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PRINCIPAL INVESTIGATOR: Branden Moriarity

CONTRACTING ORGANIZATION: Regents of the University of Minnesota
Minneapolis, MN 55455

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Branden Moriarity, Tim Starr, Emily Pomeroy, Meg Crosby, Wilaiwan Durose.

E-Mail: mori0164@umn.edu

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Regents of the University of Minnesota,
Office of Sponsored Projects Administration
450 McNamara Alumni Center
200 Oak Street SE, Minneapolis, MN 55455-2070

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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-hydroxytamofixen (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 MHCI 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.

15. SUBJECT TERMS
Au-NP: Gold Nano particles Oligo: Oligonucleotides CRC: Colorectal Cancer

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12/31/2018 Final Technical Report for DOD Grant titled:

Targeted therapy combined with immune modulation using gold nanoparticles for treating metastatic colorectal cancer

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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 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 memory 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 a 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 goals of the project were to construct gold nanoparticles (AuNPs) that carry siRNA targeting the PVT1, siRNA targeting PDL1, and a peptide antigen capable of activating circulating memory CD8+ T cells. Once made, our goal was to deliver these AuNPs to colorectal cancer (CRC) cells *in vitro*. We also sought to establish a mouse model of CRC to perform *in vivo* experiments with our AuNPs.

What was accomplished under these goals?

Mouse model of CRC: We have established a reliable mouse model of CRC. We received LSL-rtTA3/shAPC/Lgr5-CrER transgenic mice (2 males, 2 females) from Dr. Luke Dow. We bred these pairs of mice to create a colony of transgenic mice. When experimental mice were 8 weeks of age, they were treated with 4-hydroxytamofixen (4OHT) and put on continuous doxycycline via drinking water to initiate tumor formation. We were able to readily detect tumor formation after 4-6 weeks via colonoscopy. Tumor cells expressed GFP that was detectible macroscopically as well as by flow cytometry (shown in previous report).

Design AuNPs that selectively release a peptide antigen in cells expressing a cell-specific mRNA: We purchased a DNA-peptide conjugate 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 1**). 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 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.

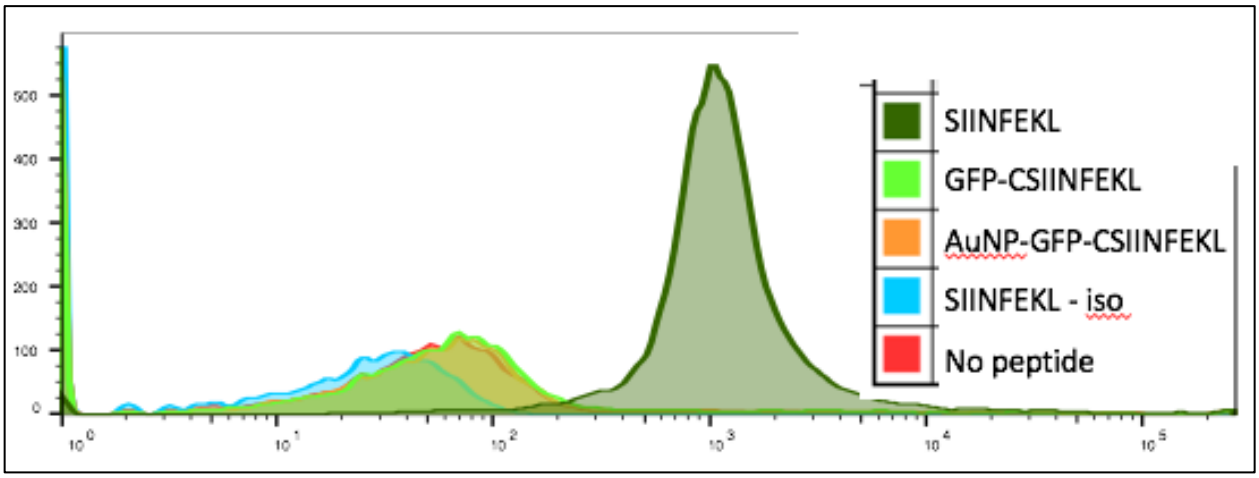


Figure 1. SIINFEKL peptide linked to a DNA oligonucleotide cannot be detected in H2Kb by flow cytometry. C57BL/6 splenocytes were pulsed with SIINFEKL, SIINFEKL linked to a DNA oligonucleotide (GFP-SIINFEKL), or AuNPs loaded with GFP-SIINFEKL. After pulse, cells were stained with an antibody specific to SIINFEKL loaded in the MHC Class II molecule H2Kb. Only the SIINFEKL peptide alone could be detected with this assay.

