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INTRODUCTION: This study investigates a function for cell surface GRP78 in regulating prostate cancer stem-like cells. We investigate the hypothesis that cell surface GRP78 drives cancer stem-like behavior by activating an Akt/GSK-3/Snail-1 signaling axis in prostate cancer stem-like cells. In Aim 2, we study the ability of GRP78-neutralizing monoclonal antibodies to eliminate prostate cancer stem-like cells in vitro and in a mouse model. These studies have the potential to identify a novel and effective therapy for prostate cancer.

BODY (Tasks refer to those outlined in approved Statement of Work):

Task 1: Establish adherent and prostasphere cultures from prostate cancer cell lines.

During the first year of this award, we optimized conditions for growing prostasphere cultures from human prostate cancer cell lines (1LN and DU145). We found that addition of methylcellulose to sphere cultures was necessary to prevent non-specific cell-cell aggregation (See Fig. 1A). We also investigated the effect of seeding density on sphere formation, and determined that a seeding density of 12,000 cells/well of a 6 well plate generated the optimal sphere density for counting (Fig. 1A and 1C). These conditions were optimal for establishing prostasphere cultures for both human prostate cancer cell lines studied (1LN and DU145) (Fig. 1A and 1C). We also showed our ability to grow secondary spheres from dissociated primary spheres (Fig. 1B), an assay reflecting the self renewing activity of cancer stem-like cells. Collectively, these results show that we have optimized cell culture conditions for addressing a role for cell surface GRP78 in regulating prostate cancer stem-like cells (remaining tasks in Statement of Work).

Fig. 1A: Optimizing sphere cultures for prostate cancer cells. 1LN cells form distinguishable spheres in media containing methylcellulose, but not in media lacking methylcellulose. 1-LN cells were seeded into wells of a 6 well tissue culture dish at the indicated seeding densities in sphere medium (RPMI1640 containing 4 µg/ml human insulin, 20 ng/ml EGF, 20 ng/ml FGF, 100 units/ml penicillin and 100 µg/ml streptomycin) either containing methylcellulose or lacking methylcellulose (1%, 400 cp). Pictures (100 X) were taken on day 4.

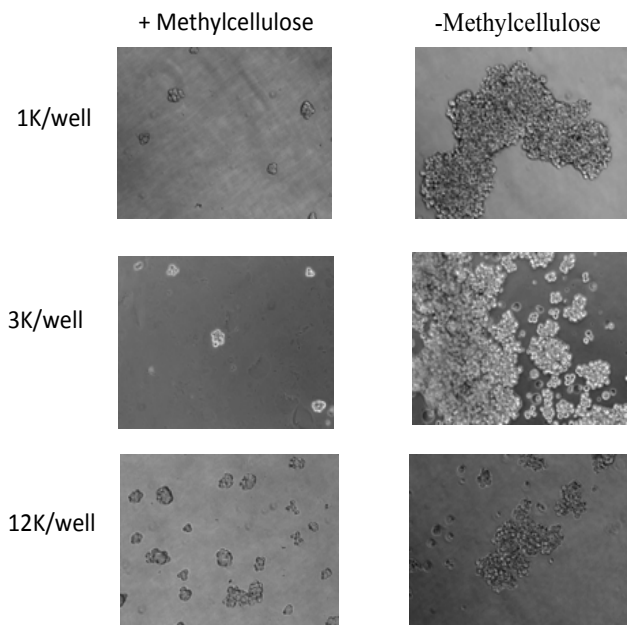


Fig. 1B: Primary 1LN spheres, when dissociated and reseeded into sphere culture form secondary spheres, indicative of self-renewing activity. Primary 1LN prostaspheres were harvested, washed with PBS and dissociated using Trypsin/EDTA. Cells were put through a 40µm cell strainer and re-plated in sphere medium in 6-well plates, as in Fig. 1A.

