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Development of Stable Cell Lines for the Production of Hematopoietic Stem Cell Targeted Exosomes

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UNDER MY SUPERVISION BY

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ENTITLED

DEVELOPMENT OF STABLE CELL LINES FOR THE PRODUCTION OF HEMATOPOIETIC STEM CELL TARGETED EXOSOMES

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DEVELOPMENT OF STABLE CELL LINES FOR THE PRODUCTION OF HEMATOPOIETIC STEM CELL TARGETED EXOSOMES

By

Anja Beard and Zach Ehlinger

SENIOR DESIGN PROJECT REPORT

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ABSTRACT

Exosomes represent a promising new approach to targeted drug delivery. Current research explores the ability of these naturally occurring nanoparticles to transport therapeutic cargo to specific tissues of the body and to subsequently enter the cells of those tissues. Human cells act as an efficient source of these nano-vesicles. Our project ultimately focuses on the establishment of a cell line that produces exosomes tagged with the RD114 protein, which allows for the targeting of hematopoietic stem cells. With the ability to deliver therapeutic cargo to these specific cells, exosomes can serve as a vehicle for many effective hematopoietic stem cell therapies. We ultimately developed two stable cell lines that produced engineered exosomes and conducted a series of tests on these nanoparticles. Results from these tests indicate that these exosomes are tagged with the desired proteins, opening several possibilities for future research.

Keywords:
Hematopoietic Stem Cells, RD114, Exosomes, Targeted Drug Delivery

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Chapter 1: Introduction

1.1 Background

Therapeutic biomolecules have been extensively developed to treat a broad range of conditions and diseases. In recent years, the delivery of those therapeutics has become a much greater concern. Exosomes are now rising to meet that concern. They are natural nanovesicles derived from living cells that can carry proteins on their surfaces and therapeutic biomolecules within their lumen [1]. Exosome-mediated therapeutic delivery can, in principle, be applied to many biomedical situations and this technique has already been utilized to treat various diseases [1, 2]. These nanoparticles can carry surface proteins that allow for targeted delivery of the therapeutic cargo to the appropriate cells of the human body [4]. By achieving efficient therapeutic delivery, exosomes may enhance the efficiency of these therapeutics in medical applications.

Diseases impacting hematopoietic stem cells (HSCs) present several dilemmas to the medical community as it seeks to develop effective treatments. They play a critical role in healthy physiological processes and give rise to the blood cell lineages of the body [5]. These lineages include the megakaryocytes and platelets that facilitate blood clotting processes, erythrocytes that transport oxygen from the lungs to peripheral tissues, and cells of the innate and acquired immune system [6]. HSCs, like other cells in the body, are susceptible to mutations. These mutations can eventually give rise to diseases that affect the cells of the blood. In response to these diseases, a few treatment approaches have been developed. In the instance of leukemia, a cancer of the blood that hinders the body's ability to fight off infections, chemotherapy or radiation can be applied [7]. Transplantation of healthy HSCs can also be performed to provide a patient with a repertoire of HSCs for basic physiological functions. Unfortunately, HSCs taken from allogeneic donors can present a risk of immunologic complications [8]. To circumvent these challenges, the most practical approach is to examine the treatment of the diseased HSCs in the body, possibly through gene therapy. In order for these therapies to be practical and effective, however, the mechanism of drug delivery must be considered.

1.2 Review of the Field

Many delivery systems have been examined as novel drug therapies have been developed. Effective drug delivery systems are needed to overcome key barriers that drugs face within the body. Drugs often demonstrate poor solubility, cause unintentional tissue damage, and decrease in activity over time. Additionally, research has been conducted to address the ongoing need of direct drug delivery to the targeted tissues and cells. Such targeted delivery ultimately reduces the negative impact of the drug on surrounding tissues while increasing the local concentration of the drug within the targeted tissues. In recent years, nanoparticle drug delivery technology has come into focus as a potential solution. Various forms of nanotechnology exist, and many of them have effectively addressed some of these barriers.

Liposomes are nanosized unilamellar phospholipid bilayer vesicles with a central cavity that can carry therapeutic cargo within the body [9]. They have been shown to maintain stability as they circulate through the body, and research has focused on ways to induce the release of the cargo from the liposome through ultrasound, heat, and light stimulation [10]. Also, because they are composed of biomolecules, they are naturally inert and induce minimal immune response from the body. These nanovesicles encounter a major limitation, however, in their inability to effectively target specific cell types. Because they lack surface biomolecules capable of governing specific interactions within the body, these nanoparticles cannot effectively distinguish between different tissue types.

Gold nanoparticles (AuNPs) are another candidate for drug delivery. They are capable of transporting drugs that ordinarily exhibit low solubility, poor pharmacokinetics, and susceptibility to enzyme degradation [11]. The surfaces of AuNPs can also be functionalized with ligands that allow them to target specific cells and tissues within the body. Further, the size of AuNPs present an important advantage. Because AuNPs are roughly equivalent in size to the targeted cells, they are able to bind to target cells at multiple receptor sites, thus enhancing their uptake into the targeted cells [12]. Some studies, however, have called into question the ability of AuNPs to induce toxic side effects through natural immune response mechanisms [13]. Ultimately, these studies have generated relatively inconsistent results that make the use of AuNP drug delivery treatments potentially problematic.

Polymeric nanoparticles are yet another potential mode of drug delivery. These nanoparticles are composed of macromolecules that carry an active therapeutic agent. These macromolecular structures gradually dissolve over time and release the therapeutic agent to the surrounding cells and tissue. This method of release can allow for sustained delivery of the drug to a targeted region. These nanoparticles are also relatively easy to synthesize, biocompatible, and biodegradable [14]. Unfortunately, they also present obstacles of their own. They are not able to effectively targeting specific cells and they tend to agglomerate within solution, thus hampering their capability for targeted drug delivery.

1.3 Critique of Current Technology

The major limitation of using liposomes for drug delivery is their lack of targeting ability. Liposomes cannot be manufactured with transmembrane proteins that span the lipid bilayer to direct the nanoparticle to a desired tissue. Instead, they can only capture biological material within their aqueous compartments. This captured material is incapable of directing the actual delivery of these nanoparticles to the proper cell types in the body. Gold nanoparticles show a variety of promising features, but they are not naturally produced. This ultimately raises a concern that they may demonstrate toxic effects *in vivo*. For polymeric nanoparticles, similarly to

liposomes, they do not deliver drugs to specific cells, but rather to a targeted region where their therapeutics are then released. In this scenario, it becomes difficult to predict the unintended effects that the released drug may impose on surrounding tissues.

Having examined the existing nanotechnologies that are currently employed for drug delivery applications, there is a clear need for a practical drug delivery system that meets a complete set of criteria. Exosomes are appealing candidates to meet these standards.

1.4 Project Goal

This project is dedicated to the development and execution of a robust and reliable gene integration protocol that will allow for efficient production of targeted nanoparticles from a stable HEK293 cell line. To overcome the disadvantages of other nanoparticles, we will explore exosomes for our project. Exosomes are naturally derived, living nanoparticles that allow for therapeutic protein transport *in vivo*. Due to their origination from living systems, exosomes are biocompatible and stable. They can also permeate biological barriers with low toxicity and immunogenicity [15]. They can carry proteins in both their aqueous interior compartment as well as in their lipid membrane. Because of their ability to carry transmembrane proteins, these 30 to 100 nm vesicles can be used to carry proteins that target specific tissues to treat various diseases.

The first step in approaching this project will be to use previously established protocols to develop and maintain a stable HEK293 cell line. This cell line will be used to run various experiments over the course of this project. The next milestone of the project will be the cell line transfection. To determine if our transfection created a stable cell line with our vector, we will monitor the cells for 40 days and confirm continued expression of the vector in the cells with a fluorescent protein. Luminescent proteins will also help us quantify the successful integration of the vector through imaging and luciferase assays. Through imaging, we will be able to observe the location of our protein within the cell. We will confirm that the proteins are on the exosomal membranes through exosome harvesting and analysis. This leads to our last step of experiments and our expected outcome. We will do uptake experiments with our exosomes and a cell line to show that our exosomes can deliver therapeutics to these cells. Then, we will produce a conclusive protocol with our proven methods that future students can use to repeat our process.

1.5 Backup Plan

The main concern with this project is the 2-3 month timeline that is required to verify the stability of our generated cell lines following their transfection. If the vectors are not successfully integrated into the cell line genome after the first or second attempted transfection, there will not be enough time to complete this process. Our alternative plan is to execute a transduction procedure using a lentivirus gene delivery process. This method of integration can be performed to integrate the viral vectors into the HEK293 cells over a shorter timeline. Transduction only

requires 1-2 weeks to confirm the integration of the target gene and the creation of a stable cell line. This will allow us to reach our project goal in a shorter amount of time if we run into complications so that we can still complete our project.

