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PRINCIPAL INVESTIGATOR: Shiaw-Yih Lin, Ph.D.

CONTRACTING ORGANIZATION: University of Texas M. D. Anderson Cancer Center  
Houston, TX 77030

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## INTRODUCTION

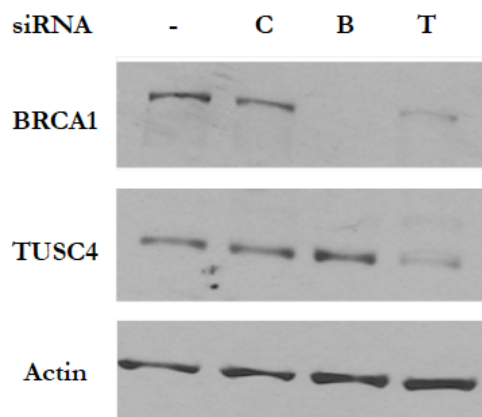
In both precancerous breast lesions and breast cancer, hyperproliferative activity due to oncogene activation or loss of tumor suppressor genes induces stalling and collapse of DNA replication forks, which in turn activates the replication stress response (RSR) to maintain genome integrity [1-4]. RSR 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. During the first two years of this project, we have validated TUSC4 as a novel RSR gene and established an RSR-defect gene signature. Here, we further investigated how TUSC4 mechanistically participates in RSR and whether TUSC4 functions as a novel tumor suppressor gene in breast cancer that suppresses mammary tumor formation in mice. In addition, we verified the RSR-defect-specific membrane proteins and developed nanoparticles that could detect RSR defect cells through these membrane markers. Finally, we have been continuing the screening and validation of drugs that target RSR-defect cells. The progress of our third year research is described below.

## BODY

The tasks involved in our second-year research include: Task 1b,c, Task 2d, Task 3b and Task 4a,b from our final version of Statement of Work.

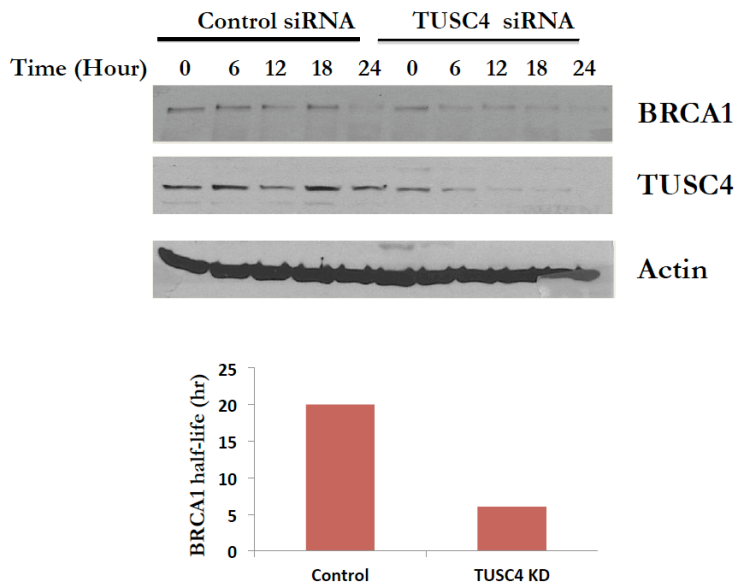
Task 1b. To study how these five RSR candidates mechanistically participate in the RSR network. As demonstrated in our previous progress report, among the five potential candidates, we have validated the function of TUSC4 and PRMT5 in response to replication stress, with TUSC4 as the most promising candidate required for homologous recombination (HR) DNA repair to resolve the replication stress.

To determine how TUSC4 is involved in HR repair, we examined the status of many key HR regulators, such as ATM, ATR, Chk1, BRIT1 and BRCA1 in TUSC4 knockdown cells. Intriguingly, we found that knockdown of TUSC4 by specific siRNAs reduced the expression of the potent tumor suppressor gene, BRCA1 (Figure 1).



**Figure 1. TUSC4 regulates the expression of BRCA1.** MCF10A cells were mocked transfected or transfected with luciferase control siRNA (C), BRCA1 siRNA (B) or TUSC4 siRNA (T). 48 hours after the transfection, cells were harvested for Western blotting and probed with antibodies against indicated proteins.

