FGFR3 showed the highest level of phosphorylation in these cells (Fig. 4B). Based on these results, our new hypothesis is that FGFR3 drives TN breast tumor cell chemoresistance.

Figure 4: Assessment of FGFR family member activity in SUM159 triple-negative tumor cells. Total cellular proteins were extracted from SUM159 breast tumor cells. Expression levels of 28 tyrosine phosphorylated receptors were determined using a phospho receptor tyrosine kinase (RTK) array (Cell Signaling Technology). **A.** Of the FGFR family members represented on this array, only FGFR3 exhibited significant tyrosine phosphorylation. **B.** Of the 28 RTKs represented on this array, FGFR3 was the most highly phosphorylated.

A.
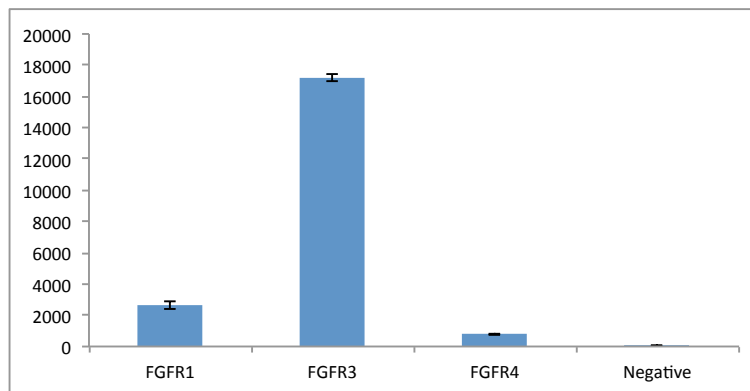
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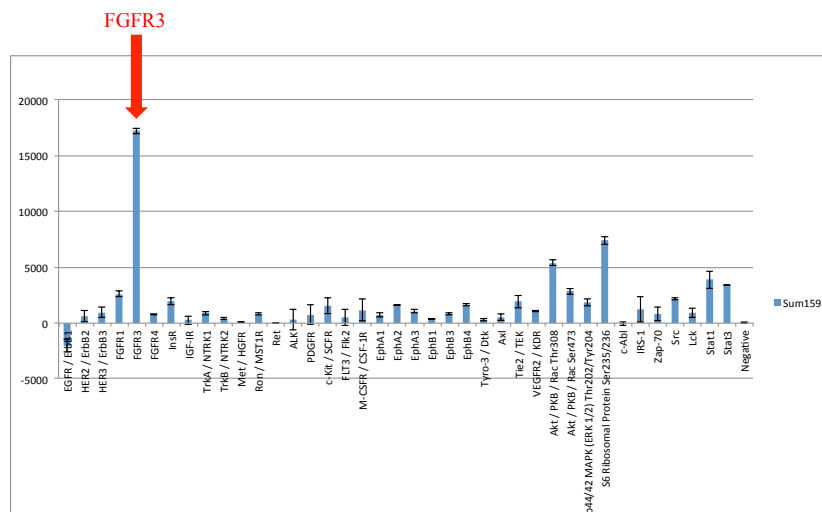
KEY RESEARCH ACCOMPLISHMENTS:

- Developed an in vitro model of triple-negative breast cancer dormancy/recurrence
- Determined optimal time (d8 post chemotherapy treatment) for studying chemoresistance pathways in TN breast cancer cells
- Determined that FGFR2 is not a driver of triple-negative breast tumor cell chemo-resistance
- Showed that Snail-1 is upregulated in chemo-residual TN tumor cells obtained 8 days after chemotherapy treatment of TN tumor cells
- Established that a small molecule inhibitor of FGF receptor family members increases TN tumor cell chemo-resistance
- Identified phosphor-FGFR3 as the most highly phosphorylated FGF receptor family member in TN tumor cells

CONCLUSION: Our studies during this grant period optimize a model for studying triple-negative breast cancer chemoresistance pathways that likely contribute to recurrent tumor growth. Our data rule out a function for FGFR2 in TN breast cancer chemo-resistance, and implicate an alternative FGFR family member (FGFR3) in driving chemo-resistance of this breast cancer subtype. These findings pave the way for studies of FGFR3 regulation of chemo-resistance in the next period of this grant funding. Ultimately, results from this period support our future studies (Aim 2) of combination therapies for TN breast cancer that include both chemotherapy and a small molecule FGFR inhibitor.

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).

Abstracts:
1.   Li, S, Payne, S, Kennedy, KM, Pizzo, SV, and **Bachelder, RE**. 2014. Identifying novel therapeutic targets for triple-negative breast cancer using an in vitro model of tumor dormancy/recurrence. Stem cells and Cancer. Keystone Symposium.

