Developing a Tool to Confirm the Successful Differentiation of Human Pluripotent Stem Cells into Cardiomyocytes

Honors Thesis
Presented to the College of Arts and Sciences,
Cornell University
In Partial Fulfillment of the Requirements for the
Biological Sciences Honors Program

by
Calvin Schuster
May 2019

Supervisor Dr. Michael I. Kotlikoff

Abstract

Heart disease has been the leading cause of death in the United States since 1921 (Greenlund et al., 2006). However, age-adjusted cardiovascular disease death rates have declined by 55% since 1950, a trend that is in part due to improvements in treatment (Greenlund et al., 2006). Researchers interested in developing effective heart disease treatments have begun using human myocardial cells derived from induced pluripotent stem cells (iPSCs) to build *in vitro* heart disease models, screen for new cardiovascular drugs or drug side effects, and study individualized cardiac therapy (Sinnecker et al., 2012). The future clinical use of iPSCs in cell therapy for cardiovascular disorders is also incredibly promising (Sinnecker et al., 2012). However, when differentiating iPSCs into cardiomyocytes, researchers currently have no tools available to confirm whether the differentiation was successful. The goal of this project was to create and test a tool that can confirm and quantify the successful differentiation of iPSCs into cardiomyocytes by coupling the GCaMP8 calcium indicator and the cardiomyocyte-specific promoter alpha myosin heavy chain (αMHC). A release of calcium ions triggers cardiomyocyte contraction (Marks, 2003). When calcium ions bind to GCaMP molecules a conformational change occurs that results in an increase in green fluorescence (Shui et al., 2014). Therefore, an αMHC-GCaMP8 cardiomyocyte cell line should produce green fluorescence during contraction. This project demonstrated the use of Lipofectamine 3000 to transfect human iPSCs with an αMHC-GCaMP8-iCAG insertion plasmid and the successful differentiation of transfected iPSCs into cardiomyocytes, and it suggests that differentiated cardiomyocytes fluoresce.

Introduction

In 2011, over 780,000 people in America died due to heart disease, demonstrating that cardiovascular diseases account for about one in every three deaths in the United States (Mozaffarian et al, 2015). However, the death rate of heart disease has been steadily declining, in part due to continual advances in heart disease treatment (Greenlund et al., 2006). Innovations in research have made these advances in treatment possible, and one major innovation in the study of heart disease and potential heart disease treatments has been the use of cardiomyocytes produced from induced pluripotent stem cells (iPSCs) (Sinnecker et al., 2012). Induced pluripotent stem cells can be derived from somatic cells, exist in an embryonic-like state, and have a high proliferative capacity (Faiella et al., 2016). Cardiomyocytes derived from iPSCs can be used in a wet lab setting to create *in vivo* heart disease models to further study heart disease or to screen for potential cardiovascular drugs or drug side effects (Sinnecker et al., 2012). Cardiomyocytes derived from iPSCs could also potentially be used in a clinical setting, which is a particularly revolutionary concept for the treatment of heart disease due to the nature of cardiomyocytes.

The heart has a limited capacity for repair and regeneration, so when cardiomyocytes die due to heart disease the dead cells will not be regenerated and replaced (Chiong et al., 2012). Thus, when a myocardial infarction occurs, and cardiomyocytes experience irreversible cell death, it greatly impacts the heart's ability to function (Chiong et al., 2012). Perfecting a method to replace the cells that were killed due to heart disease would therefore be a major leap forward for human healthcare, and this feat could potentially be accomplished through transplants of iPSCs that have been differentiated into functional myocardial cells (Martins et al., 2015).

While using stem cells in general in tissue repair and functional restoration could be a revolutionary treatment for myocardial infarction, the use of embryonic stem cells presents many ethical and political challenges, which has greatly slowed the progress of research when embryonic stem cells are involved (Sun et al., 2014). In addition, embryonic stem cells are generated from embryos, and thus do not have the same genotype as the patient; this increases the risk of the patient rejecting the transplant because their immune system could attack the foreign tissue (Sun et al., 2014). Finally, grafted embryonic stem cells only generate a small amount of cardiomyocytes, and the number of differentiated cardiomyocytes significantly compromises the ultimate success of future cell-grafting procedures (Sun et al., 2014). iPSCs could solve these issues and overcome these limitations because they are derived by reprogramming adult somatic cells and putting them in a pluripotent state (Sun et al., 2014). This circumvents the political and ethical complications of using embryonic stem cells (Faiella et al., 2016). Additionally, this reduces this risk of patients rejecting the transplants because the cells come from their own bodies (Sun et al., 2014). Finally, iPSCs have a high proliferative capacity, so they grow much more quickly than embryonic stem cells (Faiella et al., 2016)

Stem cell treatments with iPSCs as a therapeutic option after myocardial infarction can be risky, however. If undifferentiated iPSCs are injected into a heart, there is a risk of teratoma formation, or the formation of a tumor composed of many different cell types (Faiella et al., 2016). Therefore, it is crucial that successful and full differentiation occurs before cells are injected as a therapeutic intervention (Faiella et al., 2016).

This poses a critical dilemma, because currently there are no widely used tools that researchers can use to confirm the successful differentiation of iPSCs into cardiomyocytes. Therefore, the Kotlikoff Laboratory in the Department of Biomedical Sciences of Cornell

University has been attempting to solve this issue. A tool must be developed that can confirm and quantify successful iPSC differentiation so that different differentiation protocols can be assessed and then optimized in future research, to improve differentiation efficiency.

One potential way to determine if iPSCs have been successfully differentiated into cardiomyocytes and to quantify the differentiation is to couple the cardiomyocyte-specific αMHC promoter and GCaMP8 reporter. The regulation system of the alpha-myosin heavy chain gene, or αMHC, was identified and described in 1996 by Molkentin, Jobe, and Markham (Molkentin, 1996). The αMHC gene codes for a protein that is cardiac muscle-specific, meaning it is only expressed in heart cells (Molkentin, 1996). This is accomplished because components of the regulatory region of this gene direct cardiac muscle-restricted expression (Molkentin, 1996). Therefore, the use of the αMHC promoter ensures the cardiac muscle-restricted expression of a gene.