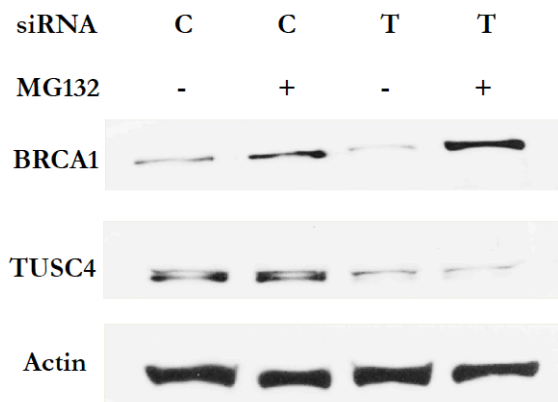
Since BRCA1 RNA level was not affected by TUSC4 knockdown (data not shown), we suspected that TUSC4 might regulate BRCA1 protein stability. To test this possibility, we compared BRCA1 protein turnover between control and TUSC4-knockdown cells in the presence of cycloheximide (CHX), which blocks protein synthesis. As shown in Figure 2, TUSC4 knockdown clearly led to a reduced half-life of BRCA1 protein from more than 20 hours to less than 6 hours, indicating an essential role of TUSC4 in the stabilization of BRCA1 protein.



**Figure 2. BRCA1 protein turnover is increased in the absence of TUSC4.**

Control or TUSC4-knockdown MCF10A cells were incubated with 10  $\mu$ g/ml of CHX for the periods of time indicated to inhibit protein synthesis. The lysates were harvested from cells and analyzed by Western blotting (top) and the half-life of BRCA1 protein in the control or TUSC4 knockdown cells was indicated (below).

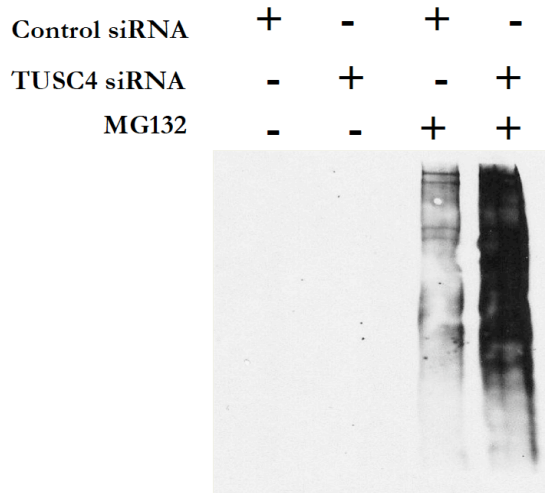
To determine if TUSC4 regulates BRCA1 protein stability through the proteasomal pathway, we treated control and TUSC4-knockdown cells with 10  $\mu$ M of the proteasome inhibitor MG132. MG132 treatment substantially increased BRCA1 protein levels in TUSC4-knockdown cells but only slightly increased BRCA1 expression levels in control cells (Figure 3) indicating that TUSC4 protects BRCA1 from proteasomal-dependent degradation.



**Figure 3. Proteasome inhibitor MG132 effectively rescues the expression level of BRCA1 in TUSC4-knockdown cells.**

Control (C) or TUSC4-knockdown (T) MCF10A cells were treated with 10  $\mu$ M of MG132 for 6 hrs and probed with antibodies against indicated proteins.

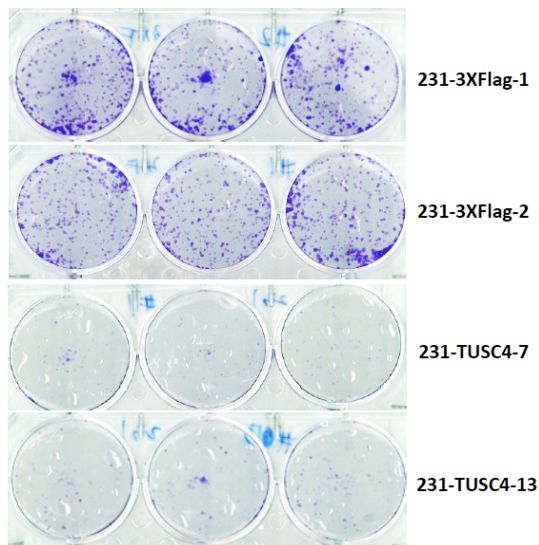
Since protein ubiquitination is a key mechanism that promotes proteasomal-dependent degradation, we thus tested if knockdown of TUSC4 increased BRCA1 ubiquitination. We performed an *in vivo* ubiquitination assay. First, MCF10A cells were cotransfected with plasmid encoding HA-tagged ubiquitin. The endogenous BRCA1 was then pulled down from cell lysates with anti-BRCA1 antibody. Subsequently, Western blotting against HA-tag was performed to detect ubiquitinated BRCA1. As predicted, we found a robust increase of polyubiquitinated BRCA1 protein in the TUSC4 knockdown cells compared to the control siRNA transfected cells with MG132 treatment (Figure 4).