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).
2.   R. E. Bachelder, S. O. Yoon, C. Franci, A. G. de Herreros, A. M. Mercurio, Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial-mesenchymal transition. *J Cell Biol* **168**, 29-33 (2005); published online EpubJan 3.
3.   L. S. Wanami, H. Y. Chen, S. Peiro, A. Garcia de Herreros, R. E. Bachelder, Vascular endothelial growth factor-A stimulates Snail expression in breast tumor cells: Implications for tumor progression. *Exp Cell Res*, (2008); published online EpubMay 17 (
4.   R. Rouzier, C. M. Perou, W. F. Symmans, N. Ibrahim, M. Cristofanilli, K. Anderson, K. R. Hess, J. Stec, M. Ayers, P. Wagner, P. Morandi, C. Fan, I. Rabiul, J. S. Ross, G. N. Hortobagyi, L. Pusztai, Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res* **11**, 5678-5685 (2005); published online EpubAug 15 (11/16/5678 [pii] 10.1158/1078-0432.CCR-04-2421).

APPENDICES:

Revised Statement of Work

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).

REVISED STATEMENT OF WORK:

Goal 1: Examine FGFR3 regulation of chemotherapy resistance in triple-negative breast tumor cells.

Task 1 (Months 13-15): Examine the ability of DNA-damaging (Adriamcyin, Cyclophosphamide) and
microtubule-targeting (Docetaxel) chemotherapies to select for triple-negative tumor cells expressing FGFR3.

Incubate two triple-negative tumor cell lines (MDA-MB-231, SUM159PT) *in vitro* for 2 d +/- chemotherapy
[Adriamycin (50, 25, 10, 5, 1 ng/mL), Docetaxel (100 nM, 50 nM, 25 nM, 10 nM, 1 nM), or the active form of
Cyclophosphamide (4-hydroperoxy-cyclophosphamide, 4-HC; 0.1, 1, 5, 10, 25 μM)] or vehicle alone. Obtain
nuclear and non-nuclear (cytoplasmic + cell membrane) extracts from chemo-residual tumor cells obtained on
d8. Immunoblot equivalent amounts of nuclear and non-nuclear proteins with antibodies specific for FGFR3,
Lamin A (nuclear loading control), or tubulin-specific (non-nuclear loading control). For the purpose of
generating preliminary data for this grant proposal, MDA-MB-231 and SUM159PT cells were already obtained
from the Duke Cell Culture facility. SUM159PT cells were provided by this facility after obtaining permission
for distribution from Dr. Gayathri Devi.

*Outcome: These studies will determine the ability of different chemotherapy regimens to enrich for FGFR3-
expressing TN tumor cells.*

Task 2 (Months 13-15): Investigate FGFR3 phosphorylation/kinase activity in triple-negative breast tumor
cells exposed to DNA-damaging and microtubule-targeting chemotherapies.

Incubate triple-negative tumor cells +/- chemotherapy as in Task 1. To measure tyrosine phosphorylated
FGFR3, immunoprecipitate FGFR3 from extracts of these cells, and immunoblot with pan phospho-tyrosine
antibody. Measure FGFR3 kinase activity by performing *in vitro* kinase assays on FGFR3 immunoprecipitates
from nuclear extracts.

*Outcome: These studies will determine the activity of FGFR3 in chemotherapy-enriched triple-negative tumor
cells.*

Task 3 (Months 16-24): Using shRNAs, investigate FGFR3/Snail-1 regulation of chemotherapy resistance
(Adriamcyin vs Cyclophosphamide vs Docetaxel) in triple-negative breast tumor cells.

Task 3a (Months 16-24): Investigate FGFR3 regulation of chemotherapy resistance in triple-negative breast
tumor cells.

Transfect TN tumor cells with FGFR3 shRNAs or a control shRNA. Incubate these cells +/- chemotherapy
(Adriamycin or Cyclophosphamide or Docetaxel) for 2d. Determine cell viability on d8 using Alamar Blue
(Invitrogen) and trypan blue assays.

Task 3b (Months 16-24): Investigate Snail-1 regulation of chemotherapy resistance in TN tumor cells.

Transfect TN tumor cells with Snail-1 shRNAs or a control shRNA. Incubate transfectants +/-chemotherapy as in Task 1. Test cell viability as in task 3A.

Outcome: We will determine whether FGFR3 and Snail-1 drive chemotherapy resistance in TN breast tumor cells.

Task 4 (Months 22-24): Test the hypothesis that FGFR3 drives AP-1-dependent Snail-1 transcription in chemotherapy-selected triple-negative breast tumor cells.

Task 4a (Months 22-24): Investigate FGFR3 regulation of c-jun expression.

Incubate FGFR3 transfectants (from task 3) +/- chemotherapy for 2d. Harvest chemoresidual tumor cells on d8. Extract RNA, and determine c-jun mRNA levels by real time-PCR. Immunoblot equivalent amounts of total cellular protein with c-jun, phospho-c-jun(Ser63), FGFR3, and actin antibodies.