Due to the issues with selective release and the issue with oligo-linked SIINFEKL, we decided to move forward with directly attaching SIINFEKL to AuNPs without the DNA oligo. To attach a peptide directly to AuNPs, the peptide must contain an amino-terminal cysteine residue. We tested the ability of SIINFEKL-specific CD8+ T cells (OT1 cells) to kill target cells loaded with SIINFEKL or control peptide (SSYSYSSL) in different formats. We tested peptide alone, peptide with N-terminal cysteine (C-peptide), and peptide attached to AuNPs (**Figure 2**).

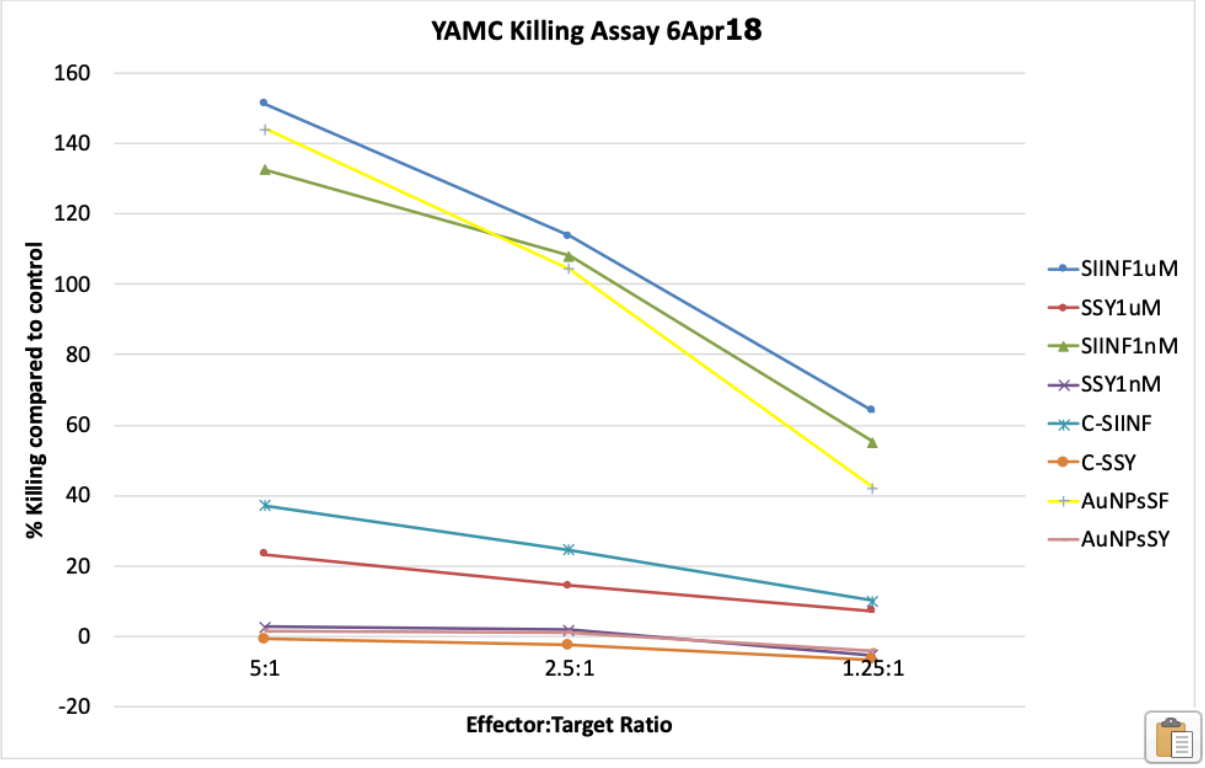


Figure 2. OT1 splenocytes were harvested from mice and activated for 5 days with SIINFEKL peptide, 30 IU/mL IL2, and CD3/CD28 Dynabeads. YAMC cells were loaded with peptide for 2 hours then washed with PBS. Target cells were incubated with effector cells at the indicated ratios and killing was measured by LDH release.