GCaMP was first developed in 2001 by Nakai, Ohkura, and Keiji as a high signal-to-noise calcium ion probe (Nakai, 2001). In other words, GCaMP sensors are calcium indicators: they have an affinity for calcium ions, which are responsible for cardiac muscle contraction (Marks, 2003). The sarcoplasmic reticulum releases intracellular calcium, thus increasing the cytosolic calcium concentration and triggering contraction (Marks, 2003). Spectral analysis has shown that GCaMP molecules that are not bound to calcium ions have an absorbance maximum of 399 nanometers, but once a calcium ion binds, a conformational change occurs and the sensors then have an absorbance of 488 nanometers instead, which is a green fluorescence (Shui et al., 2014). Therefore, αMHC-GCaMP8 expressing cardiomyocytes should produce green fluorescence during contraction. GCaMP8 specifically, along with GCaMP5, has the highest dynamic range of all the GCaMP sensors (Shui et al., 2014). GCaMP was selected instead of a

simple fluorescent reporter like green fluorescent protein (GFP) because GFP simply indicates activity of the promoter, but because GCaMP can report calcium ion release during contraction, it can determine cell phenotype as well. Because the promoter αMHC is unique to cardiomyocytes, when αMHC and GCaMP8 are coupled the sensor GCaMP8 will only be expressed in cardiomyocytes.

The conception of this project began in May 2016 when Joseph Neumeyer of the Kotlikoff Lab created an αMHC-GCaMP8 construct (Neumeyer, 2016). Then, in May 2017 Bayan Yazdi of the Kotlikoff Lab successfully used the construct to create an insertion plasmid, αMHC-GCaMP8-iCAG (Yazdi, 2017). An insertion plasmid is a circular, double-stranded piece of DNA that contains an origin of replication and can therefore replicate on its own, and a sequence that is recognized by a restriction enzyme and can therefore integrate foreign DNA as well. Insertion plasmids frequently contain selectable markers. Therefore, insertion plasmids can be used efficiently in transfections to transfer genes of interest from the plasmid into the genome of target cells, which can then undergo a selection process to confirm transfection.

The insertion plasmid used for this project was pC13N-iCAG.copGFP (Addgene plasmid #6658). This plasmid was created and described by Jizhong Zou (Cerbini, 2015). This plasmid was originally selected for this project by Neumeyer because it was designed for use in the transcription activator-like effector nucleases (TALEN) site-specific transfection process that targets the safe-harbor gene CLYBL (Cerbini, 2015). Additionally, these cell lines maintain robust reporter expression during differentiation, and targeted CLYBL insertion resulted in stronger transgene expression and less disruption of local gene expression than other common gene insertion sites (Cerbini, 2015). Therefore, Neumeyer selected iCAG because TALEN-mediated CLYBL insertion yields optimized transgene expression (Cerbini, 2015). Neumeyer

then removed the CAG-GFP cassette to create a linear vector with a sticky end and a blunt end which still had the homologous TALEN arms and neomycin gene, conferring resistance to the aminoglycoside antibiotic Geneticin (G418); following this process, Yazdi then added the αMHC-GCaMP8 construct to the plasmid to replace the GFP construct (Neumeyer, 2016; Yazdi, 2017). However, while the origins of this project focused on the iCAG plasmid to capitalize on the site-specific TALEN transfection it afforded, the continuation of this project discussed here utilized non-site specific transfection using Lipofectamine 3000 due to the reagents accessibility. A full description of the potential use of this plasmid with TALEN transfection, however, is included in the discussion.

After the successful production of the αMHC-GCaMP8-iCAG insertion plasmid, the natural progression of Neumeyer and Yazdi's work leads to the next critical steps in the process: transfecting iPSCs with αMHC-GCaMP8-iCAG, selecting for iPSCs that have successfully incorporated the construct into their genome, testing the successfully transfected iPSCs to confirm the insertion of the full construct, differentiating the transfected iPSCs into cardiomyocytes, testing transfected cells before and after differentiation to confirm that GCaMP is only being expressed after differentiation, and finally imagining the transfected cells using fluorescent imaging to confirm that they display green fluorescence in the presence of calcium ions after contraction.

Materials and Methods

Growing, Thawing, and Plating Human iPSCs

The human iPS cell line (GM25256 WTC-11, P37 from 3/18/16 and P39 from 3/23/16) used for this project was supplied by Dr. Bruce Conklin of The Gladstone Institute. All tissue culture plates were coated with Corning Matrigel Matrix (REF 356231), stored at -20 ˚C and aliquoted out to reduce freeze-thaws. The plates were coated overnight at 37 ˚C, 5% CO_2 using 0.081 mg/mL Matrigel Matrix in Knockout DMEM (Gibco REF 10829018). Matrigel Matrix samples were always kept on ice and pipette tips were pre-chilled in a freezer before use. Matrigel aliquots were thawed on ice overnight.

Thawed cells were plated in Gibco Essential 8 Medium (E8) and roughly 10 μM Y-27632 ROCK Inhibitor (R_i) (CAT #72304). When thawing cells, partial thaws were conducted where frozen cell samples were placed into a hot-bath until only a small frozen section remained, and then the sample was diluted into 9 mL of E8/R_i media. This sample was then centrifuged down at roughly 800 rpm for 3 minutes, the media was aspirated out, and then the pelleted cells were resuspended into as much E8/R_i media was necessary to plate. To plate, the Knockout DMEM was aspirated out of a Matrigel coated plate and then the cells were slowly pipetted onto the plate in a dropwise fashion. After 20-22 hours, the E8/R_i media was aspirated out and then just E8 was added to the cells. The E8 was replaced daily following this to feed the cells. E8 media was always left to warm to room temperature before use.

Splitting Cells

Cells were split at 70-80% confluency. The E8 was aspirated, the cells were gently washed with PBS twice and then incubated in Versene (CAT #15040066) for 10 minutes at room

temperature. Versene is an EDTA solution that can bind calcium and thus detach membrane proteins from the dish and each other. After this, the Versene was aspirated, E8 media was added and then aspirated out as an additional wash step, and then E8/R_i media was added and the cells were mechanically scraped with a cell scraper. Cells were then diluted into more E8/R_i media based on the necessary dilution needed, and then plated onto Matrigel coated plates. After 20-22 hours the E8/R_i media was replaced with just E8 media.

Confirming the Insertion Plasmid: Restriction Digest

For this project the αMHC-GCaMP8-iCAG insertion plasmid that Yazdi created and stored at -80 ˚C was used (Yazdi, 2017). A restriction digest was performed on a purified plasmid sample named "BY5" in order to confirm the plasmid. The sample was thawed on ice and 1 μg of the BY5 DNA, 2 μL of ThermoScientific Fermentas Fast Digest Green Buffer (10x), 1 μL of SmaI restriction enzyme, and 16 μL of sterile water were added to one 0.5 mL tube, and 1 μg of the BY5 DNA, 2 μL of NEBuffer 3.1 (10x), 1 μL of BioLabs SalI restriction enzyme, and 16 μL of sterile water were added to another 0.5 mL tube. SmaI was used to check that the band lengths approximately matched what was expected based on the sequence of the plasmid that Yazdi presents (6530, 5586, 2998, and 64 base pairs). SalI only cuts in the GCaMP8 region so it was used to confirm that GCaMP was successfully incorporated into the iCAG plasmid. These samples were vortexed and then placed in a 37 ˚C bacterial incubator for 60 minutes. Then 2 μL of 6x loading dye was added to each sample and 10 μL each of 1 kb+ DNA ladder, the SalI sample, and the SmaI sample were loaded into a 0.8% agarose gel and ran at 100 V for 45 minutes. The gels were imaged, as shown in the Figures and Tables section (Figure 1).