Task 4b (Months 22-24): Investigate FGFR3 regulation of c-jun transcription.

Incubate FGFR3 transfectants (from task 3) +/- chemotherapy. Determine c-jun promoter activity using a c-jun promoter-driven luciferase construct. Test association of FGFR3 with the c-jun promoter by chromatin immunoprecipitation (CHIP).

Task 4c (Months 22-24): Assess FGFR3 regulation of activity of AP-1.

After incubating FGFR3 transfectants (from task 3) +/- chemotherapy, measure AP-1 activity using an AP-1 reporter luciferase kit (Qiagen).

Task 4d (Months 22-24): Investigate FGFR3 regulation of Snail-1 expression.

Incubate FGFR3 transfectants (from task 3) +/- chemotherapy. Measure Snail-1 mRNA, Snail-1 protein, and Snail-1 promoter activity in these transfectants using our published methods(*2, 3*).

Task 4e (Months 22-24): Determine importance of AP-1 for chemotherapy regulation of Snail-1.

Transfect triple-negative tumor cells with c-jun shRNAs or control shRNAs. Incubate transfectants +/-chemotherapy. Confirm c-jun knockdown by immunoblotting extracted proteins with c-jun antibody. Determine the ability of chemotherapy to regulate Snail-1 expression/promoter activity in c-jun shRNA and control shRNA transfectants. Test c-jun association with the Snail-1 promoter by CHIP.

Outcome: These studies will determine if FGFR3 drives Snail-1 transcription by regulating AP-1 transcription factor.

Task 5 (Months 19-24): By immunohistochemistry (IHC), examine FGFR3 expression in tumor cells obtained from triple-negative breast cancer patients pre- and post- neoadjuvant chemotherapy treatment [Adriamcyin/Cyclophosphamide (AC) or Docetaxel/Cyclophosphamide (TC)].

Task 5a (already completed): Obtain human subjects approval.

Task 5b (Months 19-21): Optimize an IHC protocol for detecting FGFR3 in triple-negative breast cancers.

Optimize a protocol for detecting FGFR3 in triple-negative tumor cells pre- and post- chemotherapy treatment using cell blocks from tumor cell lines previously shown (by immunoblotting) to be positive [Adriamcyin-treated (25 ng/mL) SUM159PT cells] or negative (MCF7 cells) for FGFR3.

Task 5c (already completed): Identify/obtain relevant retrospective triple-negative breast cancer patient samples.
Identify and obtain from National Comprehensive Cancer Network (NCCN) relevant triple-negative breast cancer tissues [n=40, pre and post-neoadjuvant (AC) therapy (cohort 1); n=40, pre- and post- neoadjuvant (TC) therapy (cohort 2)]. Obtain TN breast cancer tissues from Dr. Marcom's neoadjuvant protocol [n=40, pre and post-neoadjuvant (AC) therapy (cohort 3); n=40, pre- and post- neoadjuvant (TC) therapy (cohort 4)]. Considering that approximately 60% of triple-negative breast cancer patients exhibit an incomplete patholgoic response to neoadjuvant chemotherapy treatment(4), we expect to obtain residual tumor cells post chemotherapy treatment from only 24 patients from each cohort of 40 patients.

Task 5d (Months 22-24): Measuring FGFR3 in triple-negative breast cancers pre- and post- neoadjuvant chemotherapy treatment.

Using the protocol optimized in Task 5b, stain the triple-negative breast cancer cases described in Task 5c for FGFR3. Score the % FGFR3(+) tumor cells for each case (pre and post chemotherapy treatment). For each of the four cohorts, determine the change in percent FGFR3(+) tumor cells from pre- to post- chemotherapy. Estimate mean change with its 80% confidence interval. Use the Wilcoxon signed-rank test (1-sided alpha of 0.025) to test whether the central tendency of this endpoint is greater than zero.

Outcome: We will determine if the percent FGFR3(+) tumor cells in triple-negative breast cancers is increased following either AC or TC neoadjuvant chemotherapy treatment.

Goal 2: Perform pre-clinical studies of a novel combination therapy (chemotherapy + FGFR inhibitor) for triple-negative breast cancer (Months 25-36).

Task 6 (Months 25-27): Assess the impact of a selective FGFR inhibitor (Novartis; NVP-BGJ398) on FGFR3 phosphorylation/kinase activity and Snail-1 expression in chemotherapy-enriched triple-negative breast tumor cells.

Incubate triple-negative tumor cells (MDA-MB-231 and SUM159PT) +/- chemotherapy (as described for Task 1) +/- selective FGFR inhibitor [NVP-BGJ398, Novartis; concentrations= 0.05, 1, 5, 10, 50 nM) in 96 well plates. Measure FGFR3 phosphorylation/kinase activity, and Snail-1 expression/promoter activity as in Goal 1.