We observed killing of target cells loaded with SIINFEKL or with AuNP-SIINFEKL delivered, but no killing of any target cells loaded with control peptide or with C-SIINFEKL. This indicates that the N-terminal cysteine likely prevents loading of the peptide, but delivery with AuNPs overcomes this and SIINFEKL can be cleaved/released from the AuNP and loaded for MHC I presentation. **These results indicate that an antigenic peptide can be delivered with AuNPs to elicit an immune response from peptide-specific CD8+ T cells.**

Identify potent and specific siRNA to Pvt1 and Pd-l1: We designed 6 siRNAs to each Pvt1 and Pd-l1 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 (shown in previous report). 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. AuNPs with siRNA attached were delivered to CT26 cells and we measured knockdown in the same manner. Results of delivery of AuNPs containing Pd-l1 siRNA are shown in **Figure 3**.

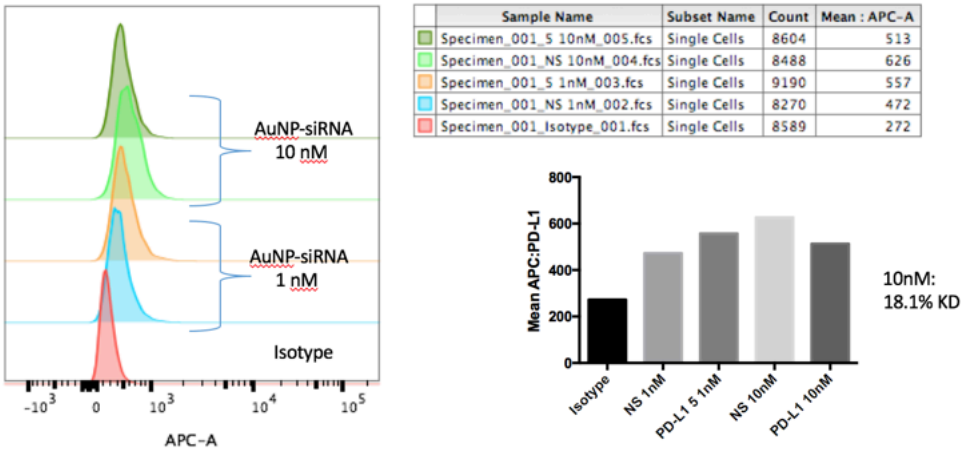


Figure 3. AuNPs containing a non-silencing siRNA (NS) or siRNA against PD-L1 were delivered to CT26 cells and knockdown was measured by flow cytometry 48 hours later. Neither 1 nM or 10 nM dose of PD-L1 siRNA AuNPs were able to significantly decrease PD-L1 expression.

Unfortunately, we did not observe significant knockdown of Pd-l1 after delivery of AuNPs containing siRNA against Pd-l1. We tried higher doses of AuNPs (30 and 300 nM) and still observed negligible knockdown of Pd-l1 (data not shown).

Although these results were not promising, we had also delivered Pd-l1 siRNA-containing AuNPs to our shAPC mouse model in parallel. We first confirmed Pd-l1 expression in colon polyps from shAPC mice (**Figure 4**).

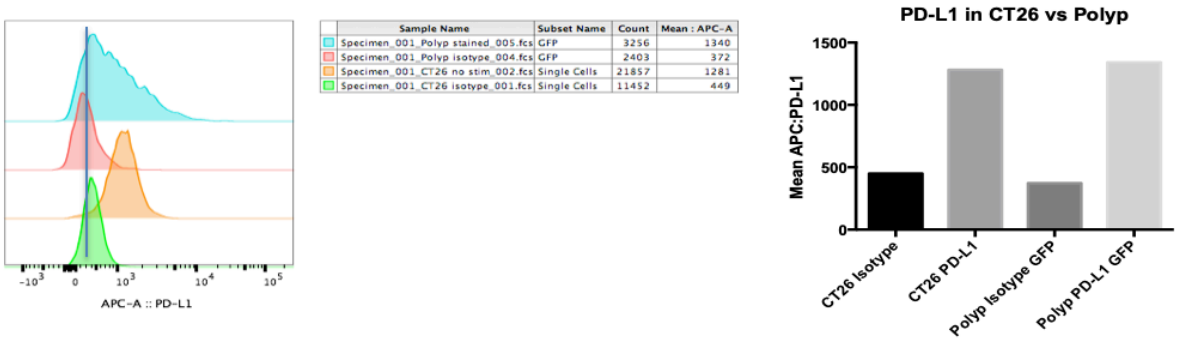
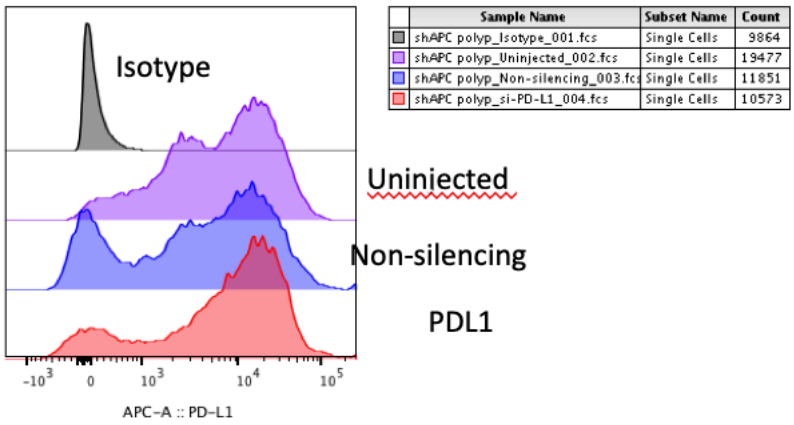