1.6 Significance

This method of exosome production can be used to develop therapeutic carriers that are designed to treat a variety of diseases within a broad range of cell types through targeted drug delivery. Exosome-mediated drug delivery also presents unique advantages over other drug delivery methods. Because these nanovesicles can be derived from human cells, they have ultimately demonstrated a lower potential to induce an immune response from the body [16]. Exosome technology has already been explored as a potential approach to the treatment of a broad range of diseases. The outcomes of this project could simplify the process by which these targeted nanoparticles are produced and consequently make the technology more accessible for clinical applications around the world.

1.7 Team and Management

1.7.1 Team Members

Table 1. Project Members

Member	Degree	Email
Anja Beard	Bioengineering - Biomolecular Track	abeard@scu.edu
Zach Ehlinger	Bioengineering - *Biomolecular Track*	zehlinger@scu.edu

Advisor:
Dr. Biao (Bill) Lu

1.7.2 Project Budget

Table 2. Project Materials and Total Costs

Item	Count	Cost ($)
HEK293 Cell Line	1	400
RD114 Viral Vectors	2	400
GFP and RFP Constructs	1	500
Cell Culture Media	1	400
Cloning Kit	1	200
E. coli Cells	1	250
Puromycin	1	500
Tissue Culture Materials	1	500
Total		3,000

The most important aspect of this project is the HEK293 cell lines. Along with this, the cell culture media is necessary to provide nutrients for our cells and keep them alive in a stable environment. Finally, the RD114 viral vectors with the GFP and RFP are essential elements to target exosomes and visualize their location throughout the cell.

1.7.3 Project Timeline

During fall quarter, we became comfortable in the laboratory by learning effective protocols and proper techniques. With the time we had over winter break we did more research and protocol development, paying specific attention to further iterature searches. This allowed us to better understand our technology and the role that it plays in targeted drug delivery for HSCs. In the winter we began our stable cell line, collected data, and finalized our protocols. This allowed us to perform exosome uptake experiments and prepare for our conference presentation throughout the beginning of spring quarter. Finally, we finished compiling and analyzing our results during the end of spring quarter to develop this draft of our final thesis.

Chapter 2: Development of Stable Cell Line

2.1 Introduction

We began with human embryonic kidney (HEK) cells. More specifically, our project utilized HEK293 cells, which come from a permanently modified cell line that is particularly amenable to the introduction and expression of new genes. Historically, these cells have been manipulated to produce recombinant proteins derived from the genes of both mammalian and non-mammalian organisms. We found this particular cell line to be relevant for our project because it contains sophisticated biochemical machinery that can execute essential post-translational modifications (17).

Simple transfection protocols represent just one of the ways in which these cells can be genetically modified for engineered protein production. We used this method to introduce a foreign construct into our HEK293 cells. This construct contained four critical domains that were essential to either the validation of our transformed cell line or to the final application of the engineered exosomes. These domains are as follows:

1. A luminescent reporter gene that could provide sensitive, quantitative information concerning the extent to which our construct was expressed.
2. A fluorescent reporter gene that could provide a quick, qualitative indication of fluorescence while also showing where the expressed proteins from our construct were localized in the cells.
3. A puromycin resistance reporter gene that could allow us to impose a selective pressure on the cell line based on the successful integration of our specific construct.
4. A gene encoding for the RD114 protein, which would become integrated in the exosome membranes to allow for specific binding of those nanoparticles to the surface antigens of hematopoietic stem cells.

We transfected our cells using our designed construct and polyethyleneimine, which enhances transfection efficiency by allowing the construct to avoid acidic lysosomal degradation as it is transported to the nucleus of the cell (18). Though transfection protocols are relatively easy to execute, they demand a certain amount of time before we can confirm that our introduced DNA has been effectively integrated into the actual genome of the cell line.

We ultimately allowed our cells to grow in puromycin culture for over 40 days. As a result of this stage in the cell culturing process, any cells that did not integrate the construct into their genomes would be killed after the many stages of cell division that would dilute the presence of our construct in the cells where no complete integration had occurred. Meanwhile, other cells integrated this DNA into their chromosomes. This integration allowed for successful replication

and transcription of our construct. Following this transcriptional process, the newly formed mRNA would then be translated into the proteins corresponding to the four domains of our construct. These cells would then finally be assessed for their ability to produce exosomes tagged with our proteins of interest, but that will be discussed in the next chapter.

2.2 Key Constraints

When approaching the process of creating a stable cell line, there are some key constraints that must be taken into consideration. The first set of constraints are with the HEK293 cells. The limitations around these cells include the conditions that the cells must be cultured in and the risks of contamination. The cells need to be kept in the proper conditions to grow and avoid cross contamination between plates. To avoid other contamination from outside sources, proper protective equipment is used. Gloves and lab coats are necessary while in contact with the culture plates and ethanol is used when moving the plates from one sterile location to another to prevent an outside contaminant. The second set of constraints are related to the issue of time. One time limitation is simply how fast the cells can grow. If the cells are not confluent enough, it could delay some of our experiments until enough cells are present. Another issue is that once the cells have been transfected with the gene, the cells must continue to produce exosomes with the target protein present for at least 40 days to become a stable cell line. If a problem comes up during this process, there may not be time to start the process over.

2.3 Design Approach

Our design approach to create the stable cell lines began with the vector construct. Our vector contains genes that produce a protein that goes to the surface of exosomes and targets HSCs. Specifically, our vector includes an RD114A gene that goes to the outside of the exosome surface once translated into a protein and promotes the uptake of these exosomes by HSCs. Next, the puromycin resistance gene and polyA tail provide selective resistance for our cells and stability for the protein respectively. And finally, the gluc and GFP/RFP act as reporter genes for our exosomes so we can quantify our protein production and visualize protein movement in the cells. These portions of the protein are inside the exosomes. As explained above, these genes are both inserted into our HEK293 cells using transfection and selected for with puromycin pressure. The cells that have taken up our gene will have a puromycin resistance so they can live in the puromycin media while the rest of the cells die under this pressure. This will allow us to culture the cells for 40 days to create a stable cell line containing the desired genes.

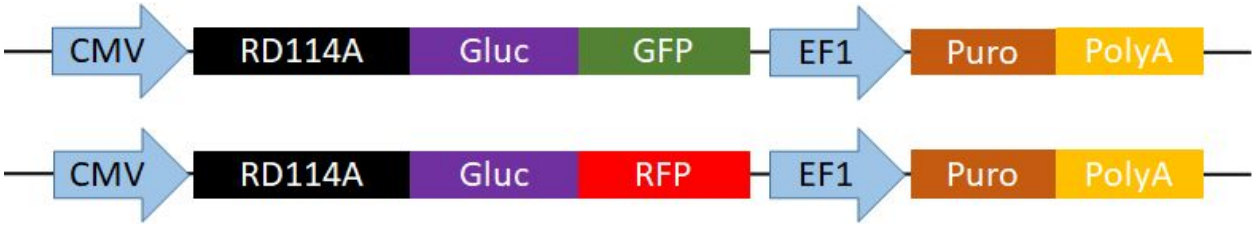

Figure 1. Vector constructs used for cell line transfection.

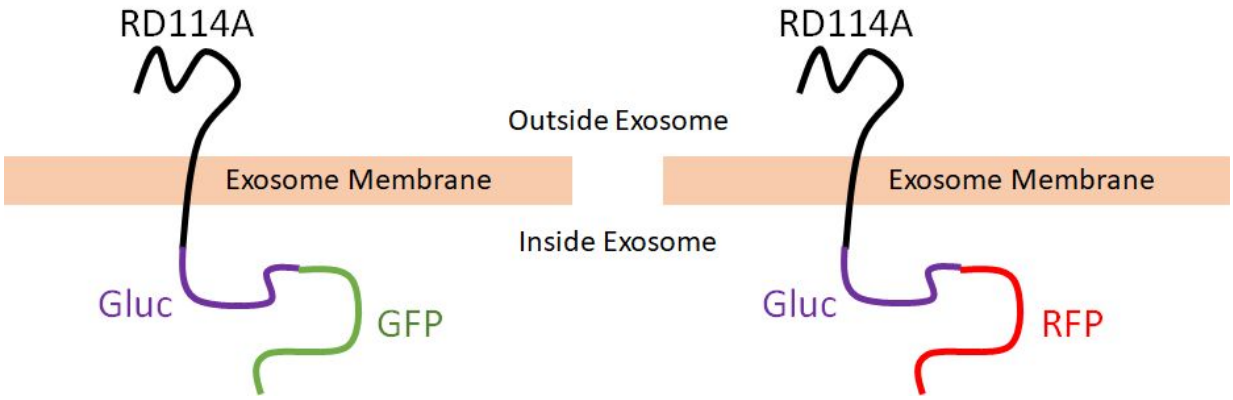

Figure 2. Schematic of protein complexes expressed within the membranes of engineered exosomes produced by the two stable cell lines.