**Figure 4. TUSC4 knockdown promotes BRCA1 ubiquitination.** Control or TUSC4-knockdown MCF10A cells were transfected with HA-ubiquitin vector. 24 hours after the transfection, cells were treated with or without 10  $\mu$ M of MG132 for 6 hrs and then harvested. Cell lysates were immunoprecipitated by anti-BRCA1 antibody and probed with anti-HA antibody.

Task 1c. To determine whether any of these five RSR candidates functions as tumor suppressor gene in breast cancer.

Previously, we have shown that depletion of TUSC4 increased the cell growth using clonogenic assay, indicating an anti-proliferative activity of TUSC4 in breast cells. To further determine if TUSC4 can suppress breast cancer *in vitro* and *in vivo*, we generated TUSC4 stable clones in MDA-MB-231 cell line, a highly dedifferentiated, invasive breast cancer cell line. As shown in the Figure 5, overexpression of TUSC4 significantly reduced growth of MDA-MB-231 cells.



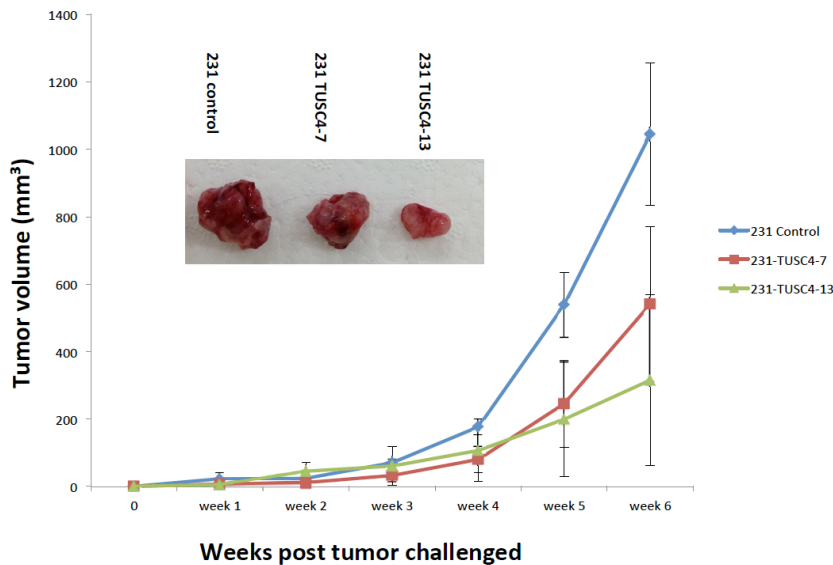
**Figure 5. Overexpression of TUSC4 inhibits breast cancer cell growth.** Representative images of clonogenic assay that measures cell growth in MDA-MB-231 cells stably transfected with the TUSC4 (clone 7 and 13) or empty vector (clone Flag-1 and Flag-2).

Next, we investigated the effect of TUSC4 expression in a xenograft mouse model. Mice were injected in the mammary glands with two independent TUSC4-overexpressing MDA-MB-231 cell lines or vector control MDA-MB-231 cells and monitored weekly for tumor formation. By week two, 3 out of 5 mice injected with TUSC4-overexpressing clones remained tumor free, whereas all 5 of the control mice had developed tumors (Table 1 and Figure 6), indicating a tumor suppressive function of TUSC4 *in vivo*.

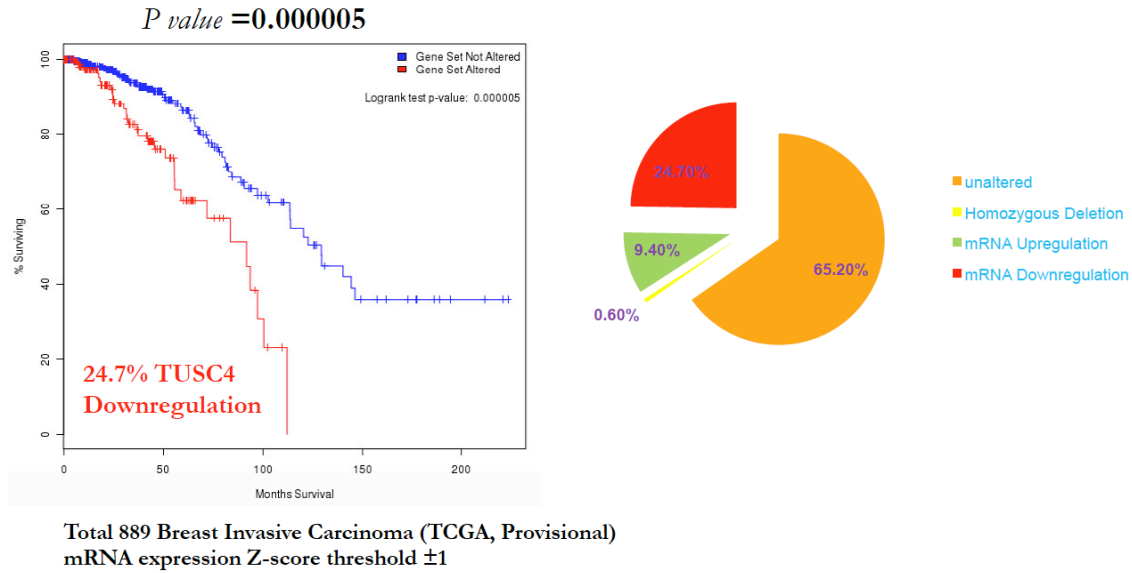
**Tumorigenicity of Orthotopically Implanted Control and TUSC4-overexpression MDA-MB-231 cells**