Figure 4. Comparison of PD-L1 levels in all live CT26 cells and live polyp cells gated for GFP (confirmation of shAPC tumor).

We injected AuNPs containing either NS siRNA or Pd-l1 siRNA directly into colon polyps. Polyps were harvested and analyzed by flow cytometry (**Figure 5**). Polyps have high levels of Pd-l1 on their surface, and there seems to be a negative population, a medium-high population, and a high population. Injection of AuNP containing Pd-l1 siRNA had no effect on the surface levels of Pd-l1.



Sample Name	Subset Name	Count
shAPC polyp_Isotype_001.fcs	Single Cells	9864
shAPC polyp_Uninjected_002.fcs	Single Cells	19477
shAPC polyp_Non-silencing_003.fcs	Single Cells	11851
shAPC polyp_si-PD-L1_004.fcs	Single Cells	10573

Figure 5. Polyps of shAPC mice were directly injected with AuNPs containing NS-siRNA or PD-L1-siRNA. Mice were sacrificed 48 hours later and injected polyps as well as uninjected controls were harvested and processed to a single-cell suspension. PD-L1 expression was analyzed by flow cytometry.

We next tried a different delivery format for our siRNA instead of AuNPs. We used PLGA nanoparticles conjugated with Pd-l1 siRNA and tested them in vitro in CT26 cells (**Figure 6**). This alternative delivery method also did not successfully accomplish PD-L1 knockdown.

