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Award Number: W81XWH-10-1-0558

TITLE: Characterizing and Targeting Replication Stress Response Defects in Breast Cancer

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REPORT DATE: August 2011

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE August 2011		2. REPORT TYPE Annual		3. DATES COVERED 15 July 2010 – 14 July 2011	
4. TITLE AND SUBTITLE Characterizing and Targeting Replication Stress Response Defects in Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0558	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Shiaw-Yih Lin, Ph.D. E-Mail: sylin@mdanderson.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of Texas M.D. Anderson Cancer Center Houston, TX 77030				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 Replication stress response (RSR) is a subset of the DNA damage response that safeguards the replication process; defects in RSR allow the survival of genomically unstable cells, ultimately leading to breast cancer. Since the initial RSR defects occur before cancer develops, RSR defects can serve as a powerful biomarker to predict the risk of cell transformation. We have generated an oncogene-induced RSR breast cell model, in which cyclin E can be conditionally induced to trigger RSR in normal breast cells. Using this model, we demonstrated that when RSR genes, such as ATM, ATR, Chk1 and the two novel RSR genes, PRMT5 and TUSC4, was depleted, cells escaped the oncogene-induced growth arrest. These results clearly demonstrate a critical role of RSR genes in preventing oncogene-induced cell transformation. In addition, we successfully validated our RSR-defect gene signature and we found that luminal but not basal-like breast cell lines are more RSR-defective. This is an important discovery and we will further pursue this study to understand the underlying mechanisms and its potential application in clinic in the future.					
15. SUBJECT TERMS Replication stress response, Cyclin E, Gene signature					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	13	19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

In both precancerous breast lesions and breast cancer, hyperproliferative activity due to oncogene activation or loss of tumor suppressor genes induces stalling and collapse of DNA replication forks, which in turn activates the replication stress response (RSR) to maintain genome integrity [1-4]. RSR is a subset of the DNA damage response that safeguards the replication process [5]; defects in RSR allow the survival and proliferation of genomically unstable cells, ultimately leading to breast cancer [4-6]. Since the initial RSR defects occur before cancer develops, RSR defects can serve as a powerful biomarker to predict the risk of cell transformation. Importantly, the presence of RSR defects distinguishes premalignant lesions and breast cancer from normal tissues, which makes these defects effective targets for both breast cancer prevention and breast cancer treatment. This project is to use cutting-edge technologies to characterize novel RSR genes and their functions in tumor suppression; identify gene signature and membrane proteins associated with defective RSR; identify drugs that target these defects; and develop RSR-defect-targeting nanoparticles for diagnostic imaging, prevention, and treatment of breast cancer.

BODY

The tasks involved in our first-year research include: Task 1a, Task 2a and Task 4a from our final version of Statement of Work.

Task 1a: To validate the RSR gene candidates that we have identified and choose five top candidates to characterize their functions in response to replication stress.

From our previous siRNA screening, we have identified many potential RSR genes. Here, we sought to validate and characterize the five promising RSR gene candidates, DNA2, TUSC4, RNF2, MCM5 and PRMT5 and determine what types of replication stress can activate these genes and what cell responses, such as cell cycle arrest and/or DNA synthesis inhibition, will be triggered by these RSR genes in response to the replication stress.

In this project, we induced replication stress in a non-transformed breast cell line, MCF10A, by overexpressing an important oncogene in breast cancer, cyclin E. We established an inducible-cyclin E 10A cell line in which cyclin E was fused to V5 tag and cloned into a tetracycline-inducible system (Invitrogen) so its expression can be induced by adding doxycycline to the medium. We named this cell line as 10A/TR-CycE. As shown in Figure 1, cyclin E was significantly induced in 10A/TR-CycE cells with addition of doxycycline (+Dox) compared to without doxycycline addition (-Dox) and to the parental MCF10A cells.

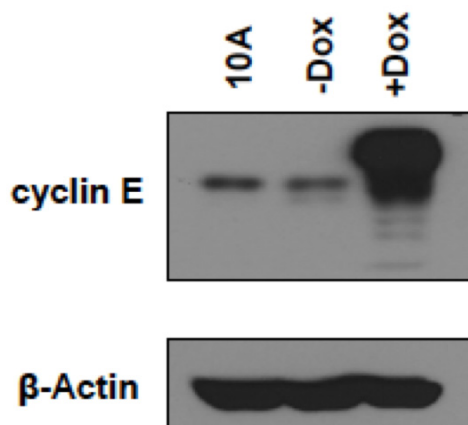


Figure 1. Establishment of cyclin E-inducible breast cell line. Cyclin E expression was induced in 10A/TR-CycE cells by doxycycline treatment.

