ENGINEERING DISEASE RESISTANCE THROUGH GENERATION OF AN iPSC-DERIVED *P. MANICULATUS* CHIMERA

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Engineering Disease Resistance through Generation of an iPSC-derived *P. Maniculatus* Chimera

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The goal of this project is to develop methods to manipulate the genome of *Peromyscus maniculatus* for the future eradication of diseases such as Hantavirus and Lyme disease that this species can transmit to humans. The initial thrust of this project is to generate induced pluripotent stem cells (iPSCs) from *P. maniculatus* mouse embryonic fibroblasts (MEFs) using an engineered Sendai Virus vector system. The Sendai Virus vector is a non-integrating viral vector that delivers four transcription factors necessary to reprogram normal somatic cells to a pluripotent, stem cell-like state. Subsequent, a plasmid will be used to insert DNA into the genome of the iPSC that codes for a fluorescent protein. In the future, the fluorescence will be used to confirm that *Mus musculus* cells are able to combine with the *P. maniculatus* cells in a chimeric blastocyst. Because many notable diseases are transmissible to humans from *P. maniculatus*, the genetic manipulation and creation of a disease resistant *P. maniculatus* using iPSCs could help to eradicate the spread of these diseases.

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NOMENCLATURE

iPSCs	Induced Pluripotent Stem Cells
MEFs	Mouse Embryonic Fibroblasts
GFP	Green Fluorescent Protein
RFP	Red Fluorescent Protein
PBS	Phosphate-Buffered Saline
LB	Luria Broth
SeV	Sendai Virus

CHAPTER I

INTRODUCTION

Peromyscus maniculatus, a North American native mouse species, is known to be a reservoir for various diseases that are transmissible to humans, such as Lyme disease and Hantavirus (Richter, Spielman et al. 1998). Genetically engineering disease resistance in *P. maniculatus* could be a powerful approach to stop the spread of these diseases, but is currently not feasible since methods for assisted reproduction in *Peromyscus* have yet to be developed.

With advances in induced pluripotent stem cell (iPSC) technology (Yamanaka 2008), there is an opportunity for creating disease resistant *P. maniculatus*. However, the generation of iPSCs from *P. maniculatus* was not previously possible due to this species being non-permissive to reprogramming to a pluripotent state under normal culture conditions. Therefore, more advanced conditions including signal pathway inhibitors must be used in order to allow the MEFs to be reprogrammed into the desired iPSCs. The iPSCs are generated from MEFs through usage of a Sendai Virus vector, which is ideal since the viral genes do not insert into the host cell genome and the transgenes coding for specific transcription factors stay in the cytoplasm (Nakanishi and Otsu 2012). The host cell eventually starts expressing endogenous versions of the transcription factors.

The Sendai Virus vector is not the only way to generate iPSCs, however. Retroviral vectors have been used to introduce reprogramming factors, and the silencing of the transgenes displays successful reprogramming. Lentiviral vectors are more efficient at gene delivery, but the transgenes are more resistant to being silenced compared to retroviral vectors. Since transgenes

can interfere with functional genes, adenoviral vectors are used because the vectors integrate with the genome at low frequencies and the transgenes do not transfer into the host genome. The downside to this is that the cells are not reprogrammed as efficiently as they would be with a retroviral vector. A Cre-excisable lentivirus system can generate iPSCs, but there is a risk of gene breaks being inserted into the genome because of the LoxP sequence. To avoid introducing genetic material into the cell's genome, synthetic mRNAs can also be used to reprogram cells to a pluripotent state. This method reprograms cells more efficiently than retroviral vectors (Seki and Fukuda 2015).

The Sendai Virus vector is optimal for this experiment because it can be used in multiple cell types from different species. Genetic background can influence cells becoming iPSCs, and the variability of the Sendai Virus is the best option to use in this case (Schnabel, Abratte et al. 2012). The virus will allow for cell differentiation since it is not retained in the host genome.