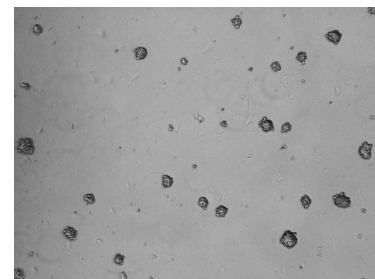
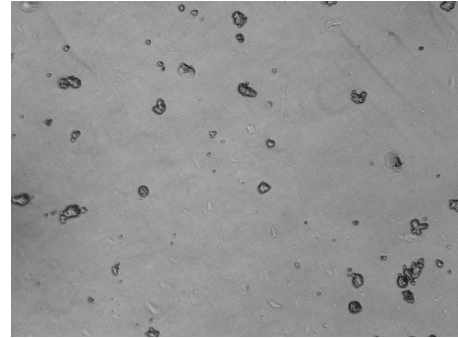


Fig. 1C: Development of sphere culture conditions for DU145 prostate cancer cells.

DU145 cells were seeded at 12,000 cells per well of 6 well plate using methylcellulose-containing sphere medium, as described in Fig. 1A. Pictures (100X) were taken on d4.



Task 2: Investigate the relative expression of cell surface GRP78 in adherent prostate cancer cells and in prostate cancer stem-like cells.

Using the prostasphere culture conditions optimized in Fig. 1, we next investigated cell surface GRP78 expression in prostasphere-derived tumor cells and in prostate cancer cell lines. As shown in Fig. 2A, GRP78 expression levels were increased in prostasphere culture compared to 2D culture conditions. *These studies demonstrate that cell surface GRP78 levels are increased in prostate cancer stem-like cells (prostatespheres) relative to that in bulk adherent prostate cancer cells (achieving goal for Tasks 1 and 2, Statement of Work).*

Upon performing these experiments, we recognized that the level of cell surface GRP78 on prostate cancer cells is too low to permit GRP78 sorting, as was originally planned in Task 6 (Statement of Work). Based on a previous report indicating that chemotherapy treatment enriches for cancer stem-like cells[1], we next investigated the hypothesis that chemotherapy treatment of human prostate cancer cells increases GRP78 cell surface expression. As shown in Fig. 2B, chemotherapy treatment of DU145 cells for 4 days significantly increased the percent cell surface GRP78-positive cells, raising the possibility that chemotherapy enriches for prostate cancer stem-like cells that express cell surface GRP78.

By working with chemotherapy-pretreated prostate cancer cells, we can obtain sufficient GRP78 levels to perform GRP78 sorting, as was originally planned in Task 6. We have optimized a chemotherapy treatment strategy for increasing cell surface GRP78 expression on prostate cancer cells, and will study in year 2 the ability of GRP78-sorted, chemotherapy-pre-treated cells to grow as self-renewing prostaspheres (Task 6, Statement of Work).

Fig. 2A: Flow cytometry for cell surface Grp78 on 1LN sphere cells.

1LN cells were harvested with 2mM EDTA from adherent tissue culture plates. 1-LN sphere cultures were established as in Fig. 1A. After 5 days, spheres were harvested with 2mM EDTA in HBSS. Cells were stained (1 ug/10⁵ cells) with anti-Grp78 antibodies (C20 and N-20) from Santa Cruz. Control is goat IgG.

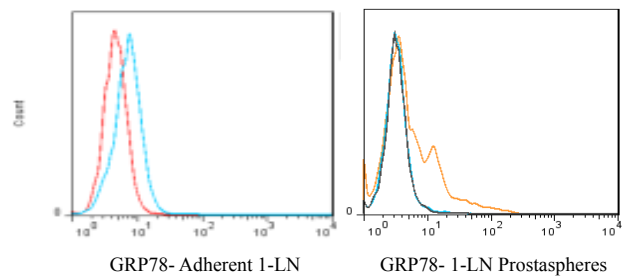
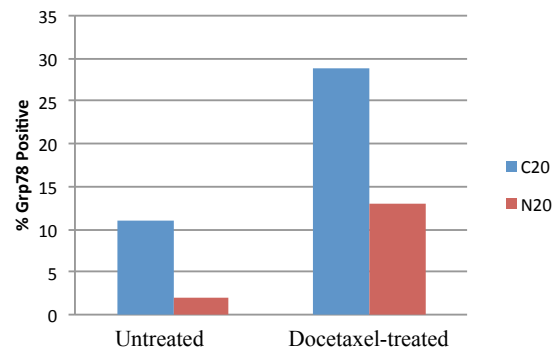


Fig. 2B: Chemotherapy treatment enriches for prostate cancer cells with increased GRP78 cell surface expression. DU145 cells were incubated with 10 nM docetaxel for 4 days. Media was removed, cells were washed with PBS and fresh media was added. Cells were incubated for an additional 6 days, then harvested and stained for Grp78 using C-terminal (C-20) or N-terminal (N-20) GRP78 antibody. % cells positive for cell surface GRP78 are indicated.