Number of Mice with Tumors (%)						
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
231 Control	3/5 (60)	5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)
TUSC4-7	1/5 (20)	2/5 (40)	3/5 (60)	3/5 (60)	3/5 (60)	3/5 (60)
TUSC4-13	2/5 (40)	2/5 (40)	2/5 (40)	2/5 (40)	2/5 (40)	2/5 (40)

**Table 1 and Figure 6. Tumorigenicity of orthotopically implanted control and TUSC4-overexpressing MDA-MB-231 cells.** Cells ( $5 \times 10^6$ ) from MDA-MB-231 control and two independent TUSC4-overexpressing MDA-MB-231 cell clones (TUSC4-7 and TUSC4-13) were injected into mammary gland of 6-week-old female nude mice. Each cell line was injected in 5 different mice and tumor volumes were measured every 2 days.



In addition to characterizing TUSC4 function *in vitro* and *in vivo*, we sought to determine if TUSC4 gene status correlated with the outcome of breast cancer patients. We analyzed TCGA data through Memorial Sloan-Kettering Cancer Center cBio cancer genomic portal. Total of 889 breast invasive carcinoma mRNA expression was plotted by Z-score with threshold  $\pm 1$ . Significantly, we found a good correlation between patients with reduced TUSC4 expression levels in their tumors and poor prognosis ( $p = 0.00005$ ), consistent with a tumor suppressive role of TUSC4 in breast cancer (Figure 7).



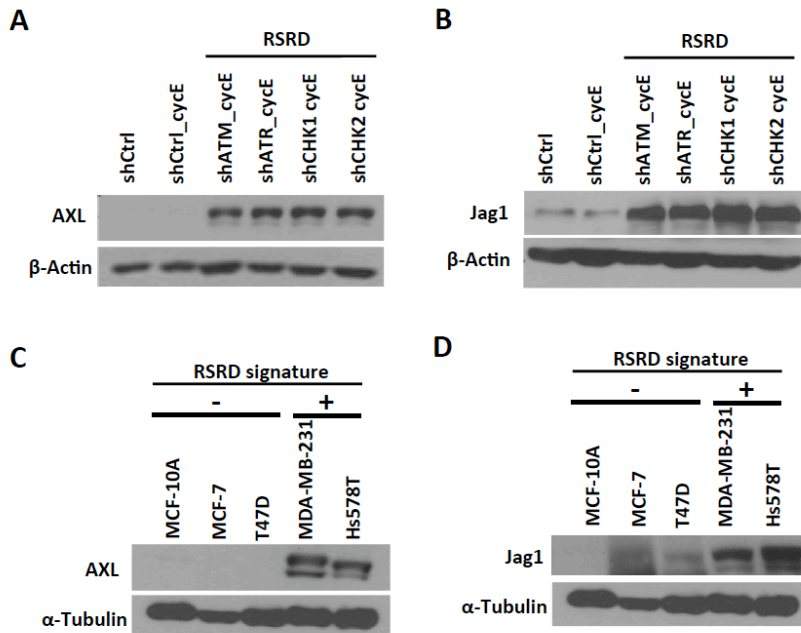
TCGA Data obtained from MSKCC  
cBio Cancer Genomics Portal

Figure 7. The expression level of TUSC4 is altered in human breast cancer and low TUSC4 level correlates with poor clinical outcomes

Task 2d. To validate the RSR-defect-specific membrane proteins.

