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INTRODUCTION

Approximately 30% of patients with early-stage breast cancer eventually develop recurrent disease and die of breast cancer. Recurrence after treatment of early-stage breast cancer and a long period of dormancy is one of the biggest challenges in breast cancer treatment [1]. Numerous studies indicate that various mechanisms of tumor dormancy exist, including cellular dormancy (quiescence) and limit tumor size by angiogenic dormancy and immunologic dormancy [2-5]. In this award, we are focused on the role of cellular dormancy in promoting cancer aggressiveness and drug resistance in recurrent breast cancer. We aim to determine the impact of dormancy on breast cancer phenotype, and to identify the molecular determinants that mediate breast cancer dormancy.

BODY

There are two major tasks proposed in this grant. During the two years of grant period, we have made significant progress on both tasks as described below.

Task 1. To determine if dormancy promotes genomic aberrations through error-prone NHEJ DNA Repair.

Breast cancer dormancy is a clinical phenomenon in which disseminated breast cancer cells remain occult after frontline therapies of the primary tumor. In this project, we established a novel *in vitro* culture system by growing cells on Petaka device at 22 °C for up to 14 days to investigate dormancy in both estrogen receptor-positive (ER+) and -negative (ER-) breast cancer cell lines. To determine whether the Petaka system is a steady *in vitro* dormancy model, cell proliferation was examined in ER+ (MCF7) and ER- (MDA-MB-231) breast cancer cell lines following Petaka incubation (Figure 1).

Cell numbers indicated that Petaka incubation led to inhibition of cell growth in either MCF7 or MDA-MB-231 cells (Figure 1A). Dormancy status is usually characterized as a balance between proliferation and apoptosis [2-5]. Therefore, we examined the proliferation marker, Ki67, and apoptotic marker, cleaved Caspase-3 in MCF7 cells following Petaka incubation (Figure 1B). Western blotting showed that Ki-67 protein levels were significantly reduced, but cleaved Caspase-3 protein level did not change in cells following Petaka incubation (Figure 1B). Additional evidence that these cells are nondividing was demonstrated with the CFSE (carboxyfluorescein diacetate succinimidyl ester) assay (Figure 1C). CFSE is a stable, cell-permeable dye that is an effective indicator of cell division. The uniform fluorescence of CFSE is diminished following cell division whereas CFSE fluorescence is sustained as cells are nondividing. CFSE intensity of the viable cells was monitored over several days of Petaka incubation (Figure 1C). The CFSE intensity for the cells incubated in the Petaka system remained high whereas the intensity of the control cells decreased overtime (Figure 1C). Overall these data indicate that the Petaka system can maintain ER+ breast cancer cells survival and trigger breast cancer cells into dormancy. We also investigated the dormancy status on MDA-MB-231 cells cultured in Petaka system. We found that like MCF7 cells, MD-MB-231 cells stopped proliferation under Petaka incubation but with slightly increase of apoptosis (data not shown). Thus, our data clearly demonstrated that Petaka incubation could promote cellular dormancy for both ER+ MCF7 cells and the ER- MD-MB-231 cells and can serve as a suitable *in vitro* model to study breast cancer dormancy.