As expected, cyclin E induction led to growth inhibition measured by clonogenic assay. As shown in Figure 2, doxycycline-induced cyclin E expression significantly inhibited cell growth in 10A/TR-CycE cells compared to without cyclin E-induction or to the MCF10A parental control cells. This growth inhibition was triggered by robust replication stress response in cells to counteract the hyper-proliferative signals from cyclin E overexpression.

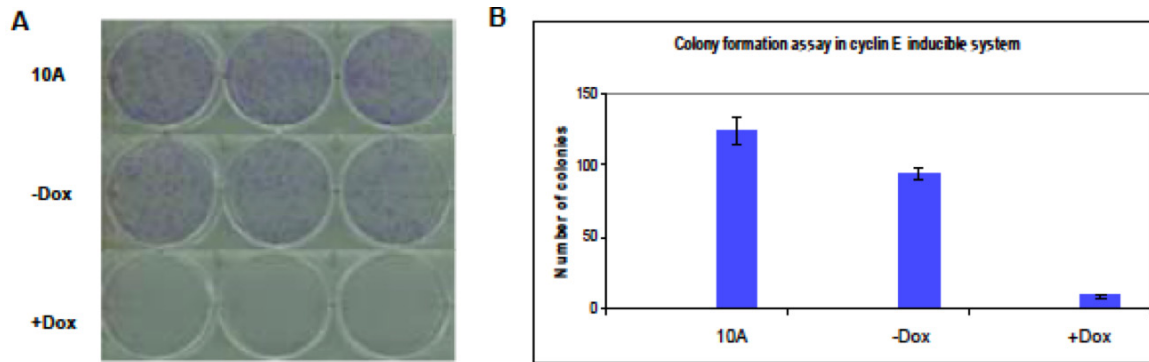


Figure 2. Cyclin E overexpression inhibits breast cell growth (A) Representative images of clonogenic assay that measures cell growth in parental MCF10A cells and in 10A/TR-CycE cells without (-Dox) or with (+Dox) doxycycline induction. (B) Quantitative summary of the clonogenic assay. Each value represents the mean $\pm$ SD of three independent experiments.

Since oncogene-induced growth inhibition mainly results from replication arrest [1-3], we expected to detect a reduction of BrdU incorporation after cyclin E induction. As shown in Figure 3, Cyclin E overexpression significantly reduced BrdU incorporation.

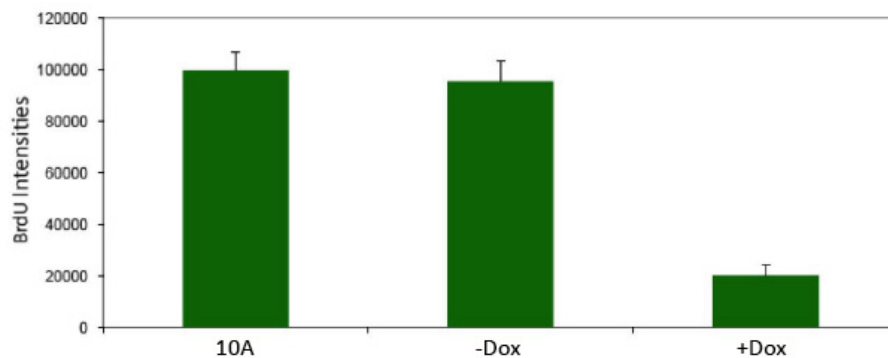


Figure 3. Cyclin E overexpression reduces BrdU incorporation. (A) BrdU incorporation was reduced when 10A/TR-CycE was treated with doxycycline (+Dox) compared to without doxycycline treatment (-Dox) and to the parental MCF10A cells. Each value represents the mean $\pm$ SD of three independent experiments.

The growth inhibition from cyclin E induction is also consistent with the molecular changes in the cells. As shown in Figure 4, cyclin E induction led to increase of γ -H2AX and p53, reflecting stress-induced DNA double-strand breaks and checkpoint activation.

