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Novel Cancer Treatment Using Engineered Exosomes to Disrupt Cancer's Immune Escape

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UNDER MY SUPERVISION BY
Abena Boateng, Carley Fowler, Maritza Soria

ENTITLED

Novel Cancer Treatment Using Engineered Exosomes to Disrupt Cancer's Immune Escape

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

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IN

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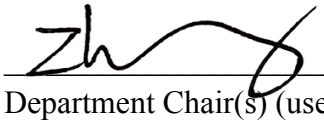

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Department Chair(s) (use separate line for each chair) date

Novel Cancer Treatment Using Engineered Exosomes to Disrupt Cancer's Immune Escape

By

Abena Boateng, Carley Fowler, Maritza Soria

Senior Design Project

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Abstract

Cancer is one of the leading causes of death in the United States[1]. This disease has impacted billions worldwide and has led to an ever-increasing burden on the healthcare system. Over the last couple of years, researchers have improved upon conventional cancer therapy that includes chemotherapy, radiation, and surgery. One novel approach is immunotherapy, which shows great potential because it has the ability to directly target cancerous cells[2]. This is a viable treatment because most cancers develop the ability to block immune pathways and evade the killer immune cells in what is known as cancer immune escape. One such pathway that is inhibited during the progression of specific cancers is the PD-L1 and PD-1 pathway thus inhibiting this pathway can be an effective treatment of advanced cancers[3]. To improve upon the efficacy of certain immunotherapies, we engineered a novel delivery method using exosomes to target this specific pathway. We focused on exosomes due to the nanovesicles ability to penetrate tissue better, the longer half-life of the treatment, and the biocompatibility of naturally derived vesicles. To complete this task, we focused on successfully integrating our exosomes expression vector in HEP-G2 and HK-2 cell lines. Next, we verified production of the engineered exosomes through the process of cotransfection using a known exosome-specific marker vector. Our results are valuable as they prove successful exosome plasmid vector integration. Furthermore, our study is a proof of concept that exosomes can be manipulated to express surface proteins; having the potential of interrupting immune evading pathways of numerous diseases.

[1] U.S. Cancer Statistics Working Group. U.S. Cancer Statistics Data Visualizations Tool, based on November 2018 submission data (1999-2016): U.S. Department of Health and Human Services, Centers for Disease Control and Prevention and National Cancer Institute; www.cdc.gov/cancer/dataviz, June 2019.

[2] Sharma P, Wagner K, Wolchok JD, Allison JP. Novel cancer immunotherapy agents with survival benefit: recent successes and next steps. Nat Rev Cancer. 2011;11:805–12.

[3] Wu Y, Chen W, Xu ZP and Gu W (2019) PD-L1 Distribution and Perspective for Cancer Immunotherapy— Blockade, Knockdown, or Inhibition. Front. Immunol. 10:2022. doi: 10.3389/fimmu.2019.02022

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1. Introduction

1.1 Background and motivation

The search for a successful cancer treatment has been an ongoing pursuit. Over the last few decades there have been many advancements but the treatments available today still cause severe side effects that diminish the quality of life for patients. For example, conventional therapies like chemotherapy and radiation lead to chemotherapy-induced nausea, vomiting, and peripheral neuropathy, as well as malabsorption, weight loss, anemia, fatigue and increased risk of sepsis[4]. Therefore research has pivoted its focus towards biologics, like immunotherapies, as novel treatment options for the future. Antibody based treatments have been proven to be more effective at reducing side effects than conventional therapies, but they still have their limitations. Oftentimes antibody therapies cause the immune system to attack healthy cells resulting in adverse effects from an overactive autoimmune response[5]. For this reason we want to explore the possibility of an engineered biocompatible nanoparticle immunotherapy, inspired by exosomes and their unique ability to penetrate tissue membrane barriers.

1.2 Literature review

1.2.1 Overview of the Immune System

The immune system, as humans experience it, has two main subdivisions. The first is the innate immune system response which refers to a variably-nonspecific and fast-acting defense mechanism. This includes physical barriers such as the skin, chemicals in the blood, and immune system cells that attack foreign cells in the body. The active cells of the innate immune system recognize general features of pathogens, like bacterial cell walls, and include cells like macrophages, neutrophils,dendritic cells, natural killer cells, mast cells, and basophils[6]. The other subdivision is the adaptive immune system response and it is much more specific and complex. Adaptive immunity is considered the second line of defense because it is initiated if the innate immune response is unable to completely combat the invading pathogen. The main cells of the adaptive immune response, T-cells and B-cells, originate in our bone marrow and mature at different locations in the body. The cells may float around in the bloodstream or lymphatic system or take up residence in an organ or tissue. The specific immune response has four defining characteristics; antigen specificity, diverse recognition range, memory immunity, and

[4] Nurgali, K., Jagoe, R. T., & Abalo, R. (2018). Editorial: Adverse Effects of Cancer Chemotherapy: Anything New to Improve Tolerance and Reduce Sequelae?. Frontiers in pharmacology, 9, 245. https://doi.org/10.3389/fphar.2018.00

[5] Bajwa, R., Cheema, A., Khan, T., Amirpour, A., Paul, A., Chaughtai, S., Patel, S., Patel, T., Bramson, J., Gupta, V., Levitt, M., Asif, A., & Hossain, M. A. (2019). Adverse Effects of Immune Checkpoint Inhibitors (Programmed Death-1 Inhibitors and Cytotoxic T-Lymphocyte-Associated Protein-4 Inhibitors): Results of a Retrospective Study. Journal of clinical medicine research, 11(4), 225–236. https://doi.org/10.14740/jocmr3750

[6] Janeway CA Jr, Travers P, Walport M, et al. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science; 2001.

self:non-self discrimination[7]. The innate immune system and adaptive immune system work together to monitor the body looking for abnormalities and maintaining the homeostatic standard.

An important concept to understand is how the immune system determines a cell to be unhealthy or pathogenic, resulting in cell death. Although the intercellular communication is much more complex, the foundational concept of how immune cells determine which cells to kill based on presented immune-evasion surface biomarkers is illustrated in figure 1 below.

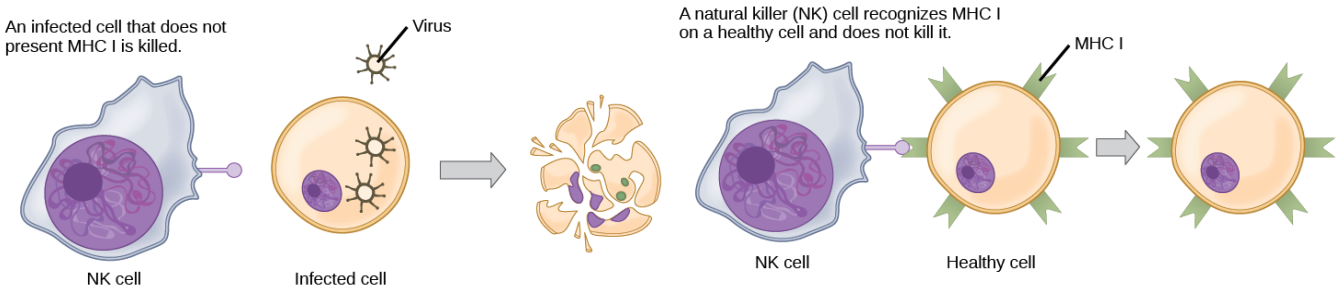

Figure 1. Natural killer (NK) cells recognize the MHC I receptor on healthy cells. If MHC I is absent, the cell is lysed. This figure is adapted from Concepts of Biology.[8]

1.2.2 Cancer is a Complex Disease to Treat

When cells become mutated, they usually appear to the immune cells as abnormal. The body then recognizes them as non-self or foreign because the proper immune-evasion surface biomarkers are not presented. By eliminating cells that have become abnormal, the immune system helps to protect against cancer. However, if the cells mutate enough so that they are able to escape the surveillance mechanisms of the immune system, and they may continue to reproduce as cancer cells[9]. Cancer is one of the leading causes of death in the United States[10]. This disease has impacted billions worldwide and has led to an ever-increasing burden on the healthcare system. Over the last couple of years, researchers have improved upon conventional cancer therapy that includes chemotherapy, radiation, and surgery but it still proves very difficult to treat.