CHAPTER II

METHODS

Sendai Virus Transduction and Clearing

In order for the *Peromyscus maniculatus* mouse embryonic fibroblasts (MEFs) to be reprogrammed into induced pluripotent stem cells (iPSCs), the Sendai Virus vector that contains genes coding for stem cell transcription factors will be used. This vector transmits the four Yamanaka factors, Oct4, Sox2, Klf4 and Myc to the MEFs. The Sendai Virus vector is ideally suited for the reprogramming of somatic cells because it does not integrate into the host genome, can infect a broad range of hosts, and replicates only in the cytoplasm of infected cells, among many other advantages. Most rodent species' somatic cells are not permissive to reprogramming to a pluripotent state, so we use additional supplements to drive the *P. maniculatus* fibroblasts to iPSCs. These virus-transfected cells will then be passaged 10-15 times; during this time, iPSC colonies will be subcloned and separated based on gender, using sex-specific primers to detect X versus Y chromosomes present in each colony. Passaging the cells will slowly eliminate the virus and allow these fibroblasts to be transformed into the desired iPSCs. Clearing of the viral vectors and expression of the Yamanaka endogenous stem cell markers is verified by PCR with primers for each gene.

Subcloning

To allow individual iPSC colonies to continue their growth, they are removed from the original 6-well plate and placed into a new 6-well plate in a process called subcloning. The

colonies can also be used to collect cDNA which is analyzed using PCR. To start the subcloning process, phosphate-buffered saline (PBS) and 2iS medium are warmed to 37°C. Next, 300µL of trypsin is added to a 12-well plate where the colonies will be broken up. A 0.1% gelatin-coated plate with 1mL of 2iS medium will be prepared for the newly subcloned cells. The media is aspirated out of the original 6-well plate, and then each well is rinsed with 1mL of PBS twice. The first individual colony is picked up with a wide-bore pipette tip at a setting of 50µL. The colony is released from the gelatin plate with a small twist, and is placed into the 300µL of trypsin. The plate is incubated for 4 minutes to allow the trypsin to start breaking up the colony, then 700µL of 2iS medium is added to the mixture and pipetted to break up the rest of the colony. This mixture is added to the new 6-well plate and the process is repeated for all of the colonies being subcloned.

Reverse Transcription PCR

Reverse transcription PCR is used to obtain cDNA from iPSCs. The primers used include the Sendai Virus Transgene Primers, SEV, KOC, KLF4, and C-MYC, and the Murine Endogenous Stem Cell Markers, Oct 3/4, Klf4, c-Myc, and Sox-2. The PCR is used to check for the elimination of the Sendai Virus vectors with specific transgene primers, and to verify the pluripotency with murine-specific endogenous stem cell markers.