Outcome: These studies will determine whether an FGFR small molecule inhibits FGFR3 activity and Snail-1 expression in chemotherapy-treated triple-negative tumor cells.

Task 7 (Months 28-30): Investigate relative efficacy of combination therapy [chemotherapy + selective FGFR inhibitor (Novartis; NVP-BGJ398)] versus chemotherapy alone in eliminating TN tumor cells *in vitro*.

Incubate tumor cells *in vitro* +/- chemotherapy +/- NVP-BGJ398 as in Task 6. Determine cell viability on d8 post chemotherapy treatment as in Task 3a. Count recurrent colnoies on d18 post chemotherapy-treatment, following the protocol of our published dormancy/recurrence model(1).

Outcome: These studies will determine whether an FGFR small molecule inhibitor reduces the number of chemo-residual tumor cells/recurrent colonies in an in vitro model of tumor dormancy/recurrence.

Task 8 (Months 25-36): Determine efficacy of combination therapy (chemotherapy + FGFR inhibitor) in eliminating triple-negative tumor cells in an orthotopic mouse model.

Task 8a (Months 30): Obtain approval to perform animal work.

Task 8b (Months 31-32): Establish triple-negative tumors in nude mice.
Inject female nude mice (4 weeks old) in the mammary fat pad with 10^6 MDA -MB-231 cells. Evaluate tumor volume with calipers 3x/week. Once tumors reach 200 mm^3, start treatments (See Task 8c). These studies will employ 180 nude mice (30 mice/treatment x 6 treatments).

Task 8c (Months 33): Treatment of nude mice with combination therapy (chemotherapy + selective FGFR inhibitor).
Randomize mice with tumors (200 mm^3) into six treatment groups (30 mice per group): 1) vehicle alone, 2) 4 mg/kg Adriamycin [intravenous (IV)], 3) NVP-BGJ398 (15 mg/kg; oral gavage), 4) NVP-BGJ398 (45 mg/kg; oral gavage), 5) Adriamycin (4 mg/kg; IV) + NVP-BGJ398 (15 mg/kg; oral gavage), and 6) Adriamycin (4 mg/kg; IV) + NVP-BGJ398 (45 mg/kg; oral gavage). Repeat Adriamycin treatments weekly for 14 days. Repeat NVP-BGJ398 treatments daily. Measure tumor volume with calipers 3x/wk for the duration of treatment (14 days). At the end of treatment, excise and snap freeze any residual tumors detected.

Task 8d (Month 34): Perform statistical analysis to determine if combination therapy is more effective than chemotherapy alone in reducing triple-negative tumor volume.

We hypothesize that the median tumor volume at the end of 2 weeks will be significantly smaller in:
a) group 2 (Adria) than in group 1 (vehicle)
b) group 5 (Adria + 15 mg/kg FGFR inhibitor) than in group 2 (Adria)
c) group 6 (Adria + 45 mg/kg FGFR inhibitor) than in group 2 (Adria)
d) group 5 (Adria + 15 mg/kg FGFR inhibitor) than in group 3 (15 mg/kg FGFR inhibitor)
e) group 6 (Adria + 45 mg/kg FGFR inhibitor) than in group 4 (45 mg/kg FGFR inhibitor).

Perform the Wilcoxon rank-sum test with a 1-sided alpha of 0.01 (0.05/5) to test for the significance of each of these five hypothesized group differences. At the end of the study, plot median tumor volume against time for each of the six treatment groups. Use the Wilcoxon rank-sum test to test for a group difference at each of the individual time points.

Task 8e (Months 35-36): Analyze residual tumor cells.

Extract nuclear proteins from residual tumor cells. Immunoprecipitate FGFR3 from equivalent amounts of nuclear extracts, and immunoblot these immunoprecipitated proteins with anti-phospho-tyrosine. Measure FGFR3 and Snail-1 expression in nuclear extracts by immunoblotting.

Outcome: Results will establish in an orthotopic mouse model whether a novel combination therapy (Adriamycin + FGFR inhibitor) is more effective than chemotherapy alone in eliminating triple-negative breast tumor cells. They will also determine the ability of this FGFR inhibitor, when combined with chemotherapy, to reduce levels of tyrosine phosphorylated FGFR3 and Snail-1 in triple-negative tumor cells.