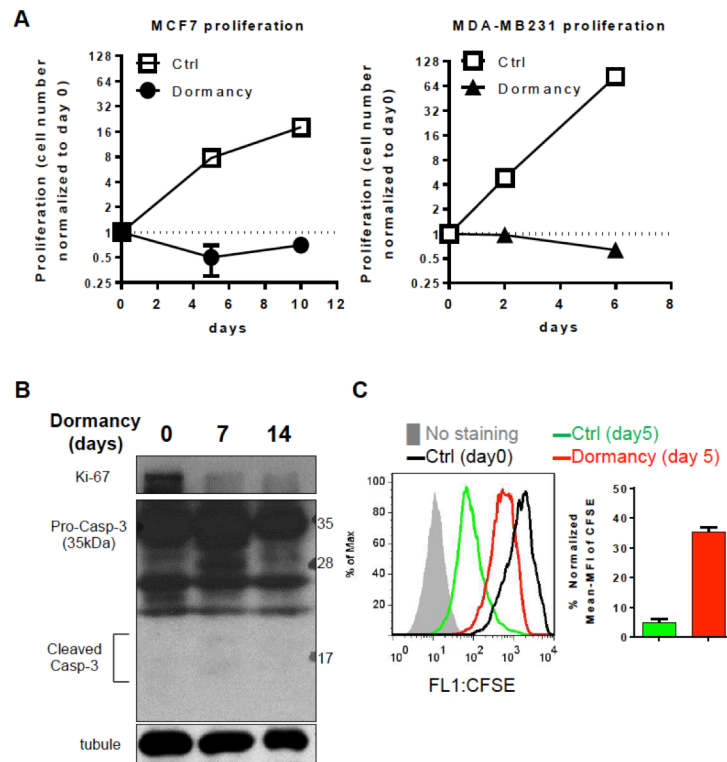


Figure 1. Establishment of *in vitro* ER+ and ER- breast cancer dormancy model. (A) The Petaka device was used to trigger MCF7 (ER+) and MDA-MB-231(ER-) cells into dormancy by incubating them at room temperature for 5 and 10 days. The proliferation rate was significantly reduced in ER+ and ER- cells incubated in the Petaka device. Each value was normalized to the control (day 0) and represents the mean $\pm$ s.d. from three independent experiments. (B) MCF7 cells were triggered into dormancy using the Petaka incubation method for 14 days and Western blotting demonstrated reduced proliferation using the Ki-67 marker. However there was no expression of the apoptotic marker, cleaved Caspase-3. Tubulin was used as the loading control. (C) (Left) A flow cytometry plot illustrated that MCF7 cells induced into dormancy retained CFSE (carboxyfluorescein diacetate succinimidyl ester) labeling as indicated by the red line. The ER+ cells are CFSE-positive directly after labeling (Ctrl, day 0, black line) whereas the labeling was lost overtime in proliferating cells (Ctrl, day 5, green line). (Right) Quantitative analysis of the CFSE mean fluorescence intensity was determined within the control and dormant cells after 5 days at room temperature. Results are shown as mean $\pm$ s.d. from three independent experiments,

To investigate the potential mechanisms of how dormant cells can survive after primary therapies, breast cancer cells were pretreated with DNA damage reagents, Doxorubicin (Doxo, 250 nM) or irradiation (IR, 8 Gy), and then triggered into dormant status. Cell viability results demonstrated that dormant ER+ MCF7 breast cancer cells were more resistant to IR or Doxo (Figure 2A). Treatments for breast cancer usually involves IR or the chemotherapy agent, Doxo, which kill cells by inducing DNA double strand breaks (DSBs). Therefore, we hypothesized that dormant cells may be more tolerate to DSBs after DNA-damage reagent treatment. Neutral-pH comet assays were applied to determine the number of DSBs in cells undergoing dormancy (Figure 2B and 2C). In dormant ER+ MCF7 breast cancer cells, Doxo-treated or IR-treated cells had a decreased number of DSBs based on quantitation of tail moment of comet assays indicating that a higher portion of cells had intact DNA. (Figure 2B-D). These results suggest that dormancy status can facilitate DNA damage repair. Next, we sought to determine if dormant cells utilize homologous recombination (HR) or non-homologous end joining (NHEJ) repair pathways. Western blotting indicated that phosphorylated-DNA-PKcs protein level, a well known marker for NHEJ activity, was upregulated whereas Rad51, a known protein involved in HR, was reduced (Figure 2E). These results suggest that NHEJ repair may be predominantly active in ER+ dormant cells

and possibly plays a critical role in protecting dormant ER⁺ breast cancer cells from DNA-damage induced cell death.