In the past year, we have successfully validated two RSR-defect-specific membrane proteins, AXL and Jag1. We found that these two proteins were overexpressed in MCF10A cells knocked down with key

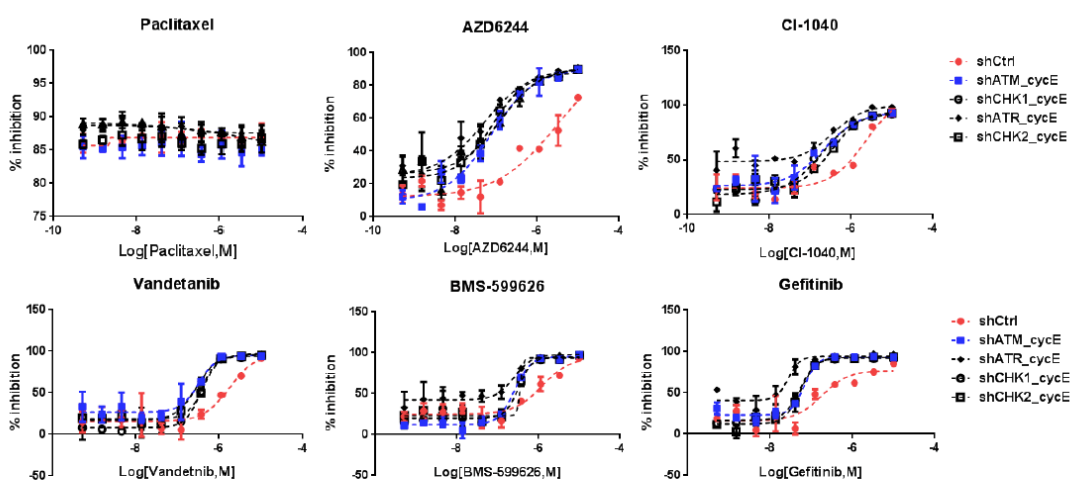
RSR genes, such as ATM, ATR, CHK1 and CHK2 (Figure 8A and 8B). The expression levels of these two membrane proteins could distinguish breast cell lines with RSR defect from cell lines without RSR defect. As shown in the Figure 8C and 8D, AXL and Jag1 were highly expressed in breast cancer lines that possess RSR defect (RSRD) gene signature (MDA-MB-231 and Hs578T) but not the cell lines without RSR defect gene signature (MCF-7, T47D and MCF10A). These data clearly validated AXL and Jag1 as promising biomarkers for RSR defective cells.



**Figure 8. AXL and Jag1 are membrane proteins upregulated in RSR-defect breast cells.** AXL and Jag1 were overexpressed in MCF10A cells when key RSR genes, such as ATM, ATR, CHK1 or CHK2 were stably knocked down (A and B). AXL and Jag1 were overexpressed in breast cells which present RSR defect gene signature such as MDA-MB-231 cells and Hs578T cells (C and D).

**Task 3b. Pilot study to test 5 top compound candidates selected from Task 3a for their effects on cancer cells.**

In collaboration with High-Throughput Screening Core Facility at the John S. Dunn Gulf Coast Consortia, we have identified top five compound candidates that can preferentially kill RSR defective breast cancer cells, including AZD-6244, CI-1040, Vandetanib, BMS-599626, and Gefitinib. As shown in the Figure 9 and Table 2, all these five candidate compounds can preferentially inhibit growth of RSR-defect cells when comparing the dose response curve and the half maximal inhibitory concentration ( $IC_{50}$ ) with the RSR intact control cells.



**Table 2.** The half maximal inhibitory concentration ( $IC_{50}$ ) of the 5 top compound candidates on RSR intact control (RSRI) and various RSR defective (RSRD) MCF10A cell lines. The  $IC_{50}$  is shown at  $\mu M$ .

