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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Prostate cancer cells were transfected with claudin siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen) as well as the appropriate controls including vehicle, non-targeting siRNA (siSC5) (negative control) and glyceraldehyde-3-phosphate dehydrogenase (GAPD) (positive control). A time course and concentration curve was completed to determine the maximum down-regulation of claudin-3 and claudin-4 expression. In addition, cytotoxicity assays were performed to determine the number of viable prostate cancer cells upon claudin-3 siRNA or claudin-4 siRNA treatment. We were able to get great knockdown, which translated into a decrease in the number of viable prostate cancer cells by testing each target with 4 independent siRNAs for claudin-3 and claudin-4. In looking at our claudin-4 siRNA data, we treated prostate cancer cells in vitro for 72 hours, at concentrations of 25, 50 and 100 nM (Fig 2, top). As can be seen from our data, there was approximately a 60% decrease in cell viability upon treatment with claudin-4 siRNA. In addition, our immunohistochemical data demonstrate that claudin-3 and claudin-4 are expressed in subsets of aggressive prostate cancer. Finally, we produced our first two batches of nanoparticles during year 1 and we were able to show that these nanoparticles bind to prostate cancer cells.

15. SUBJECT TERMS
Prostate cancer, superparamagnetic iron oxide, nanoparticle, magnetic resonance imaging, targeting, diagnostic, therapeutic, Claudin, *Clostridium perfringens enterotoxin*

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1. INTRODUCTION

Prostate cancer is one of the most common malignancies in men. Distant metastases are a poor prognostic predictor with limited survival. Hence, there is a need for effective diagnostic tools that can track small clusters of metastatic prostate cancer cells. In addition, more effective therapeutic tools also are required to treat metastatic prostate cancer once it is located. Poly(lactide-co-glycolide) (PLGA) is a biocompatible, degradable polymer that is approved by the FDA, and is used in the development of our nanoparticle system. Our nanoparticles encapsulate superparamagnetic iron oxide (SPIO) contrast for magnetic resonance (MR) imaging. These nanoparticles are being designed to target tight junction proteins, claudin-3 and claudin-4, which have been found to be altered in many human cancers including ovarian, breast, pancreas, and prostate cancer. Therefore, the goal of this project is to develop a nanoparticle system that has the specificity to target and treat metastatic prostate cancer.

2. KEYWORDS

Prostate cancer, superparamagnetic iron oxide, nanoparticle, magnetic resonance imaging, targeting, diagnostic, therapeutic, Claudin, *Clostridium perfringens enterotoxin*

3. ACCOMPLISHMENTS

➔ **What were the major goals of the project?**

There was a peer and programmatic review with recommendations prior to the start of the project, which included suggestions to perform additional work regarding the specificity of the claudin proteins. Since we have already shown that DU145 and PC3 prostate cancer cells express claudin-3 and claudin-4, we performed siRNA knockdown experiments to confirm specificity. This data will be important for the treatment phase when using the nanoparticles. In addition, another main goal was to determine the expression of claudin-3 and claudin-4 in advanced prostate cancers specimens.

➔ **What was accomplished under these goals?**

Firstly, we performed a western blot to characterize our three prostate cancer cell lines, LNCaP, DU145 and PC3, which are being used in this project. We showed that prostate specific antigen (PSA) is expressed in the LNCaP cells, but absent in the DU145 cells whereas AMACR (P504S) is expressed in all prostate cancer cell lines (Fig 1). These results coincide with previous published data and further confirm our in vitro models. Prostate cancer cells were transfected with four independent claudin-3 and claudin-4 siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen). For these experiments we also included the appropriate control siRNAs. A time course and concentration curve was completed to determine the maximum down-regulation of

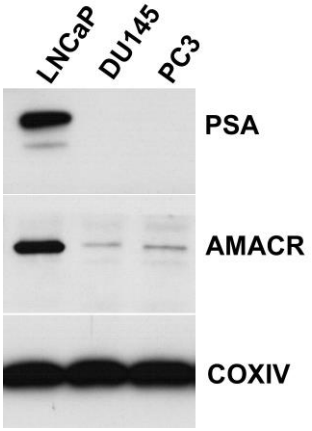

Fig 1. Characterization of three prostate cancer cell lines by western blot. COXIV is used as a loading control.