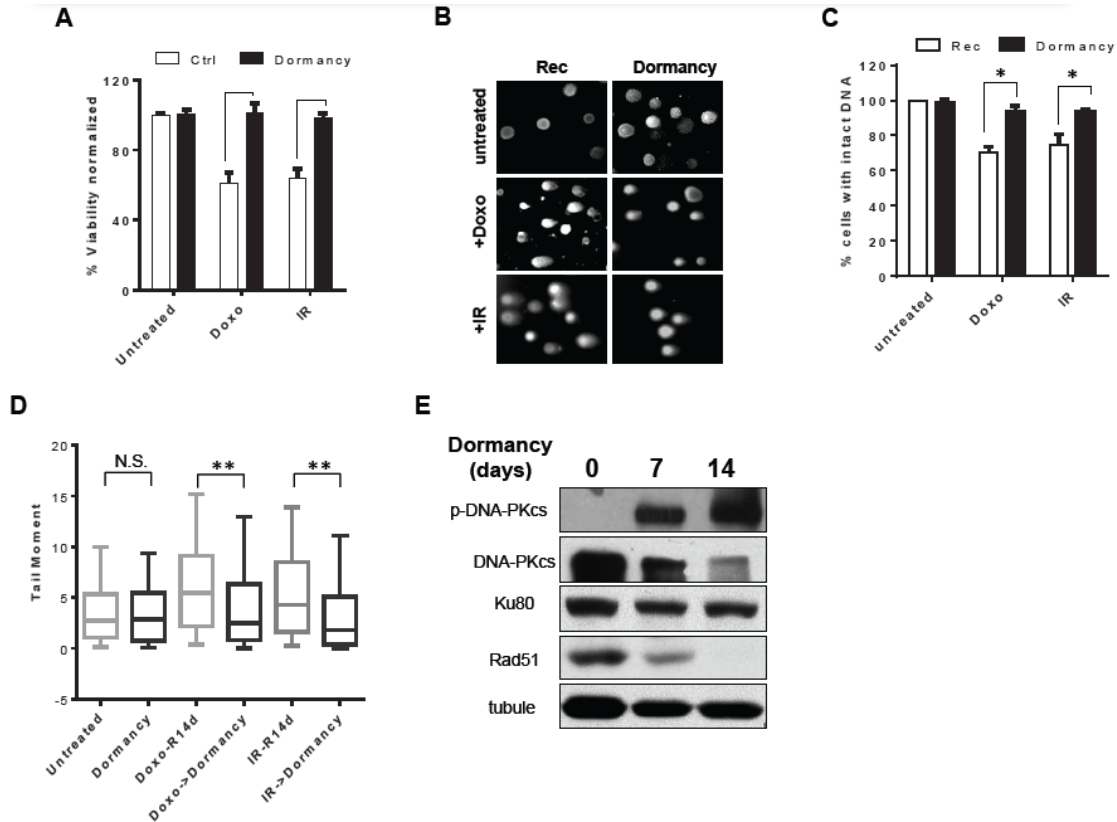


Figure 2. Dormant ER⁺ breast cancer cells reduced IR- or Doxo-induced DSB breaks. (A) ER⁺ (MCF7) cells were pretreated with doxorubicin (Doxo, 250 nM) or exposed with irradiation (IR, 8Gy), and then triggered into dormancy or normal growth condition for 14 days. The percent of viability was calculated for each treatment (A) and neutral comet assays (B) were applied to determine the levels of double strand break of DNAs. (C) Quantification of double-stranded DNA breaks by a neutral comet assay. At least 100 cells were scored in each sample and each value each value represents the mean $\pm$ s.d of three independent experiments. A Student t-test with a $p < 0.05$ was used to determine significance. (D) Tail moment was calculated. A Box whisker plot was plotted to determine the variation between the tail moments. Differences between distributions were assessed using the Mann-Whitney rank sum test. ** $P < 0.001$, N.S., not significant. (E) Western blot in ER⁺ cells for proteins involved in the NHEJ pathway. Phosphorylated-DNA-PKcs protein level was upregulated whereas Rad51, a known protein involved in HR, was reduced. These results suggest that NHEJ repair is predominantly active in ER⁺ dormant cells.

To further confirm a higher NHEJ activity in dormant ER⁺ MCF7 cells compared to the proliferative control cells, we performed an NHEJ report assay [6] to compare the NHEJ activity in both cell populations. In this assay, BamHI-cleaved pCSCMV-tdTomato was used as a target for NHEJ and pEGFP-C1 as a control plasmid. Briefly, MCF7 cells were co-transfected with pCSCMV-tdTomato plasmid (Plasmid 30530; Addgene, Cambridge, MA, USA) digested with BamHI, which cleaves between the promoter and the tdTomato open reading frame, and pEGFP-C1 of DNA. Cells were then either induced for dormancy or grown in the regular culture condition. The counts of tdTomato-expressing and GFP-expressing cells were estimated with LSRFortessa (BD Biosciences) and data were analyzed with FlowJo Version 10 (Tree Star, Ashland, OR). As shown in the Figure 3, compared to the control cells, dormancy led to a three-fold increase of NHEJ report activity, indicating a higher NHEJ activity during dormancy.

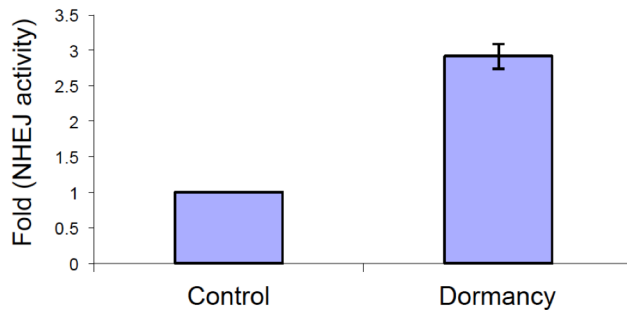


Figure 3. NHEJ activity is increased in dormant ER+ MCF7 cells. NHEJ activity was reported as the ratio of cells that were double positive for red (dTomato+) and green (GFP+) fluorescence to the total cells that were only positive for green fluorescence (GFP+). The level of NHEJ activity in normal culture condition was set as 1.

We also examined whether dormancy status protects ER- MDA-MB-231 breast cancer cells from DNA damage. In contrast to dormant ER+ breast cancer cells, dormant ER- breast cancer cells did not reduce IR or Doxo-induced cell death (data not shown). Neutral-pH comet assay reveal that dormant status in ER- breast cancer indeed led to a reduced of DNA repair reflected by higher tail moments in cells (Figure 4). Overall, our data demonstrated that dormant ER+ breast cancer cells can facilitate NHEJ repair, however ER- breast cancer cells do not undergo effective DNA repair during dormancy.