Increasing BY5 Plasmid Stock via Bacterial Cultures

BY5 transfected *E. coli* cells, which Yazdi created and froze down in glycerol and stored in a -80 ˚C freezer, were plated onto LB-kanamycin plates using the sterile loop technique (Yazdi, 2017). The plates were then stored in a 37 ˚C bacterial incubator overnight. The iCAG plasmid possesses a kanamycin resistant gene, so the use of this antibiotic selected for cells which successfully incorporated the insertion plasmid because only cells that successfully took up the full plasmid or a derivation of the plasmid could grow on the LB-kanamycin plates (Yazdi, 2017). The next day, single colonies were observed on the plates and autoclaved pipette tips were used to select single colonies from each plate via sterile technique. Each pipette tip was then dropped into a small culture tube that contained 5 mL of liquid LB media and 1.5 μL of 10 mg/mL kanamycin. These tubes were placed into a 37 ˚C, 243 rpm shaker overnight. The next day a ThermoScientific GeneJET Plasmid Miniprep Kit was used to perform minipreps to purify and isolate the plasmids from each liquid media culture. The purified samples were then stored in a -20 ˚C freezer. A NanoDrop machine was used to measure the DNA concentration of each sample.

Transfection

Lipofectamine 3000 (Invitrogen) was used to chemically transfect the iPSCs following the protocol provided online by ThermoFisher Scientific. Cells were transfected at 70-80% confluency. 125 μL Opti-MEM was added to two tubes, 3.75 μL of Lipofectamine 3000 was added to one of these tubes and 7.5 μL of Lipofectamine 3000 was added to the other. 250 μL Opti-MEM was added to a third tube along with 10 μL P3000 and 5 μg of BY5 DNA. 125 μL of this DNA solution was then added to each of the first two tubes, and the new solutions were

incubated at room temperature for 10 minutes. Then 250 μL of each solution was added to one well on a 6-well plate of iPSCs. This was allowed to incubate for 3 days for the first transfection and 2 days for the second transfection, and then for the second transfection each well was split into two wells and were returned to just E8 media until selection with G418 began. Both 3.75 μL and 7.5 μL of Lipofectamine 3000 were used to assess whether one concentration was more successful than the other.

Selection of Successfully Transfected iPSCs

The aminoglycoside antibiotic Geneticin (G418) was used for selection because the αMHC-GCaMP8-iCAG insertion plasmid has a neomycin gene that confers resistance to G418 (Yazdi, 2017). Therefore, G418 exposure selects for successfully transfected cells by killing cells that were not transfected and therefore do not have G418 resistance. Based on the kill curve that was conducted (Figure 2, Table 1), 9 μg/mL of G418 in E8 was originally used for the selection process. This amount was later increased to 11 μg/mL and then 15 μg/mL based on the observed cell death rate of the untransfected control cells during the experimental selection process. During the first transfection, cells were exposed to G418 selection pressure for 25 days. For the second selection, cells were exposed to G418 selection pressure for 15 days. Throughout the selection process, anytime the cells were split the G418 selection pressure was removed for the day of the split and the day after to ensure the healthy re-adherence of the cells.

Conducting a Kill Curve

Two 12-well plates were seeded with iPSCs, and when they reached roughly 75% confluency the kill curve process was initiated. Triplicates of each concentration of G418 in E8

were created: 1 μg/mL, 3 μg/mL, 4.5 μg/mL, 6 μg/mL, 7.5 μg/mL, 9 μg/mL, and 11 μg/mL. Three wells were reserved as negative controls, which were just exposed to E8 media without G418. See Table 1 and Figure 2 for observations and results. The kill curve was conducted to determine the optimal concentration of G418 needed to kill non-resistant cells without killing the resistant ones.

Confirming Transfection: DNA Extraction, PCR, and Gel Electrophoresis

In any situation throughout this project where DNA was tested to confirm the presence or absence of particular sequences of DNA, first a genomic DNA extraction was performed. A sample of cells was removed during the splitting or freezing process and centrifuged to pellet the cells. Then the E8/R_i or differentiation media was aspirated out and the pellet was resuspended in 50 μL of PBS. 300 μL of cell lysis solution and 6 μL of proteinase K was then added to these cells, and the sample was pipetted and then vortexed to mix. Samples were then placed on ice to chill. Then 100 μL of protein precipitation solution was added to each sample and samples were vortexed for 10 seconds and then centrifuged at 13,300 rpm for 3 minutes. Supernatants were decanted into new tubes containing 300 μL isopropanol each, and these tubes were inverted to mix and then centrifuged for 1 minute at 13,300 rpm, and then the supernatant was poured off and the tubes were drained upside down on a paper towel. Then 300 μL 70% ethanol was added to each tube to wash the DNA, and the tubes were centrifuged for an additional 1 minute and then drained again. Samples were then allowed to airdry at room temperature for roughly 30 minutes, and finally 100 μL hydration solution was added to each sample.

Once samples were allowed to rehydrate overnight, the genomic DNA could be used in polymerase chain reactions (PCR), a process that amplifies a particular segment of DNA, if the

segment of DNA is present in the DNA sample, based on the selected primers. For this project, a PCR was performed for both GCaMP and GAPDH for each DNA sample extracted from a given set of experimental cells. This assessed the presence of the GCaMP gene in each transfected sample. GAPDH, which is a housekeeping gene that all cells possess, was used as a control to demonstrate that DNA was present in general in the given sample and to show relatively how much DNA in general was extracted. For each PCR, a positive and a negative control were included. The positive controls for both the GCaMP PCR and the GAPDH PCR used a DNA sample that was confirmed to possess the GCaMP gene. No additional DNA was added to the negative control. See Figure 3 for the results. For the PCR process, 2x Dream Taq Green PCR Master Mix was used, GAPDH sense (Eurofin) and GAPDH antisense (Eurofin) primers were used, and GCaMP calmprobefor (Eurofin) and calmproberev (Eurofin) primers were used. All primers were used at a concentration of around 300 nM.