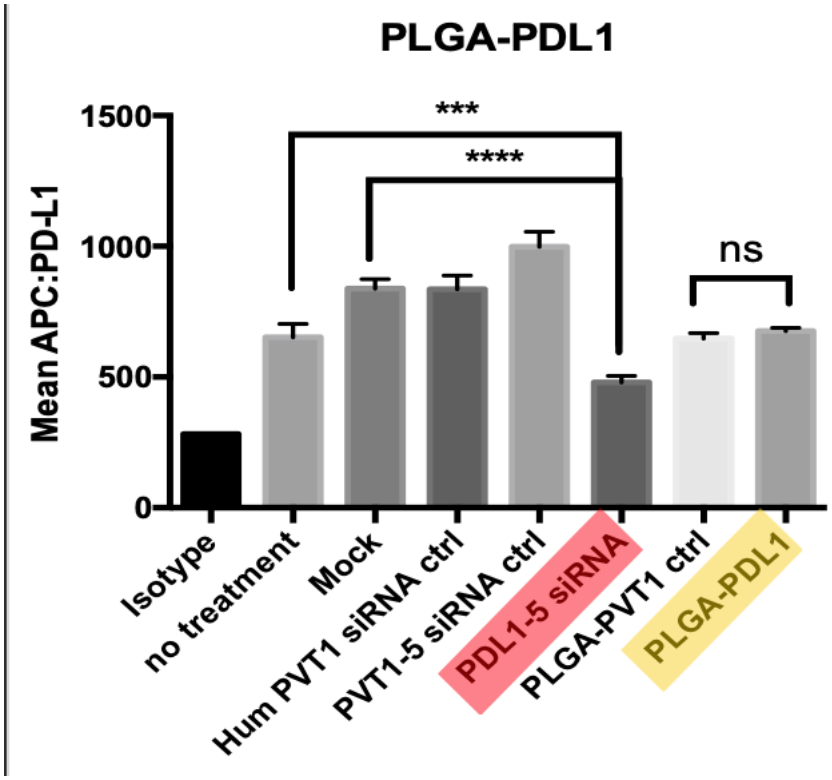


Figure 6. Flow cytometry for PD-L1 in CT26 cells 72 hours after transfection with siRNA or incubation with PLGA nanoparticles. We used human PVT1 siRNA and mouse PVT1 siRNA as controls (should have no effect on PD-L1 expression) as we did not have PLGA nanoparticles conjugated with NS siRNA. The naked siRNA worked to knockdown surface levels of PD-L1, but the PLGA nanoparticles delivering PD-L1 siRNA did not knockdown levels of PD-L1.

We thought the propothiol attachment to the siRNA used to conjucgate siRNA to AuNPs might disrupt the siRNA activity. To assess for a functional effect of Pvt1 knockdown, we performed a transwell migration assay. We delivered Pvt1 siRNA alone, Pvt1 siRNA with the propothiol

attachment, and Pvt1 siRNA-loaded AuNPs to CT26 cells and then assessed migration (**Figure 7**). We observed a reduction in migration using siRNA, less of an effect using siRNA with the attachment, and a significant increase in migration in cells treated with AuNPs.