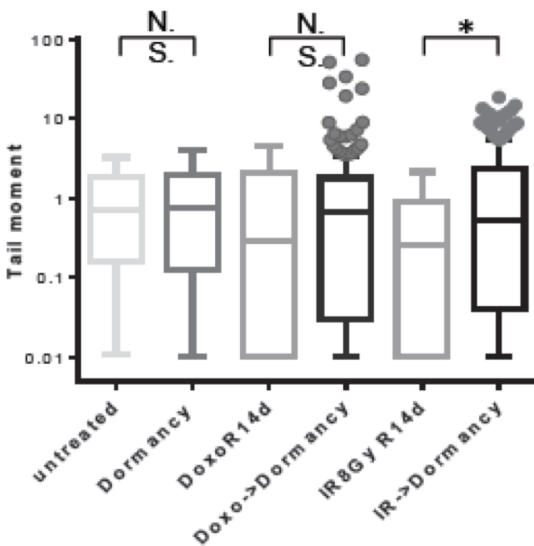


Figure 4. Dormancy didn't protect ER- breast cancer cells from DSB breaks. Tail moment was calculated. A Box whisker plot was plotted to determine the variation between the tail moments. Differences between distributions were assessed using the Mann-Whitney rank sum test. *P < 0.01, N.S., not significant.

Dormant cancer cells are suggested to be implicated in the recurrence and metastasis of breast cancer after they exit from dormancy. Therefore, we sought to determine whether cancer cells that exited from dormancy are more malignant using this *in vitro* dormancy model. Five different populations of MCF7 cells were established to investigate the effects of dormancy, including cells that have recovered from dormancy without the treatment of IR or doxorubicin (Dormancy-R), IR-treated (IRDormancy-R) and Doxo-treated (DoxoDormancy-R) cells forced into dormancy and later recovered, as well as cells recovering from IR (IR-R) or Doxo (Doxo-R) treatment without dormancy induction (Figure 5). Given that ER- breast cancer cells were extremely sensitive to doxorubicin, we had difficulties in development of ER- breast cancer cells recovered after doxorubicin treatment. To determine if the cell populations that exited from dormancy are more drug resistant and more malignant, we first re-challenged these cells with higher doxorubicin dose, and also determined their radiosensitization. In ER+ breast cancer cells, cells without pretreatment with DNA damage reagents had no change in sensitivity to doxorubicin or irradiation after exit from dormancy. Interestingly, cells pretreated with IR or Doxo after exit from dormancy (IRDormancy-R and DoxoDormancy-R) became more resistant to

doxorubicin and irradiation, compared to cells only treated with DNA damaging reagents (IR-R and Doxo-R) (Figure. 5A and 5B). In addition, anchorage-independent soft agar colony formation assays reveal that cells pretreated with IR or Doxo after exit from dormancy (IRDormancy-R and DoxoDormancy-R) had significantly increased soft agar colony formation ability, compared to control cells. (Figure 5C). In contrast, in ER- breast cancer cells, exit from dormancy did not promote cells to be more resistant to doxorubicin or irradiation (Figure 5D and 5E). Interestingly, IR-pretreated cells that exited dormancy had significantly reduced soft agar colony formation ability (Figure 5F). In sum, these data demonstrate that exit from dormancy enhances the malignancy of ER+ breast cancer cells, but attenuates the malignancy of ER- breast cancer cells.