GCaMP PCRs can confirm the presence of the GCaMP gene, but cannot confirm that the full αMHC-GCaMP8 constructed was successfully integrated. In order to confirm that the unique αMHC promoter was integrated along with the GCaMP gene, an additional PCR was performed using primers that would include DNA from the αMHC promoter on one end and from the GCaMP gene on the other end. Therefore, a PCR was performed using αMyHCfor3 as the forward primer and αMyHCrev2 as the reverse primer. Based on the αMHC-GCaMP8 sequence, the PCR product size was expected to be 1,313 base pairs. Based on the melting temperature of the primers and the size of the expected product, the PCR protocol was: one cycle of 95 ˚C for 2 minutes, 30 cycles of 95 ˚C for 30 seconds, 56 ˚C for 30 seconds, and 72 ˚C for 90 seconds, and then one cycle of 72 ˚C for 5 minutes. Then cells were then held at 10 ˚C until the gel was run.

After PCRs were performed, gel electrophoresis was performed and then the gels were imaged to assess the presence or absence of GCaMP or of the particular sequence of DNA being assessed. 1% agarose gels were created, and samples were run at 100 V for 25 minutes. Sybr Safe was used for imaging. See Figure 3, Figure 4, and Figure 5 for the results.

Differentiation

iPSCs were differentiated into cardiomyocytes by following the Conklin Lab 15-day protocol, "Human iPSC-Cardiomyocyte Production Conklin Lab SOP V1.5 (Combination V1.4 and D6 Split)" (Yazdi, 2017; Neumeyer, 2016). Following this protocol, cells were exposed to 6 μM of CHIR 99021 (Tocris), a glycogen synthase kinase 3 inhibitor, in a solution of RPMI (Life Technologies) and 1x B27 minus insulin (Life Technologies) for 48 hours. Then the media was changed, and the cells were incubated in 5 μM of IWP2 (Tocris), an inhibitor of Wnt processing and secretion, in a solution of RPMI/B27(-) for 48 hours. Then the media was changed to just RPMI/B27(-) for 48 hours. Then the cells were split in a 1:1 ratio. For the first transfection of this project, cells were split according to the Conklin plan. For the second transfection, cells were not split to minimize the amount of time before beating. From here, the RPMI/B27(-) media was changed twice more following the 48-hour exposure pattern. Then, on day 11 the media was changed to RPMI (Life Technologies) and 1x B27 plus insulin (Life Technologies) instead. This new media was then changed every 3-4 days. For the first transfection, only the RPMI/B27(-) media was used. For the second transfection, the Conklin protocol was followed and the RPMI/B27(+) switch occurred.

Imaging for Fluorescence

An Olympus MVX10 microscope and Andor iXon camera were used to visualize the fluorescence of the cells. A Bio-Rad ZOE Fluorescent Cell Imager was used to visualize the cells as well, and images were taken using this microscope (Figure 6).

RT-qPCR

Quantitative reverse transcription PCRs (RT-qPCRs) were performed to assess the expression of the GCaMP gene after transfection but before differentiation, and after both transfection and differentiation in order to confirm that, due to the cardiac-specific αMHC promoter, GCaMP was only being expressed after cells became cardiomyocytes. Isolation of total RNA, including the optional on-column DNase digestion, was performed following the "QIAGEN RNeasy Mini Handbook" protocols and using the QIAGEN RNeasy Mini Kit. Reverse transcription of RNA to complementary DNA (cDNA) was then conducted using the Applied Biosystems by ThermoFisher Scientific High Capacity RNA-to-cDNA Kit (LOT 00635123). The Applied Biosystems TaqMan Universal PCR Master Mix, Applied Biosystems custom TaqMan Gene Expression Assay GreenFP, and Applied Biosystems Human GAPDH TaqMan Gene Expression Assay were all used along with the Bio-Rad iCycler iQ Real-Time PCR Detection System to conduct RT-qPCR. The GreenFP (GFP) TaqMan Assay works for GCaMP as well, so it was used to detect GCaMP expression.

Freezing Cells

When creating back-up samples or storing cells for prolonged periods of time, cells were frozen down at roughly 60% confluency. The freezing medium consisted of ES cell qualified

fetal bovine serum (FBS) and 10% DMSO, and was filter sterilized before use using a syringe filter. First cells were washed with PBS, then left in Versene for 10 minutes. The Versene was aspirated and the cells were scraped, added to 10 mL E8, and centrifuged for 3 minutes at roughly 800 rpm. The E8 was then aspirated and the pellets were resuspended in freezing medium. About 1 mL of this solution per tube was added to cryotubes, and then the tubes were moved into a freezing container in isopropanol, which was then stored in a -80 ˚C freezer for one to three days before being moved to a liquid nitrogen freezer for longer term storage.

Results

Confirming the Insertion Plasmid: Restriction Digest

At the very beginning of this project, one of the first steps was to confirm that the insertion plasmid from Yazdi, an αMHC-GCaMP8-iCAG insertion plasmid that he created and stored at -80 ˚C, was the sequence that it was supposed to be. This vector had been sequenced before by Yazdi, but because this was a new project and the sequence is so central to the project, it was confirmed again via a restriction digest on the purified plasmid sample named "BY5" to properly set the foundation for the rest of the experiment. The expected band sizes according to Yazdi's plasmid sequence for the SmaI restriction enzyme digest were 6530, 5586, 2998, and 64 base pairs (Yazdi, 2017). The restriction digest and gel that were run reflected these values, confirming the insertion plasmid (Figure 1). Bands were observed at around 6,500 base pairs, 5,600 base pairs, and 3,000 base pairs. The 1 kb+ ladder that was used for this gel is designed for sizing bands between 100 and 15,000 base pairs in size; the expected 64 base pair band was not

observed; it likely ran off the end of the gel due to its small size. There was a band for the SalI digest as well which confirmed the incorporation of GCaMP into the iCAG plasmid (Figure 1).

Kill Curve

After confirming the sequence of the plasmid, the next preparatory step for this project was to gage the optimal concentration of G418 that would be used for selection. This was accomplished by conducting a kill curve. After 10 days, almost complete cell death had occurred with 9 μg/mL G418 in E8, so this concentration was originally used for the selection process based on the kill curve results. This amount was later increased to 11 μg/mL and then 15 μg/mL based on the observed cell death rate of the untransfected control cells during the experimental selection process. See Figure 2 and Table 1 for specific results.

The protocol used in this project to conduct the kill curve utilized a subjective, qualitative assessment of confluency: the confluency was estimated after the wells were observed under a microscope. However, in future experiments this process could be conducted qualitatively instead, to increase overall accuracy, which would make finding the optimal concentration of G418 more precise. For example, a method known as area fraction output could be used with any cell culture plastic container, such as cell culture plates, to assess confluency objectively by imaging with a camera and phase contrast light microscope and then using image analysis software such as ImageJ to quantify confluency (Busschots, 2014).