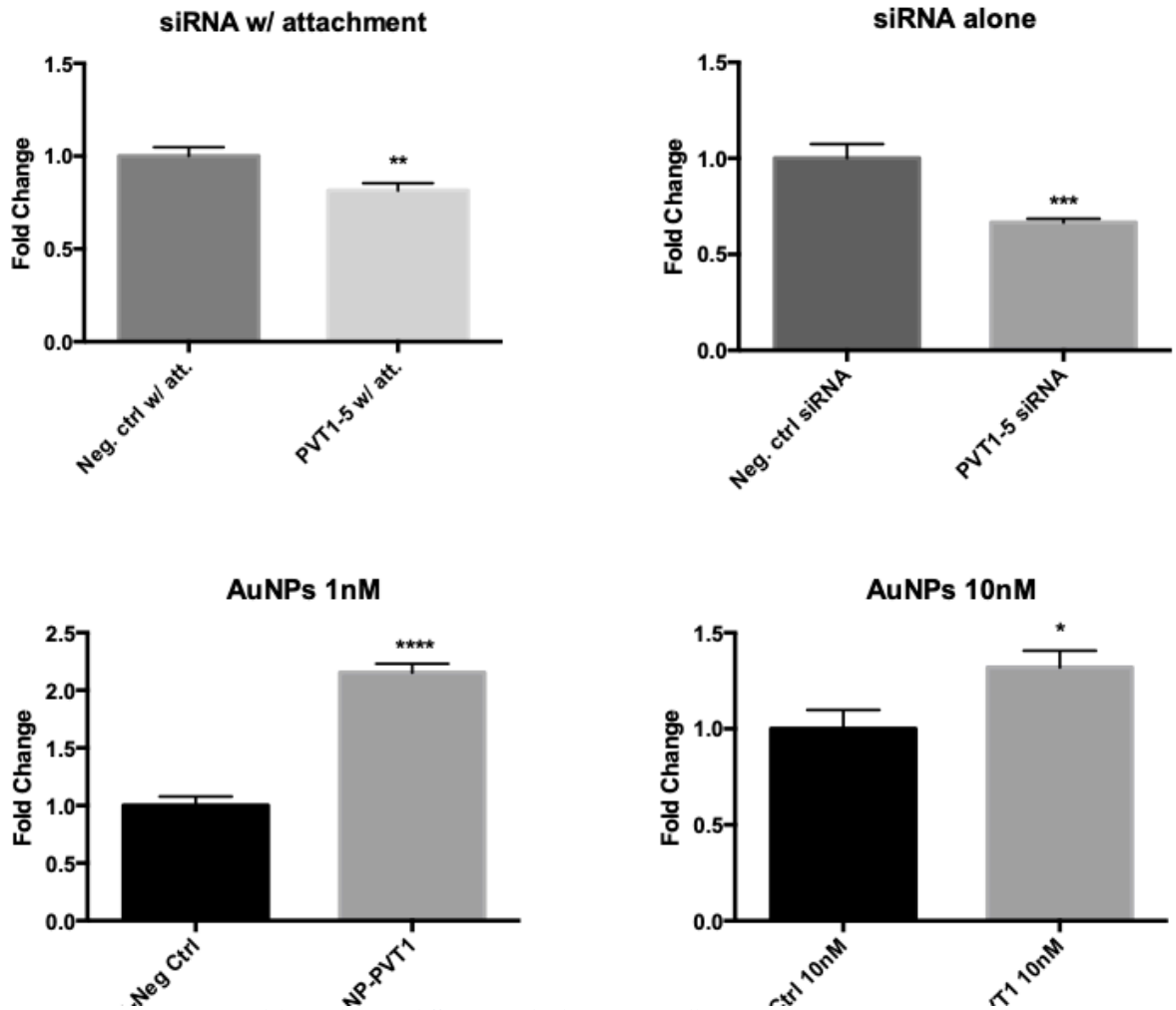


Figure 7. PVT1 siRNA was delivered using different methods to CT26 cells.

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?

Margaret Crosby and Wiliawan Durose presented the results of this research as a poster at the 7th and 8th Annual University of Minnesota Masonic Cancer Center Research Symposium. Margaret Crosby also presented this research at the 2018 Genome Writers Guild Conference.

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. Our project sought a new way to deliver targeted drugs (peptides and siRNA) using gold nanoparticles (AuNPs) as a method for treating colorectal cancer (CRC).

Our results indicate that delivery of siRNA using AuNPs is not a feasible approach for targeting cancer-specific genes. The siRNA we designed worked well on its own to knockdown target genes in CRC cells. However, delivery of siRNA using AuNPs had no effect on target gene expression.

We also sought to deliver an immunogenic peptide via AuNPs to activate pre-existing CD8+ memory T cells, eliciting an immune response toward the tumor. Our proof of principle experiments used the model peptide antigen SIINFEKL. We were able to deliver the peptide using AuNPs and demonstrated enhanced killing of target cells that received the peptide by peptide-specific T cells. Our results also indicate that AuNPs are internalized and cleaved to the appropriate peptide as demonstrated by the ineffectiveness of loading target cells with C-SIINFEKL. This lays the groundwork for an in vivo model of using a peptide antigen to activated memory T cells against a tumor. The advantage of using pre-existing, high functioning memory T cells that already exist in vaccinated humans helps eliminate the cost and potential for autoimmunity associated with cellular based therapies.

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

We initially intended to deliver an immunogenic peptide using selective release – a peptide attached to a DNA oligo that would only be released in cells expressing specific mRNA, based on the NanoFlare technology. During our work we confirmed that selective release was not a viable

option and NanoFlares didn't work as previously reported (cargo was released with high efficiency irrespective of mRNA expression). We thus directly attached the peptide directly to AuNPs.

We tried to validate delivery of siRNA using AuNPs in numerous ways and did not observe the expected results, despite confirmation of siRNA alone.

Changes that had a significant impact on expenditures

Nothing to report

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

Nothing to report

Products

Publications, conference papers, and presentations

Conference presentations:

- Margaret Crosby
  - 7th Annual Masonic Cancer Center Research Symposium (2016)
  - 8th Annual Masonic Cancer Center Research Symposium (2018)
  - Genome Writers Guild Conference: The Next Revolution – Genome Engineering 2018
- Wiliawan Durose
  - 8th Annual Masonic Cancer Center Research Symposium (2018)

Other publications, conference papers, and presentations.

Nothing to report.

Participants & Other Collaborating Organizations

Name:	*Branden Moriarity, PhD*
Project Role:	*Co-PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-3031-3767*
Nearest person month worked:	*1.5*
Contribution to Project:	*Co-lead the project, personnel management spending time planning experiments, trouble shooting, analyzing data, and writing manuscripts.*

Funding Support:	*DOD Idea Award*

Name:	*Tim Starr, PhD*
Project Role:	*Co-PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-6308-3451*
Nearest person month worked:	*1.5*
Contribution to Project:	*Co-lead the project, personnel management spending time planning experiments, trouble shooting, analyzing data, and writing manuscripts.*
Funding Support:	*DOD Idea Award*

Name:	*Wilaiwan Durose, PhD*
Project Role:	*Post-doctoral fellow*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-9463-9689*
Nearest person month worked:	*12*
Contribution to Project:	*Planned and carried out experiments. Managed mouse colony and breeding to develop murine model of CRC. Also generated all AuNP for experiments.*
Funding Support:	*DOD Idea Award*

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

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.