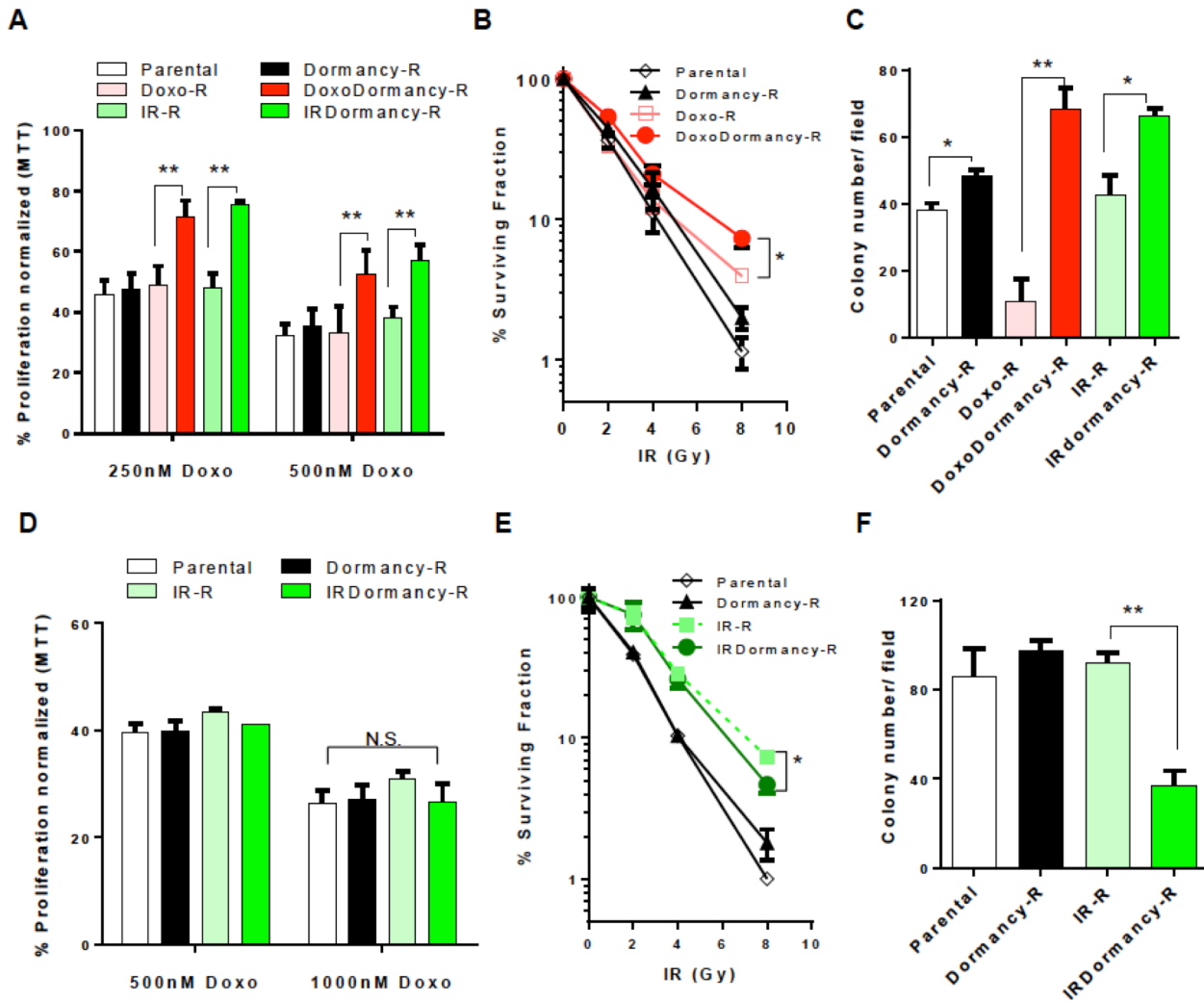


Figure 5. Exit from dormancy promotes malignancy in ER+ but attenuates malignancy in ER- breast cancer cells. ER+ (MCF7) and ER- (MDA-MB-231) cells were treated with DNA damaging reagents, induced into dormancy for 14 days and then forced to exit dormancy. The six treatments are as follows: Parental (Control) - cells without treatment; Dormancy-R- cells recovered after exit from dormancy; Doxo-R- cell recovered from Doxo treatment (250-1000 nM, 48 or 72h). DoxoDormancy-R- Doxo-pretreated cells following dormancy recovered after exit from dormancy; IR-R-: cells recovered from IR treatment (8Gy); IRDormancy-R: IR-pretreated cells following dormancy recovered after exit from dormancy. (A) After exiting dormancy, the MCF7 cell lines were re-challenged with Doxo (250 nM or 500 nM for 72h) and proliferation rate was analyzed by MTT assay. Each value is relative to the parental (untreated) cell line. (B) Log-linear survival curves for the MCF7 cell lines cell lines exposed with irradiation (IR) as the indicated dose (0, 2, 4 and 8Gy). Radiosensitization was assessed by clonogenic survival assay. (C) The MCF7 cell lines were cultured in soft agar and the foci numbers were counted. (D) The indicated MDA-MB-231 cell lines after exit from dormancy were re-challenged with Doxo (500 nM or 1000 nM for 72h), and proliferation rate was analyzed by MTT assay. Each value is relative to the value in the untreated cells. (E) Log-linear survival curves for the indicated MDA-MB-231 cell lines exposed with various doses of irradiation (0, 2, 4 and 8Gy). Radio-sensitization was assessed by clonogenic survival assay (mean $\pm$ standard deviation, n=3). (F) The MDA-MB-231 cell lines were cultured in soft agar and the colony numbers were counted. Significance was determined using a Student t-test, **p<0.001, *p<0.01, N.S.: not significant.

Task 2. To identify the underlying mechanisms and molecular determinants that mediate breast cancer cell dormancy.