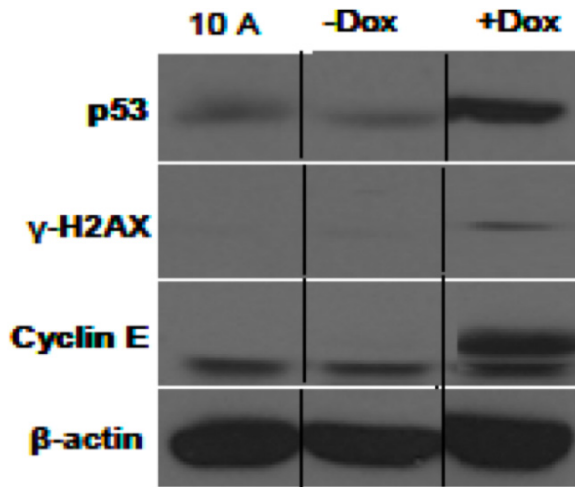


Figure 4. Cyclin E overexpression leads to DNA damage and p53 stabilization. Cell lysates from MCF10A or 10A/TR-CycE cells treated with (+Dox) or without (-Dox) doxycycline were harvested and immunoblotted with antibodies against indicated molecules.

With the oncogene-induced RSR system established, we next sought to validate the five promising RSR candidate genes that we previously identified through siRNA screen. It has been known that knockdown of RSR genes can overcome the oncogene-induced growth inhibition and therefore resume the BrdU incorporation (i.e., DNA synthesis) in non-transformed cells [2,3]. We first sought to reproduce these data in our system by knocking down the known RSR genes such as ATM, ATR, and Chk1 as the positive control [2,3]. As shown in Figure 5, knockdown of these known RSR genes resumed BrdU incorporation compared to the control siRNA treatment.

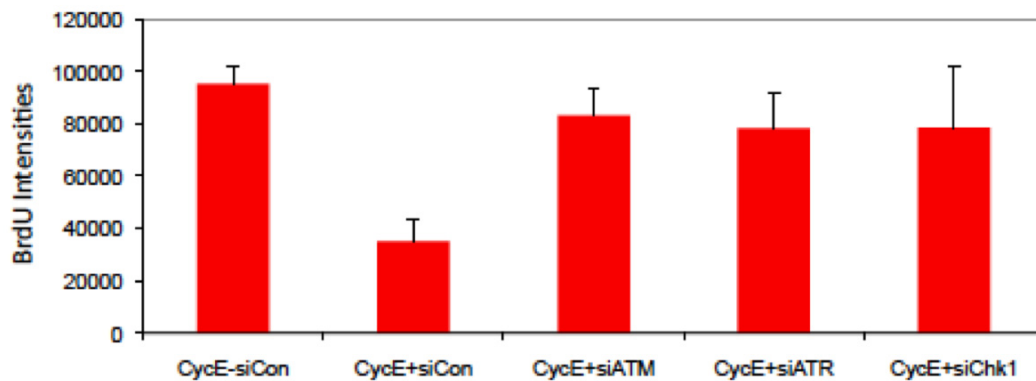


Figure 5. Knockdown of known RSR genes rescues BrdU incorporation in Cyclin E overexpressed cells. Cyclin E-uninduced (CycE-) or Cyclin E-induced (CycE+) 10A/TR-CycE cells were transfected with indicated siRNA and measured with the levels of BrdU incorporations. Each value represents the mean $\pm$ SD of three independent experiments.

We then knocked down our five RSR candidates in 10A/TR-CycE cells after cyclin E induction. Using SMARTpool siRNAs from Dharmacon, we successfully depleted the expression of all these candidate genes (Figure 6). Due to the lack of proper antibody against TUSC4, we confirmed TUSC4 depletion by RT-PCR (data not shown).

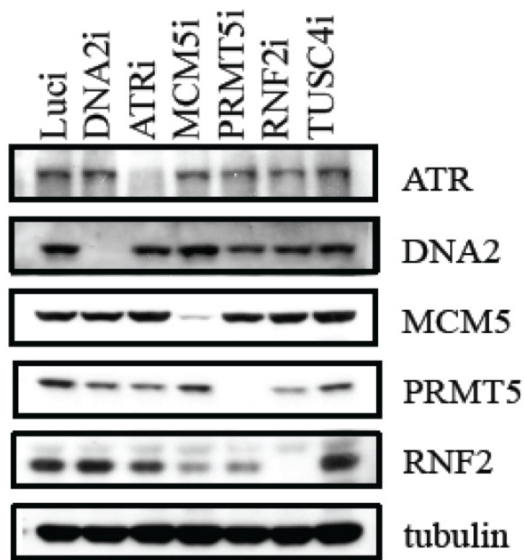


Figure 6. siRNAs effectively knock down the expression of the RSR gene candidates. 10A/TR-CycE cells were transfected with the indicated siRNAs and the lysates were harvested and immunoblotted with antibodies against the individual molecules.

As shown in Figure 7, depletion of PRMT5 and TUSC4 but not RNF2, MCM5 or DNA2 rescued the RSR-induced growth inhibition measured by BrdU incorporation, indicating only PRMT5 and TUSC4 are genuine RSR genes.