2.4 Supporting Analyses

This stage of our experiment gave rise to a stable cell line that seemed to successfully express our construct throughout the cell culture. We observed relatively high levels of fluorescence under a fluorescent microscope immediately following the transfection. After 40 days of growth in puromycin media, fluorescence decreased, but was still present. This suggests that, even though expression of our gene seemed to decrease over time, our construct was still visibly present in the cell line.

2.5 Expected Results

To create our stable cell line, our cells must survive for 40 days under pressure and continue to produce our desired protein. To prove that this has happened, we cultured our cells with puromycin expected only the cells containing our vector construct to survive. We could then support this evidence with microscope imaging of the GFP and RFP proteins in the cells. If our cells were still producing our protein, we would expect to see either green or red fluorescence present in the cells.

2.6 Backup Plan

Should transfection methods not have been successful in generating a stable cell line that expressed our desired proteins, we could then have turned to transduction methods. These

methods would allow us to obtain a verified stable cell line in less time, but they would ultimately require additional steps to successfully package a lentivirus gene delivery system for our HEK293 cell line.

2.7 Materials and Methods

2.7.1 Cell Culture Passaging

Materials:

- 70% Ethanol
- Light-Duty Tissue Wiper
- 1000μL pipette
- Aspirator
- Pipette Controller
- Aspirating tips
- 5mL Pipette tips
- 2mL Pipette tips
- Dulbecco's Modified Eagle's Medium (DMEM)
- Trypsin
- Phosphate Buffered Saline (PBS)
- 100x20 mm cell culture plate

Methods:

1. Take cell culture plates out of the 37°C incubator and check the confluency of the cell under the microscope. If the confluency is 70% or above, the cell culture can be passaged.
2. Collect DMEM, PBS, and trypsin tubes from stock and place them in the 37°C water bath.
3. Prepare the hood by spraying down the surface with 70% ethanol and wiping it with a light-duty tissue wiper.
4. Collect and spray the following equipment with 70% ethanol and place inside the hood:
    a. 1000μL pipette
    b. Aspirator
    c. Pipette Controller
    d. 3 Aspirating tips
    e. 3 5mL Pipette tips
    f. 1 2mL Pipette tips
    g. DMEM
    h. Trypsin
    i. PBS

5. Attach an aspirating tip to the aspirator and aspirate off the DMEM while the plate is tilted at a 45 degree angle minimizing the cell exposure.
6. Add 3mL of PBS to the wall of the plate at a 45 degree angle. Set the plate down.
7. Repeat aspirating technique to remove PBS.
8. Add 1.5mL of trypsin to the plate and place in the incubator for 2 minutes.
9. Remove plate from the incubator and add 5mL of DMEM to the plate in the hood.
10. Collect all the liquid with the suspended cells from the plate using the pipette tip and put it in a 15mL tube.
11. Centrifuge the 15mL tube with the cell suspension at 1500rpm for 5 minutes.
12. Back in the hood, aspirate off the supernatant.
13. Add 7mL of DMEM to a clean 100x20mm cell culture plate and resuspend the cells in the 15mL tube in 4mL of DMEM.
14. Add 250μL of resuspended cells to the clean cell culture plate.
15. Check cell culture plate for the presence of suspended cells. If cells are present, place cell plate in the incubator to grow.

2.7.2 Mammalian Cell Transfection

Materials:

- Micropipettes
- 6-well plate
- 1.5 mL microcentrifuge tubes
- Plasmid
- Dulbecco's Modified Eagle's Medium (DMEM)
- PEI transfection reagent
- Opti-MEM + 1% PBS

Methods:

1. Pipette 2 mL of DMEM into each well of a 6 well plate.
2. Transfer 45 μL of the final passaging suspension from the original HEK293 cell culture to each well of the plate.
3. For each well that is to be transfected, obtain two microcentrifuge tubes. Add 100 μL of Opti-MEM + 1% PBS to both tubes.
4. Then add 10 μL of PEI reagent to one tube and 2 μg of plasmid to the other tube. Use the pipette to mix both solutions.
5. Finally, transfer the contents of the tube containing Opti-MEM + 1% PBS and PEI reagent to the other tube. Mix the contents of this tube together and wait for 20 minutes before proceeding to the next step.
6. Add 220 μL of the mixture in the tube to the well that is to be transfected with that plasmid.

7. Place the plate in the incubator and allow the cells to grow for two days before performing other experiments with these transfected cells.

2.8 Results

To verify that our stable cell lines contained our desired genes and produced the proteins of interest, green and red fluorescent images were taken of the cells after 40 days in puromycin. A confocal microscope was used to take fluorescent and phase images of both cell lines. Photograph editing software was then used to overlay these images. The images from these tests are shown in Figure 3.

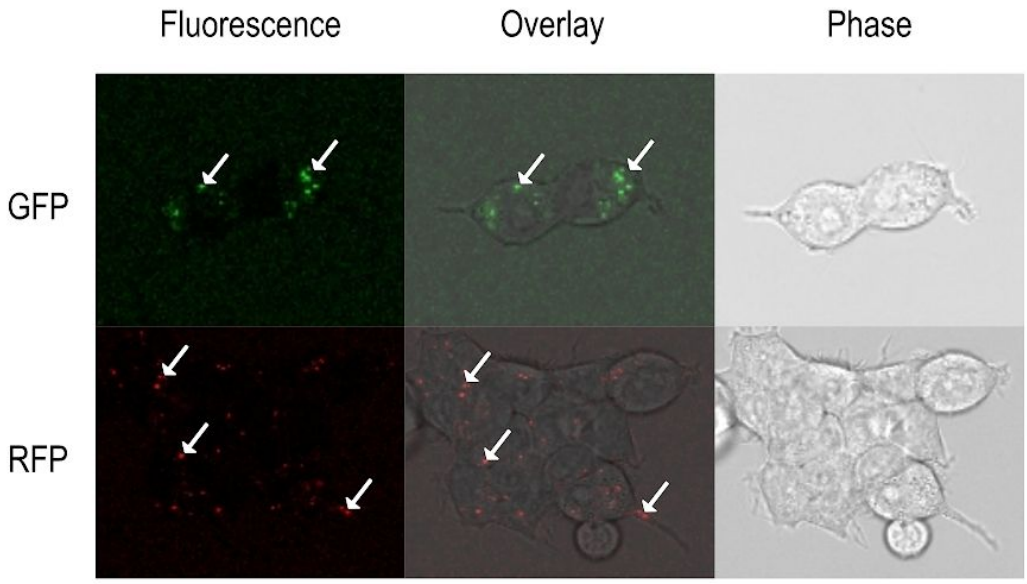

Figure 3. Fluorescent and phase confocal microscopy images of the stable cell lines.

These images indicate that the cells survived in puromycin after 40 days. The white arrows in the fluorescent and overlay images indicate the presence of GFP and RFP in the cells.

2.9 Discussion

The results seen in Figure 3 show that the cells survived in the puromycin media after 40 days, demonstrating that the puromycin resistance gene is present in the cells. The images also show green and red fluorescence dots inside the living cells, proving that the GFP and RFP genes are being translated in the cells. These results show that the puromycin resistance gene and fluorescent protein genes are both present in the cell genome and continue to be translated by the cell. Ultimately, this indicates that the cell lines are stable.

Chapter 3: Hematopoietic Stem Cell Targeted Exosome Production

3.1 Introduction

Following the transfection of our cells with our construct, we turned our attention to exosome analysis so that we could validate whether our HEK293 cell line could produce exosomes with the relevant proteins of interest. Exosomes are produced through a sequence of endocytosis, invagination, and exocytosis (19). The outer membrane of the cells first creates pockets leading into the cytoplasm of the cell. The surface of the cell membrane then joins together to produce a small vesicle within the interior of the cell. This vesicle is called an endosome.

Once inside the cell, a similar process occurs in the membrane of the endosome. Even smaller pockets form within its membrane that eventually give rise to smaller vesicles within the larger endosome. The ultimate product of this invagination stage is a multivesicular body with a collection of naturally produced nanoparticles contained within the larger carrier. These nanoparticles contain material from the interior of the cell, which is critical to the final purpose of exosomes both in the natural world and in its engineering applications.