Cancer is the uncontrolled growth of abnormal cells in the body and these cells have distinct characteristics that make them so difficult to treat. A main hurdle when trying to treat cancer is that it develops a kind of immortality. In culture malignant cells have shown to live for an

[7] Charles Janeway, Paul Travers, Mark Walport and Mark Schlomchik. Immunobiology. (2004) 6th Edition. Garland Publishing, NY, NY

[8] Concepts of Biology - 1st Canadian Edition by Charles Molnar and Jane Gair

[9] The Immune System. (n.d.). Retrieved from https://www.cancerquest.org/cancer-biology/immune-system

[10] U.S. Cancer Statistics Working Group. U.S. Cancer Statistics Data Visualizations Tool, based on November 2018 submission data (1999-2016): U.S. Department of Health and Human Services, Centers for Disease Control and Prevention and National Cancer Institute; www.cdc.gov/cancer/dataviz, June 2019.

indefinite number of population doublings which is alarming compared to their normal diploid cell counterparts. It is not clear what limits the life expectancy of normal diploid cells in culture, but it may be related to the continual shortening of chromosome telomeres each time cells divide. Cancerous cells on the other hand are known to have elevated levels of telomerase that maintain telomere length and extend their overall possible life and probability to become tumors.[11] Cancer also does not exhibit unique biomarkers , making it difficult to find possible drug targets that would not cause adverse healthy cell death. The most difficult hurdle when trying to treat cancer is its ability to evade the immune system, an ability also known as immune escape.

1.2.3 Overview of Immune escape and the PD1 PD-L1 pathway mechanisms

Under normal conditions, the immune system can activate an anticancer immune response and induce cancer cell death, known as the cancer immunity cycle[7]. First, tumor cells produce mutated antigens that are captured and phagocytosed by antigen-presenting cells (APCs) called dendritic cells. Next, the dendritic cells prime T cells with the tumor antigen, stimulating its differentiation into activated cytotoxic T cells. These activated cytotoxic T cells then travel to the tumor, infiltrate its microenvironment, recognize, and bind to the surface of cancer cells. Lastly, the bound killer T cells release cytotoxins, inducing apoptosis in their target cancer cells. This process is depicted in figure 2 below; and this process would be an ideal response

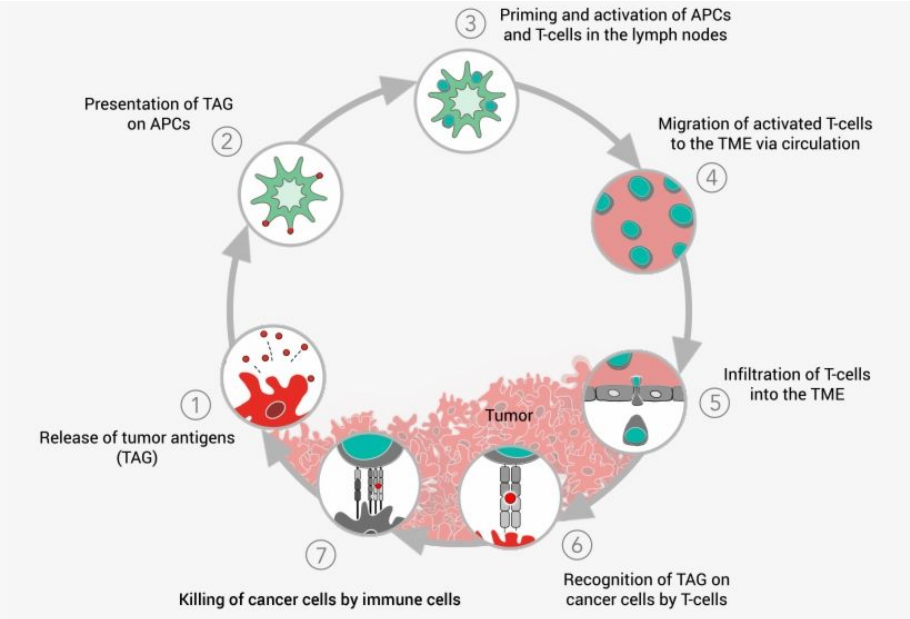

Figure 2. Cancer-immunity Cycle - Immuno Oncology Tumor Micro Environment. Image pulled from DMCA complaint PNG library[12]

[11] Ruddon RW. What Makes a Cancer Cell a Cancer Cell? In: Kufe DW, Pollock RE, Weichselbaum RR, et al., editors. Holland-Frei Cancer Medicine. 6th edition. Hamilton (ON): BC Decker; 2003.

[12] https://www.seekpng.com/ipng/u2e6t4a9u2q8e6i1_cancer-immunity-cycle-immuno-oncology-tumor-micro-environment/" target="_blank">Cancer-immunity Cycle - Immuno Oncology Tumor Micro Environment

Although there are several ways that tumor cells may get around the immune defenses of the body. Many cancers produce chemical messengers that inhibit the actions of immune cells. Other cancers have defects in the way that antigens are presented on their cell surface. An example of this can be seen in the T-cells tightly regulated PD1-PDL1 pathway mechanisms. This pathways helps to ensure activation or deactivation of the immune system at the appropriate times to minimize autoimmune reactions[13]. PDL1 is expressed on the surface of healthy cells to avoid their death during an immune response. However, many cancers have evolved to express PD-L1 on their surface as well to hijack these built in controls to prevent the immune response and avoid cell death, this is called immune escape.

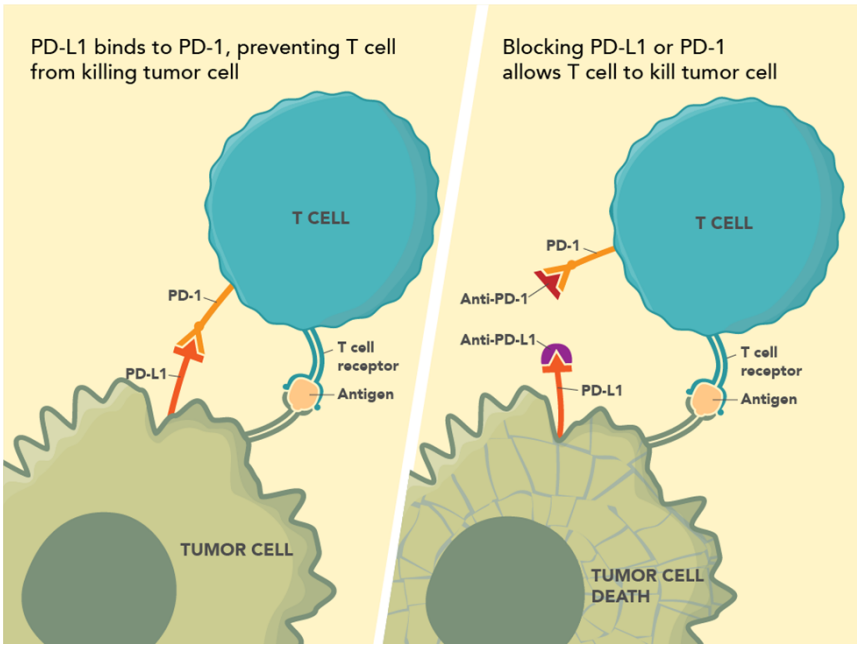

Figure 3. PD-1/PD-L1 Mechanism. The interaction between a T cell and a Tumor Cells via the PD-1/PD-L1 pathway[14]. This image is adapted and modified from the FDA.