Future Directions: The proposed work studies the ability of a novel combination therapy (chemotherapy + FGFR inhibitor) to eliminate human triple-negative breast tumor cells more effectively than chemotherapy alone. We will test the efficacy of this combination therapy both *in vitro* and in an orthotopic mouse model. These pre-clinical studies will provide an essential foundation for a future clinical trial of this combination therapy in triple-negative breast cancer patients. Based on his extensive experience with clinical trials, our collaborator (Paul Kelly Marcom, M.D.) can rapidly translate these findings into human clinical trials. These trials will test efficacy of this combination therapy (compared to efficacy of chemotherapy alone) in: 1) promoting a complete pathologic response in triple-negative breast cancer patients, and 2) prolonging patient survival.

For this proposal, our studies of combination therapy in the orthotopic mouse model are limited to Adriamycin + FGFR inhibitor. If our *in vitro* studies show that other combination therapies (Docetaxel + FGFR inhibitor; cyclophosphamide + FGFR inhibitor) are more effective than the respective chemotherapy alone in eliminating triple-negative tumor cells, we will in future studies test these alternative combination therapies in an orthotopic mouse model, paving the way for clinical trials of FGFR inhibitors in combination with other chemotherapy regimens as an effective treatment strategy for triple-negative breast cancer.

Study Site/Key Personnel: All studies will be performed at Duke University Medical Center, Durham, N.C. The PI is Dr. Robin Bachelder. Drs. Joseph Geradts, Mark Dewhirst, and Paul Marcom will serve as co-investigators. Dr. Bercedis Peterson will serve as Statistician. These studies will include animal use, and involve the use of human subjects and human anatomical substances.

PLOS ONE

Model of Tumor Dormancy/Recurrence after Short-Term Chemotherapy

Shenduo Li[1], Margaret Kennedy[1], Sturgis Payne[1], Kelly Kennedy[1], Victoria L. Seewaldt[2], Salvatore V. Pizzo[1], Robin E. Bachelder[1]*

1 Department of Pathology, Duke University Medical Center, Durham, North Carolina, United States of America, 2 Department of Medicine, Duke University Medical Center, Durham, North Carolina, United States of America

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.