Finally, the endosome fuses with the exterior membrane of the cell to release the contained exosomes to the extracellular environment. These nanoparticles then have the opportunity to fuse to the outer membranes of other cells to deliver their cargo.

3.2 Key Constraints

The main constraints of targeted exosome production lies in the actual harvesting of these nanoparticles. The exosomes must be produced by HEK293 cells that contain the target gene in order for them to be tagged with the desired protein. This means that the cells must be transfected or the stable cell line must be established before the exosomes can be harvested. Additionally, harvesting a pure, concentrated sample of exosomes can be difficult due to other small particles and the cell culture's inherent level of exosome production.

3.3 Design Approach

For this step of the project, our goal was to collect and isolate exosomes from our stable cell line to test them for exosomal qualities and the presence of our proteins. To do this, we harvested the exosomes from the media of transient and stable cell line plates, centrifuged them, and resuspended them at a relatively high concentrations. This allowed us to check the properties of the particles to confirm that they were the desired, engineered exosomes.

3.4 Supporting Analyses

This stage of our project provided us with evidence of the successful production of exosomes from our stable cell line that were tagged with the our proteins of interest. The produced and

isolated exosomes were of the correct size, contained surface markers that are characteristic of HEK293 cell exosomes, exhibited luciferase activity, and demonstrated fluorescence in the cytoplasms of the cells.

3.5 Expected Results

In this series of experiments, there were several ways in which we intended to analyze our harvested exosomes. The first method used was a Nanoparticle Tracking Analysis (NTA) test. This indicated the size of the particles present in our exosome sample. We expected to see a significant peak at 30-100 nm and no other peaks on the graph. Next, we conducted an enzyme-linked immunosorbent assay (ELISA), which provided insight into the surface proteins on the nanoparticles. We expected to see evidence that the CD63 surface antigen was present on the surfaces of these nanoparticles. After proving that our nanoparticles were exosomes, we analyzed for the presence of the engineered proteins in the exosomes. To do this, we used confocal microscope imaging to visualize the living cells in puromycin media and analyzed for the localization of the fluorescent reporter genes in the cells. We also performed a luciferase assay to determine whether our luciferase gene was integrated into the cell and expressed within the exosomes.

3.6 Backup Plan

Our goal was to collect results using exosomes from our stable cell line; however, if our cell line was not yet stable, we could also harvest exosomes by transfecting a new HEK293 cell line with our gene and collecting the exosomes produced by these cells at their transient phase of expression.

3.7 Materials and Methods

3.7.1 Exosome Harvest

Materials:

- 145x20 mm cell culture plate
- 15 mL tubes
- Pipette controller
- Micropipettes
- DMEM
- Plasmid
- Dulbecco's Modified Eagle's Medium (DMEM)
- PEI transfection reagent
- Opti-MEM + 1% PBS
- UltraCULTURE + 1% P/S Glut
- 30 mL syringe
- Sterile syringe filter with 0.2 μm polyethersulfone membrane

- Exo-TC exosome precipitation solution
- Phosphate-buffered saline (PBS)

Methods:

Day One

1. Complete a passage of a cell culture.
2. Pipette 20 mL of DMEM to the 145x20 mm cell culture plate.
3. Add about 2 mL of the final passaging suspension to the the plate.
4. Place the plate in the incubator.

Day Two

1. If the cell line has already been transfected with the plasmid of interest, allow the cells to grow on this day.
2. If the cell line must be transfected, obtain two 15 mL tubes. Add 1 mL of Opti-MEM + 1% PBS to both tubes.
3. Then add 100 μL of PEI reagent to one tube and 20 μg of plasmid to the other tube. Use the pipette to mix both solutions.
4. Finally, transfer the contents of the tube containing Opti-MEM + 1% PBS and PEI reagent to the other tube. Mix the contents of this tube together and wait for 20 minutes before proceeding to the next step.
5. Add 2 mL of the mixture in the tube to the plate.
6. Place the plate in the incubator.

Day Three

1. Take cell culture plates out of the incubator and place in clean hood.
2. Aspirate off the existing DMEM by tilting the plate at a 45 degree angle away to minimize cell exposure.
3. Add 20mL of UltraCULTURE + 1% P/S Glut to the cell culture plate and place back in the incubator

Day Four

1. Allow cells to grow.

Day Five

1. Transfer all media from the plate to a 50 mL tube and spin the tube in the centrifuge at 1,500 g's for 10 minutes.
2. Obtain a new 50 mL tube and open the 30 mL syringe.
3. Screw the sterile syringe filter with the 0.2 μm polyethersulfone membrane onto the barrel of the syringe. Fit the syringe filter and barrel into the top of the 50 mL tube. Pull the plunger out of the syringe barrel.
4. Next, transfer the media from the tube that was spun in the centrifuge to the barrel of the syringe.

5. Using the plunger of the syringe, push the media through the filter of the syringe and into the 50 mL tube to filter out the cell debris.
6. Add 5 mL of Exo-TC to the 50 mL tube and invert the tube a few times to ensure proper mixing.
7. Place the tube in the refrigerator overnight.

Day Six

1. Spin the 50 mL tube in the centrifuge at 3,000 g's for one hour and 30 minutes.
2. Pour off the media into the sink and add 150 μL of specific PBS to the tube, mixing the solution in the tube to effectively resuspend the pellet.

3.7.2 Exosomal Luciferase Assay

Materials:

- Exosome Samples
- Luciferase Substrate
- 96-well plate
- 200μL pipette
- Plate reader

Methods:

1. Collect exosome samples from exosome harvest, luciferase substrate, 96-well plate, and 200μL pipette.
2. Vortex the exosome samples thoroughly.
3. Add 20μL of each sample to three wells skipping a column of wells between samples to minimize interference from neighboring samples.
4. Add 100μL of luciferase substrate to the sample wells and place well plate into the plate reader.
5. Run luciferase assay program on the computer and save results.

3.7.3 Enzyme-Linked Immunosorbent Assay

Materials:

- ExoELISA-ULTRA protein
- Coating Buffer
- Microcentrifuge tubes
- Vortexer
- 96-well Plate
- Micropipettes
- Blocking Buffer
- Super-sensitive TMB ELISA substrate
- Stop buffer

- Plate reader
- Wash Buffer

Methods:

Creating Exosome Protein Standard Curve

1. Thaw ExoELISA-ULTRA protein standard on ice.
2. In a microcentrifuge tube, create a 1:1000 dilution by add 1μL of protein standard to 1mL of coating buffer and vortex to mix the solution.
3. Using this first dilution, perform serial dilutions 6 more times using 60μL of the previous dilution and 60μL of coating buffer. Vortex each dilution.

ExoELISA Assay

1. In a 96-well plate, add 50μL of prepared protein standards and 50μL of exosome samples to the wells.
2. Cover the plate with film(or cover) and incubate the plate for 1 hour at 37°C.
3. After incubation, remove the liquid and wash the plate 3 times for 5 minutes using 100μL of 1x Wash Buffer.
4. To prepare the CD63 primary antibody, dilute it using a 1:100 dilution in Blocking Buffer.
5. Add 50μL of the primary antibody dilution to each well and incubate at room temperature for 1 hour with shaking.
6. After incubation, was the plate 3 times for 5 minutes using 100μL of 1x Wash Buffer.
7. To prepare the secondary antibody, dilute it using a 1:5,000 dilution in Blocking Buffer.
8. Add 50μL of the secondary antibody dilution to each well and incubate at room temperature for 1 hour with shaking.
9. After incubation, was the plate 3 times for 5 minutes using 100μL of 1x Wash Buffer.
10. Add 50μL of room temperature Super-sensitive TMB ELISA substrate to each well and incubate at room temperature for 15 minutes with shaking.
11. Add 50μL of Stop buffer to each well and read with a spectrophotometric plate reader at 450 nm immediately to provide a fixed endpoint for the assay.

3.8 Results

After creating the stable cell lines, the nanoparticles produced were analysed to determine if they were exosomes and if the desired protein was present on these exosomes. The first test was a Hoechst stain of both cell lines. This allowed us to visualize where the GFP and RFP were present within the cells. Next, luciferase assays were run on the exosomes harvested from the transient cell lines and on the exosomes harvested from the stable cell lines, after 40 days had passed in puromycin media. This provided results on the presence of the gLuc gene in the cell lines. Finally, the nanoparticles were analyzed with NTA and ELISA tests to further characterize their properties.