CHAPTER III

RESULTS AND DISCUSSION

Sendai Virus Vector

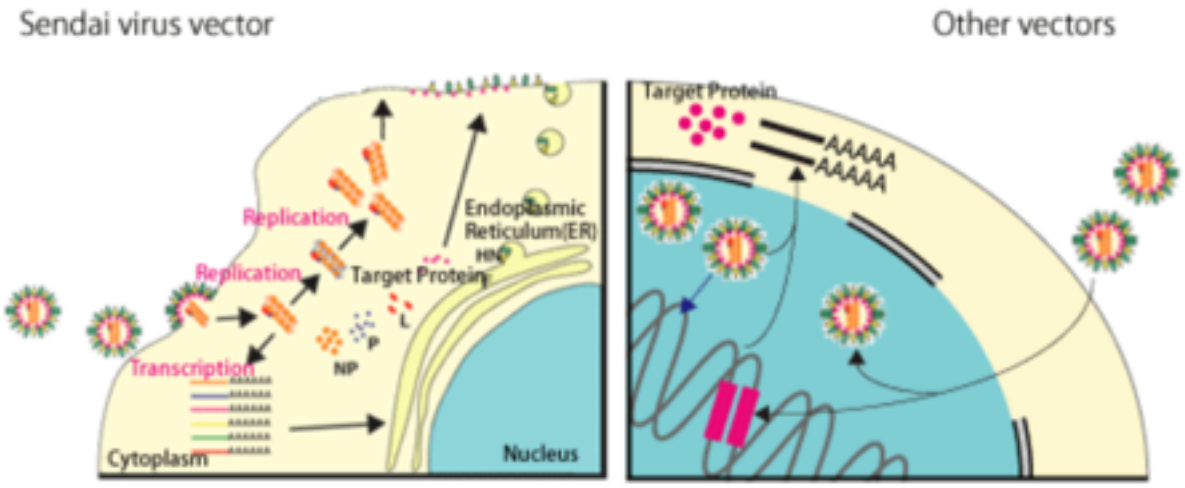

Figure 1. Non-integration of Sendai Virus Vector into host genome

As shown in Figure 1, the Sendai Virus vector does not integrate into the host genome, unlike other vectors. The Sendai virus vector undergoes transcription and replication all within the cytoplasm of the infected cells, which allows the vector to deliver and express key genetic factors necessary for the reprogramming of the somatic cells. Temperature-sensitive point mutations are introduced in viral polymerase genes *P* and *L* so that they could be easily removed at non-permissive temperatures (Yamanaka 2008). Therefore, the Sendai Virus is ideally suited for reprogramming a genetic reference population of MEFs that can accurately represent the diversity present in the human population.