Task 3: Determine relative activities of Akt and its substrate GSK-3 in adherent prostate cancer cells and in prostate cancer stem-like cells.

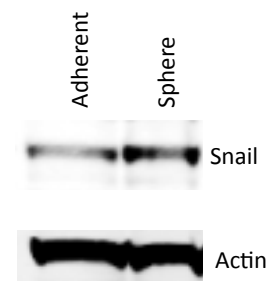
We will investigate these signaling pathways in year 2.

Task 4: Investigate the relative expression of Snail-1, a GSK-3 target, in adherent prostate cancer cells and in prostate cancer stem-like cells.

To begin to address the hypothesis that Snail-1 expression is driven by cell surface GRP78 expression in prostate cancer stem-like cells, we determined Snail-1 protein levels in 1LN prostate tumor cells grown in 2D culture, as well as the same cells grown as prostaspheres (using the methods described in Fig.1). As shown in Fig. 4, Snail-1 expression was significantly increased in 1LN cells cultured as prostaspheres compared to the levels observed in 1LN cells grown in monolayer culture. These findings show that increased cell surface GRP78 expression in prostaspheres (compared to adherent prostate tumor cells) is associated with increased expression of Snail-1.

This work addresses the goal of Task 4 in the Statement of Work (testing the hypothesis that Snail-1 expression levels are increased in prostate cancer stem-like cells relative to that in bulk adherent prostate cancer cells).

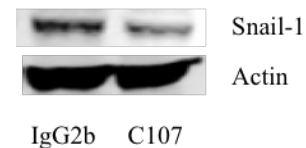
Fig. 4: Snail-1 expression is increased in 1LN cells grown in sphere culture compared to that in 1LN cells grown as monolayer. Total cellular proteins were extracted from 1LN 2D culture (Adherent) and from 1LN grown in sphere culture as in Fig. 1A (Sphere). Equivalent amounts of protein were subjected to SDS-PAGE and probed with a Snail-1 antibody (Cell Signaling) or Actin antibody (Sigma), followed by IRdye-conjugated secondary antibody. Proteins were detected by Odyssey infrared imaging.



Task 5: Determine the effect of incubating human prostate cancer stem-like cells with monoclonal antibodies specific for N-terminal and C-terminal domains of cell surface GRP78 on Akt/GSK-3/Snail-1 signaling.

To directly address the hypothesis that cell surface GRP78 drives Snail-1 expression in human prostate cancer cells, we incubated 1LN cells with a C-terminal GRP78-neutralizing monoclonal antibody (or isotype control antibody). After 24 hours, total cellular proteins were extracted, and Snail-1 levels were assessed by immunoblotting. As shown in Fig. 5, this GRP78 neutralizing antibody significantly reduced Snail-1 protein levels, supporting the hypothesis that cell surface GRP78 drives Snail-1 expression in prostate tumor cells. ***These findings address in part the goal of Task 5 (to determine if antibodies directed against the N-terminus or C-terminus of cell surface GRP78 impact an Akt/GSK-3/Snail-1 signaling axis in androgen independent prostate cancer stem-like cells. Studies in year 2 will: 1) determine the impact of C-terminal GRP78 antibodies on Akt and GSK-3 activity, and 2) explore the impact of N-terminal antibodies on the AKT/GSK-3/Snail-1 signaling axis (Task 5, Statement of Work).***

Fig. 5: Snail-1 expression is reduced in prostate cancer cells incubated with a C-terminal GRP78-specific monoclonal antibody. 1-LN cells were incubated for 24 h with a C-terminal GRP78 antibody (C107) or isotype control antibody (IgG2b) at 1µg/mL. Equivalent amounts of total cellular protein were immunoblotted with Snail-1 or actin antibodies, as described for Fig. 4A.



