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TITLE: Targeted Therapy Combined with Immune Modulation Using Gold Nanoparticles for Treating Metastatic Colorectal Cancer

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14. ABSTRACT During this reporting period, we have established a reliable mouse model of CRC (LSL-rtTA3/shAPC/Lgr5-CrER). We bred these mice to create a colony of transgenic mice. When experimental mice are 8 weeks of age, they are treated with 4-hydroxytamoxifen (4OHT) and put on continuous doxycycline via drinking water to initiate tumor formation. We are able to readily detect tumor formation after 4-6 weeks via colonoscopy. We have also detected PD-L1 expression in colon polyps from these mice. We tested the ability of this peptide to be loaded into a MHC I molecule in splenocytes harvested from C57BL/6 mice. The control SIINFEKL (without a DNA oligo attached) is detectable by this antibody, but unfortunately GFP-SIINFEKL added at the same concentration cannot be detected. We are trouble shooting this issue and seeking alternative approaches. We also designed siRNAs to each PVT1 and PD-L1 and successful knockdown of targets was confirmed by RT-qPCR, flow cytometry, and/or Western blot. Of the siRNAs for each target, the two most potent siRNAs were selected and ordered with a propylthiol modification at the 3' end and attached to AuNPs. These AuNPs with siRNA attached were delivered to CT26 cells and knockdown of targets was confirmed in the same manner as described above. We are currently working on delivering siRNA-coated AuNPs to our mouse model of CRC.					
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Introduction

Our long-term objective is to develop an effective treatment for metastatic colorectal cancer. Our idea is to use gold nanoparticles to deliver agents capable of directly attacking the cancer cells and activating the immune system. The three specific agents we will attach to the gold nanoparticles are two small-interfering RNA molecules (siRNAs) and a peptide antigen. One of the siRNAs is designed to disable an important oncogene (MYC), while the second siRNA is designed to turn off a gene that tumor cells use to block the immune system from attacking the cancer (PDL1). The peptide antigen is designed to activate pre-existing killer CD8 T cells present in the patient and cause these cells to attack the cancer cells. We are performing these experiments using both *in vitro* and *in vivo* models consisting of mouse and human cells and transgenic mouse models. These experiments will determine the effectiveness of this approach and, if successful, will lead to Phase I clinical trials.

Keywords

Metastatic colorectal cancer

Gold nanoparticles

Small interfering RNA

Immune checkpoint inhibitor

MYC oncogene

PVT1 long non-coding RNA

PDL1 Programmed Cell Death Ligand 1 gene

APC Adenomatous Polyposis Coli gene

Peptide Antigen

Memory CD8 T cells

Accomplishments

What were the major goals of the project?

The major goal of this project is to construct gold nanoparticles (AuNPs) that carry siRNA targeting the oncogene PVT1, an siRNA targeting the immune checkpoint PD-L1, and a peptide antigen capable of activating circulating memory CD8 T cells (eg. a flu or measles peptide). Once these AuNPs are made, our goal is to treat colorectal cancer (CRC) tumors with these AuNPs, thereby initiating a three-pronged attack that will control tumor growth.

What was accomplished under these goals?

Mouse model of CRC: During this reporting period, we have established a reliable mouse model of CRC. We received 2 male and 2 female LSL-rtTA3/shAPC/Lgr5-CrER transgenic mice (Figure 1, top) from a collaborator, Dr. Luke Dow. We bred these pairs of mice to create a colony of transgenic mice, and continue to breed them as needed. When experimental mice are 8 weeks of age, they are treated with 4-hydroxytamoxifen (4OHT) and put on continuous doxycycline via drinking water to initiate tumor formation. We are able to readily detect tumor formation after 4-6 weeks via colonoscopy (Figure 1, middle). We have also detected PD-L1 expression in colon polyps from these mice (Figure 1, bottom).