Reprogramming of MEFs into iPSCs

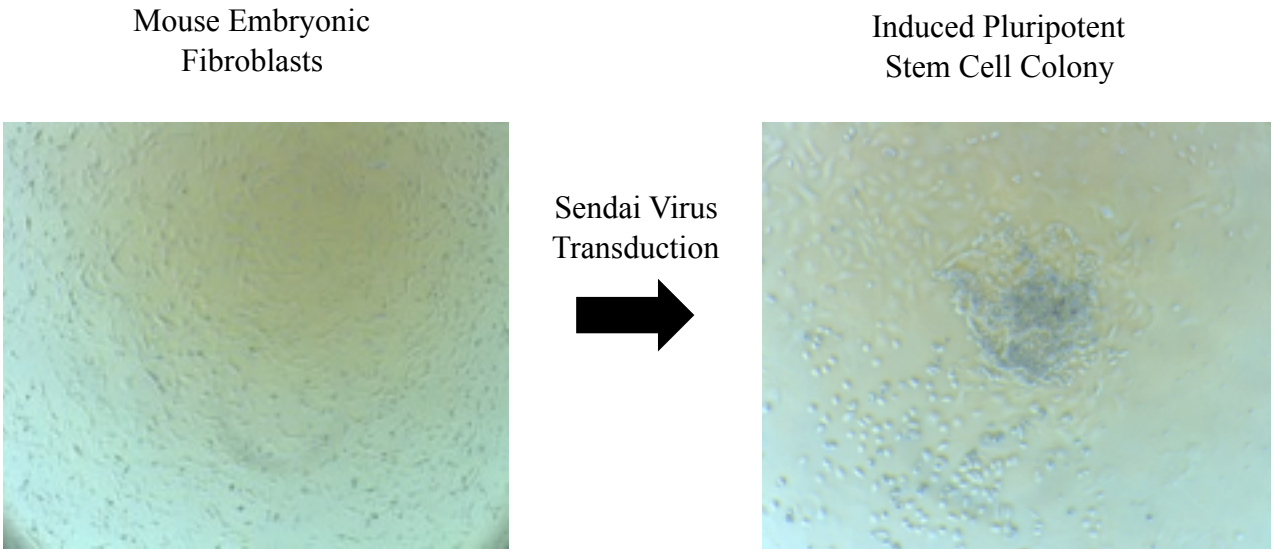

Figure 2. Morphological change from MEFs to iPSCs

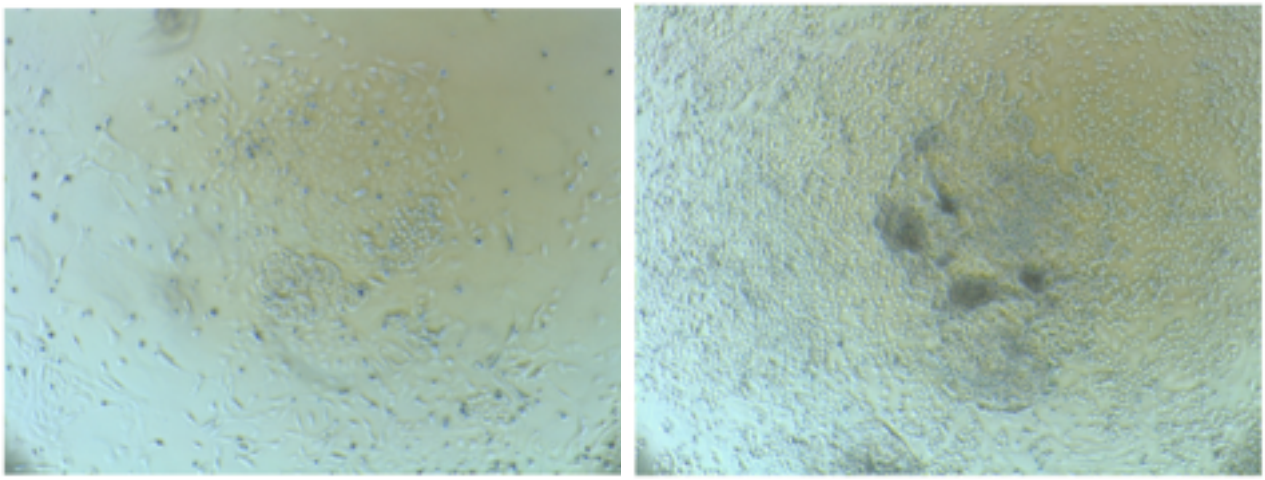

Figure 3a. iPSC reprogramming post SeV transduction. Left, Day 7; Right, Day 18.

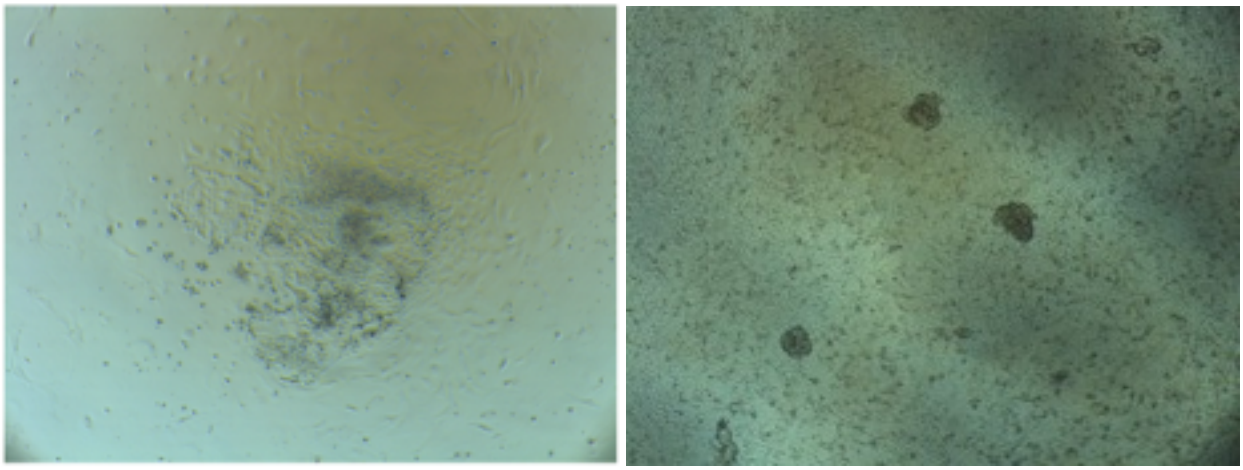

Figure 3b. iPSC reprogramming post SeV transduction. Left, Day 24; Right, Week 8.

The Sendai Virus vector is able to successfully reprogram MEFs into iPSCs. Figure 2 shows the morphological distinction between the MEFs and the reprogrammed iPSCs. Fibroblast cells are elongated and attach to the bottom of the plate, while iPSCs are rounder and aggregate into colonies. This is one way to confirm the successful generation of iPSCs. Figures 3a and 3b indicate the change post Sendai Virus transduction up to 8 weeks. Seven days after transduction, the fibroblasts are still present, but more rounded cells are seen on the plate. This indicates that the cells are in the process of becoming iPSCs. Eighteen days post transduction shows the aggregation of cells and the formation of what will become a colony. As more time goes by, there is a decrease of MEFs and an increase of iPSCs, which indicates successful reprogramming. By week 8, there seems to be no sign of MEFs.