1.2.4 PD-1 PD-L1 dependent cancers

Since 1992 when Yasumasa Ishida, Tasuku Honjo and colleagues at Kyoto University discovered the PD-1 receptor there has been vast research on which cancers typically hijack this programmed death pathway in order to hide inside the body. Understanding what cancers are dependent on the PD-1 and

[13] Cancer Immunotherapy: PD-1 and Beyond. (2020). Retrieved https://www.smartpatients.com/targets/pd-1.
[14] Center for Drug Evaluation and Research. (2019, February 22). Determining Clinical Benefits of Treatment with Checkpoint Inhibitors. Retrieved May 30, 2020, from https://www.fda.gov/drugs/regulatory-science-action/impact-story-determining-clinical-benefit-treatment- beyond-progression-immune-checkpoint-inhibitors

PD-1 mechanism can improve the survival rate of cancer patients as the cancer treatments prove to work better when the therapy also targets this specific pathway. As of this year, the use of checkpoint inhibitors are possibly the most understood and successful immunotherapy discovered at this point in cancer therapy. Some cancers that manipulate the PD-1 pathway include bladder cancer, breast cancer, lung cancer, kidney cancer, skin cancer, colorectal cancer, head and neck cancer, lymphoma, cervical cancer, esophageal cancer, and stomach cancer[15]. Moreover, there are specific uses of this immunotherapy that affects the PD-1/PD-L1 checkpoint such as squamous cell carcinoma of the head and neck[16], glioma tumors[17], EGFR-Driven Lung Tumors[18], and chronic infection-induced cytotoxic T lymphocyte exhaustion[19]. Thus, it is imperative to optimize immunotherapies that target PD-1/PD-L1 as they can be used to fight numerous cancer types.

1.2.5 Immunotherapy

Our novel approach involves immunotherapy, which helps the immune system to fight the cancer. There are also many types of immunotherapies which include cancer vaccines, nonspecific immunotherapies, monoclonal antibodies, and checkpoint inhibitors, with the latter two being our strategic focus[20]. Moreover, the benefits of these immunotherapies over conventional therapies are that they can help to bolster treatments by being used in combination with other therapies and that they are effective when other treatments are not[20]. In addition, cancer is less likely to return due to immunomemory, and immunotherapy results in fewer systemic side effects related to conventional therapies[20]. These benefits all greatly improve patient outcomes.

1.2.6 Exosomes have therapeutic potential

Once thought to be a system of cellular waste elimination, exosomes have recently been characterized as a model for cell-cell communication, revitalizing interest in their drug delivery capacity.[21] Exosomes are naturally occurring nano-biovesicles secreted by the cells in our body that can transport cell-specific cargo. Unlike liposomes and other synthetic nanoparticle vehicles, exosomes contain transmembrane and membrane-bound proteins that could promote the

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[15] Luke, J., & Cancer Research Institute. (2020). Immunomodulators: Checkpoint Inhibitors, Cytokines, Agonists, Adjuvants. Retrieved May 27, 2020, from https://www.cancerresearch.org/immunotherapy/treatment-types/immunomodulators

[16] Zandberg, D. P., & Strome, S. E. (2014). The role of the PD-L1:PD-1 pathway in squamous cell carcinoma of the head and neck. *Oral oncology*, *50*(7), 627–632. https://doi.org/10.1016/j.oraloncology.2014.04.003

[17] Filippova N, Yang X, An Z, Nabors LB, Pereboeva L. Blocking PD1/PDL1 Interactions Together with MLN4924 Therapy is a Potential Strategy for Glioma Treatment. *J Cancer Sci Ther*. 2018;10(8):190-197. doi:10.4172/1948-5956.1000543

[18] Akbay, E. A., Koyama, S., Carretero, J., Altabef, A., Tchaicha, J. H., Christensen, C. L., … Wong, K.-K. (2013). Activation of the PD-1 Pathway Contributes to Immune Escape in EGFR-Driven Lung Tumors. *Cancer Discovery*, *3*(12), 1355 LP – 1363. https://doi.org/10.1158/2159-8290.CD-13-0310

[19] Hofmeyer, K. A., Jeon, H., & Zang, X. (2011). The PD-1/PD-L1 (B7-H1) Pathway in Chronic Infection-Induced Cytotoxic T Lymphocyte Exhaustion. *Journal of Biomedicine and Biotechnology*, *2011*, 451694. https://doi.org/10.1155/2011/451694

[20] Media, H. (2019, March 25). Immunotherapy: Side Effects, Risks & Benefits. Retrieved from https://www.cancertherapyadvisor.com/home/tools/fact-sheets/immunotherapy-side-effects-risks-and-benefits/

[21] Lasser C, O'Neil SE, Shelke GV, Sihlbom C, Hansson SF, Gho YS, Lundback B, Lotvall J. Exosomes in the nose induce immune cell trafficking and harbour an altered protein cargo in chronic airway inflammation. J Transl Med. 2016;14(1):181.

endocytosis and delivery of their internal content.[22] Exosomes carrying cell-specific cargo of proteins, lipids, and nucleic acids may act as a novel intercellular communication mechanism. This concept is based on the observation that exosomes released from parental cells may interact with target cells, leading to the subsequent influence of target cell behavior and phenotypic features[23]. Exosomes have been pursued because they show targeted delivery within the body and are even able to pass the blood-brain barrier. They may display distinct tissue/cell homing as a result of their high expression of adhesion molecules such as integrins and tetraspanins, giving them their potential target capability[24]. Once exosomes are internalized there is horizontal genetic transfer of their content to the cytoplasm of the target cells.

Knowing this we would like to utilize the exosomes homing abilities to inhibit a cancer cell's ability to escape the body's immune response by blocking the escape pathway via surface expression of PDL1-scFV, blocking the PD-L1 and PD-1 pathway. In the future, there should be an investigation into the engineered exosomes potential to deliver target-cell nanomedicine, a build off of our project that would make the treatment even more effective.

1.2.7 Exosome Biogenesis

Exosomes are produced in the endosomal compartment of most eukaryotic cells. They originate from multivesicular bodies (MVBs), a specialised subset of endosomes that contain membrane-bound intraluminal vesicles (ILVs) which form by budding into the lumen of these MVBs[25]. The content of MVBS, ILVs, can either be degraded through fusion with lysosomes or released into the extracellular space as exosomes through fusion with the plasma membrane[25, 26].

1.2.8 Tetraspanins

Tetraspanins are membrane-bound proteins with a unique structure that play an important role in cellular signal transduction[27]. Simply, tetraspanins organize membrane proteins for cell-to-cell communication. Thus, they are paramount in the creation of exosomes, nanovesicle-shuttles for signal molecules.The transmembrane structure of tetraspanins allows for inner and outer surface display modifications, which is necessary for engineering exosomes as well as enables the study of exosomes via fluorescent probes.[28] For our project, in the exosomes' expression vector exploits CD8 tetraspanin

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[22] Gonzalez-Calero L, Martin-Lorenzo M, Alvarez-Llamas G. Exosomes: a potential key target in cardio-renal syndrome. Front Immunol. 2014;5:465.

[23] Schneider, A., & Simons, M. (2013). Exosomes: vesicular carriers for intercellular communication in neurodegenerative disorders. Cell and tissue research, 352(1), 33–47. https://doi.org/10.1007/s00441-012-1428-2

[24] Zoller M. Tetraspanins: push and pull in suppressing and promoting metastasis. Nat Rev Cancer. 2009;9(1):40–55. doi: 10.1038/nrc2543.

[25] Multivesicular bodies. (n.d.). Retrieved from https://www.nature.com/subjects/multivesicular-bodies

[26] Exosome (vesicle). (2020, May 19). Retrieved from https://en.wikipedia.org/wiki/Exosome_(vesicle)

[27] Termini, C. M., & Gillette, J. M. (2017). Tetraspanins Function as Regulators of Cellular Signaling . *Frontiers in Cell and Developmental Biology* , Vol. 5, p. 34. Retrieved from https://www.frontiersin.org/article/10.3389/fcell.2017.00034

[28] Stickney Z, Losacco J, McDevitt S, Zhang Z, Lu B. Development of exosome surface display technology in living human cells. *Biochem Biophys Res Commun*. 2016;472(1):53-59. doi:10.1016/j.bbrc.2016.02.058

while we use CD63, a tetraspanin specific to exosomes, to verify the secretion of the engineered exosomes.