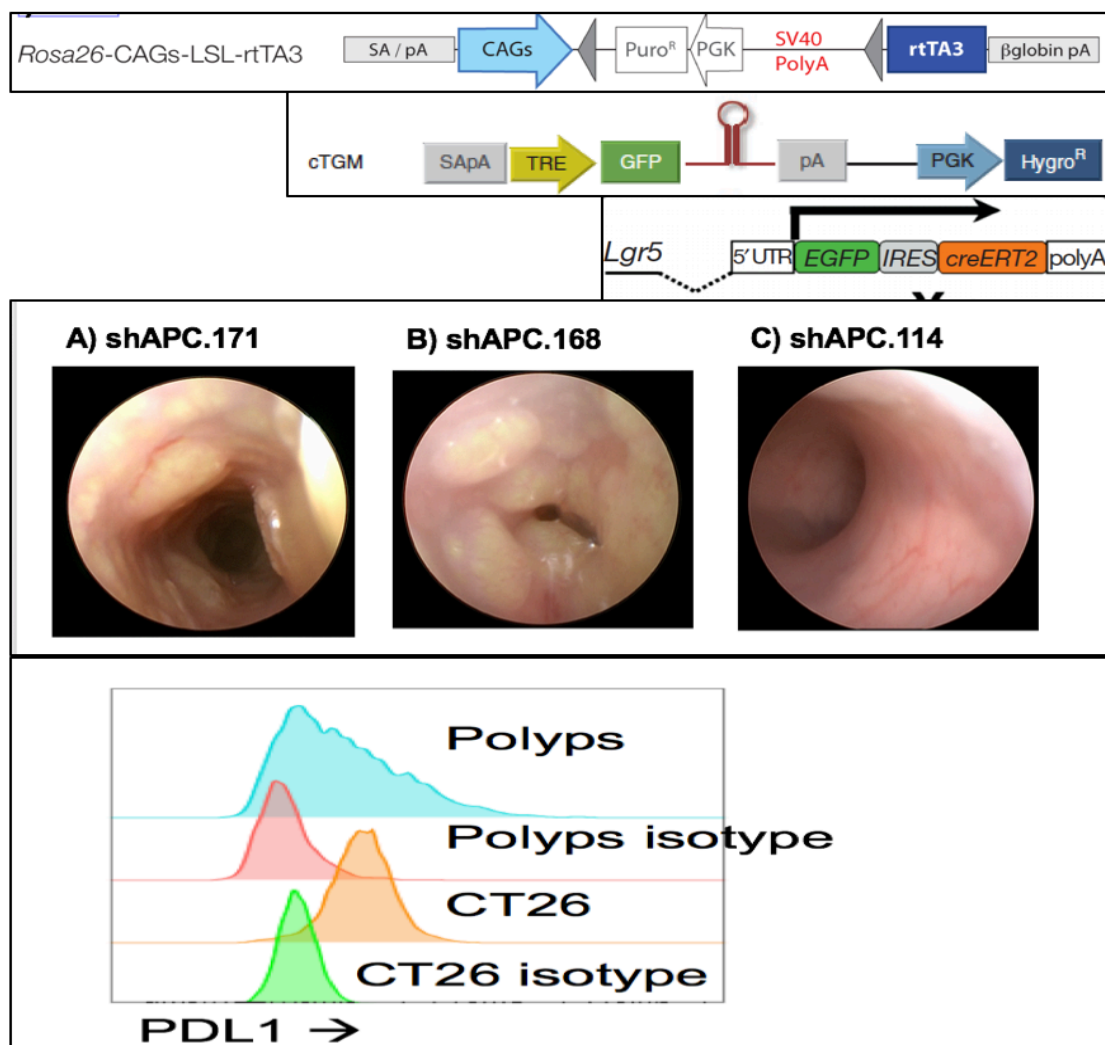
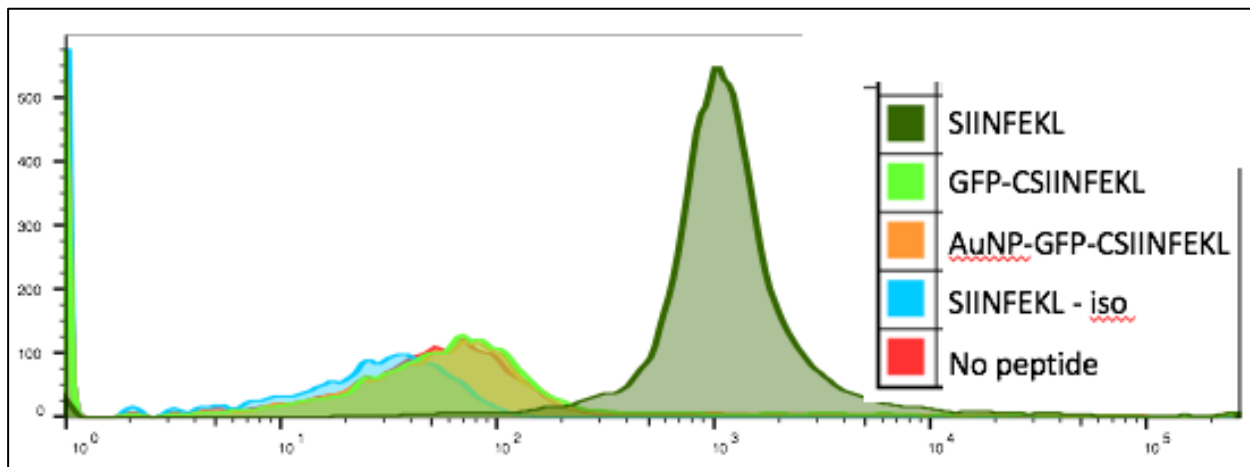