PCR Results

The restriction digest to confirm the plasmid and the kill curve to assess the optimal G418 concentration for selection set the stage for the experimental work of this project to begin,

and this work started with a transfection of iPSCs using Lipofectamine 3000. The cells were transfected with the αMHC-GCaMP8-iCAG insertion plasmid, and then the successfully transfected cells were selected for using G418. The success of the transfection was then gaged with PCR analysis that tested for: the presence of GAPDH, to confirm that DNA was successfully extracted during the preparation of DNA samples; the presence of GCaMP, to confirm that transfected cells did take up and integrate the GCaMP gene; and the presence of a sequence of DNA that was partially in the GCaMP gene and partially in the αMHC promoter, to confirm that both the cardiomyocyte specific promoter and the gene of interest were taken up together.

The GAPDH PCR did not display a band for the negative control but did display bands for the positive control and all of the experimental samples, showing that the DNA extractions were successful, and that DNA was present in all the samples (Figure 3). The first GCaMP PCR did not display a band for the negative control but did display bands for the positive control and all of the transfected cell samples except for sample 2, which demonstrates that the GCaMP gene was present in all of the transfected cells except for one of the two wells of cells from the second transfection that was exposed to 3.75 μL of Lipofectamine 3000 (Figure 3). To test this again, an additional PCR was conducted after a new DNA sample was created using cells from the same well as the original sample 2. This PCR did display a band for GCaMP for sample 2 (Figure 4). This demonstrates that cells from that well did have the GCaMP gene, so the lack of a band in Figure 3 for GCaMP for sample 2 was likely due to a loading error. The αMyHCfor3 and αMyHCrev2 PCR displayed bands for samples 1, 2, 3, 4, and 5. This suggests that both the αMHC promoter and the GCaMP gene were taken up.

Sample 1 was DNA from iPS cells from the first transfection, which used 7.5 μL of Lipofectamine 3000 and transfected for three days. Samples 2 and 3 were from two different wells of cells from the second transfection that were exposed to 3.75 μL of Lipofectamine 3000 and were transfected for two days. Samples 4 and 5 were two different wells of cells from the second transfection that were exposed to 7.5 μL of Lipofectamine 3000 and were transfected for 2 days.

RT-qPCR Results

Once the PCR and gel electrophoresis analysis confirmed that the transfected cells had successfully taken up the αMHC-GCaMP8 construct, the next step was to confirm that the cardiomyocyte specific promoter αMHC was working successfully, meaning that the GCaMP gene was only being expressed in transfected cells that were differentiated into cardiomyocytes. Therefore, comparative RT-qPCR analysis was carried to test differentiated cells compared to undifferentiated cells to assess relative expression of the GCaMP gene.

The endogenous control gene used to normalize the data was GAPDH. The normalized ΔC_T value for transfected but undifferentiated iPSCs was 11.8 (Table 2). The normalized ΔC_T value for transfected and differentiated cells was 11.5 (Table 2). These values resulted in a $\Delta\Delta C_T$ value of -0.3, because $\Delta\Delta C_T$ was the experimental ΔC_T value, or the one for transfected cardiomyocytes, minus the control ΔC_T value, or the one for transfected iPSCs. This value was then used to calculate the expression fold change using the equation: $2^{-\Delta\Delta CT}$ (Table 2). The calculated expression fold change was 1.23, suggesting that GCaMP was expressed roughly 20% more in the differentiated cells as compared to the undifferentiated cells. This suggests that there was more message present for GCaMP for the differentiated cells.

An additional RT-qPCR was conducted for just undifferentiated, transfected iPSCs to assess whether the use of the αMHC promoter was successful in preventing the expression of GCaMP in non-cardiomyocyte cells, despite the presence of the gene in the cells based on regular PCR analysis. The GFP C_T, which represents the expression of GCaMP because the GFP TaqMan assay works for GCaMP as well, for these cells was 31.5. A control sample was included as well, where the cells were not exposed to reverse transcriptase, so the RNA was never converted to cDNA and therefore will not work within the qPCR reaction. The control sample C_T was 31. For RT-qPCR, the higher the C_T value is the less gene of interest was expressed in the original sample; therefore, the fact that the C_T value was even higher than that of the negative control shows that the transfected iPSCs, which did have the GCaMP gene based on PCR analysis, were not expressing the GCaMP gene because no significant amount of GCaMP mRNA was present. The raw data for this RT-qPCR can be observed in Table 3.

It is important to note that because only one comparative RT-qPCR was conducted and the ΔC_T values for the transfected and differentiated cells as compared to transfected and undifferentiated cells were similar, that while there is a slight trend that suggests increased GCaMP expression in differentiated as compared to undifferentiated cells, these results are not statistically significant and therefore should be considered critically. Additional RT-qPCRs must be run to further assess this observation.

Differentiation and Cell Imaging Results

After comparative RT-qPCR analysis suggested that differentiated cells were expressing GCaMP more than the undifferentiated cells, the differentiated cells were then imaged to confirm that GCaMP expression in cardiomyocytes led to an increase in fluoresce and to see if beating,

caused by a release of calcium ions, led to an increase in fluorescence intensity with each beat. Following the Conklin Lab 15-day differentiation protocol, untransfected iPSCs began to beat after 9 days. Following the same protocol, transfected iPSCs began to beat after 18 days, showing that differentiation of cells that underwent the transfection process was successful.

When using the Olympus MVX10 microscope and Andor iXon camera, examples of green fluorescence were observed in the transfected cells that went through the differentiation process. Green fluorescence was not observed in the untransfected iPSCs that went through the same selection and differentiation process, nor was it observed in iPSCs that were successfully transfected according to PCR analysis but were not put through the differentiation process.

When using the Bio-Rad ZOE Fluorescent Cell Imager, more unreliable background fluorescence was observed than for the Olympus MVX10 microscope, but in general transfected and differentiated cells displayed fluorescence whereas differentiated but untransfected cells and transfected but undifferentiated cells generally did not display meaningful fluorescence. In Figure 6, three sets of example images are displayed: the brightfield image and green fluorescent image of the same section of cells side by side for transfected and differentiated cells, transfected and undifferentiated cells, and untransfected differentiated cells (Figure 6). For the transfected and differentiated cells, based on the appearance of the section it is believed that the fluorescence observed was in an area of cells that was beating, but this cannot be confirmed with absolute certainty because the cells stopped beating by the time the image was taken.

This imaging process suggests that differentiation of transfected iPSCs into cardiomyocytes led to an increase in fluorescence, however due to technical complications with imaging, a beating and fluorescing section of cells was not found with the Olympus MVX10 microscope. A beating and fluorescing section of cells was found with the Bio-Rad ZOE

Fluorescent Cell Imager on one occasion, and it appeared like fluoresce was possibly increasing with each contraction as well, but it was unclear whether this effect was caused by the release of calcium ions combining with the GCaMP proteins or whether it was caused by the consistently existing fluorescence collecting closer together upon contraction. Therefore, more imaging with quantitative microscopes must be conducted to further confirm the success of this tool. Specifically, microscopes equipped with an incubator that could keep the cells at 37 ˚C and 5% CO_2 during imaging would help increase the amount of time that cells could be imaged without compromising the health of the cells.