1.3 Current Checkpoint Inhibiting Immunotherapies and their drawbacks

Anti PD-1 or anti PD-L1 antibodies can prevent the cancer tumor cell from binding to PD-1 and thus allow T cells to remain active. Table 1 depicts the mAb checkpoint inhibitors that are currently being used as cancer immunotherapy. For example Keytruda, an anti-PD1 inhibitor is specifically FDA- approved for the treatment of metastatic non-small cell lung cancer and head and neck squamous cell carcinoma. And Tecentriq is an anti PD-L1 inhibitor FDA approved to treat cancers like urothelial carcinoma and non-small cell lung cancer. These drugs have been shown to be helpful in treating several types of cancer, and new cancer types are being added as more studies show these drugs to be effective[29]. However, one drawback of these immunotherapies is that it can over activate the immune system and cause off targeting where the immune system attacks healthy cells resulting in serious adverse effects known as immune-related adverse events (irAEs)[5, 30]. The spectrum of organ systems affected by irAEs is very broad, as such toxicities can affect almost any organ, with varying frequencies and severities[30]. Examples of these toxicities range from minor symptoms of inflammation (e.g., fever) to major conditions similar to autoimmune disorders like pneumonitis (inflamed lungs), hepatitis (inflamed kidneys), encephalitis (inflamed brain), and myocarditis (inflamed heart muscle)[30]. Other limitations include rapid clearance rates and low tissue penetration[31].

[29] Immune Checkpoint Inhibitors and Their Side Effects. (2019, December 27). Retrieved from https://www.cancer.org/treatment/treatments-and-side-effects/treatment-types/immunotherapy/immune-checkpoint-inhibitors.html

[30] Martins, F., Sofiya, L., Sykiotis, G.P. et al. Adverse effects of immune-checkpoint inhibitors: epidemiology, management and surveillance. Nat Rev Clin Oncol 16, 563–580 (2019). https://doi.org/10.1038/s41571-019-0218-0

[31] Duong N, Curley K, Brown A, Campanelli A, Do MA, Levy D, Tantry A, Marriott G, Lu B. Decoy exosomes as a novel biologic reagent to antagonize inflammation. Int J Nanomedicine. 2019;14:3413-3425 https://doi.org/10.2147/IJN.S196975

Table 1. *Current PD-1 & PD-L1 Checkpoint Inhibiting Immunotherapies*[15]

	Immunotherapies	Cancers
Anti PD-1	-Pembrolizumab (Keytruda)	-metastatic non-small cell lung cancer -head and neck squamous cell carcinoma
	-Nivolumab (Opdivo)	-melanoma -squamous cell lung cancer -renal cell carcinoma -Hodgkin's Lymphoma
	-Cemiplimab (Libtayo)	-cutaneous or locally advanced squamous cell carcinoma
Anti PD-L1	-Atezolizumab (Tecentriq)	-urothelial carcinoma -non-small cell lung cancer
	-Avelumab (Bavencio)	-metastatic merkel-cell carcinoma
	-Durvalumab (Imfinzi)	-urothelial carcinoma -unresectable non-small cell lung cancer

1.4 Project Goals and Constraints

The ultimate goal of our project is to advance immunotherapy by synthesizing an engineered exosome equipped with a specialized biomarker that will disrupt the PD-1 and PD-L1 pathway between T-cells and cancer cells and in turn, promote cancer cell death more effectively than the current antibody-based checkpoint inhibiting treatments.

The first step in this long-term goal and the specific aim of our senior design project is to successfully engineer an exosome and show that the plasmid vector construct could be integrated and expressed.

We achieved our specific aim in three phases. In the first phase, we established protocols to grow and maintain healthy cells. In the second phase, we used a transfection method to integrate the plasmid construct into two cell lines. Lastly, in the third phase, we used a co-transfection technique to verify the generation of engineered exosomes in our cells.

1.4.1 Phase 1: Learning Mammalian cell culture to Generate Healthy Cells

All group members were new to working in a wet lab environment with mammalian cell culture procedures, so our first project goal was to learn and practice proper mammalian cell culture protocols as well as aseptic technique in order to grow and maintain healthy cells essential for the next phases.

1.4.2 Phase 2: Plasmid vector uptake into 2 cell lines

Next, we aimed to present the successful integration of the plasmid vector into a mammalian cell line. We selected HepG2, a human liver cancer cell line, to complete a PEI transfection and used GFP imaging techniques to check for plasmid vector uptake.

In order to further promote the validity of our findings we made it a goal to show proper plasmid vector expression in a second mammalian cell line. We chose HK-2, human kidney cells, and used GFP imaging techniques to check for plasmid vector uptake.

1.4.1 Phase 3: Verify vector is incorporated into exosomes

Once plasmid vector uptake was established, we wanted to confirm whether or not the engineered exosomes were modified with the vector construct as intended. To verify this we used a co-transfection procedure that would allow us to image the vector construct uptake locations and separately image the intracelular exosome locations so that we can overlay them and check for co-occurence.

1.5 Back-Up Plan

Although we practiced aseptic technique, research with mammalian cell lines is susceptible to bacterial or fungal contamination. Therefore, we kept frozen copies of our cell lines in order to not jeopardize our project timeline in case of this unforeseen circumstance.

1.6 Significance

The successful completion of this study would result in a novel cancer immunotherapy as this therapy would be one of the first exosome-based therapies to be used for cancer treatment. The significance of this treatment is the use of engineered exosomes. These unique nanovesicles increase the efficacy of cancer therapies due to the natural stability of exosomes in the body, the ability to penetrate tissue barriers, and biocompatibility of the vesicales to not induce adverse immune responses. The technology utilized by our proposed exosome therapy could also act as a

precedent design to be applied to a number of other therapeutic uses, such as gene therapies and targeted drug delivery.

1.7 Team Management

We have shared equal responsibility as a team to maintain our cell lines, design and perform experiments, and analyze data. In addition, Dr. Lu and Annie Brown provided guidance through regular correspondence and weekly meetings.

1.8 Budget

Table 2. *Project Budget*

Item	Brand	Cost
DMEM	Gibco	$222.00
Trypsin-EDTA	Gibco	$237.00
PBS	Thermo Fisher Scientific	$253.00
K-SFM	Thermo Fisher Scientific/Gibco	$112.00
BPE	Thermo Fisher Scientific/Gibco	$59.50
EGF	Thermo Fisher Scientific/Gibco	$107.00
100 mm Plates	Greiner Bio One	$524.63
FuGENE HD	Promega	$482.00
PD-L1 Plasmid Prep	Genscript	$700.00
Fetal Bovine Serum	GE Healthcare	$352.00
Opti-MEM Media	Gibco	$21.98
Serum-free Media (Ultraculture)	Lonza	$70.00
	Total Cost:	**$ 3,141.11**

1.9 Research Timeline

Table 3. *Project Timeline*

	Fall Quarter	Winter Quarter	Spring Quarter
Literature Review	X	X	X
Phase 1	X	X	
Phase 2		X	
Phase 3		X	
Write Thesis		X	X
Present Results			X

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2. Learning Mammalian Cell Culture to Maintain Healthy Cells (Research Phase I)

2.1 Design description

Cells and their culture are integral to biomedical research. The ability to reliably and efficiently assess their wellness is a critical aspect of high-quality and reproducible experimentation. Knowing this our phase 1 is to establish protocols to generate healthy cells because all research going forward depends on the success of maintaining several stable cell lines; and adequate confluency between plate passages. All group members were new to working in a wet lab environment with mammalian cell culture procedures so we planned to maintain 2 cell lines through several passages to ensure our ability to generate healthy cells on an ongoing basis.

2.2 Key constraints

The key constraints include the volatility that comes with working with biologics, even when the same procedure is followed, the cells can react differently. This would need vigilant communication among group members in order to ensure that our cell culture was healthy since thawing and restarting a cell line would offset the research timeline by several weeks.