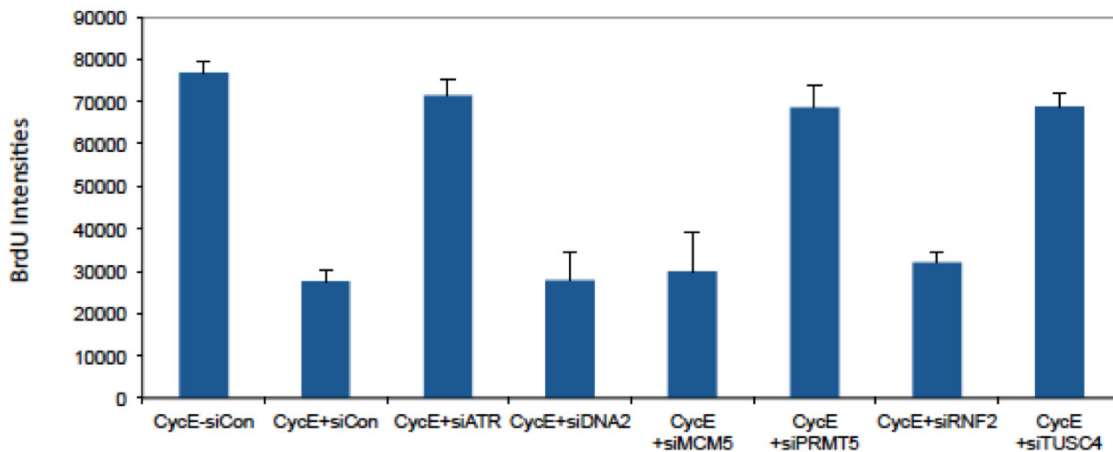


Figure 7. Knockdown of PRMT5 or TUSC4 rescues BrdU incorporation in Cyclin E overexpressed cells. Cyclin E-uninduced (CycE-) or Cyclin E-induced (CycE+) 10A/TR-CycE cells were transfected with indicated siRNA and measured with the levels of BrdU incorporations. Each value represents the mean $\pm$ SD of three independent experiments.

In addition to being involved in oncogene-induced cell growth inhibition, many RSR genes have also been shown to be required for replication re-start after replication stress induced by replication stalling agents such as hydroxyurea [7]. As shown in Figure 8, like ATR, a known RSR gene, depletion of TUSC4 or PRMT5 but not DNA2, MCM5 or RNF2 inhibited the replication re-start as indicated by delayed exit of cells from S phase. All of these data clearly indicate both TUSC4 and PRMT5 to be genuine RSR genes.