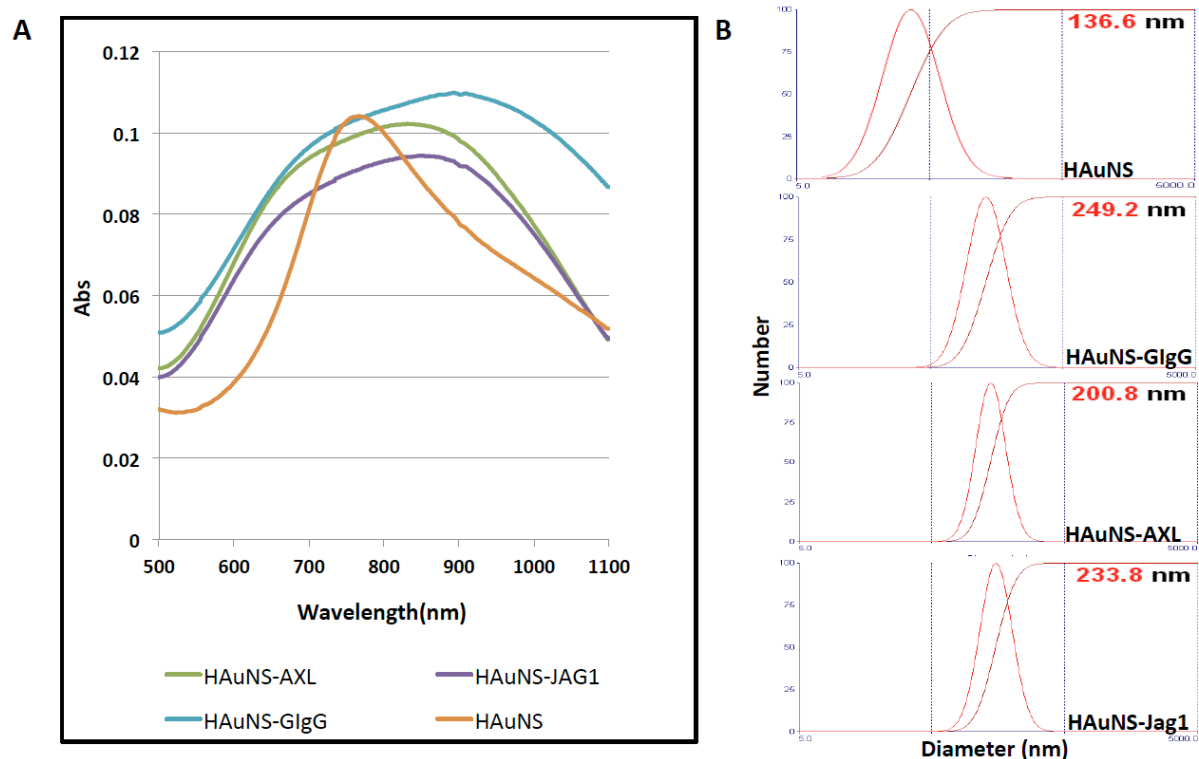
	shCtrl (RSRI)	shATM_cycE (RSRD)	shATR_cycE (RSRD)	shCHK1_cycE (RSRD)	shCHK2_cycE (RSRD)
AZD6244	2.956	0.07439	0.05201	0.07981	0.1273
CI-1040	2.29	0.2182	0.3548	0.188	0.3304
Vandetanib	1.685	0.3265	0.2943	0.3667	0.4231
BMS-599626	1.23	0.2553	0.2523	0.3247	0.3587
Gefitinib	0.1818	0.06765	0.02813	0.05316	0.05477

**Figure 9 and Table 2. Dose-response curves and  $IC_{50}$  of 5 top compound candidates that specifically kill RSR-defect cells.** The RSR-intact (RSRI) control MCF10A cells and the RSR-defect (RSRD) MCF10A derivative cell lines were treated with the five candidate drugs and Paclitaxel as the positive control. Percentage of inhibition (%) was calculated by the ratio of the total cell numbers between compound-treated and DMSO-treated dishes 48 hours after the treatments (Figure 9). Table 2 showed the  $IC_{50}$  of the indicated treatments ( $\mu M$ ).

In the next six months, we will test whether these five compound candidates can specifically kill breast cancer cells with RSR defects *in vivo*.

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.