claudin-3 and claudin-4 expression. In addition, cytotoxicity assays were performed to determine the viability of prostate cancer cells upon claudin-3 siRNA or claudin-4 siRNA treatment. For our data, the prostate cancer cells were treated with claudin-4 siRNA for 72 hours at concentrations of 25, 50 and 100 nM (Fig 2, top). Claudin-4 was targeted with 4 independent siRNAs to ensure the great knockdown was achieved. From this data, there was approximately a 60% decrease in cell viability upon treatment with claudin-4 siRNA. On the other hand, when PC3 cells were treated with claudin-3 siRNA for 48 hrs there was a 20-25% decrease in cell viability (Fig 2, bottom).

From the goals of year 1, we have completed the majority of our immunohistochemical experiments regarding the levels of claudin-3 and claudin-4. Our analysis is underway and is expected to be completed at the beginning of year 2. Initial analyses suggest that claudin-3 and claudin-4 are found in advanced subsets of prostate cancer. As can be seen from Fig 3, there are high levels of claudin-4 present in the advanced (Gleason 9) prostate cancer specimens, which is represented by the red staining.

Additionally, in collaboration with Dr. Tarek Fahmy, Associate Professor of Biomedical Engineering at Yale University,

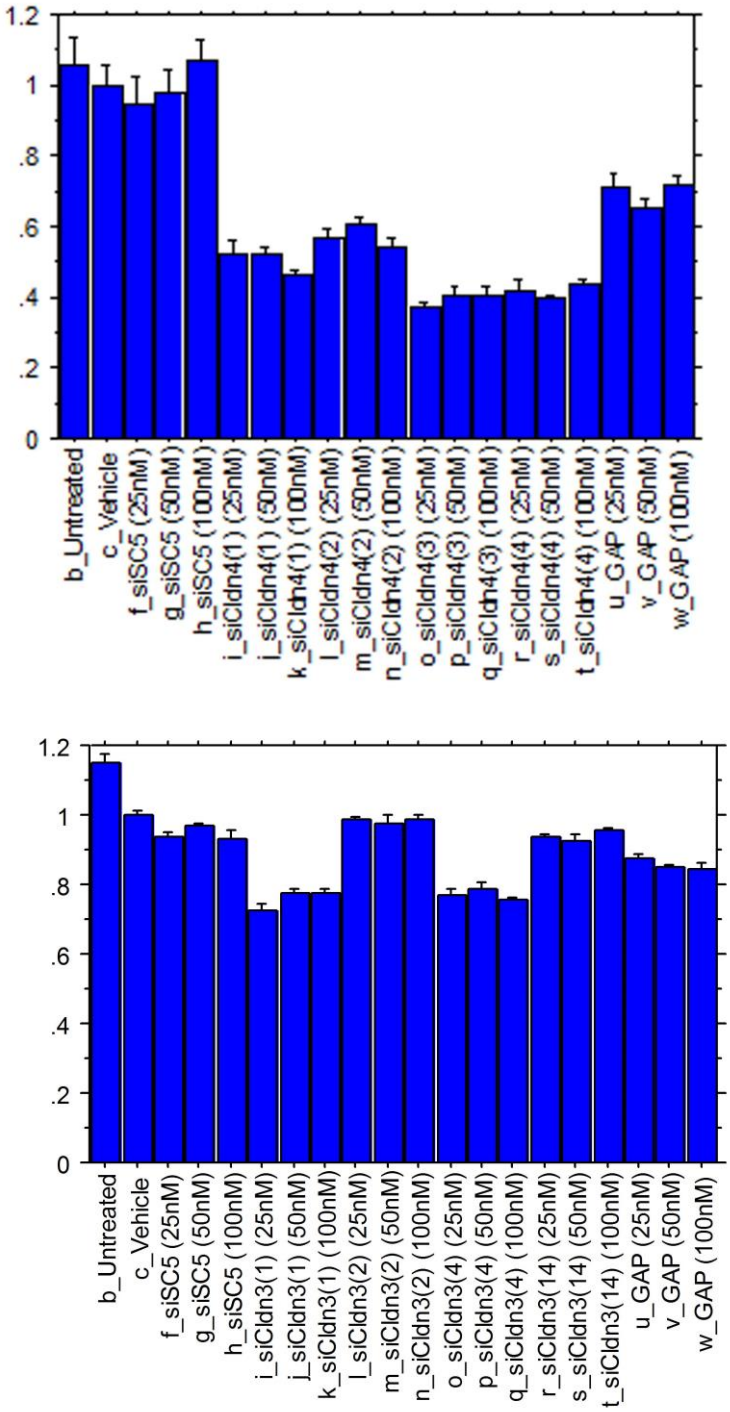