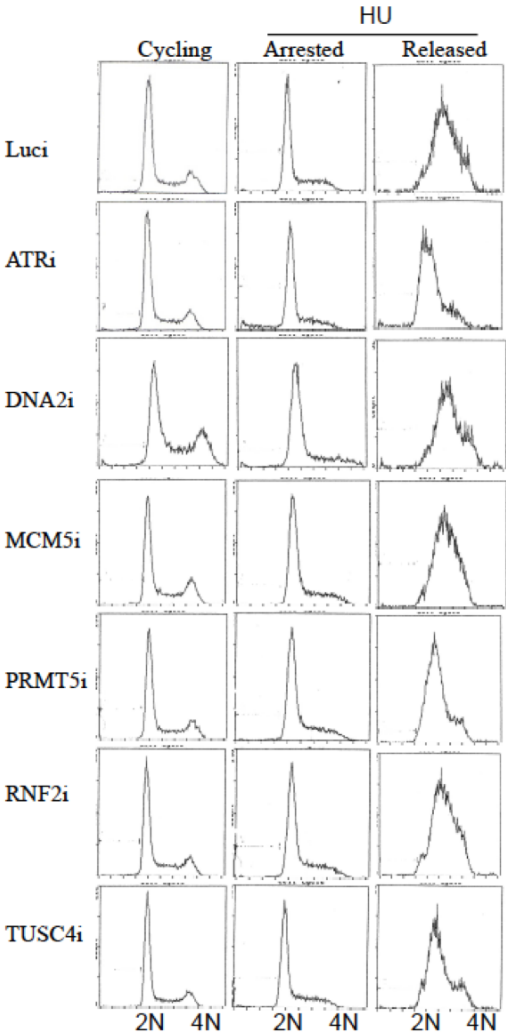
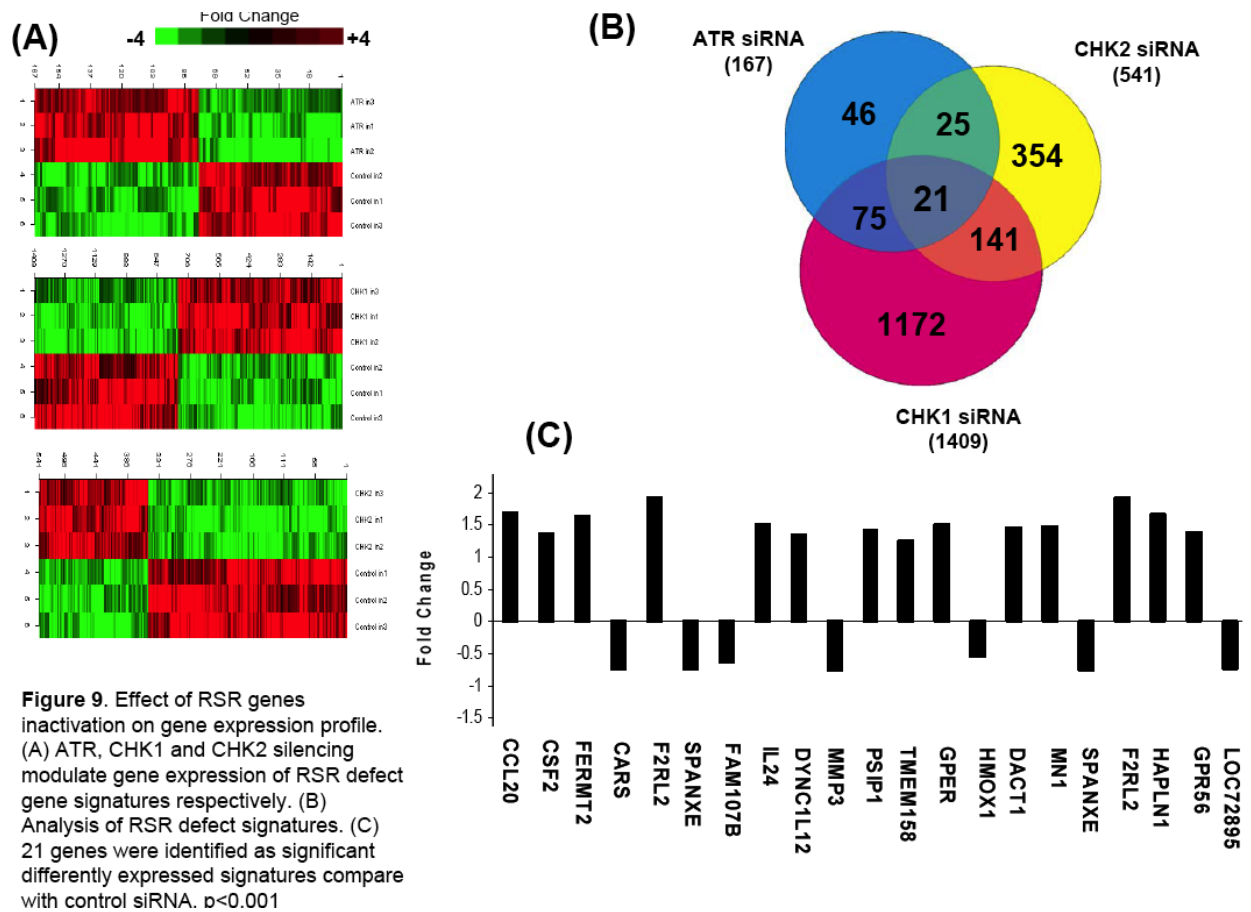


Figure 8. ATR, PRMT5 or TUSC4 are required for cells to complete DNA synthesis after replication stress. MCF10A cells were transfected with luciferase control or indicated siRNAs, treated with 3 mM HU for 20 h (arrested), and released into 1 μ g/ml nocodazole for 10 h (released). DNA content was analyzed by using flow cytometry.

Task 2a. To identify patterns of “RSR-defect” gene signature from a collection of highly characterized breast cancer cell lines.

By microarray analysis, we have previously identified a gene-expression signature that may represent cells lacking RSR. This gene signature was done by knocking down three RSR genes in the cyclin E-induced MCF10A cells and compared their common gene expression changes with the control (see Figure 9). We established a 21-gene signature that changes when all these RSR genes were depleted individually. We name this 21-gene set as RSR-defect gene signature.



We then compared the gene expression patterns of a large collection of common breast cancer cell lines with our gene signature. As expected, as shown in the Figure 10, the two normal breast cell lines, MCF10A and HBL100 lack the expression of RSR-defect gene signature (green), consistent with their intact RSR status. Indeed, our preliminary studies also confirmed MDA-MB-436 and MDA-MB-231 cells to be RSR-intact, and T47D and BT474 cells to be RSR-defect by BrdU incorporation assay as our signature predicted (data not shown).