Passaging Cells

Throughout the life cycle of the cells, they need to be passaged on a regular basis to ensure proper growth and colony formation. The first type of passaging used is bulk passaging. This method is used before and after the cells are transfected with the Sendai Virus Vector to allow for continued growth. Once the cells are about 70% confluent, they are passaged to various dilutions depending on the cell density. Passage dilutions vary from 1:2 for plates with fewer cells to 1:50 for plates that are completely confluent. The passaging allows for the cells to have room to divide, and to eliminate the Sendai Virus Vector.

Once colonies form, the cells are passaged through subcloning. Using a wide-bore pipet, individual colonies are picked up from the plate and broken up in trypsin. Once the cells are added to a new plate, they are able to form new colonies. This method is used to eliminate the MEFs that failed to be transduced with the Sendai Virus Vector. After multiple rounds of subcloning, no MEFs remain and the stem cells can continue to grow. The problem with excessive MEFs is that they grow rapidly and take up space that stem cells could be growing and forming colonies in, and subcloning eliminates this problem. Subcloning is also used to eliminate the Sendai Virus Vector.

MEF depletion is used when subcloning fails to eliminate most, if not all, of the MEFs. When colonies are subcloned at a small size, MEFs can be subcloned along with the stem cells. This allows for the continued growth of both MEFs and stem cells, when only stem cell growth is desired. To overcome the excessive population of MEFs, a process called MEF depletion is used to isolate the stem cells. Trypsin is added to the cells to release them from the plate, then media is added to deactivate the trypsin. The media-cell mixture is added to a new gelatin-coated

plate and allowed to incubate anywhere from 30 minutes to 2 hours, depending on how long it takes the MEFs to attach to the plate. Since MEFs attach more quickly than stem cells, the media only contains stem cells. This media is placed onto a new plate and the stem cells are allowed to grow without influence from MEFs.

After multiple passaging of the cells, it was discovered that there weren't many iPSC colonies growing in the plates. This could be due to the fact that the MEFs that are still present are overcrowding the plate and won't allow the iPSCs room to attach. In order to get rid of excess MEFs found in the plates, MEF depletion was performed.