Discussion

The goal of this project was to create and test a genetic tool that can confirm and quantify the successful differentiation of iPSCs into cardiomyocytes by coupling the GCaMP8 calcium indicator and the cardiomyocyte-specific αMHC promoter. Joseph Neumeyer began this process by creating an αMHC-GCaMP8 construct and Bayan Yazdi continued the process by using the construct to finalize an αMHC-GCaMP8-iCAG insertion plasmid (Neumeyer, 2016; Yazdi, 2017). This project continued the work they began by demonstrating the successful use of Lipofectamine 3000 to transfect human iPSCs with the αMHC-GCaMP8-iCAG insertion plasmid and the successful differentiation of iPSC that went through the transfection process into cardiomyocytes, and by suggesting an increased expression of GCaMP in transfected cardiomyocytes as compared to transfected but undifferentiated iPSCs and suggesting that

fluorescence occurs only in differentiated cardiomyocytes and that fluorescence intensity could be increasing with contraction of the cells.

First, after putting iPSCs through a transfection protocol that utilizes Lipofectamine 3000, cells were tested for the presence of both the GCaMP gene and the αMHC promoter via PCR and gel electrophoresis. As the GCaMP PCR and αMyHCfor3 and αMyHCrev2 PCR demonstrate, this process of transfecting was successful in incorporating the construct of interest into the iPSCs (Figure 3, Figure 4, Figure 5). Then, cells were maintained in their pluripotent state as stem cells throughout the G418 selection process and were then differentiated into cardiomyocytes. The successful differentiation demonstrates that a stable cell line can be maintained after the use of Lipofectamine 3000 as a chemical transfection agent and the use of the antibiotic G418 as a selection reagent. Therefore, it was shown that the αMHC-GCaMP8-iCAG insertion plasmid can be inserted successfully into iPSCs without disrupting their pluripotent status.

Next, the successful differentiation of transfected iPSCs into cardiomyocytes was confirmed. This was confirmed in several ways. First, after the differentiation process, beating was observed among the transfected cells, and beating is characteristic of cardiomyocytes. Additionally, fluorescence was observed among transfected and differentiated cells (Figure 6). Because αMHC is cardiomyocyte-specific and is coupled with the GCaMP gene in the insertion plasmid, this suggests that the cells were fluorescing because they had successfully become cardiomyocytes, which allowed for the GCaMP gene to be expressed and therefore led to fluorescence. However, while these results are promising, due to some observable background fluoresce when using the Bio-Rad ZOE Fluorescent Cell Imager more imaging should be conducted to further assess the success of this tool (Figure 6).

Finally, RT-qPCR was executed for transfected cells both before and after differentiation, and the results, although not definitive due to the small size of the effect and the fact that comparative RT-qPCR was only run once, show a trend that suggests that the transfected cells were successfully differentiated into cardiomyocytes. This is because the results suggest that there was more GCaMP messenger RNA present for the differentiated cells as compared to the undifferentiated ones, which, if the cardiac-specific αMHC promoter was working successfully, would only occur if the transfected cells had successfully become cardiomyocytes. Based on the data, the calculated expression fold change, which was calculated using the equation $2^{-\Delta\Delta CT}$, was 1.23 (Table 2). This suggests that GCaMP was expressed roughly 23% more in the differentiated cells as compared to the undifferentiated cells. However, once again more RT-qPCR data must be collected in order to confirm this possible trend.

Finally, this project tested the idea that after the uptake of the αMHC-GCaMP8 construct, cardiomyocytes would fluoresce and would only begin to fluoresce after successfully becoming cardiomyocytes. First, after transfection, selection, differentiation, and imaging: fluorescence was observed among transfected and differentiated cells (Figure 6). Fluorescence was not observed, however, among the untransfected control cells that otherwise went through all the same conditions, including differentiation, as the transfected cells. This shows that cardiomyocytes with the construct fluoresce, and that only cells with the construct will fluoresce when they become cardiomyocytes. Additionally, after imaging it was observed that undifferentiated cells which were successfully transfected based on PCR results did not fluoresce. This demonstrates that cells with the construct will only begin to fluoresce once they become cardiomyocytes. However, this result must be assessed further with more imaging with quantitative microscopes that present less complicating background fluorescence before this

result can accepted with confidence. Finally, as previously discussed and as Table 3 shows, according to RT-qPCR analysis of undifferentiated, transfected iPSCs, there was even less expression of GCaMP in undifferentiated cells than in the negative control that did not possess cDNA because it was not exposed to reverse transcriptase, which supports the idea that even when the GCaMP gene is present in cells, it will not be expressed until the cells become cardiomyocytes (Table 3). This suggests that the coupling of αMHC and GCaMP was successful. More RT-qPCR analysis must be conducted, however, in terms of the comparative RT-qPCR that was conducted (Table 2) to assess whether the observed slight increase in expression of GCaMP in differentiated cells as compared to undifferentiated cells was statistically significant.

The cumulative results of this project suggest promising evidence that using Lipofectamine 3000 to transfect iPSCs with an αMHC-GCaMP8-iCAG insertion plasmid and then using fluorescent imaging after differentiation could be an effective way to confirm the successful differentiation of iPSCs into cardiomyocytes. Crucially, this proof of concept project shows that Lipofectamine 3000 can be used to transfect iPSCs without disrupting their pluripotent status and iPSCs transfected with the αMHC-GCaMP8-iCAG plasmid can then be differentiated into cardiomyocytes, and the collected evidence suggests, tentatively, that cardiomyocytes that possess the αMHC-GCaMP8 construct will fluoresce while undifferentiated transfected cells will not, and transfected cells will only fluoresce when they have become cardiomyocytes. In short, the preliminary data of this project suggests that the use of the αMHC-GCaMP8-iCAG insertion plasmid and fluorescent imaging could be a successful genetic tool that researchers can use in the lab to confirm and quantify the differentiation of iPSCs into cardiomyocytes.

Because this was a proof of concept experiment, future research could begin to work towards perfecting the efficiency of this tool. Specifically, other transfection methods could be explored. When choosing Lipofectamine 3000, both accessibility and cost were taken into consideration, as well as transfection efficiency. Lipofectamine is a common chemical transfection reagent, however it is not site-specific in terms of gene integration. Therefore, the gene of interest can be integrated anywhere into the cell genomes. Some potential site-specific transfection methods that could be explored in this context are transcription activator-like effector nucleases (TALEN) or CRISPR-Cas9. Electroporation could be explored as well, as a way of introducing DNA into the cells without using a chemical transfection reagent. Both CRISPR-Cas9 and electroporation technology have both been used together to successfully transfect human stem cells in previous research, so these tools could be very effective (Xu et al., 2018).