2.3 Expected Results

Informed by healthy mammalian cell description in literature we would expect to see

- plates that had adequate nutrients (media that was pinkish in color)
- Cells that were "starfished" or adhered to the cell plate bottom and not floating to show that they were alive and in condition to be used in phases 2 and 3
- Cell plate confluency of about 70-80% observed as percent of plate area covered with healthy cells

2.4 Materials and methods

Table 4. *Materials for Passaging Cells*

Material	Company	Brand Model #
*DMEM + 10% FBS + PS media or K-SFM+BPE+EGF+ 1% PS	N/A	N/A
Phosphate Buffered Saline pH 7.4 (PBS)	Thermo Fisher Scientific/Gibco	AM9625
*Trypsin 0.25% with phenol red or Trypsin 0.05% with phenol red	Thermo Fisher Scientific/Gibco	15050065
15 mL conical-bottom centrifuge tubes	VWR	89039-666
Dish 150mmx20mm	Thermo Fisher Scientific	150468

Methods

1. Check cell plate confluency (70%-80%)
2. Aspirate old media from plate
3. Wash with 3 mL of PBS. Pipet gently into the side of the plate.
4. Aspirate PBS.
5. Add 1.5 mL of trypsin to the plate to detach cells.
    - HEP-G2: 0.25% Trypsin*

- HK-2: 0.05 % Trypsin

6. Incubate at 37°C for 2 minutes.
7. To deactivate trypsin add 4.5mL of...
   - HEP-G2: DMEM + 10% FBS + PS media*
   - HK-2: K-SFM+BPE+EGF+ 1% PS media
8. Collect cells and media in a 15 mL centrifuge tube, spin at 1500 RPM for 5 minutes.
9. Aspirate off supernatant.
10. Resuspend the pellet in 4 ml of the appropriate cell media.
11. Plate 6 ml on a new cell plate
12. Drop cell suspension solution onto plate (amount depends on size of cell plate and the desired density on the new cell plate)
13. Incubate at 37°C for 24-72 hours.

2.5 Results

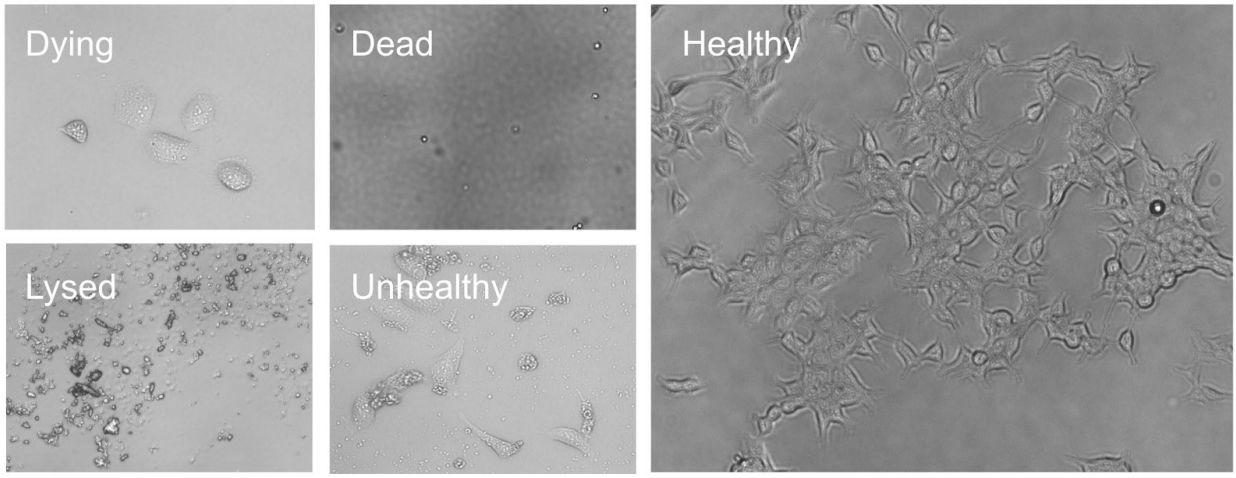

Figure 4. Cell Morphology and Health Status. Images show the difference between Dead and Healthy cells. Phase images taken at 20x magnification for Dying, Lysed, and Unhealthy. Dead and Healthy images at 10x magnification.

We accomplished Phase 1 by learning aseptic technique and mammalian cell culture. As a result, we were able to understand the difference between unhealthy and healthy cells. The data above represents some images we collected during this phase that showcase our progression to mastery. The first image shows dying cells demonstrated by their round shape. The Next image depicts dead cells which are not only round, but are also floating in solution. In the third image, the cells are lysed; this happened during one of our failed transfection experiments. The fourth image represents an overall unhealthy cell culture, which contains both healthy but mostly dying cells.

Next, the last image showcases very healthy and viable cells which have a nice stretched shape and great confluency across the plate.This is what we would want to see on the cell plate before we continue on to phase 2 and 3. Hence, the importance of Phase 1 was to prove our ability to grow healthy and viable mammalian cells.

In phase 1 we also came to learn of the phenotype characteristics of both the HEP-G2 and HK-2 cells when healthy. The figure below shows how HEP-G2 cells had more circular symmetry when "star-fiched" and had more distinctive cell edges while HK-2 cells were more elongated when adhered to the cell plate and have less distinctive cell edges.

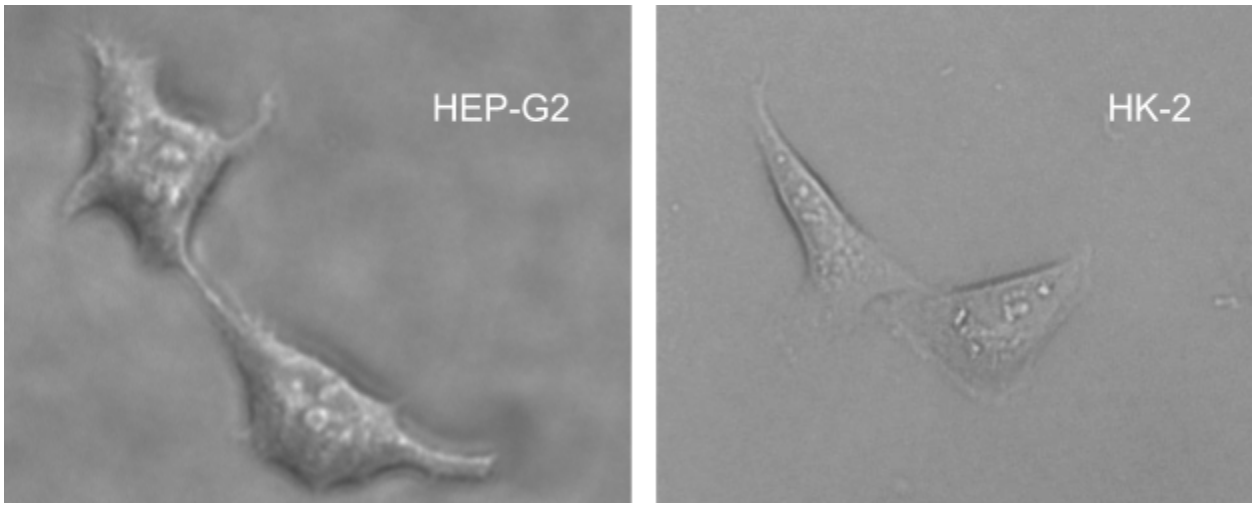

Figure 5. Phenotype Characteristics of Both the HEP-G2 and HK-2 cells when healthy.

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3. Designing and Constructing Vectors of Engineering Exosomes (Research Phase II)

3.1 Design description

A confluent plate of healthy and viable mammalian cells generated in Phase I is important for transfection. This will allow for our vector construct to be uptaken by the cells ultimately leading to the production of our engineered exosomes. We wanted to implement this phase in two mammalian cell lines, HEP-G2 (a human liver cancer line) and HK (a human kidney cell line) to prove the feasibility of our design concept.

3.1.1 Vector Construct

Our expression cassette (Fig 6. A), which includes many components integral for the proper expression of our vector construct, is contained within a pUC57 plasmid vector.