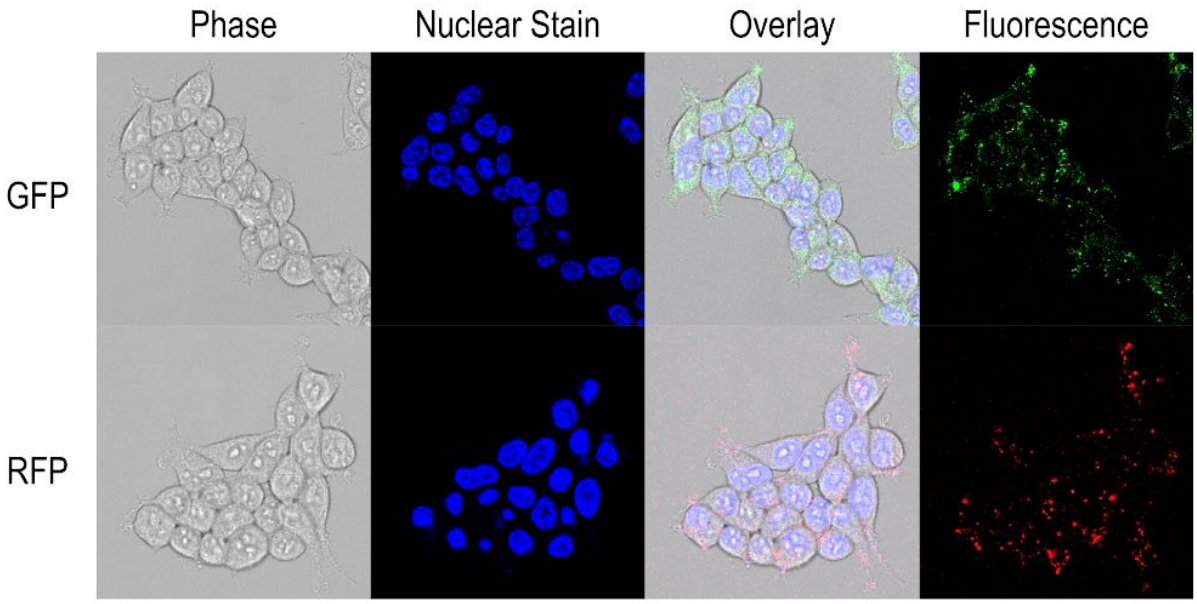

Figure 4. Fluorescent, phase, and nuclear stain confocal microscope images of the stable cell lines.

Figure 4 illustrates the localization of the exosome by staining the nucleus of the cells and overlaying these images with fluorescent and phase images of the cells. This indicates the location of the proteins in comparison to the nucleus of the cell.

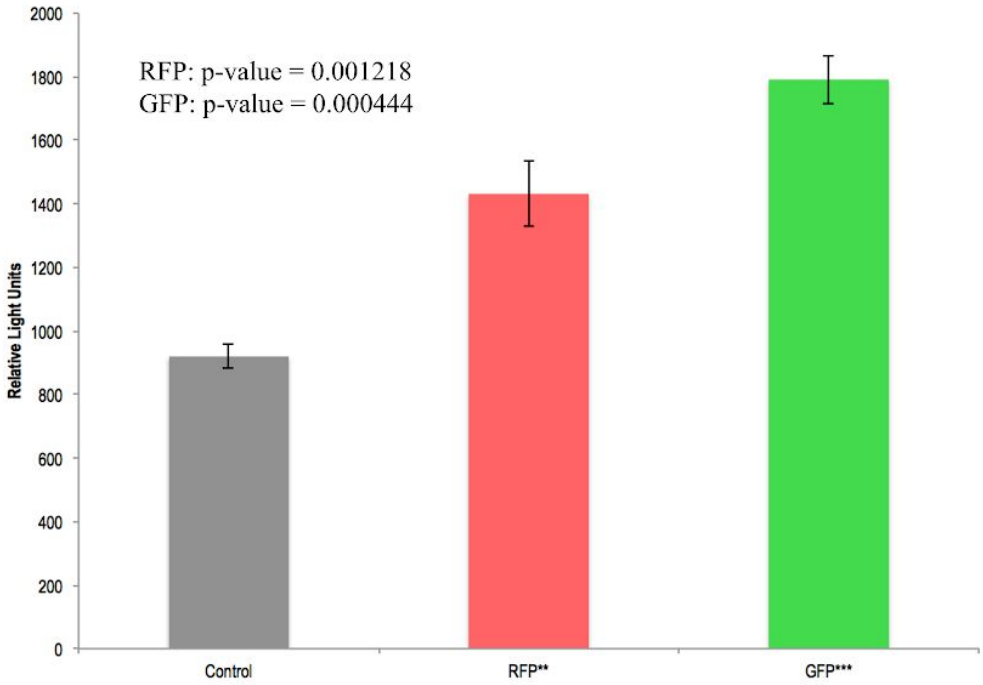

Figure 5. Luciferase analysis of exosomes harvested from the transient cell lines.

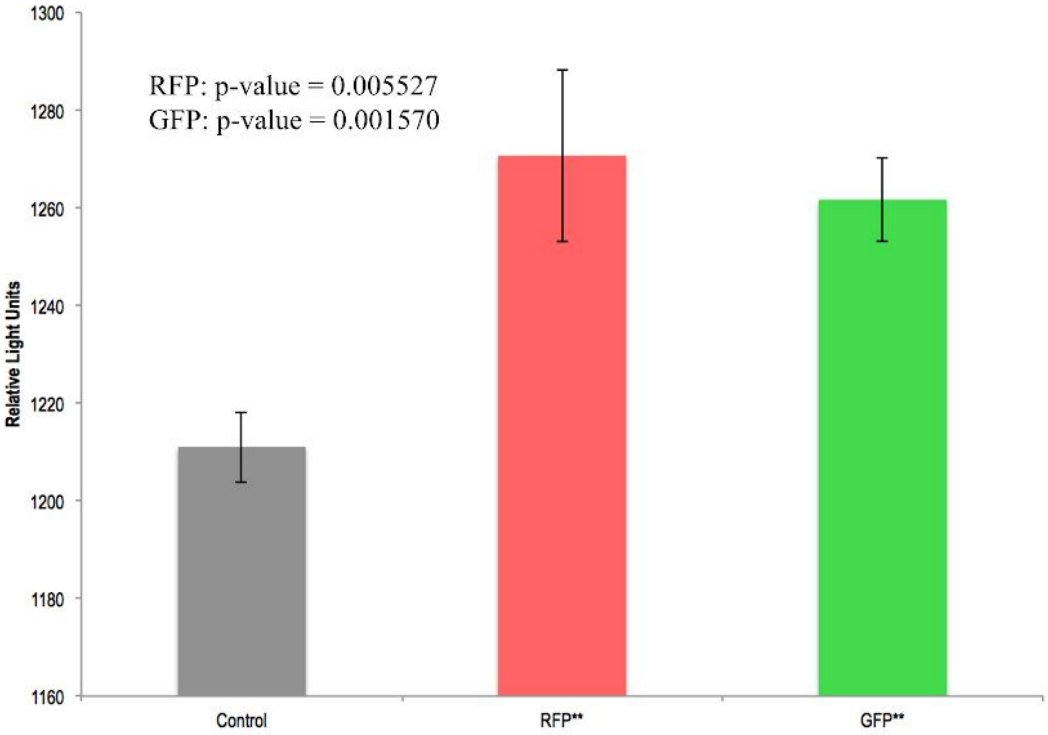

Figure 6. Luciferase analysis of exosomes harvested from the stable cell lines.

The luciferase assays for both the transient cell lines and the stable cell lines show a significantly higher expression of the gLuc gene than the control. This is known because the p-values for all four samples, relative to the control, are well below 0.05 which indicates a significant difference from the control.