More interestingly, we found that majority of luminal but not the basal-like breast cancer cell lines harbor this gene signature. The mechanism behind this interesting observation is currently unknown but we suspect that due to the intrinsic nature of genomic instability in basal-like breast cancer cells, oncogene-induced replication stress may not have played a major role in transformation of these cancer cells. Thus, these cells may still remain RSR intact. We will test this very interesting hypothesis in our future study.

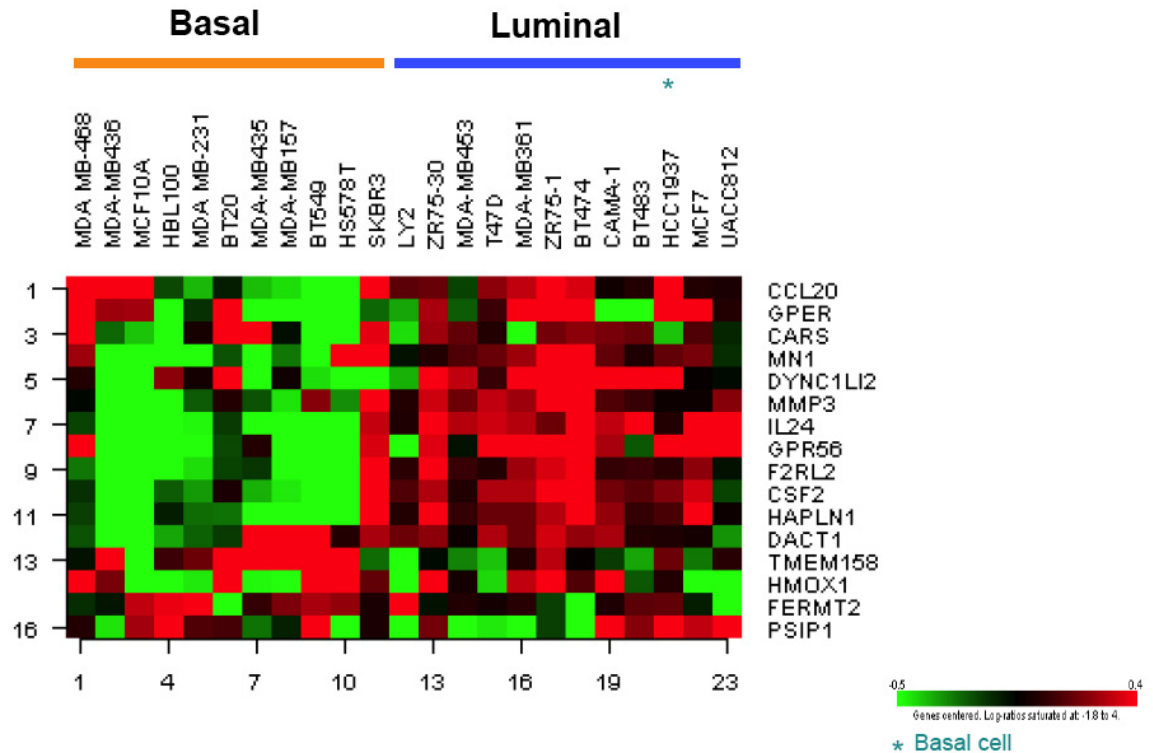


Figure 10. To identify patterns of “RSR-defect” gene signatures from a collection of highly characterized breast cancer cell lines. Two major groups, basal and luminal breast cancer cell lines were used to generate the hierarchical cluster. Luminal cell lines clearly shows higher expression levels of RSR-defect gene signatures than basal cell lines.

Due to the removal of Dr. Meric-Bernstam as a collaborator during the contract negotiation, we therefore didn’t further pursue our study on analyzing the potential clinical reference of our RSR defect gene signature at this stage of project.

Task 4a. To develop nano-imaging technology to detect RSR-defective breast cancer cells through binding of nano-imaging particles to the RSR-defect-specific membrane proteins.

In collaboration with our colleague Dr. Chun Li, an outstanding leader in nanotechnology, we aimed to develop nanoparticles that can carry in vivo imaging agents to target breast cancer cells with RSR-defect-specific membrane proteins for cancer detection. During the first 30 months of this project, Dr. Li will be working on optimizing the nanoparticle conjugation protocol so this protocol can be applied later when the RSR-defect membrane makers are identified.