Figure 1. Mouse model of CRC. Transgenes expressed in shAPC mice (top). Middle: Endoscopy images demonstrating multiple polyps present in the colon of two shAPC mice (A & B) 7 weeks after administration of 4-hydroxytamoxifen followed by doxycycline, and one control mouse (C). Bottom: Colon polyps were harvested from (AGE?) shAPC mouse. Single cells were dissociated and analyzed for PD-L1 expression by flow cytometry.

Design AuNPs that selectively release a peptide antigen in cells expressing a cell-specific mRNA: We purchased a DNA-peptide molecule from Cell Mosaic, with the DNA being 12 bases in the sense strand of GFP, and the peptide being the ovalbumin antigen SIINFEKL (GFP-SIINFEKL). We tested the ability of this peptide to be loaded into a major histocompatibility complex I (MHC I) molecule in splenocytes harvested from C57BL/6 mice. To test peptide loading, we used a commercially available PE-fluorophore-labeled antibody that detects SIINFEKL in MHC I. Control SIINFEKL (without a DNA oligo attached) is detectable by this antibody, but GFP-SIINFEKL added at the same concentration cannot be detected (Figure 2). There are three possible explanations for this: 1) The DNA attached to the peptide causes steric hindrance such that it cannot be loaded into MHC I; 2) The DNA attached to the peptide causes



PE-anti-H2Kb-SIINFEKL →

this molecule to be degraded inside the cell rather than processed for MHCI loading; 3) The antibody used to detect SIINFEKL-MHCI complex cannot bind to GFP-SIINFEKL. To address the latter possibility, we are currently testing the ability of SIINFEKL-specific CD8 T cells (OTI cells) to kill splenocytes loaded with GFP-SIINFEKL. If we observe specific killing of peptide-presenting cells by OTI cells, we will

Figure 2. SIINFEKL peptide linked to a DNA oligonucleotide cannot be detected in H2Kb by flow cytometry. C57BL/6 splenocytes were pulsed with SIINFEKL, SIINFEKL linked to a DNA oligonucleotide (GFP-SIINFEKL), or AuNPs loaded with GFP-SIINFEKL. After pulse, cells were stained with an antibody specific to SIINFEKL loaded in the MHC Class II molecule H2Kb. Only the SIINFEKL peptide alone could be detected with this assay.