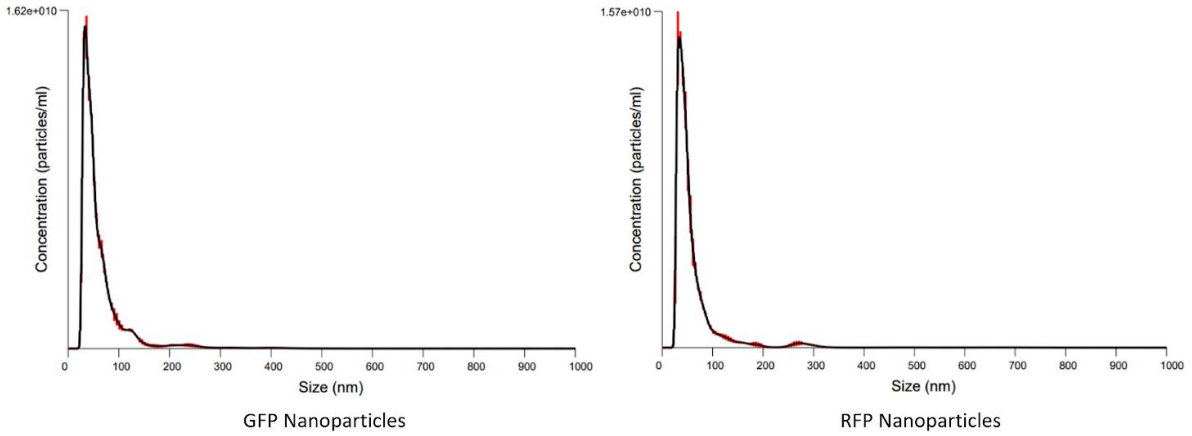

Figure 7. Nanoparticle Tracking Analysis (NTA) of exosomes harvested from the GFP and RFP cell lines.

The mean diameter of exosomes from the GFP cell line was 62.6 nm, with a standard deviation of 1.1 nm. The mean diameter of the exosomes from the RFP cell line was 62.1 nm, with a standard deviation of 3.2 nm. Three trial runs were conducted for each sample. The expected range of exosome diameters is between 30 nm and 100 nm and both of these samples fall well within this range. Additionally, both samples produced a single, sharp peak. This ultimately indicates that the exosomes isolated from these cell lines were relatively pure.

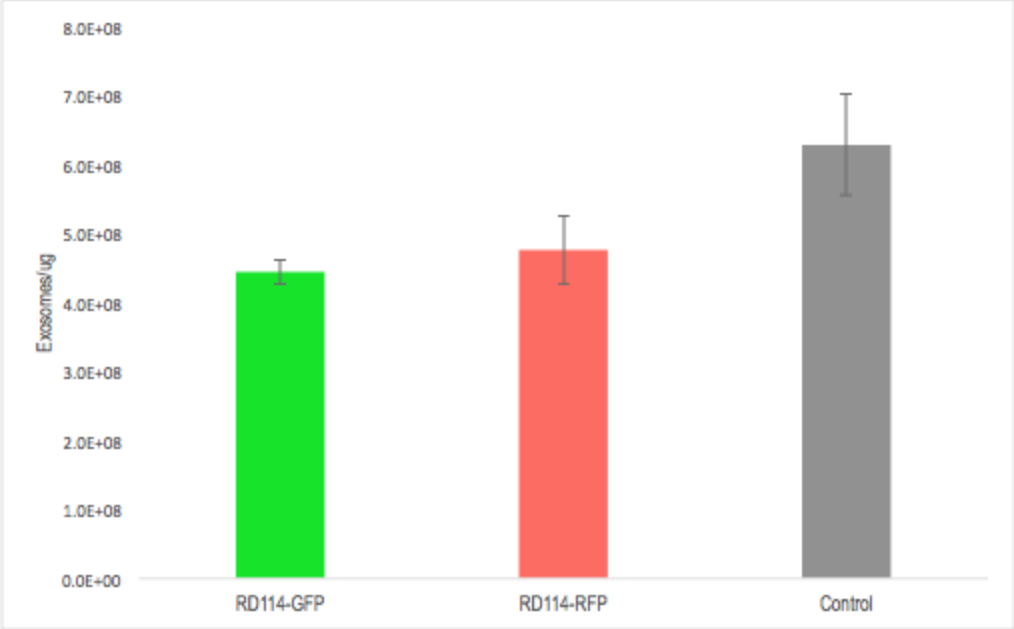

Figure 8. ELISA results for exosomes harvested from both the GFP and RFP transient cell lines.

3.9 Discussion

These analytical approaches demonstrate that the exosomes produced from our stable cell lines exhibit the expected properties. The Hoechst stain, when viewed under the confocal microscope, reveals that the engineered proteins are located in the cytosols of the cell line. This confirms that, at a minimum, the fluorescent reporter gene of our construct was successfully expressed. The luciferase assays were conducted with the isolated exosomes from both the transient and stable cell lines. In both assays, significantly higher luciferase activity was observed in the GFP and RFP stable cell lines' exosomes relative to a control. This finding indicates that the protein corresponding to the inserted construct was, in fact, present in the exosomes. Luciferase expression was, however, lower in the stable cell lines' exosomes than it was in the transient cell lines' exosomes. This drop in luciferase expression suggests that the expression of the construct decreased over time. Such a result may be due to the CMV promoter that was included in our construct. This promoter may allow for high levels of protein expression soon after transfection,

but it may not be capable of providing those levels of protein expression for a sustained period of time.

Further tests were conducted to characterize these isolated exosomes. The Nanoparticle Tracking Analysis revealed that these exosomes were of the expected size. The results show a peak ranging from 30 to 100 nm, which corresponds to the known size of these nanoparticles. Additionally, these results showed that the sample was pure, as one clearly defined peak was generated from this test. The ELISA assay was used to screen for the presence of CD63, a protein common to exosomes. The obtained graph shows that this protein was present in the obtained sample, indicating that the isolated nanoparticles were, in fact, exosomes.

Chapter 4: Tagged Exosome Uptake by Hematopoietic Stem Cells

4.1 Introduction

After isolating the exosomes from our cell lines and characterizing them according to the properties discussed in previous chapters, the next critical step was to assess these exosomes for their ability to undergo uptake into other living cells. For the scope of this project, a test was only conducted to see if these nanoparticles were capable of entering an unmodified HEK293 cell line. Upon incubating this cell line with the isolated exosome from the transfected cell lines, the locations of the exosomes could be monitored simply by confocal microscopy, where exosome uptake was confirmed through the fluorescence of the cultured cells. This provided basic insight into the therapeutic potential of these nanoparticles and suggested future directions that may be of interest as this project moves forward.

4.2 Key Constraints

The ideal uptake experiment would include the cell line that this system is intended to target. Unfortunately, for a variety of reasons, HSCs are relatively difficult to procure for these tests. If the exosomes are only exposed to a single cell line, they will ultimately enter those cells over time. After all, the exosomes are comprised of a phospholipid bilayer that is sourced from living cells, thus allowing for their surfaces to fuse. This constraint limits the conclusions that we are able to draw from these findings and leads us to consider future steps that might address these remaining questions.

4.3 Design Approach

The design approach to conduct uptake experiments relies heavily on our harvested exosomes from the stable cell lines. We were able to plate HEK293 cells with the exosomes from the GFP and RFP cell lines to determine if the exosomes would enter the cells over time.

4.4 Supporting Analyses

This stage of the experiment revealed the proclivity of these engineered nanoparticles to enter another cell line. Though no selectivity could be observed, the exosomes were capable of entering the cells that they were cultured with over 72 hours. This may generate questions concerning the selective capabilities of these nanoparticles once they enter a living system. Future *in vivo* testing would need to be designed in such a way that would allow for the explicit observation of exosome uptake into targeted cells (HSCs, in this case) over healthy tissues. This basic property is, after all, paramount to the purpose of this project.

4.5 Expected Results

For these experiments, the uptake of the exosomes by the HEK293 cells was verified by imaging the cells with a confocal microscope. This showed if the exosomes were inside the cells or not. For these experiments, the exosomes were expected to be seen inside the cells.

4.6 Backup Plan

The backup plan for the uptake experiments was to introduce the exosomes to another cell line. Although we could not acquire HSCs to do our uptake, we had access to a U87 cell line that we could conduct these experiments on. The U87 cell line is a human glioblastoma cell line that would have similar results to the HEK293 cell line when used for an uptake experiment.

4.7 Materials and Methods

4.7.1 Uptake Experiment

Materials:

- Harvested Exosomes
- Micropipettes
- Four quadrant glass bottom confocal plate
- 1.5 mL microcentrifuge tubes
- UltraCULTURE + 1% P/S Glut

Methods:

1. Plate HEK293 cell line in three quadrants of four quadrant glass bottom confocal plate.
2. Dilute GFP, RFP, and control harvested exosomes to a concentration 0.3μg/μL using UltraCULTURE to make 200μL of solution.
3. Plate each of the the 200μL of dilutions of exosomes onto one of the three quadrants of the HEK293 plate.
4. Image the cells at 24, 48, and 72 hours with the confocal microscope to observe the uptake of the exosomes.

4.8 Results

In the uptake experiments, images are taken with the confocal microscope at 24, 48, and 72 hours in order to visualize the exosomes presence in the cells. These images are taken of the GFP and RFP harvested exosomes against control exosomes.