This construct will be termed PDL1-GFP. The first component is a CMV promoter, known for its strength in driving gene expression. Next, the vector has a signal peptide which sends the targeted receptor to the membrane of the exosomes. Following the signal peptide, is the sequence for PDL1-scFV a truncated version of anti PDL1 that will present on the surface of the exosomes to bind to PDL1 on cancer cells. The vector also includes a CD8 hinge region to help with the flexibility of the external portion of the transmembrane protein and promote binding efficiency with the PDL1 on cancer cells. The tVSGS sequence is an intramembrane protein. This acts as a link allowing the receptor to be bound to the external surface of the exosomes and a fluorescent reporter molecule like eGFP, the next component which will allow us to verify vector construct (Fig 6.B) uptake and the successful generation of our engineered exosomes (Fig 6.C) through imaging, to be bound to the internal membrane. Also, the vector includes an antibiotic resistance component, expressed by the SV-40 promoter which can be used in the future for transient expression or establishing a stable cell line. Lastly, the poly(A) tail protects our construct from enzymatic degradation in the cytoplasm and aids in transcription termination, export of the mRNA from the nucleus, and translation.

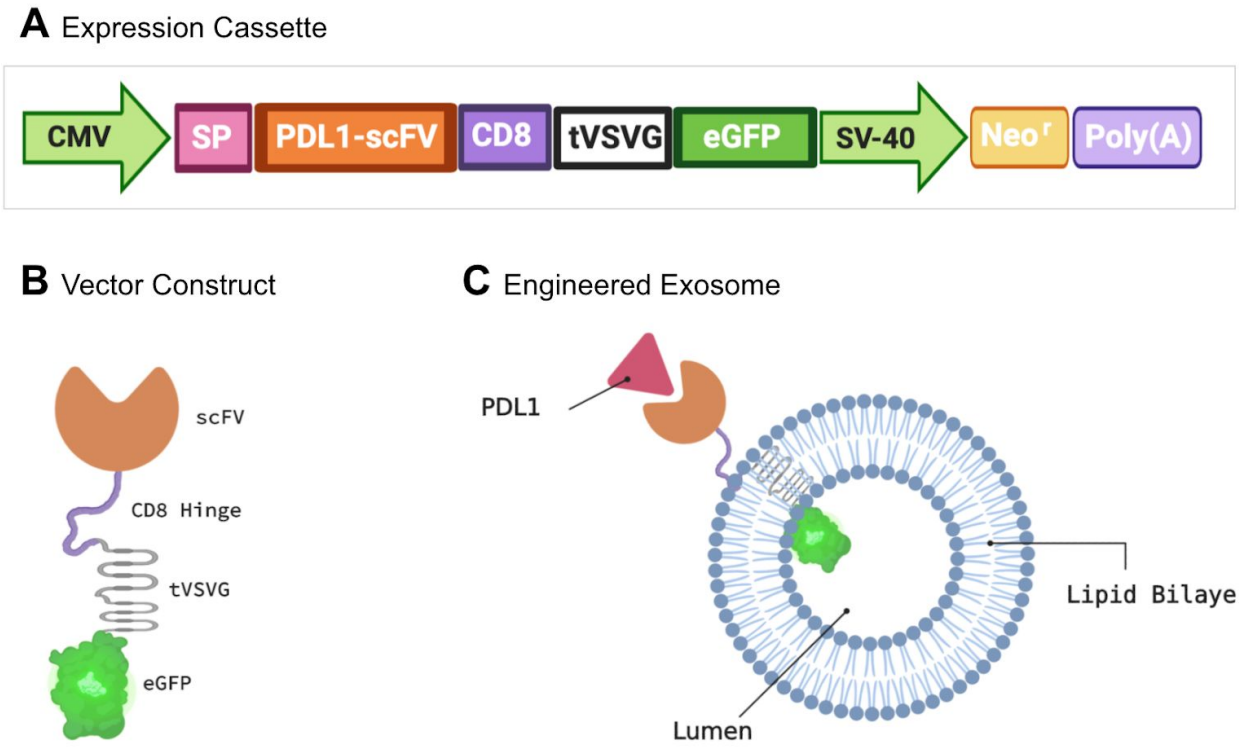

Figure 6. Illustration of the Design-Construct. (A) expression cassette (PDL1-GFP) (B) vector construct, and (C) engineered exosome.

3.2 Key constraints

The key constraints include cell health, confluency, and amount of transfection agent. It's important that our cells are healthy, that the cell plate is substantially populated at 70-80% confluency, and the transfection agents are measured in the right proportions for high transfection efficiency of our vector construct.

3.3 Expected results

Through imaging, we expect to see green fluorescence which will allow us to assume successful transfection and integration of our vector construct with the cells' exosomes.

3.4 Materials and Methods

Table 5. *Materials for Transfecting Cells*

Material	Company	Brand Model #
*HEP-G2 or HK cells		
Polyethylenimine (PEI)	Thermo Fisher Scientific/Gibco	BMS1003
Opti-MEM	Thermo Fisher Scientific/Gibco	51985034
DNA sample (PDL1_pcDNA3.1(+)-C)	Genscript	N/A
6 well plates	Sigma-Aldrich/ Greiner	CLS3516
Microcentrifuge tubes	Sigma-Aldrich/ Eppendorf Safe-Lock	T9661

1. Follow Passaging HEP-G2 Cells Protocol
2. *Adding Cell Suspension Solution
    - HEP-G2: Add 30-40 µl of cell suspension solution from step 10 to two well of a 6-well cell culture plate
    - HK: Add 500-1000 µl of cell suspension solution from step 10 to two well of a 6-well cell culture plate

3. Label one of the wells the control and the other one the transfected cells
4. Incubate at 37°C for 24 hours.
5. Check cells for 70-80% confluency.
6. Add 200 µL of OPTIMEM to a microcentrifuge tube
7.*Adding PEI & DNA
   - HEP-G2: Add 10 µL of PEI and then 2 ug of DNA to the tube
   - HK: Add 2.5 µL of PEI and then 2 ug of DNA to the tube
8. Let the solution sit for 20 minutes
9.Add the entire contents of the solution dropwise to the well designated for transfection.
10. Incubate at 37°C for 24 hours 38

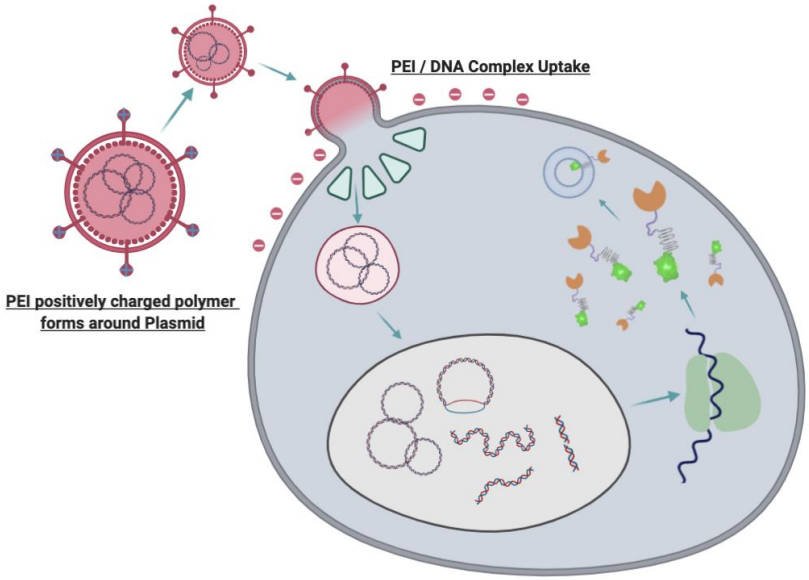

Figure 7. Mechanism of PEI. For transfection, we combined Optimum, growth media, PEI, a positively charged polymer, and our plasmid to create a transfection solution (Steps 6 & 7 above). Since the plasmid DNA and cell membrane are both negatively charged, encapsulating the DNA with a positively charged polymer like PEI allows for the plasmid to enter the cell. Without PEI, the DNA would not be able to enter the cell due to charge repulsion. Once the DNA is in the cell, the ribosomes can generate our vector construct which can then be incorporated into exosomes.