Elimination of SeV and Verification of Pluripotency

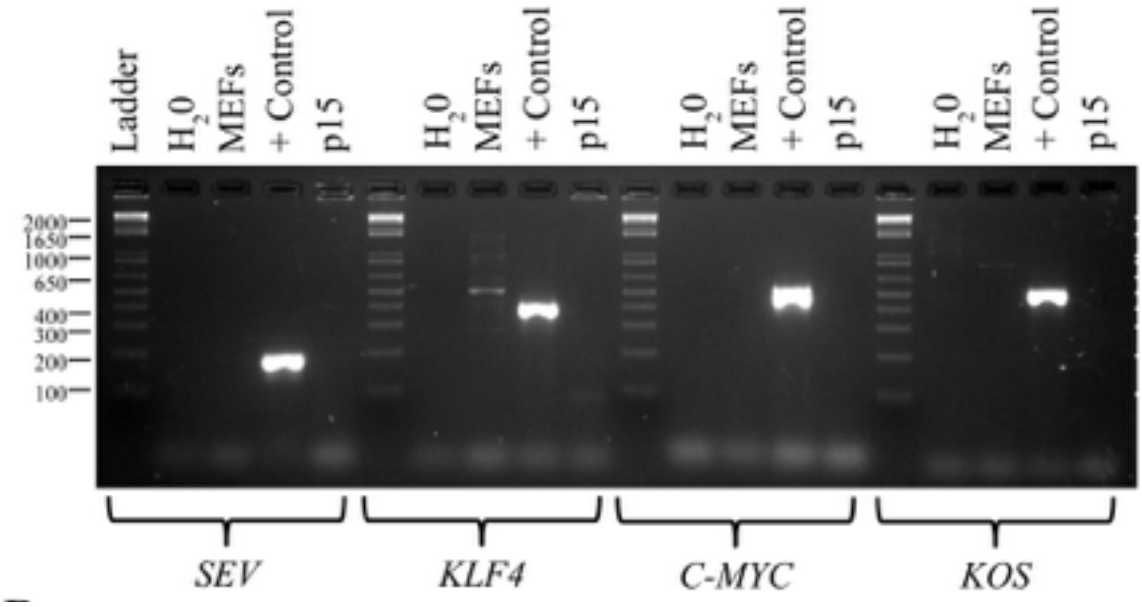

Figure 4a. Sendai Virus Transgene Primers

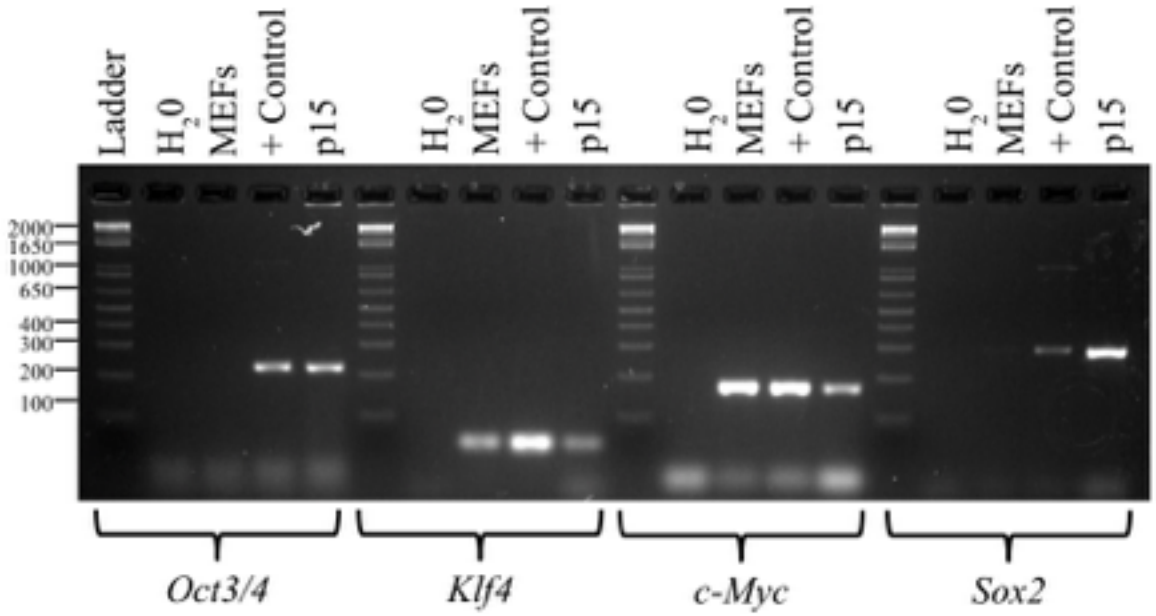

Figure 4b. Endogenous Murine Stem Cell Markers

In the gel images, the MEFs serve as the negative control and should not be expressing Sendai primers. This is because they have never been transduced. The SeV transgenes are human genes so they definitely should not be expressing SeV transgenes. The p15 lane in figure 4a indicates that a parallel cell line at passage 15 has been cleared of the Sendai Virus transgenes that were introduced to the cells during transduction. Expression of murine endogenous stem cell markers is verified in Figure 4b. Although there are bands in the MEF lanes, they key point to note is that there are not bands present in all of them. The MEFs are expressing two out of the four genes because not all genes are specific for one type of cell. The *Klf4* and *c-Myc* markers that the MEFs are expressing are good indicator of stem cells, but they are also involved in proliferation of other cells. The fact that the iPSCs (p15) are expressing all four markers verifies that they are stem cells.

CHAPTER IV

FUTURE PLANS

Bacterial Transformation

It is important to streak the bacteria on a Luria Broth (LB) agar plate in order to isolate single colonies of bacteria. To begin, an LB agar plate was obtained with the antibiotic ampicillin (100 ug/mL). Using a sterile loop, the bacteria from the punctured stab culture was inoculated onto the agar plate using the streak technique. The plate was then incubated overnight for 12-18 hours at 37°C. In order to obtain a large amount of bacteria containing the plasmid, an overnight liquid culture of the plasmid must be inoculated. First, the liquid LB is prepared using NaCl, tryptone, yeast extract, and dH2O. It is then autoclaved and stored at room temperature. When ready, the broth is added to a tube with the appropriate antibiotic at the desired concentration. A single colony from the streaked LB agar plate is selected and inoculated into the tube of LB and antibiotic. The culture is then swirled, covered loosely with aluminum foil, and incubated at 37°C for 12-18 hours in a shaking incubator. The plasmid DNA can then be stored for a long period of time using a glycerol stock. Once ready, the plasmid DNA is isolated from the bacterial cells through lysis and separation of the proteins, RNA, and chromosomal SNA from the plasmid DNA.