Task 6a: Sort for cell surface GRP78(+) and cell surface GRP78 (-) prostate cancer cells.

We were unable to accomplish this task in year 1 because GRP78 levels were not high enough on human prostate cancer cell lines to perform sorting. We showed that chemotherapy treatment of these cell lines significantly increased cell surface GRP78 levels (See Fig. 2B). We are currently sorting these chemotherapy-treated cells into GRP78(+) and GRP78(-) populations. These sorted populations will be compared with regard to their sphere forming ability (Task 6b, Statement of Work). Determining whether chemotherapy-treated prostate tumor cells express increased cell surface GRP78, and whether these treated cells exhibit increased stem-like behavior, will have important implications for patient treatment.

Task 6b: Determine the relative ability of these sorted populations to grow as self-renewing prostaspheres. We are delayed in performing this task for the reasons described in Task 6a.

Supplementary Tasks:

Supplementary Task 1: Characterization of additional C-terminal GRP78-directed monoclonal antibodies generated by using a C terminal GRP78 peptide immunization approach.

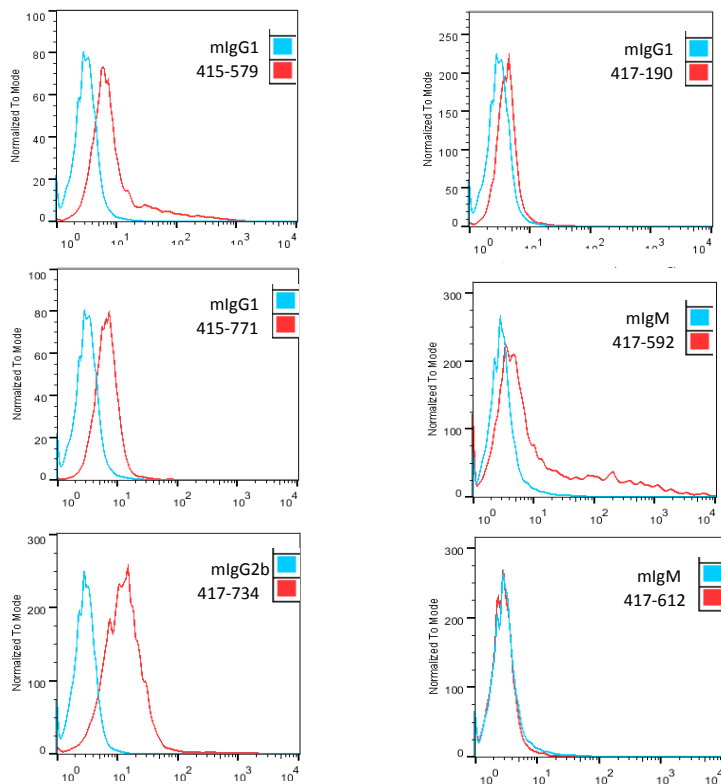
In July 2011, prior to the start of the current funding (September 2012), our lab attempted to produce C-terminal GRP78 monoclonal antibodies using a GRP78 C-terminal fragment immunization approach [under Duke Institutional Animal Care and Use (IACUC)-approved animal protocol A244-09-08]. During the current funding period, we studied antigen-specificity of hybridoma supernatants from six hybridoma clones generated by this immunization strategy. Based on their reactivity with recombinant GRP78 (Fig. 1A), all six of these clones were chosen for further analysis. As shown in Supplementary Fig. 1B, five of these six hybridoma supernatants (415-579, 415-771, 417-734, 417-190, 417-592) recognized cell surface GRP78. In years 2 and 3 of the current proposal, these antibodies will be expanded and purified for further characterization as follows: 1) Task 5 (Months 13-16)- Determine the effect of incubating human prostate cancer stem-like cells with monoclonal antibodies specific for N-terminal and C-terminal domains of cell surface GRP78 on Akt/GSK-3/Snail-1 signaling, and 2) Task 9 (Months 25-32)- Assess the effect of antibodies specific for N-terminal and C-terminal domains of cell surface GRP78 on prostate cancer stem-like cell growth.

Supplementary Fig. 1A: Assessment of GRP78 reactivity of hybridoma supernatants.