Table 6. *Materials for Imaging Cells*

Material	Company	Brand Model #
Transfected Cell	N/A	N/A
Olympus fluorescence microscope	Olympus-Life Science	

1. Follow Passaging and Transfecting Cells Protocols
2. Use the Olympus fluorescence microscope and Cell Sense Standard software
3. Capture Phase Images
4. Flip the tab on the microscope to create a black background to see the only the fluorescent cells by canceling out the white light
5. Capture of the image of the fluorescence cells.
6. Repeat the process for desired cells at different magnifications

3.5 Results

3.5.1 HEP-G2

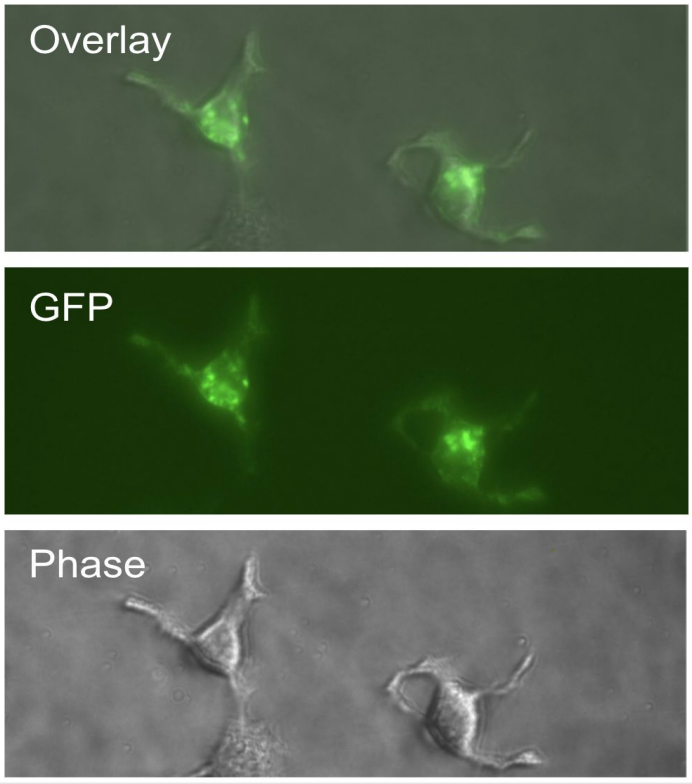

Figure 8. Transfected HEP-G2 Cells. Cells transfected with PDL1-GFP. Engineered exosomes marked by GFP. Images taken at 40x.

These are the results for our transfections into HEPG2. The bottom image is the phase image which confirms the production of healthy cells. The second image demonstrates that our plasmid was integrated due to the green fluorescence produced from GFP. Lastly, The overlaid image of the phase and green fluorescence (shown at the top) allows us to assume that our construct is concentrated in the right compartment, the cell cytoplasm. These images are what we expected, and serve as the initial data that we can use to assume our plasmid was transfected and the engineered exosomes were successfully generated.

3.5.2 HK-2

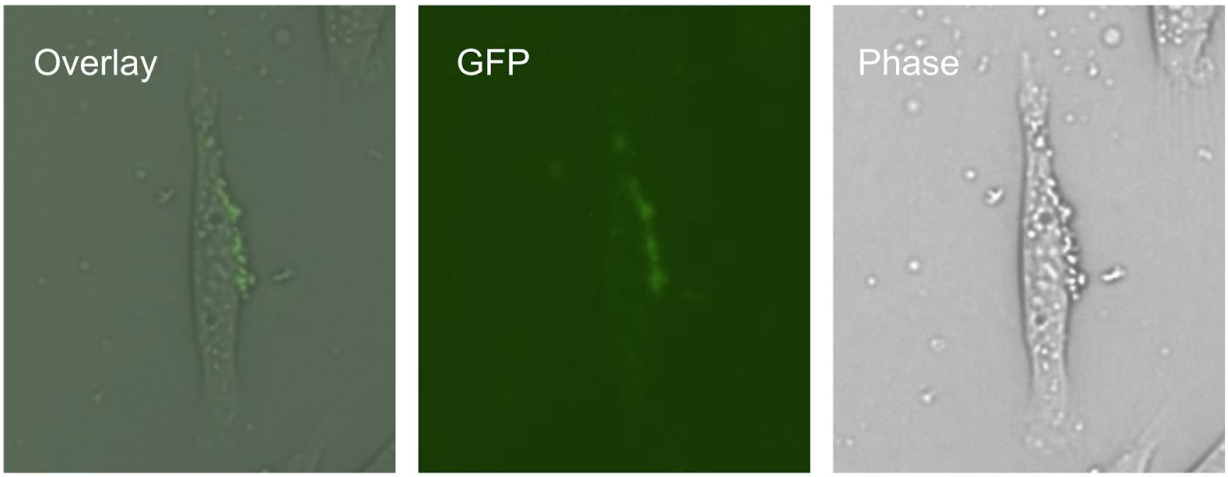

Figure 9. Transfected HK-2 Cells. Cells transfected with PDL1-GFP. Engineered exosomes marked by GFP. Images taken at 40x.

Transfection of our vector construct into another mammalian cell line, HK-2, was important to strengthen our proof of concept by further proving its feasibility. The results are analogous to HEP-G2. The phase image (on the right) represents a healthy and stretched HK-2 cell necessary for transfection.From the image in the center, green fluorescence can be seen on the right side of the cell. Lastly, the overlaid image of the phase and GFP images (on the left), showcase that our vector construct was produced and has integrated into the cell's exosomes within the correct cellular compartment.

4. Verification of Vector Uptake to Make Engineered Exosomes (Research Phase III)

4.1 Design description

The imaging of the GFP successfully showed that the exosome construct was transfected into the both cell lines, HEP-G2 and HK-2. To further verify the transfection of the cells and to confirm the creation of exosomes, we performed a co-transfection with CD63-RFP on the HEP-G2 cell line. This RGF plasmid contains an designated exosomes marker that will be used to confirm the development of the engineered exosomes within the cells. The design goal was the cotransfection with the RGF plasmid will allow us to demonstrate that the construct, PD-L1-GFP, had produced engineered exosomes if the plasmid were colocalized. Hence, the resulting RFP and GFP images were overlaid to clearly show that the exosomes were successfully created with desired surface protein.

4.2 Key constraints

The key constraints for this part of the project involved the requirements for successful transfection. These requirements are that the cell cultures were able to reach the necessary confluency, there were enough transfection reagents to transfect cells, and there was an adequate number of cells alive after transfection. All of these factors will influence the quality of the transfection and the images.

4.3 Expected results

The expected results are clear GFP and RFP images where the green and red fluorescence are colocalized in the cell. Once the images are overlaid the images will produce a yellow fluorescence to confirm that both plasmids were transfected and the cell is producing exosomes.

4.4 Materials and methods

Table 7. *Additional Materials Needed for Co-Transfecting HEP-G2 Cells*

Material	Company	Brand Model #
Exosome Specific Marker Plasmid (CD63-RFP)	Genscript	N/A

Methods

1. Passage cells following the Passaging Mammalian Cells procedure in Section 2.4
    a. Plate cells in the 2 of the 6 wells on the plate
        i. Designate a well for control and a well for both the exosome expression vector and the exosome marker vector
    b. Incubate until 70-80% confluency
2. Transfect the cells following Steps 1-6 of the Transfection procedure in Section 3.4
    a. For Step 7, Add 10µL of PEI and 1µL of each plasmid below to a microcentrifuge tube:
        i. Expression Vector: PDL1_pcDNA3.1(+)-C
        ii. Exosome Marker Vector: CD63-RFP
    b. Follow the remaining steps, steps 8-10 of the Transfection Procedure in Section 3.4
3. Image the cells following the Imaging procedure in Section 3.4
    a. Use the Olympus fluorescence microscope (Waltham, MA)
    b. Capture phase contrast, GFP, and RFP images of each well