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.
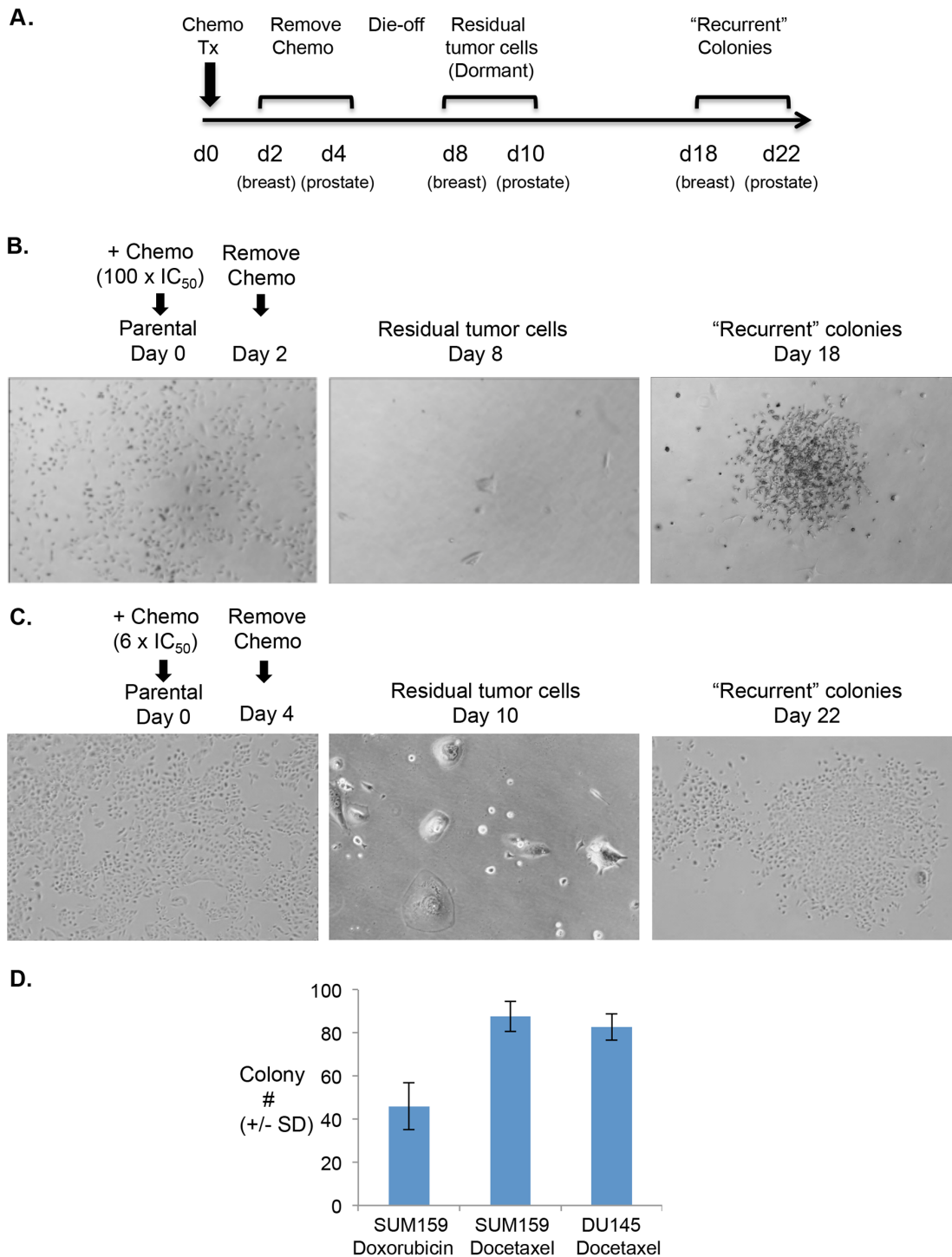
Figure 1. *In vitro* model of tumor dormancy/recurrence after short-term chemotherapy treatment. A. Schematic of experimental tumor dormancy/recurrence model. Breast (SUM159) or prostate (DU145) tumor cells were treated short term (breast 2 d; prostate 4 d) with chemotherapy *in vitro*. After 8 d (breast) 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 IC$_{50}$) 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 IC$_{50}$; 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.
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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 (10^5 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 re-plated 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 immuno-blotted with the following