Prior to the start date of the current proposal, we generated additional C-terminal GRP78 antibodies using a GRP78 C-terminal fragment immunization approach. Mice were immunized with Grp78 C-30 fragment conjugated to KLH. Spleen cells from hyper-immunized mice were fused with P3X63Ag8.653 or SP2/OAG14 myelomas using polyethylene glycol. During the current funding period, supernatants from six viable hybridomas were selected and screened for antigen specific antibodies by ELISA. ELISA reactivity with recombinant GRP78 for these six clones is shown.

Sample	Fold Above Background
415-579	84.1
415-771	8.8
417-190	23.2
417-592	58.2
417-612	5.4
417-734	2.4

Supplementary Fig. 1B: Determining reactivity of hybridoma supernatants with cell surface GRP78 by flow cytometry. 1LN cells (10^5) were stained with the indicated antibodies (50 μ L hybridoma supernatant), and analyzed by flow cytometry.



KEY RESEARCH ACCOMPLISHMENTS:

- Optimized primary and secondary prostasphere cultures for determining self-renewing activity of human prostate cancer cells. Determined that addition of methylcellulose to sphere cultures was necessary to prevent non-specific aggregation of cells.
- Determined that cell surface GRP78 expression levels are increased in prostasphere culture compared to levels in adherent culture, suggesting that GRP78 levels are increased in prostate cancer stem-like cells.
- Optimized a method for enriching for prostate cancer cells with increased cell surface GRP78 expression by short-term chemotherapy exposure. This method was necessary to obtain sufficient levels of cell surface GRP78 to perform cell sorting.
- Determined that Snail-1 expression is elevated in prostaspheres relative to adherent prostate cancer cells.
- Obtained evidence that incubation of prostate cancer cells with a C-terminal GRP78 antibody reduces Snail-1 expression levels.
- Characterized binding properties of six additional C-terminal GRP78 antibodies (hybridomas generated prior to initiation of this grant funding period). Based on their binding properties, five of these antibodies will be expanded and tested for: 1) their ability to inhibit Akt/GSK-3/Snail-1 signaling (Task 5, Statement of Work), and 2) their ability to inhibit prostate cancer stem-like cell growth (Task 9, Statement of Work).

REPORTABLE OUTCOMES TO DATE:

- Presentation: 2012 Duke University Medical Center, Prostate Cancer Research Forum “Targeting Prostate Cancer Stem-like cells through cell surface-expressed GRP78”

CONCLUSIONS: Our findings for period one indicate that cell surface GRP78 expression is increased in androgen-independent prostate cancer cells when they are grown in prostasphere culture compared to when they are grown in adherent culture. This finding suggests that cell surface GRP78 is expressed on prostate cancer stem-like cells. We also observed increased expression of Snail-1, a transcription factor associated with “stemness”, in prostaspheres compared to that in adherent prostate cancer cells. Notably, a C-terminal GRP78 antibody reduced Snail-1 in prostate cancer cells, suggesting that cell surface GRP78 drives Snail-1 expression.

Studies in period one also indicate that chemotherapy treatment of prostate cancer cells enriches for a sub-population of cells with increased cell surface GRP78 expression. This chemotherapy pre-treatment strategy is necessary to obtain sufficient levels of cell surface GRP78 for sorting. We are currently performing cell surface GRP78 sorting on these chemotherapy-treated

prostate cancer cell populations to address the relative ability of GRP78(+) and GRP78(-) populations to grow as self-renewing prostaspheres. These studies will form a foundation for our studies in years 2 and 3, investigating: 1) a function for Snail-1 in the driving cell surface GRP78+ prostate cancer stem-like cell growth, 2) the ability of antibodies specific for C-terminal domains of GRP78 to suppress Snail-1 expression and self-renewing activity of prostate cancer stem-like cells, and 3) the ability of these antibodies to suppress tumor initiating activity of GRP78(+) prostate cancer cells in a mouse model. These studies will be clinically important because: 1) they are expected to show that chemotherapy treatment enriches for prostate cancer stem-like cells, 2) they will identify a novel therapeutic target (i.e. cell surface GRP78) on these prostate cancer stem-like cells.

REFERENCES:

1. Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, et al. (2008) Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *Journal of the National Cancer Institute* 100: 672-679.