4.5 Results

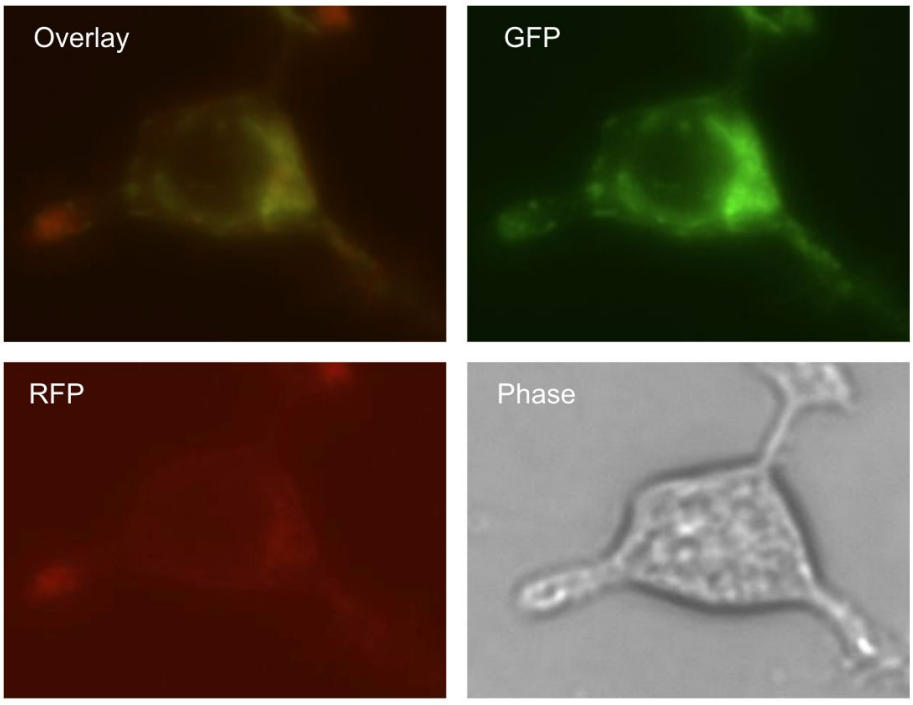

Figure 10: HEPG2 cells cotransfected with PDL1-GFP and CD63-RFP. Modified exosomes represented by GFP and verified with RFP. Images taken at 40x.

5. Discussion and Conclusion

Our study demonstrates that the delivery of an expression vector to a cell can encourage the cellular production of exosomes with specialized biomarkers. The engineered exosomes with specific surface markers have the capability to target certain cellular pathways. In this study, the PD-L1 ligand on the surface of cancer cells is the ultimate target of the exosomes in order to disrupt this immune checkpoint pathway that is evaded by cancer cells. In Phase 1 of our study, we demonstrated the use of mammalian cell culture techniques to maintain healthy HEP-G2 and HK-2 cell lines. In Phase 2, we confirmed with fluorescent images the successful transfection of the expression vector in both cell lines to produce engineered exosomes. Lastly in Phase 3, we verified the cells produced the engineered exosomes with the colocalization of an exosome-specific marker and the expression vector via fluorescent images. Together, these results are a proof of concept that mammalian cells can express the vector and produce exosomes with engineered surface proteins.

Future directions for the next Senior design team include harvesting and isolating the exosomes to pull them from solution in order to conduct characterization using methods like the nanodrop to determine size and western blot to confirm that our protein is in the right orientation and actually on the surface of the exosome. Next, the efficacy of the engineered exosomes needs to be tested by utilizing a binding assay with cells that express pdl1 on their surface. And lastly, if this is successful, can be replicated, and adequately peer-reviewed, this project may move on into the lengthy drug development process which will include pre-clinical, clinical, and post-market studies.

Moreover, broader applications of this therapy involve functionalizing different receptors on its surface to target different biomarkers, and the loading of hydrophilic drugs in its core, or hydrophobic drugs within its membrane for targeted drug delivery.

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6. Engineering Standards and Realistic Constraints

6.1 Social impact

The novelty of exosomes, as biocompatibile nanovesicles with surface modification capability, will improve upon current immunotherapies that target the PD-1/PD-L1 pathway. We believe that these superior nanovesicles will increase the efficacy of cancer treatments in turn increase the survival rate of cancer patients. This belief stems from studies that have shown exosomes to survive longer in the body, to penetrate tissues better, and to not induce a negative response due to the biocompatibility of the vesicles. Specifically, the increased half-life of the engineered exosomes will decrease the required times a patient must receive treatment. Hence, engineered exosomes could lower the cost of treating cancer while the increased efficacy of therapy could ultimately improve the survival rate of cancer patients.

6.2 Health and Safety

As this study proposes a novel cancer treatment, it would have to tolerate numerous tests before it would be a viable option for cancer patients. At this point in the study, the administration to humans is a long term goal that would be met after many smaller milestones are achieved. Specifically to our study, the next step to proving the safety of the engineered exosomes would be testing the toxicity of the treatment in in vitro models. Then, later tests would move to animal models and hopefully end in a clinical trial and FDA approval of the novel cancer treatment.

6.3 Manufacturability

At this moment in the research, a main focus to improve the manufacturability to increase cell confluency as a higher cell count would lead to a higher transfection efficacy and a higher production of exosomes. We propose improving the cell culture method to establish a better environment for cell growth, especially Hk-2. Secondly, to improve feasibility the optimal conditions for successful transfection with a high efficiency must be discovered. We suggest working toward establishing a stable cell line with the PD-L1 exosomes expression vector in the HK-2 cells. Lastly, the next procedure that must be optimized is the harvesting of the exosomes process. While we did not reach this step in our study, it is a key step in order to move forward with these engineered exosomes as a feasible therapeutic. Once these initial processes are established future work with bioreactors could significantly increase exosome production. Thus, the manufacturability of this cancer treatment relies on optimizing all steps of the cellular production of engineered exosomes.

6.4 Economic

Due to the complexity of cancer, it can be a very hard and expensive disease to treat. The American Cancer Society predicts that treating cancer will result in an $80.2 billion impact on the American healthcare system[32]. This is because the available cancer treatments require repeated treatment sessions and may never cure the patient of cancer. Thus, these engineered exosomes have the capability to decrease medical costs by lowering the number of treatments because of their longer half-life and increase efficacy compared to conventional cancer therapies. Also, it must be noted that the biocompatibility of the nanovesicles could lower treatment cost by decreasing the need for extra medications to limit the adverse immune responses.

6.5 Ethical implications

Cancer impacts people all over the world. The need for a cure and not a treatment is more imperative than ever before. That is why we believe in this project as this novel therapy using engineered exosomes could be the solution to this worldwide problem. We understand that people are fighting cancer very successfully, however, they only enter remission and have to live with the disease. Thus, this project along with other similar studies are working to develop a novel cancer treatment that will improve a patient's quality of life, which is the ethical responsibility of all people in this field.

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[32] American Cancer Society, Cancer Facts & Figures 2020.

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Appendix

A1: Making Media: DMEM + 10% FBS + PS

Table A1-1: Materials for Making Media

Material	Company	Brand Model #
Dulbecco's Modified Eagle Medium with L-Glutamine (DMEM)	Thermo Fisher Scientific/Gibco	11965092
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific/Gibco	10438034
Penicillin-Streptomycin (PS)	Thermo Fisher Scientific/Gibco	15140122

1. Add 50 mL of FBS into a 500ml bottle of DMEM with L-Glutamine.
2. Add 5 mL of PS to the combined FBS and DMEM.
3. Mix thoroughly.
4. Store at 4°C until needed.

A2: Making Media: K-SFM+BPE+EGF+ 1% PS

Table A2-1: Materials for Making Media

Material	Company	Brand Model #
Keratinocyte SFM (K-SFM)	Thermo Fisher Scientific/Gibco	17005042
Bovine Pituitary Extract (BPE)	Thermo Fisher Scientific/Gibco	13028014
EGF Recombinant Human Protein Solution (EGF)	Thermo Fisher Scientific/Gibco	PHG0311L

Penicillin-Streptomycin (PS)	Thermo Fisher Scientific/Gibco	15140122

1. Add 25 mg BPE to a 500ml bottle of K-SFM.
2. Add 0.005 mg of EGF to the combined FBS and DMEM.
3. Add 5 ml of PS to the solution
3. Mix thoroughly.
4. Store at 4°C until needed.