Fig 2. Cytotoxicity assay of prostate cancer cells. LNCaP (top) was treated for 72 hrs with various concentrations of claudin-4 siRNA whereas PC3 (bottom) was treated for 48 hrs with various concentrations of claudin-3 siRNA. Vehicle control, non-targeting siSC5 (negative) and GAP (positive) controls were included.

we have already generated the two batches of nanoparticles. This part of the project is currently ahead of schedule as it was not slated to start until year 2. In vitro testing has begun with the nanoparticles encapsulated with dye and iron oxide. The nanoparticles (Fig 4) were incubated with LNCaP prostate cancer cells for 2 hr before being fixed and imaged using confocal microscopy. We implemented three types of analyses to visualize the nanoparticles, including shadow, transparent, and surface, which can be seen in Fig 4.

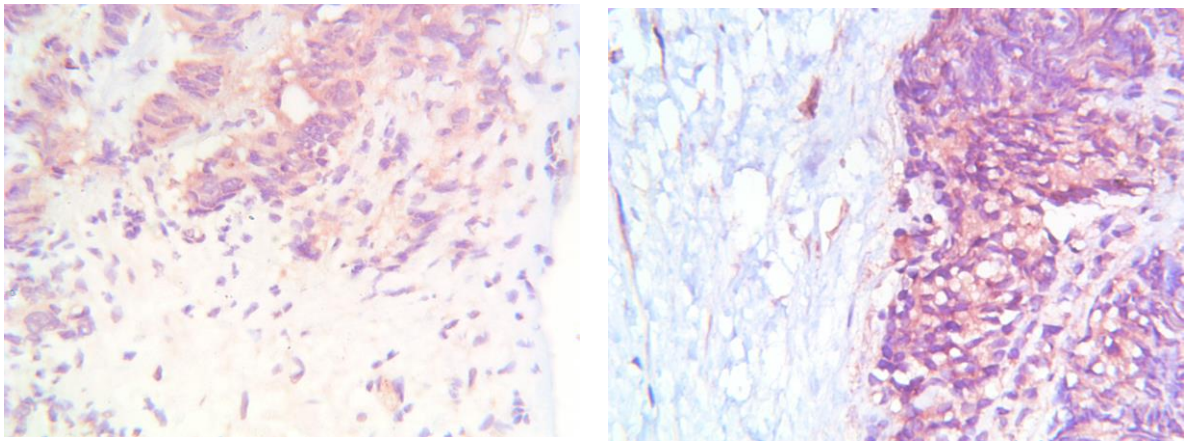

Fig 3. Claudin-4 (red color) is expressed in Gleason 9 high grade tumors 400X (left panel) and 600X (right panel). These sections were countered stained with hematoxylin.

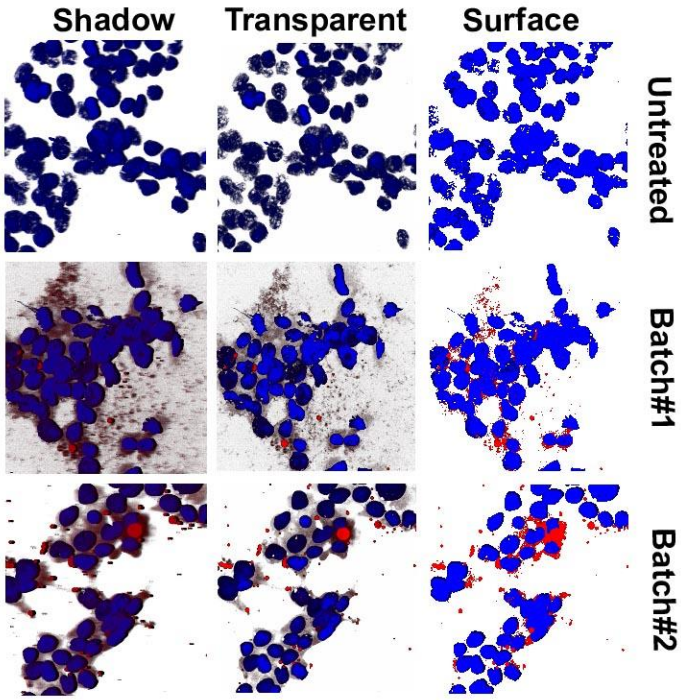

Fig 4. Untreated, batch#1 (nanoparticles), batch#2 (nanoparticles) treated for 2 hours with LNCaP prostate cancer cells. The nuclei are stained with DAPI and the nanoparticles are encapsulated with dye (Red).