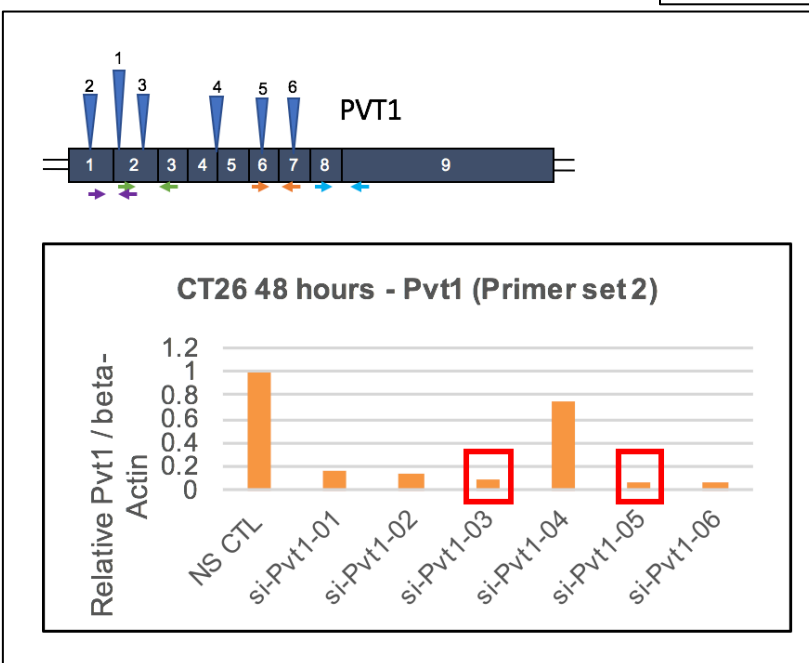


Figure 3. Design of siRNA targeting Pvt1 and confirmation of knockdown by RT-qPCR. Six siRNAs were designed along the Pvt1 transcript (top). These siRNAs were transfected into CT26 cells and Pvt1 expression was assessed 48 hours later by RT-qPCR (bottom).

conclude that DNA-conjugated peptides can readily be loaded into MHCI and we can continue to use this model. If this proves unsuccessful, we will address the first two possibilities and re-design the DNA-peptide conjugates.

Identify potent and specific mRNA to PVT1 and PD-L1: We designed 6 siRNAs to each PVT1 (Figure 3) and PD-L1 (Figure 4), and ordered them from Integrated DNA Technologies (IDT). These were transfected into a mouse CRC cell line (CT26)

and knockdown of targets was confirmed by

RT-qPCR, flow cytometry, and/or Western blot (Figures 3 and 4). Of the 6 siRNAs for each target, the two most potent siRNAs were selected and ordered with a propylthiol modification at the 3' end and attached to AuNPs. These AuNPs with siRNA attached were delivered to CT26 cells and knockdown of targets was confirmed in the same manner as described above. Because of these results, we currently working on delivering siRNA-coated AuNPs to our mouse model of CRC.