To search for the potential mechanisms and key genes involved in dormancy-promoting malignancy, we applied mRNA microarray analysis in the ER+ and ER- breast cancer cell lines. There were 383 and 248 differentially expressed genes in ER+ MCF7, or ER- MDA-MB-231, cells that were treated with DNA damaging reagents, driven into dormancy, and then allowed to recover (Figure 6A and 6B). Of those genes, 168 and 106 genes were

up-regulated and 215 and 142 were down-regulated in the ER+ or ER- dormancy promoting malignancy (Figure 6A and 6B).

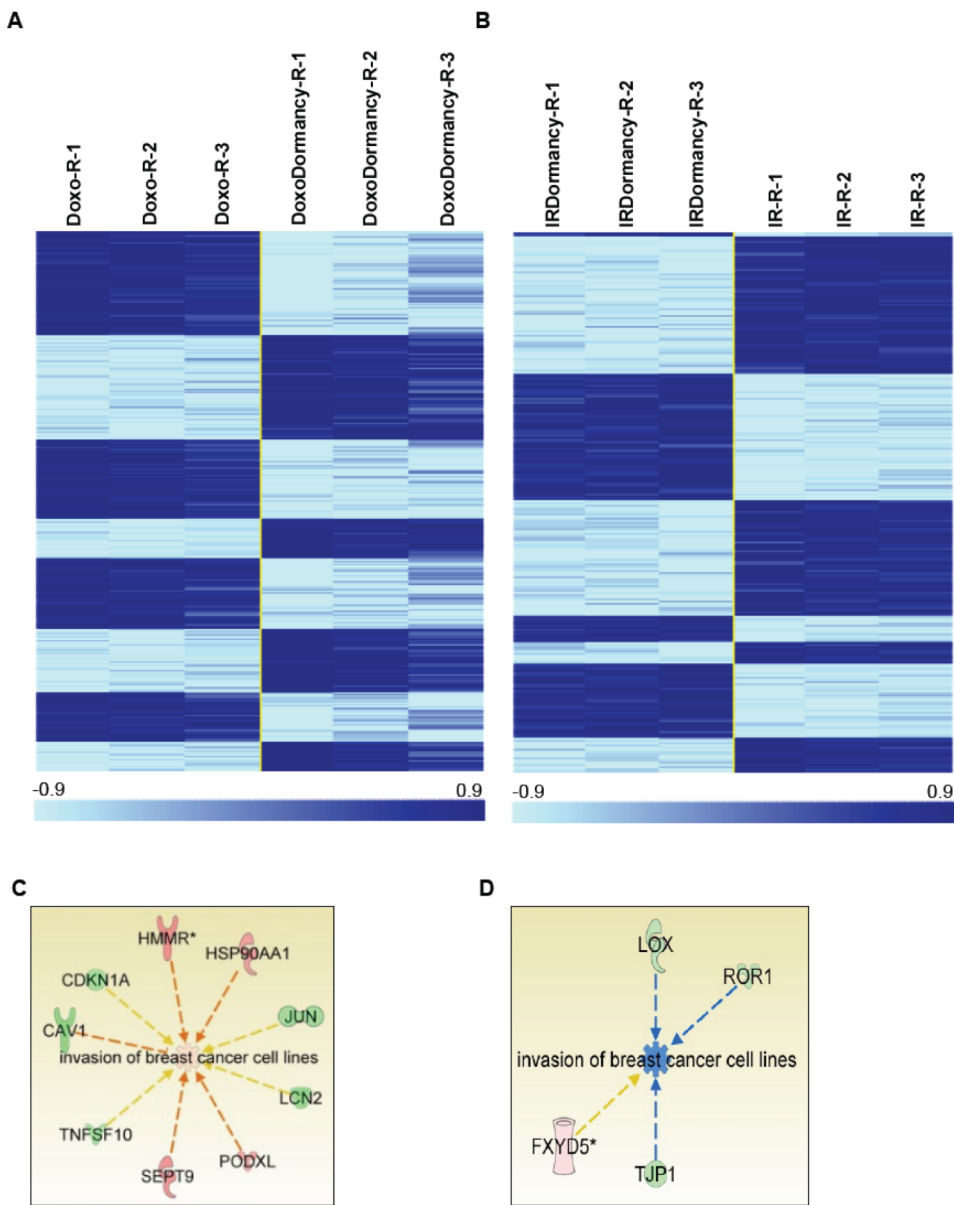


Figure 6. ER+ cells increase but ER- cells decrease malignancy via transcriptome reprogramming after exiting dormancy. Heatmap of differentially expressed genes in (A) MCF7 or (B) MDA-MB-231 cells treated with DNA damaging reagents, driven into dormancy, and then permitted to exit dormancy. Significance was determined by a Two-sample T-test (with random variance model) with a p-value= 0.001 comparing cells that were only treated with DNA damaging agents and then allowed to recover to cells that were forced into dormancy prior to recovery. IPA analysis revealed that invasion of breast cancer cell lines was activated in ER+ (C) but repressed in ER- (D) cells following dormancy, compared to untreated or parental cells.