➔ **What opportunities for training and professional development has the project provided?**
This project involves an MR imaging component. Dr. Gigi Galiana, an Assistant Professor of Diagnostic Radiology at Yale University, is providing the technical training and support required in performing MR imaging. We have already begun training and MR imaging optimization is scheduled for next month.

➔ **How were the results disseminated to communities of interest?**
There is nothing to report for this reporting period. I expect to attend the IMPACT conference during the summer of 2016 (year 2). At that time I will disseminate our findings from the first portion of this project.

➔ **What do you plan to do during the next reporting period to accomplish the goals?**
For the next reporting period, the immunohistochemical analysis of claudin-3 and -4 will be completed. Also, a prostate cancer mouse model will be established, including confirmation of metastatic sites. Pharmacokinetics and biodistribution experiments will be completed with functionalized and non-functionalized nanoparticles encapsulating iron oxide or dye.

4. IMPACT

➔ **What was the impact on the development of the principal discipline(s) of the project?**
There is nothing to report for this reporting period.

➔ **What was the impact on other disciplines? What was the impact on technology transfer?**
There is nothing to report for this reporting period.

➔ **What was the impact on technology transfer?**
There is nothing to report for this reporting period.

➔ **What was the impact on society beyond science and technology?**
There is nothing to report for this reporting period.

5. CHANGES / PROBLEMS

➔ **Changes in approach and reasons for change**
There is nothing to report for this reporting period.

➔ **Actual or anticipated problems or delays and actions or plans to resolve them**
There is nothing to report for this reporting period.

➔ **Changes that had a significant impact on expenditures**
There is nothing to report for this reporting period.

➔ **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
There is nothing to report for this reporting period.

➔ **Significant changes in use or care of human subjects**
There is nothing to report for this reporting period.

➔ **Significant changes in use or care of vertebrate animals**
There is nothing to report for this reporting period.

➔ **Significant changes in use of biohazards and/or select agents**
There is nothing to report for this reporting period.

6. PRODUCTS

➔ **Publications, conference papers, and presentations**
There is nothing to report for this reporting period.

➔ **Journal publications**
There is nothing to report for this reporting period.

➔ **Books or other non-periodical, one-time publications**
There is nothing to report for this reporting period.

➔ **Other publications, conference papers, and presentations.**
There is nothing to report for this reporting period.

➔ **Website(s) or other Internet site(s)**
There is nothing to report for this reporting period.

➔ **Technologies or techniques**
There is nothing to report for this reporting period.

➔ **Inventions, patent applications, and/or licenses**
There is nothing to report for this reporting period.

➔ **Other Products**
There is nothing to report for this reporting period.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

➔ **What individuals have worked on the project?**

Name:	Darryl Martin
Project Role:	PI

Researcher Identifier (e.g. ORCID ID):	14-002280
Nearest person month worked:	9
Contribution to Project:	My role in this proposal is to ensure all work is completed on time, and to comply with all reporting guidelines. Using my technical background I lead all development efforts using magnetic iron-oxide nanoparticles, the complexes with iron-oxide, and hold research meetings with the whole team to ensure work is completed in timely manner. In addition, I will disseminate our finding at the upcoming IMPACT conference as well as through published manuscripts.

Name:	Robert Weiss
Project Role:	Faculty mentor
Nearest person month worked:	1
Contribution to Project:	Dr. Weiss' role for this reporting period is to serve as a technical collaborator and mentor.
Funding support:	Departmental

➔ **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
No

➔ **What other organizations were involved as partners?**
Not applicable

8. SPECIAL REPORTING REQUIREMENTS

➔ **Collaborative awards**
Not applicable

➔ **Quad charts**
Not applicable

9. APPENDICES

None