Dr. Li has been using antibody against epidermal growth factor receptor (EGFR) as a testing model. To demonstrate the feasibility of coating homing ligand to gold nanoparticles, Dr. Li’s lab has synthesized gold nanoshells encapsulated with superparamagnetic iron oxide (SPIO), and successfully coated these nanoparticles with cetuximab or C225 monoclonal EGFR antibody to form C225-SPIO@Au NS. These gold nanoshells have an absorption peak in the near infrared (~800 nm), which results in preferential heating of these particles versus tissue upon NIR laser irradiation. These targeted multifunctional nanoparticles demonstrated contained T2 MRI contrast agent that allowed MR imaging.

Specific targeting of the synthesized C225-SPIO@Au NS was tested *in vitro* using A431 cells and oral cancer cells, FaDu, OSC-19, all of which overexpress epidermal growth factor receptors (EGFR). Figure 11 shows the selective binding achieved using C225-SPIO@Au NS but not with the non-targeting PEG-SPIO@Au NS and blocking group (excess of cetuximab + C225-SPIO@Au NS).

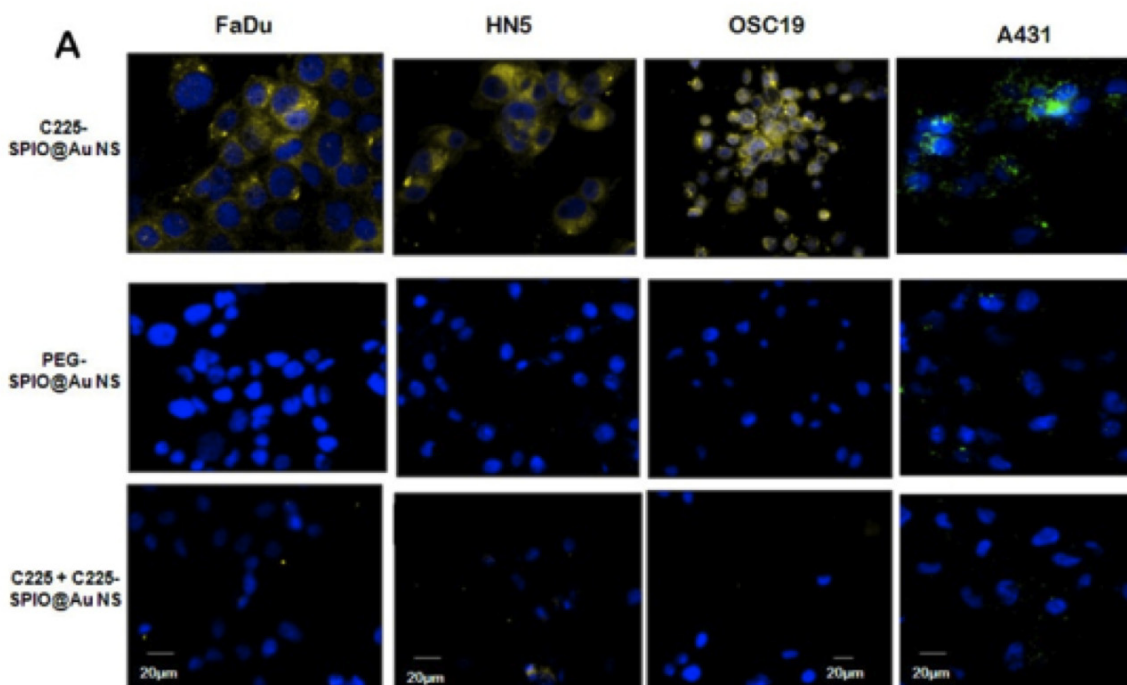


Figure 11. Selective binding of anti-EGFR-conjugated SPIO@Au NS to EGFR positive cells. FaDu, HN5, OSC19, and A431 cells were seeded onto a 96-well plate and incubated with C225- SPIO@Au NS (100 μ L, 1.0×10^{11} particles/mL), PEG-SPIO@Au NS (100 μ L, 1.0×10^{11} particles/mL), or C225 (500 μ g/mL) plus C225- SPIO@Au NS for 30 min at 37°C. Only cells incubated with C225- SPIO@Au NS had a strong light-scattering signal with the EGFR positive cell lines. Cells were stained with DAPI for visualization of cell nuclei (blue). Light-scattering images of nanoshells were pseudocolored green. Bar, 20 μ m.

The selective photothermal ablation of the nanoshells was also evaluated using A431 cells. Figure 12 shows that without laser treatment (images on top), there were no cell death and among the groups that were treated with laser at a power of 4W/cm² for 3 minutes (images below), only the cells treated with C225-SPIO@Au NS had cell killing. These results show the *in vitro* selective photothermal ablation of C225-SPIO@Au NS.