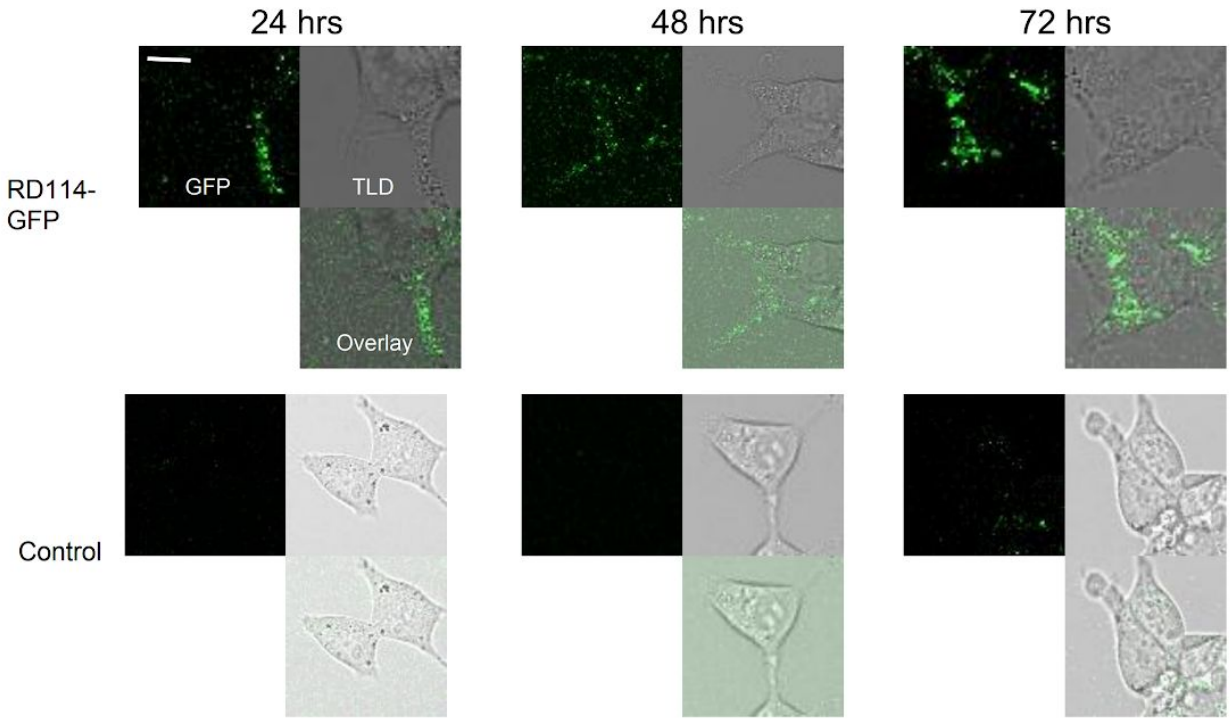

Figure 9. Confocal images of exosomes harvested from the GFP and control cell lines plated with HEK293 cells. Images were taken after 24, 48, and 72 hours.

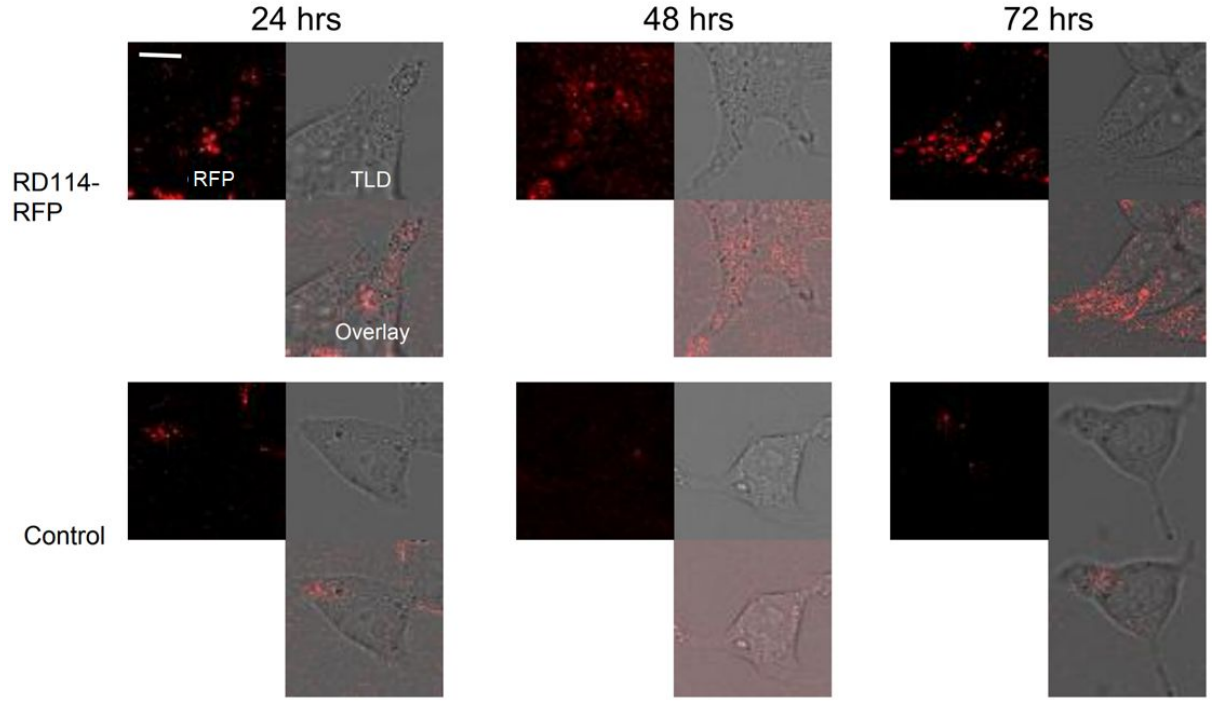

Figure 10. Confocal images of exosomes harvested from the RFP and control cell lines plated with HEK293 cells. Images were taken after 24, 48, and 72 hours.

These images show that the GFP and RFP exosomes did enter the cells within 24 hours and they continued to enter the cells throughout the 72 hour period.

4.9 Discussion

The findings of these experiments provide assurance that these engineered exosomes can enter living cells and be visualized upon uptake. This ultimately indicates that future experiments can be successfully conducted upon the acquirement of proper cell lines. Observing the capability of these exosomes to enter a living system provides a proof of concept that illustrates the basic functionality of these nanoparticles. With that in mind, though, more tests must be conducted to determine whether these nanoparticles can selectively enter HSCs over other cell lines. Once those tests are conducted, further engineering must be conducted to successfully load these nanoparticle with therapeutic cargo.

Chapter 5: Conclusion

5.1 Conclusions and Summary

The goal of this project was to develop a stable cell line that was capable of producing targeted exosomes carrying the protein complex of our engineered construct. Through the analysis of the cell lines and their corresponding isolated exosomes, we were able to show that the transfected cell lines produced modified living nanoparticles. These nanoparticles exhibited the expected properties that are universal to exosomes and the characteristics associated with the reporter genes that were included in our initial construct. All of these findings collectively indicate that the exosomes produced from these cell lines carry the proteins corresponding to the inserted genes. Additionally, the harvested exosomes from these cell lines are capable of entering live cells. More work must be done, however, to explore the capability of these exosomes to selectively enter the HSC target cell over other cell types.

5.2 Future Steps

5.2.1 Experimental Uptake with Other Cell Lines

During this project, we were able to create a stable cell line that produced modified exosomes and test them with uptake experiments using a HEK293 cell line. The next step in this project would be to test these modified exosomes on other cell lines, including HSCs, to confirm that they can deliver their proteins to different cell lines. Once this is shown to work for multiple cell types, the exosomes should be introduced to HSCs with another cell line present to see if they selectively enter HSCs over other cell types. To prove this, the engineered exosomes would need to be introduced to plates with both HSCs and another cell line present and then similar observations would be made over 72 hours. In these 72 hours, we would expect the exosomes to preferentially enter the HSCs so the HSCs would have significantly more exosomes inside of them over the other cell line. This would show that the exosomes specifically target HSCs and could potentially be used to deliver drugs to these cells over other cell types in the body.

5.2.2 Personalized Treatment

A step that can be studied more in the future is around personalizing the patient's treatment. Our engineered exosomes have shown that they can enter a HEK293 cell line, but there is still plenty of research to be done before they can be used to deliver therapeutics to HSCs in a patient. One potential treatment option for consideration is the creation of a delivery system specific for the patient. One way this could be done is by using the own patient's HSCs to produce the exosomes that will be administered to them. The patient's HSCs would have to be extracted and cultured similarly to the HEK293 cells for this project. Once the HSCs have been cultured, the therapeutic DNA would be inserted into the genome and the HCSs would start producing modified exosomes. Since these exosomes would be created using the patient's own cells, there would be no question of exosome compatibility for the patient and therefore it be a safer treatment option.