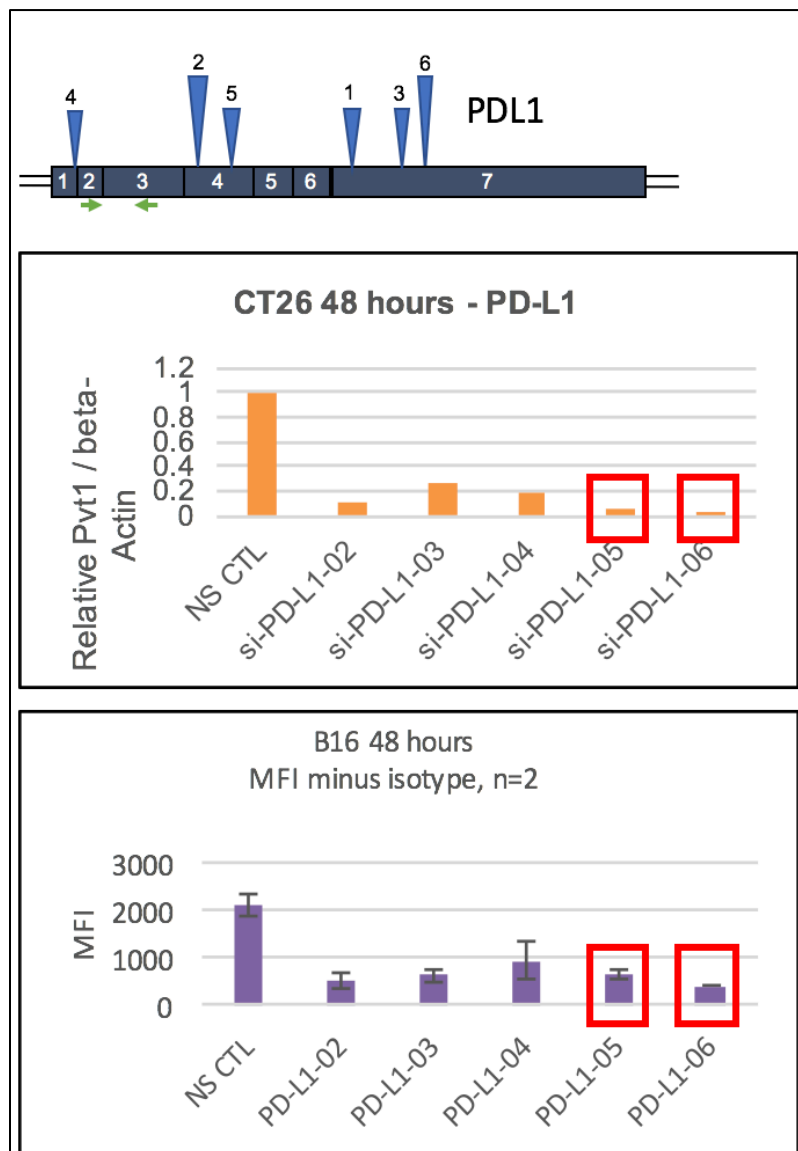


Figure 4. Design of siRNA targeting PD-L1 and confirmation of knockdown by qRT-PCR and flow cytometry. Six siRNAs were designed along the PD-L1 transcript (top). These siRNAs were transfected into CT26 cells and PD-L1 expression was assessed 48 hours later by qRT-PCR (middle). Knockdown was also confirmed at the protein level by flow cytometry (bottom).

we are now working to validate their effectiveness *in vivo*. We have started by injecting AuNPs coated with siRNA targeting PD-L1, or control non-specific siRNA, to shAPC mice via tail vein. Three days after injection, we will harvest colon tumors from the mice and assess knockdown of PD-L1 by RT-qPCR and flow cytometry. We will then repeat this experiment with AuNPs containing siRNAs targeting Pvt1, or containing both siRNAs together. We are troubleshooting

What opportunities for training and professional development has the project provided?

A postdoctoral fellow and technician have done the bulk of the work on this project. They have each brought unique skills to the project, and have developed many new skills through their research for this project and through their collaboration.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

Because we have validated our siRNA-coated AuNPs *in vitro*,

the issues with the peptide antigen, and will proceed as described in the Changes/Problems section below.

Impact

What was the impact on the development of the principal discipline(s) of the project?

Traditional cancer treatment such as surgery, chemo, and radiation therapies aim to destroy rapidly dividing cells. It can add years to a patient's life, but new methods that are more effective and less harmful for treating colon cancer need to be developed. These new types of drugs interfere with the development of cancer at the molecular level. Because these drugs target specific molecules, they are called molecularly-targeted treatments. Our project is working on a new way of attaching the molecularly-targeted drugs (peptides and siRNA) to the gold nanoparticles. Part of our project is to use immunogenic peptides to activate pre-existing CD8 T cells. The peptides will stimulate the body's immune system to target and attack cancer cells. Another part of our research includes coating these gold nanoparticles with siRNAs which degrade specific mRNA targets in cancer cells (PVT1 and PD-L1). This new approach to treat colon cancer is will have an impact in several ways.