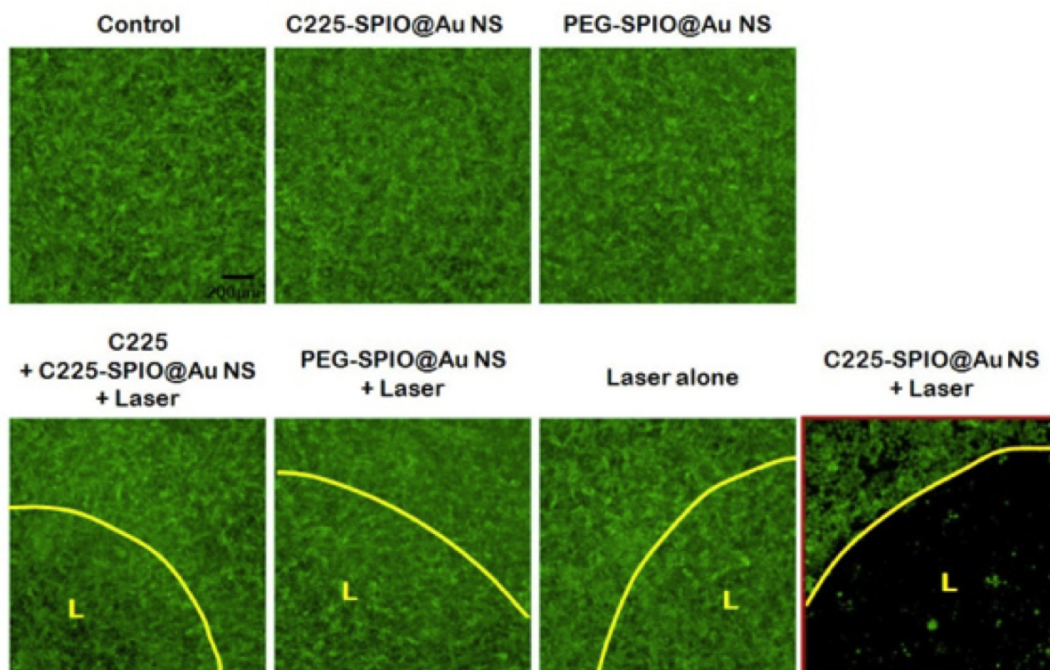


Figure 12. Cell viability after various treatments. Cells remained viable with no apparent death observed (cells were stained green with calcein) when treated with C225-SPIO@Au NS without laser, PEG-SPIO@Au NS and C225 + C225-SPIO@Au NS with and without NIR laser treatment, and laser alone. In contrast, most cells were dead after treatment with C225-SPIO@Au NS plus NIR laser. Power= 4 W/cm^2 for 3 mins.

KEY RESEARCH ACCOMPLISHMENTS

- (1) Establishment of a reliable oncogene-induced RSR breast cell model.
- (2) Identification of two novel RSR genes, PRMT5 and TUSC4 in response to the replication stress induced by oncogene or replication stalling.
- (3) Systematic analysis of RSR gene signature in a large collection of common breast cell lines from ATCC.
- (4) Discovery of the difference of RSR status between luminal and basal-like breast cell lines.
- (5) Successful development of gold nanoparticles conjugated with homing ligand to target on membrane protein such as EGFR.

REPORTABLE OUTCOMES

Part of the our study has led to an abstract publication and a poster presentation at Department of Defense Breast Cancer Research Program's 2011 Era of Hope conference at the Orlando World Center Marriott in Orlando, Florida.

CONCLUSION

In the first year of this project, we have successfully generated an inducible breast cell model to apply oncogenic stress in normal breast cells. This oncogenic stress derived from the induced cyclin E expression led to replication stress response in cells that, in turn, arrested DNA synthesis and inhibited

cell growth. This replication stress response, indeed, is considered as one of the first-line defense mechanisms that counteract the hyper-proliferative effect from oncogenic signals.

Our data also confirmed that when genuine RSR genes, such as ATM, ATR, Chk1 and the two novel RSR genes, PRMT5 and TUSC4, was depleted, cells escaped the DNA synthesis inhibition and growth arrest. These results clearly demonstrate a critical role of RSR genes in preventing oncogene-induced cell transformation.

In addition, we successfully validated our RSR-defect gene signature in a large collection of breast cell lines. Notably, our data appear to suggest that luminal but not basal-like cell lines are more RSR-defective. This is an important discovery and we will further pursue this study to understand the underlying mechanisms and its potential application in clinic.

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