The disease and functions analysis within Ingenuity Pathway Analysis (IPA) results suggested that the malignant ER+ breast cancer cell line has an increase in invasion ability and proliferation whereas ER- breast cancer cell line has potential in decreased invasion ability (Figure 6C, 6D and Table 1). IPA analysis also indicated potential key genes involved in promoting cellular proliferation associated with malignancy in ER+ breast cancer cells, such as replication factor C (activator 1) 3 (*Rfc3*), septin 9 (*Sept9*), and Interferon Regulatory Factor 1 (*Ifr1*). IPA analysis also revealed the possible genes involved in attenuated ER- breast

cancer malignancy such as tight junction protein 1 (*Tjp1*) and receptor tyrosine kinase-like orphan receptor 1 (*Ror1*).

MCF7 cells		
Diseases or Functions Annotation	Activation z-score	p-Value
proliferation of breast cancer cell lines	2.598	2.24E-04
proliferation of cells	2.514	5.82E-05
invasion of breast cancer cell lines	0.277	7.17E-03
apoptosis of breast cancer cell lines	-2.123	2.78E-05
MDA-MB-231 cells		
Diseases or Functions Annotation	Activation z-score	p-Value
invasion of breast cancer cell lines	-1.072	2.36E-02

Table 1. IPA analysis of IR- or Doxo-pretreated MCF7 and MDA-MB-231 cells after exiting dormancy

NHEJ is an error-prone DNA damage repair pathway, and can promote more genomic instability during tumorigenesis [7]. NHEJ is predominantly active during dormancy for double strand breaks repair in ER+ breast cancer cells. Therefore, we hypothesize that ER+ breast cancer cells exit from dormancy has more genomic instability that may lead to more malignant phenotype. Whole exome sequencing (WES) analysis was used to analyze DNA extracted from the isogenic ER+ breast cancer cell lines: parental, dormancy-R, Doxo-R, and DoxoDormancy-R. After mapping with parental cells, somatic mutation sites were identified (Table 2). The summary of WES analysis indicated that Doxo-treated cells exiting from dormancy (DoxoDormancy-R, 1.47/per Mb) have a higher mutation rate than dormant cells not treated with DNA damaging reagents (Dormancy-R, 1.30/per Mb) and cells that were only treated with Doxo (Doxo-R, 1.22/ per Mb) without entering the dormancy (Table 2).

Samples	samples based sequencing	Ctrl based sequence d	sample exome coverage	Ctrl exome coverage	Callable positions	all SNP sites	nonsilence mutations	mutation rate (per Mb)	coding indels	all SNP counts
Dormancy-R	1.0E+10	1.4E+10	142	150	1.4E+08	187	154	1.30	33	3995
DoxoR	9.9E+09	1.4E+10	138	150	1.4E+08	175	147	1.22	28	3812
DoxoDormancy-R	1.1E+10	1.4E+10	146	150	1.5E+08	226	195	1.47	31	4563

Table 2. Dormancy increases somatic mutations in malignant ER+ breast cancer cells. Whole exome sequencing (WES) analysis was performed on genomic DNA isolated from ER+ (MCF7) cell lines. Preliminary results indicate that 317 somatic mutations were identified. These results suggest that the malignant cell line, DoxoDormancy-R cells, has a higher mutation rate than the control cells. SNP: single-nucleotide polymorphism; Indel: insertion and deletion.

Of these mutations, a list of seven candidate genes was identified to possible play of a role in ER+ breast cancer malignancy (Table 3).

Gene Name	cDNA shift	peptide shift	SNPs Name	AlleleA	AlleleB	Exon
frameshift deletion						
MYO16	c.2525delT	p.F842fs	chr13 109661393	T	-	exon22
TNRC6B	c.1566delG	p.Q522fs	chr22 40697195	G	-	exon16
nonsynonymous SNV						
CDC27	c.T1667G	p.V556G	chr17 45216160	A	C	exon13 COSMIC
	c.T1665G	p.D555E	chr17 45216162	A	C	exon13 COSMIC
	c.C1645T	p.L549F	chr17 45216182	G	A	exon13
	c.T1424G	p.M475R	chr17 45219364	A	C	exon12 COSMIC
	c.C1035A	p.S345R	chr17 45229225	G	T	exon9
	c.G794A	p.G265D	chr17 45234327	C	T	exon7 COSMIC
GLIPR1L2	c.G720C	p.K240N	chr12 75816819	G	C	exon4 COSMIC
NF1	c.T2297C	p.I766T	chr17 29554281	T	C	exon19
MARVELD3	c.A1145G	p.Y382C	chr16 71674842	A	G	exon3
stopgain SNV						
GLCE	c.G1854C	p.X618Y	chr15 69561583	G	C	exon5

Table 3. Whole exome sequencing analysis in malignant MCF7 cells exiting from dormancy.