- The advantage of using pre-existing, high functioning memory T-cells that already exist in vaccinated humans helps eliminate the costly and potential for autoimmunity associated with cellular based therapies.
- The targeted therapies we are developing act on specific molecular targets that are associated with cancer, whereas most standard chemotherapies and radiation therapies act on all rapidly dividing normal and cancerous cells.
- Targeted therapies using siRNA can have high efficacy. siRNA can cause dramatic suppression of gene expression with just several copies.
- The new tumor targets, novel ligands, new strategies for targeting, particle stabilization and use of gold nanoparticles for drug delivery will positively impact drug delivery at the tumor level while decreasing toxicity to normal tissues.

By using all of these strategies in combination it provides the highest opportunity for curing patients with metastatic CRC. In the future, nanoparticle technology and the development of targeted cancer therapy will continue to expand. We believe that by using many different agents to both allow the body to recognize the cancer cells as "bad" and to give the immune system a jump start that we will be able to effectively treat CRC.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

Changes/Problems

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Despite successful validation of our immunogenic peptide itself, we were unable to detect MHC presentation of the DNA-peptide conjugate when introduced to splenocytes. To verify whether the absence of signal observed by flow cytometry was attributed to the lack of MHC presentation, we are in the process of performing a non-radioactive cytotoxic T-lymphocyte (CTL) killing assay and a chromium release assay. If this proves unsuccessful, we will reorder the oligo-peptide conjugate, and adjust the location where the DNA oligo associates with the peptide to improve intercellular processing and presentation on the MHC. If continued troubleshooting of this proves unsuccessful, a fallback option of conjugating the peptide antigen directly to the gold nanoparticle should still achieve the desired effect of increasing CD8 T cell targeting to gold nanoparticle bearing tumors. Though this would lose the extreme specificity of our proposed design, this approach would still result in targeted killing as the gold nanoparticles preferentially hone to tumor sites.

Changes that had a significant impact on expenditures

One measure we are taking to reduce the cost of reagents is synthesizing our own gold nanoparticles rather than purchasing them commercially. The synthesis protocol is relatively easy and though it required an initial equipment purchase of ~\$100, this method allows us to make 1L of gold nanoparticles for ~\$122 compared to the \$1,800 cost from the company for the same amount. This new method will be especially useful as we will require many gold nanoparticles for our animal studies, and allow funds to be used in other areas of the experiment.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

A minor change in animal care is the introduction of doxycycline through food chow in addition to drinking water. The dose of doxycycline from the drinking water alone, as originally described, was too low to induce the tumor polyps. However, introducing doxycycline through the drinking water in combination with doxycycline feed provided the appropriate dosage to result in tumor formation.

Products

Publications, conference papers, and presentations

Nothing to report.

Other publications, conference papers, and presentations.

Nothing to report.

Participants & Other Collaborating Organizations

Name:	<i>Branden Moriarity, PhD</i>
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Funding Support:	<i>DOD Idea Award</i>

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Funding Support:	<i>DOD Idea Award</i>

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Nearest person month worked:	<i>12</i>
Contribution to Project:	<i>Planning and carrying out experiments. Managing mouse colony and breeding to develop murine model of CRC. Also generating all AuNP for experiments.</i>
Funding Support:	<i>DOD Idea Award</i>

Name:	<i>Margaret Crosby</i>
Project Role:	<i>Technician</i>
Researcher Identifier (e.g. ORCID ID):	<i>Not Applicable</i>
Nearest person month worked:	<i>12</i>
Contribution to Project:	<i>Planning and carrying out experiments. Performing all in vitro testing of AuNPs and analysis of siRNA knockdown in cultured cell lines.</i>
Funding Support:	<i>DOD Idea Award</i>

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to report.

Special Reporting Requirements

Not Applicable

Appendices

Nothing to report.