The TALEN process could be tested as another transfection method option as well, specifically because, as previously discussed, the created plasmid was designed to work successfully with a TALEN transfection protocol that targets the safe-harbor gene CLYBL. This method of genome editing and engineering makes use of TALENs, which work as pairs with binding sites on opposite DNA strands that are separated by a small fragment of 12 to 25 base pairs called a spacer sequence (Nemudryi et al., 2014). After the artificial nucleases in the nucleus bind to target sites, then the FokI domains, which are restriction endonuclease domains, at the C-termini of the protein dimerize, which causes a double-stranded break in the spacer sequence (Nemudryi et al., 2014). With this method, a double-stranded break can in theory be made in any region of the genome where the recognition sites are known; the only limitation is that a thymine is needed before the 5’ end of the target sequence (Nemudryi et al., 2014). After

the double-stranded break is made, the αMHC-GCaMP8-iCAG plasmid can be inserted as a double-stranded piece of linear DNA (Yazdi, 2017). TALEN has also been demonstrated, in previous research, to be able to transfect iPSCs that were then successfully differentiated into cardiomyocytes, showing that TALEN could be a promising tool in this context as well (Cerbini et al., 2015).

Once cells have been successfully transfected and selected, and are then differentiated into cardiomyocytes, one potential next step would be to perfect a way to then isolate the cells that are cardiomyocytes from undifferentiated iPSCs and iPSCs that differentiated into other cell types. Therefore, future research could explore the potential use of flow cytometry to isolate the fluorescent cardiomyocyte cells. Fluorescent-activated cell sorting is a technique that can purify cells by using a genetically modified fluorescent protein marker and a flow cytometer (Basu et al., 2010). In this process, cells in suspension are passed in front of a laser in droplet form, and the fluorescent detection system of the tool uses the laser to detect cells that are expressing the specified fluorescence (Basu et al., 2010). When cells of interest are detected, the flow cytometer applies a charge to the droplet containing the cell and the droplet is then collected due to electrostatic deflection (Basu et al., 2010). Therefore, flow cytometry could be used together with the αMHC-GCaMP8-iCAG insertion plasmid tool that this project explores in order to isolate and purify a sample of cardiomyocytes after differentiation.

This αMHC-GCaMP8-iCAG insertion plasmid tool could have far reaching impacts on research fields utilizing iPSCs to create cardiomyocyte research models. Specifically, this could be a great tool for studying, measuring the success of, and perfecting different cardiomyocyte differentiation methods. This tool could also be used to explore other ways to increase the efficiency of differentiation in an *in vitro* research setting, such as by creating and researching

drugs that could increase cardiomyocyte differentiating efficiency. However, it should be noted that while the future use of iPSCs in clinical therapies for heart disease is very promising, the use of this particular tool in a clinical setting could be dangerous and unethical because it would mean introducing a foreign transgene, GCaMP8, into a human patient. More research and conversations about the clinical ethics of transgenics in human medicine should be explored before introducing foreign DNA into a human patient is considered.

The results of this project suggest that this specific genetic tool can be used in the context of iPSC-cardiomyocyte research to confirm and quantify the successful differentiation of iPSCs into cardiomyocytes. However, it is also vital to note that the concept of this tool can extend beyond the scope of just cardiomyocyte research. The general method that this project assesses involves: confirming and quantifying the successful differentiation of iPSCs into a particular cell type by following a protocol of transfection, selection, and then differentiation using an insertion plasmid with a gene construct that confers resistance to an antibiotic, utilizes a cell-specific promoter so that the gene of interest is uniquely expressed in a particular cell type only, and includes a gene that causes fluoresce within the cell type of interest. This general method could be applied to other cell types that iPSCs can become beyond cardiomyocytes, as long as a cell-specific promoter and gene to cause fluorescence in that cell type are accessible. Therefore, the results of this project suggest that this type of tool could be applicable in countless other research fields working with different commonly used cell types. The possibilities of creatively using this type of tool in cell imaging research are broad and exciting, warranting further exploration and study.

Figures and Tables

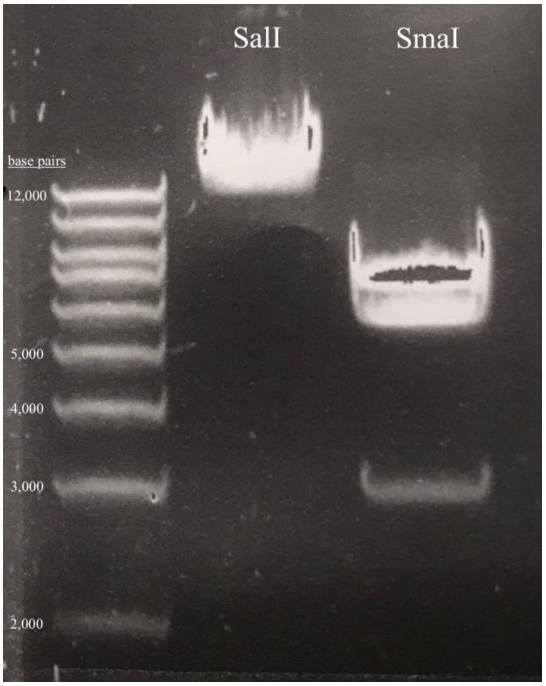

Figure 1. A gel showing the band sizes resulting after a restriction digest of the BY5 insertion plasmid using restriction enzymes SalI and SmaI and a 1 kb+ ladder.