Chapter 6: Engineering Standards

6.1 Manufacturability Concerns

This project's focus of creating a stable cell line that can produce targeted exosome is directly related to manufacturing of our product. Once a stable cell line is established, it will be able to continue to make exosomes with the targeted therapeutic attached to them. HEK293 cells are fairly resilient cells and would be relatively cheap to maintain with the use of larger bioreactors and a system for extracting exosomes. This gives our project the potential to be manufacturable in the future.

6.2 Ethical Concerns

The main ethical issues around this project is the human embryonic kidney cells we are working with. Although the cell line is a clone of cells taken from a legally aborted fetus, it still raises a lot of questions about the implications of experimenting on human cells. There is a concern that because the cells are from a fetus, there was no consent and that the use of them devalues human life. Not only does this affect the researchers working with the cells, but it can also affect the people using the product. These are topics that we must consider when using human embryonic cells.

6.3 Health and Safety Concerns

Health and safety concerns are important to address when creating a new product because they can be an issue for both the researcher and the consumer. For the researcher, it is important that proper safety precautions are taken to avoid harm. This includes wearing clothing that protects your skin such as gloves, lab coats, long pants and closed toe shoes. It is also necessary to use biosafety hoods with ventilation and ethanol between sterile locations for the researcher safety and to limit the opportunity for contamination of the cells. As for the concerns of the consumer, this project with a focus on targeted drug delivery, could help eliminate long and painful treatments with extensive side effects. It would allow a therapeutic to be delivered directly to the cells of interest and improve the health of the patient.

6.4 Environmental Concerns

Increasing use of nanoparticle technology has encouraged research regarding the potential environmental impact of such materials. Unfortunately, questions surrounding these topics remain largely unanswered. Nanoparticle environmental impact is ultimately governed by the collective effects of their various properties, including their chemical composition, size, and structure. Other nanoparticles have been engineered from compounds that are actually toxic to living organisms when they reach critical physiological levels (López-Serrano). Therefore, many studies must be conducted to understand bioaccumulation of nanoparticles within the environment. Exosomes, however, overcome this potential concern. Because they are naturally

produced, the body is capable of degrading these nanoparticles, thus preventing harmful accumulation of toxic material in the environment.

Additionally, the manufacturing process outlined in this project is relatively free of environmentally hazardous materials. Any hazardous materials that were produced through these procedures, however, was properly disposed of by the SCU Department of Bioengineering.

6.5 Political Concerns

The ethical concerns of this specific exosome application may be of greater concern in later stages of development when this technology may be considered for use in other countries across the globe. Policies will obviously vary greatly from those of the United States. Perhaps the utilization of human embryonic kidney cells could be perceived as ethically problematic and even prevent this technology from being adopted. The simple fact of the matter is that all large populations will likely include patients suffering from defective hematopoietic stem cell function, regardless of geography. Should the ethical concerns become an insurmountable barrier, however, then perhaps it will become necessary to successfully replicate this technology in a different cell line that does not elicit as many ethical concerns.

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Appendix

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Development of Stable Cell Line for the Production of Hematopoietic Stem Cell Targeted Exosomes

Anja Beard and Zach Ehlinger
Advisor: Dr. Bill Lu

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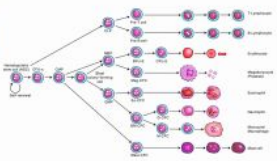
Hematopoietic Stem Cells (HSCs)

- Give rise to all blood cell lineages
- Progeny responsible for oxygen transport, immune response, and more
- Therapeutic target



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Nanoparticle Drug Delivery

Nanoparticle	Advantages	Limitations
Gold nanoparticles	Targeted drug delivery Multivalent Resistant to enzyme degradation	Limited biocompatibility Toxicity
Polymeric nanoparticles	Easy to synthesize Biodegradable Sustained drug release	Agglomeration Non-targeted drug delivery Toxicity
Liposomes	Biocompatible Stable Inert	Non-targeted drug delivery

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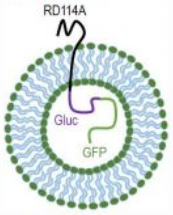
What is a therapeutic exosome?

- A naturally secreted nanoparticle
- Composed of a lipid bilayer
- Can transport proteins and nucleic acids
- Contains targeting proteins embedded in the membrane



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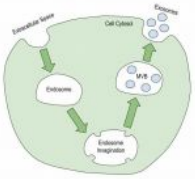
Exosome Production

- Exosomes are produced through a process of
  - Endosome formation
  - Endosome invagination
  - Endosome fusion with the cell plasma membrane



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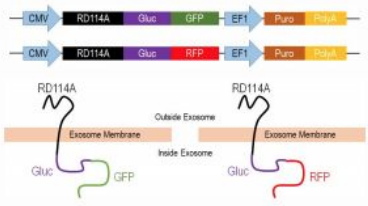
Design Approach



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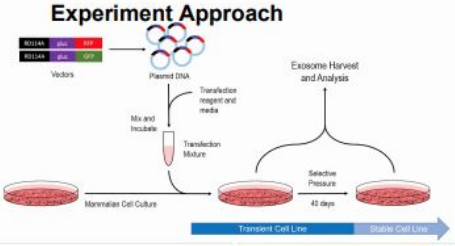
Experiment Approach



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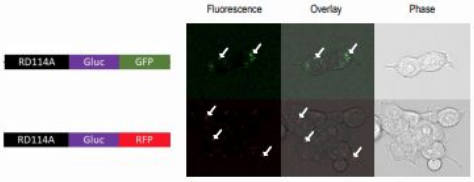
Stable Cell Line



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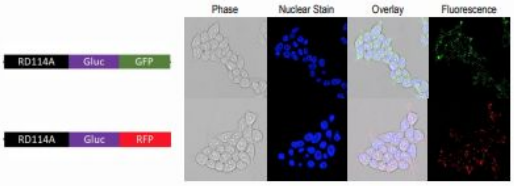
Exosome Localization Imaging



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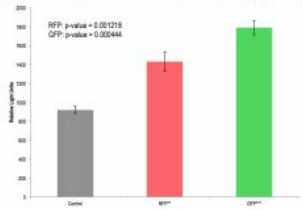
Exosome Luciferase Assay - Transient



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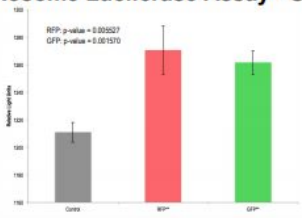
Exosome Luciferase Assay - Stable



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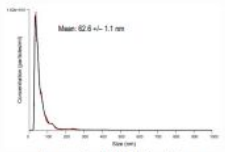
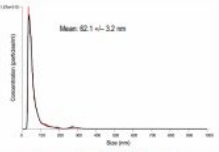
Exosome Size Analysis



Exosomes from GFP stable cell line



Exosomes from RFP stable cell line

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Concluding Remarks

- What has been accomplished?
  - Selected for cells transfected with our constructs
  - Isolated exosomes produced from the cell lines at their transient and stable stages
  - Validated the expression of multiple reporter genes
- What remains?
  - Validating the expression of the RD114 protein
- What is the significance?

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Engineering Standards

- Ethics
- Health and Safety
- Manufacturability
- Environmental considerations
- Political considerations

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Future Steps

- Conduct uptake experiments to test for selective entry into HSCs over other cell types
- Explore this technique in other cell lines
- Develop technology for applications in personalized medicine

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Questions?

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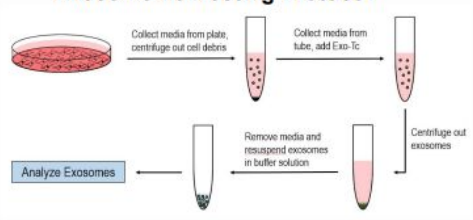
Exosome Harvesting Protocol



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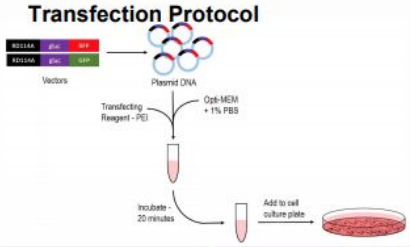
Transfection Protocol



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Luciferase Assay

- Luminescent reporter
- Quantitative results
- Smallest known luciferase
- High sensitivity