primary antibodies, followed by the approprimate 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-^{3}H]-Thymidine (Perkin Elmer) for 4–6 hs before harvesting onto glass-fiber filters. [^{3}H]-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 hs, 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 µg/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-^{3}H]-Thymidine was added (0.5 µCi/well) 6 h before harvesting onto glass-fiber filters. [^{3}H]-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 µg/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
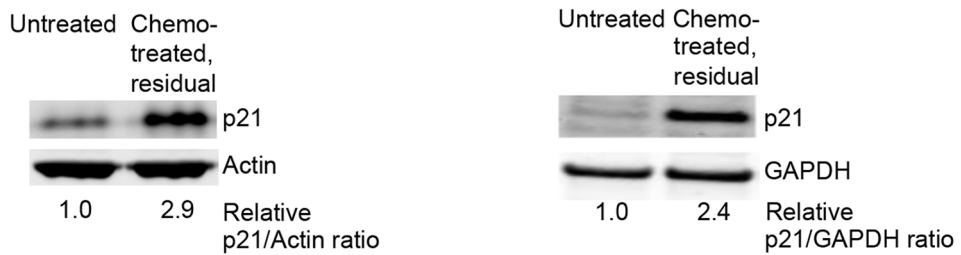
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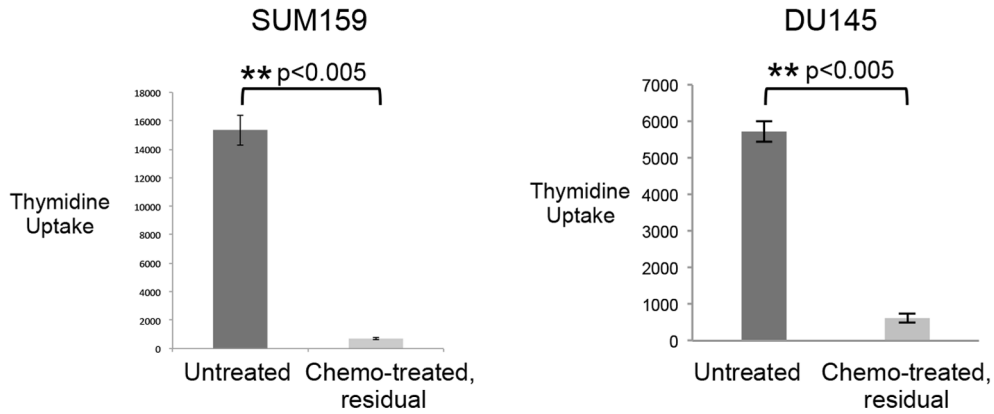
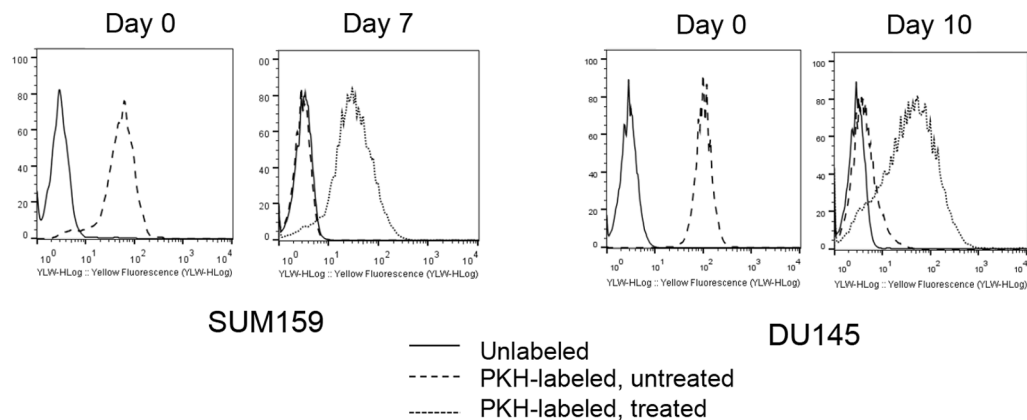**B.**
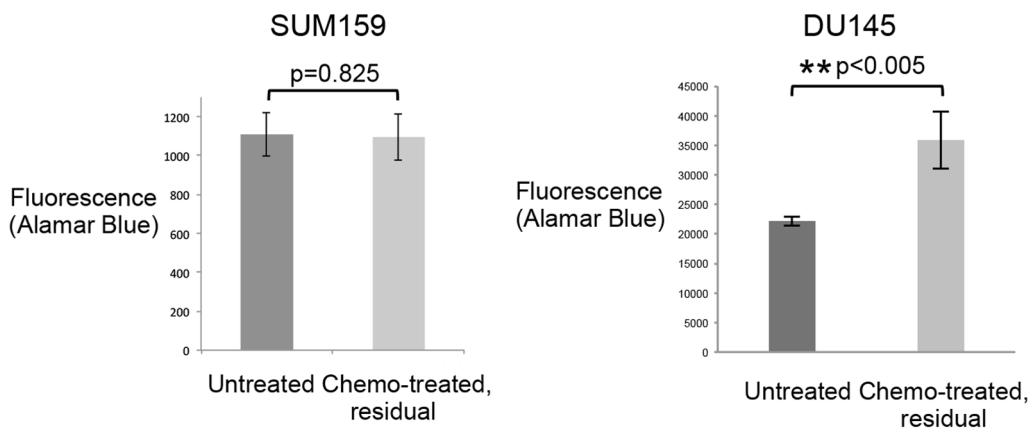
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D.