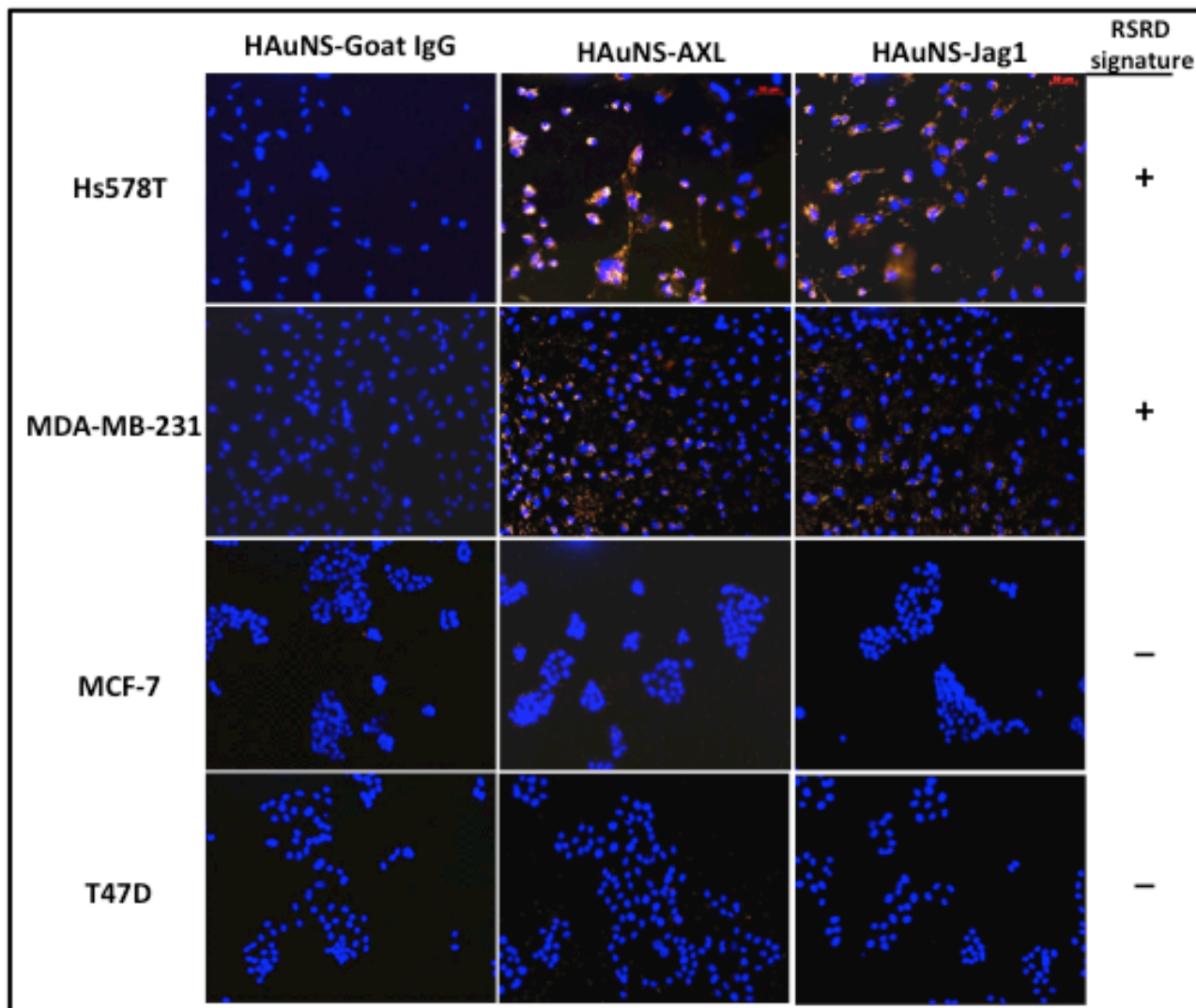
In collaboration with our colleague Dr. Chun Li, an outstanding leader in nanotechnology, we aimed to develop nanoparticles that can carry *in vivo* imaging agents to target breast cancer cells with RSR-defect-specific membrane proteins for cancer detection. In the past year, we have significantly moved this part of project forward. Based on the two RSR-defect-specific membrane proteins that we identified (described above), we conjugated human AXL affinity purified goat polyclonal antibody (R&D system, AF154) and human Jag1 affinity purified goat polyclonal antibody (R&D system, AF1277) to hallow gold nanoparticle (HAuNS). We also conjugated goat IgG (GIgG) to HAuNS as the negative control. After conjugation, we measure the UV-vis absorption of HAuNS-AXL, HAuNS-Jag1, and HAuNS-GIgG. By the comparison between HAuNS alone with HAuNS-Ab, the peak shift from 800 nm to 900 nm (Figure 10A), indicating that all three HAuNS-Abs were larger than HAuNS alone. We further measured the actual size of the particles by Raman spectroscopy and confirmed the increased sizes of the particles after conjugation (all three HAuNS-Abs have the effective diameters of 200-250 nm and the HAuNS alone has a diameter of 136 nm) (Figure 10B). These data clearly indicated a successful conjugation of antibodies to HAuNS.



**Figure 10. Antibodies against RSR-defect-specific membrane markers, AXL and Jag1, are successfully conjugated on hallow gold nanoparticle (HAuNS)** (A) The results of UV-Vis spectroscopy showed the spectrum of HAuNS alone and antibody-conjugated HAuNS. (B) The results of Raman spectroscopy showed the particle size of HAuNS alone and antibody-conjugated HAuNS. The red number indicates effective diameter.

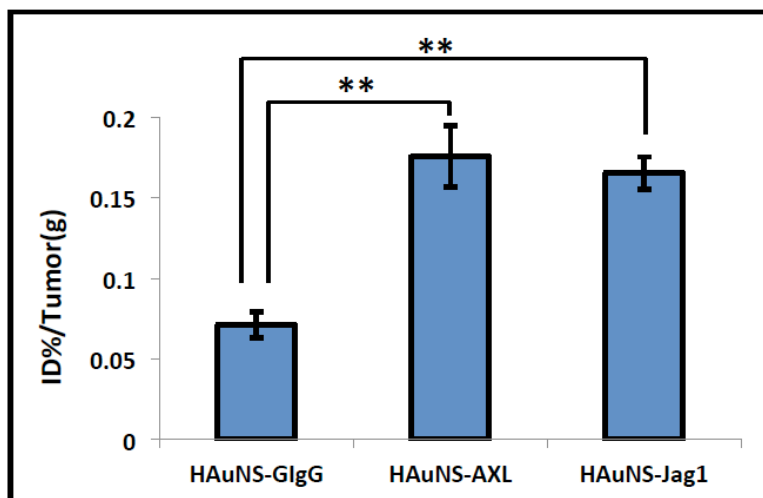
Since the conjugation process has been successful, we next tested whether the AXL and Jag1 antibodies still maintain biological activity and can specifically detect RSR-defect breast cancer cells. We incubated HAuNS-AXL, HAuNS-Jag1, and HAuNS-GIgG with two RSR-intact (MCF-7 and T47D) and two RSR-defect (MDA-MB-231 and Hs578T) breast cancer cell lines. After incubation, the