Two examples of the unique somatic mutations in the malignant MCF7 cells (DoxoDormancy-R) were further confirmed using Sanger sequencing. Sequencing chromatograms results were compared between the MCF7 cell lines and mutation site in DoxoDormancy-R cells are marked as red star (Figure 7).

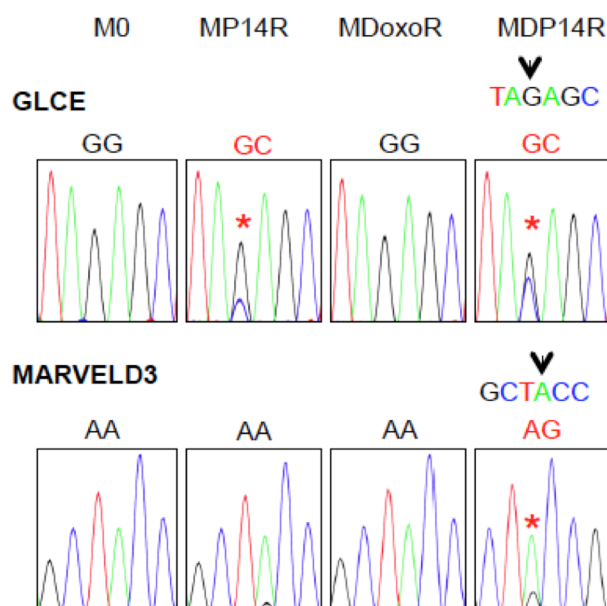


Figure 7. Sanger sequencing confirms somatic mutations in the ER+ (MCF7) cells. Two examples of the unique somatic mutations in the malignant MCF7 cells (DoxoDormancy-R) were confirmed using Sanger sequencing. Sequencing chromatograms results were compared between the MCF7 cell lines and mutation site in DoxoDormancy-R cells are marked as red star.

These results suggest that dormancy led IR- or Doxo-pretreated ER+ breast cancer cells more gene mutations, likely through its higher error-prone NHEJ repair activity. Further investigation on the altered functions of these mutated gene candidates would help us to identify the underlying mechanisms that promote malignancy and drug resistance of the recurrent ER+ breast cancer and the effective therapeutics for treatment.

KEY RESEARCH ACCOMPLISHMENTS

- (1) We established and tested a suitable cell dormancy model by Petaka incubation at 22 °C for up to 14 days.
- (2) Our studies indicated that dormancy of ER+ MCF7 but not ER- MDA-MB-321 cells reduced IR- or Doxorubicin-induced DNA double strand breaks by increasing NHEJ DNA repair.
- (3) We found that exit from dormancy promotes drug resistance and malignancy in ER+ breast cancer cells but attenuates malignancy in ER- breast cancer cells.
- (4) By transcriptome analysis and IPA, we demonstrated that ER+ cells increase but ER- cells decrease malignancy via transcriptome reprogramming after exiting dormancy. We have identified the potential pathways involved in this process.
- (5) By whole exome sequencing, we found that dormancy increases somatic mutations in malignant ER+ breast cancer cells.
- (6) We have identified mutations of 7 gene candidates which may enhance the malignancy of recurrent ER+ MCF7 cells.

REPORTABLE OUTCOMES

The interesting findings from our study have been presented by PI in an invited seminar at Baylor College of Medicine. PI has also been invited to present these interesting findings at an international conference in Taiwan next summer. In addition, a postdoctoral fellow in PI's lab will present this work at Exploring DNA Repair Pathways as Targets for Cancer Therapy' conference next February. Currently, a manuscript based on the outcomes of this study is in preparation for submission.

CONCLUSION

In this award, we have been focusing on the role of cellular dormancy in promoting cancer aggressiveness and drug resistance in recurrent breast cancer. We aimed to determine the impact of dormancy on breast cancer phenotype, and to identify the molecular determinants that mediate breast cancer dormancy. During this two-year of grant period, we have successfully established a reliable *in vitro* breast cancer dormancy cell model. Using this model, we tested and confirmed the hypothesis that we originally proposed in our grant proposal. As what we hypothesized, we found that ER+ but not ER- dormant breast cancer cells repaired double strand DNA breaks likely through the increased activity of the error-prone NHEJ pathway in cells, which led to higher gene mutations and transcriptome reprogramming. After exited from dormancy, these ER+ but not ER- breast cancer cells became more drug-resistant and more malignant compared to the parental cells. More importantly, our work on whole exome sequencing and transcriptome analysis revealed several potential molecules involved in dormancy-mediated recurrence and malignancy which may serve as important diagnosis biomarkers and therapeutic targets for breast cancer recurrence prevention and therapy in the future.

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