Purification of Plasmid

To purify the plasmid that will be introduced into the *P. maniculatus* iPSCs, a colony of bacteria is streaked from a plate and added to the LB medium. The tube is then incubated at 37°C

and vigorously shaken for 12-16 hours. The bacterial cells are harvested by centrifugation at >8,000 RPM for 3 minutes, then the supernatant is discarded. The pellet of bacterial cells is resuspended in 250μL of Buffer P1 and then transferred to a microcentrifuge tube. Next, 250μL of Buffer P2 are added and the tube is gently inverted 4-6 times. The solution should look viscous and slightly clear. Then, 350μL of Buffer N3 is added and the use is immediately inverted 4-6 times. The solution should look cloudy. The tube is then centrifuged for 10 minutes at 13,000 RPM, and a white pellet should form. A QIAprep spin column is added to a 2mL collection tube, and the supernate from the centrifugation is added to the column. This is centrifuged for 30-60 seconds and the flow-through is discarded. Following centrifugation, 0.75mL of Buffer PE is added to the column and centrifuged again for 30-60 seconds. The flow-through is again discarded and the column is centrifuged for one more minute to remove residual wash buffer. Then the column is placed in a clean microcentrifuge tube and can then be used to elute DNA.

Restriction Digests

Restriction analysis is used to verify that the correct plasmid is present. To start, the restriction digests were prepared, each containing: 7uL sterile water, 2uL 10X FastDigest buffer, 10uL of plasmid DNA, and 1uL of the appropriate Fast Digest enzyme. These came to a total volume of 20uL. For double digests, 6uL of sterile water was used with 1uL of each of the two enzymes. The digests were mixed gently, centrifuged, and placed on a 37°C heat block for 10 minutes to incubate. Next, 4uL of 6X loading dye was added to each of the samples. The samples

were loaded onto a 2% agarose gel and run at 120 V. Once the correct plasmid is confirmed, the appropriate restriction enzyme is used to linearize the plasmid for use in transfection.

Antibiotic Sensitivity

Before the plasmid is added to the cells, gentamicin is added to a subset of stem cells to verify their sensitivity to the antibiotic. Once the cells are transfected with the plasmid, they are exposed to the antibiotic once again. The cells that survive the antibiotic exposure have successfully taken up the plasmid and are therefore antibiotic resistant.

Transfection of Fluorescent Plasmid

Future experiments will include injection of *P. maniculatus* iPSCs into a *Mus musculus* blastocyst to form a chimera that can later breed with normal *P. maniculatus*. In order to verify that, in the future, *P. maniculatus* iPSCs injected into a *Mus musculus* blastocyst are indeed integrated into the blastocyst, green fluorescent protein (GFP) and red fluorescent protein (RFP) will be used to distinguish the two individuals' cells. A plasmid that expresses RFP will be used to insert the DNA into the cell, which then will insert itself into the cell's genome. A non-cytoxic chemical will be used to permeabilize the membrane for the DNA to enter the cell.

Creating a Chimera

Fluorescence will be used to ensure that the blastocyst will integrate with the *P. maniculatus* iPSCs. Once the iPSCs are fully reprogrammed and cleared of the viral vectors, future plans are transfection of the fluorescence-expressing iPSCs into a *Mus musculus*

blastocyst. Because of the considerable genetic resources available for this common lab mouse, it is likely that using a *M. musculus* female for implantation of a *P. maniculatus* iPSC-injected blastocyst will aid in the successful generation of a chimera (Guo, Wu et al. 2014). Disease resistant genes will eventually be introduced into the iPSCs to be expressed in the chimera, which should then be able to successfully reproduce with wild *P. maniculatus* and transmit disease resistance in the future.

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