cells were stained with DAPI and the dark field images were obtained. The results of dark field images showed that both HAuNS-AXL and HAuNS-Jag1 were retained only on the surface of RSR-defect breast cancer cell lines (Figure 11). Taken together, we have successfully conjugated antibodies to HAuNS that can specifically bind to RSR-defect cells without disrupting the biological activity of antibodies.



**Figure 11. AXL- and Jag1-conjugated HAuNS can specifically bind to RSR-defect breast cancer cell lines.** Various breast cancer cell lines, including both RSR-intact and RSR-defect cells, were incubated with either AXL- or Jag1-conjugated HAuNS for two hours. After fixed and stained with DAPI, the images were obtained by dark field microscopy. HAuNS-Goat IgG was used as negative control. Scale bar is 50  $\mu$ m.

To assess the tumor targeting efficiency of HAuNS-AXL and HAuNS-Jag1 *in vivo*, we used MDA-MB-231 human breast cancer xenograft model and injected  $^{111}\text{In}$ -labeled HAuNS-AXL, HAuNS-Jag1, or HAuNS-GIgG into mice by intravenous injection. The tumors were dissected out and measured the radioactivity by scintillation counter 24 hours after injection. By calculating percentage of the injected dose per gram of tissue (%ID/g), the results indicated that HAuNP-AXL and HAuNS-Jag1 could target RSR-defect tumors (Figure 12).



**Figure 12. AXL- and Jag1-conjugated HAuNS can target RSR-defect (MDA-MB-231) breast tumor.** Comparison of  $^{111}\text{In}$ -labeled HAuNS-GlgG, HAuNS-AXL and HAuNS-Jag1 uptakes in MDA-MB-231 tumors 24 hours after intravenous injection. The results were measured by percentage of the injected dose per gram of tissue (%ID/g). Error bar indicates mean  $\pm$  SEM ( $n=6$ ). \*\* indicates  $P<0.001$ .

Task 4b. To develop nanoparticles to kill RSR-defective breast cancer cells through their binding to the RSR-defect-specific membrane proteins on cancer cells.

We have created the nanoparticles that can specifically bind to RSR-defect breast cells. We will continue to optimize these nanoparticles and conjugate them with therapeutic agents in the next two years to develop nano-targeting agents to kill RSR-defect breast cells for cancer prevention.

## KEY RESEARCH ACCOMPLISHMENTS

- (1) We demonstrated that TUSC4 might regulate HR repair by stabilizing BRCA1 protein from proteasomal-dependent degradation.
- (2) We demonstrated the functions of TUSC4 in suppressing breast cell growth *in vitro* and tumorigenicity *in vivo*.
- (3) Through the analysis of TCGA data, we found that low-expression of TUSC4 in breast cancer was correlated with poor cancer prognosis.
- (4) We identified and validated AXL and Jag1 as two new RSR-defect-specific membrane markers.
- (5) We identified 5 top compound candidates that preferentially killed RSR-defect breast cancer cells.
- (6) We successfully conjugated antibodies against AXL or Jag1 to hollow gold nanoparticles (HAuNP) and demonstrated their specific targeting to RSR-defect breast cancer cells..

## REPORTABLE OUTCOMES

The progress of this project in the past year has led to one manuscript under review (Nature Communications) and one manuscript in preparation. Our intriguing findings have also allowed me to be invited for presentations at 4<sup>th</sup> International Conference on Biomarkers & Clinical Research in Philadelphia in July and at the upcoming 3<sup>rd</sup> World Congress on Cancer Science & Therapy.

## CONCLUSION

During the third year of this project, we have made significant progress in several of our proposed tasks. We found that TUSC4 is a potent tumor suppressor gene in breast cancer with an important function in stabilizing BRCA1 protein.

In addition, we identified and validated AXL and Jag1 as two novel RSR-defect-specific membrane proteins and have successfully conjugated the antibodies against these two molecules to hollow gold nanoparticles. We also demonstrated the specific binding of these nanoparticles to RSR-defect breast cells.

Finally, we have identified and *in vitro* validated 5 top compound candidates that preferentially killed RSR-defect breast cells.

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