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≤0.05 (*); p≤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 μg/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.
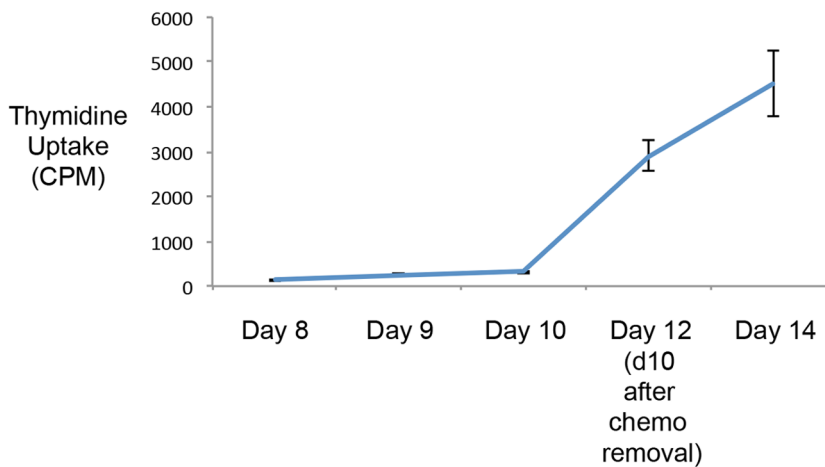doi:10.1371/journal.pone.0098021.g002
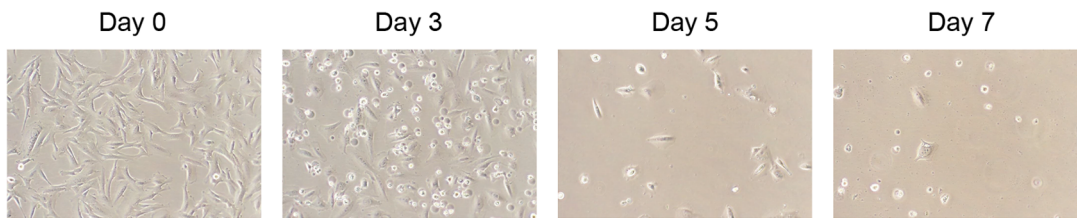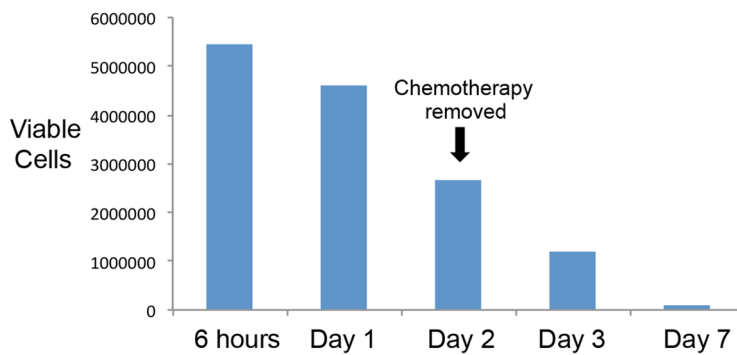
Figure 3. Kinetics of ''recurrent'' colony growth. SUM159 tumor cells were incubated with Doxorubicin (2d) as indicated in Fig. 1. **A and B.** Kinetics of cell die-off were assessed by imaging representative fields (**A**) as well as by counting viable cells using trypan blue (**B**) at the indicated 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.
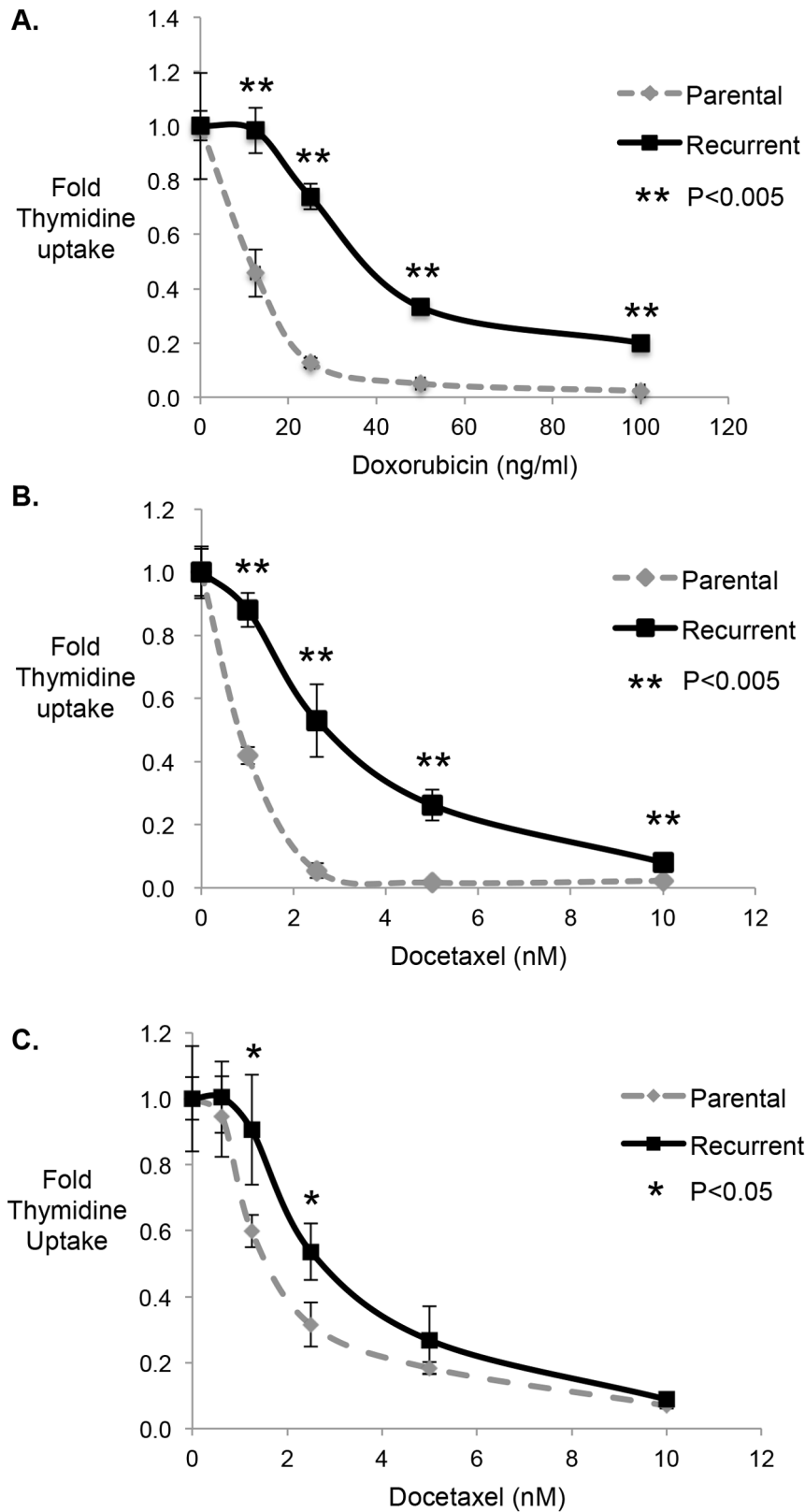doi:10.1371/journal.pone.0098021.g003

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.
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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 anthracycilne) (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.

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.

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