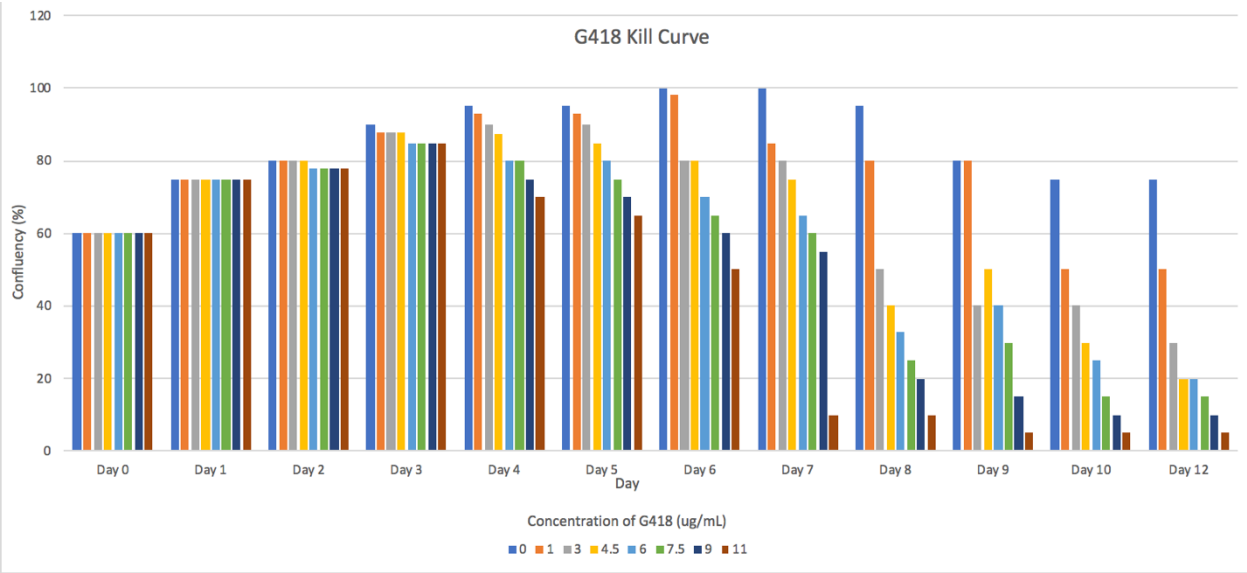

Figure 2. A kill curve bar graph showing the confluency of iPSCs as a function of time in days. Untransfected iPSCs were exposed to varying concentrations of the antibiotic G418 over the span of 12 days. Confluency values are approximate estimates.

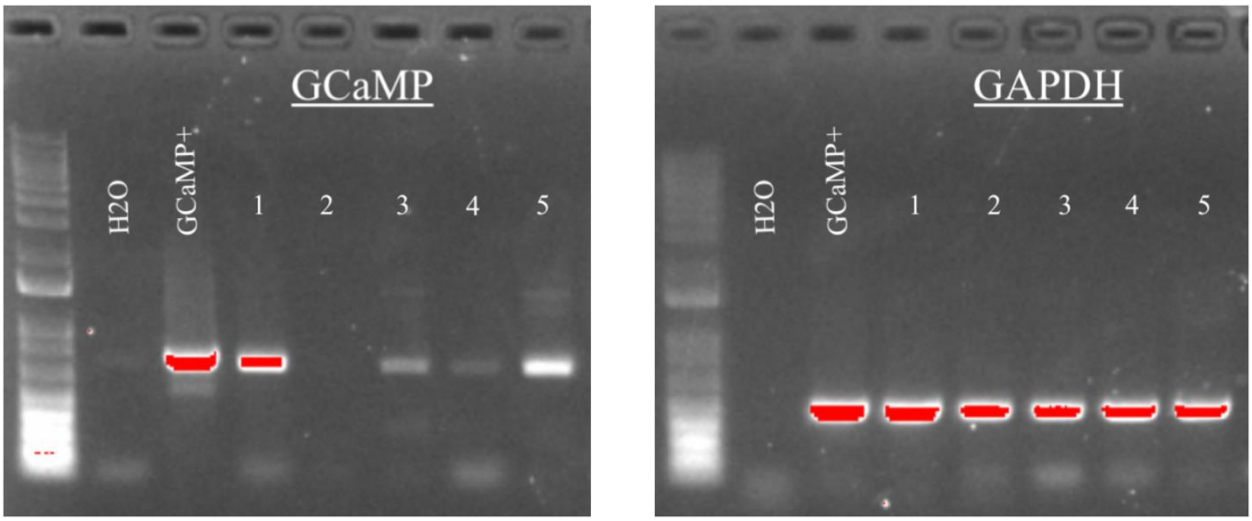

Figure 3. A gel showing samples from a GCaMP PCR and a GAPDH PCR. From left to right the samples are: a 1 kb+ ladder, a negative control where no DNA sample was added (H2O), a positive control of DNA containing the GCaMP gene (GCaMP+), DNA from iPS cells from one transfection (1; 7.5 μL of Lipofectamine 3000, three days of transfecting), two wells of cells from a second transfection (2, 3; 3.75 μL of Lipofectamine 3000, two days of transfecting), and two additional wells from the second transfection (4, 5; 7.5 μL of Lipofectamine 3000, two days of transfecting).

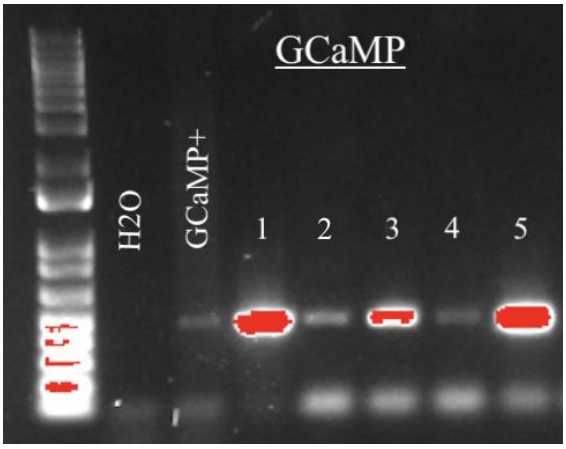

Figure 4. A gel showing samples from a repeat GCaMP PCR with a different DNA extraction for cells from sample 2. From left to right the samples are: a 1 kb+ ladder, a negative control where no DNA sample was added (H2O), a positive control of DNA containing the GCaMP gene (GCaMP+), DNA from iPS cells from one transfection (1; 7.5 μL of Lipofectamine 3000, three days of transfecting), two wells of cells from a second transfection (2, 3; 3.75 μL of Lipofectamine 3000, two days of transfecting), and two additional wells from the second transfection (4, 5; 7.5 μL of Lipofectamine 3000, two days of transfecting).

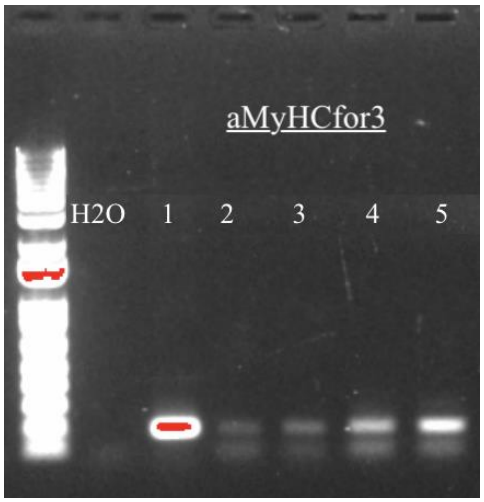

Figure 5. A gel showing samples from a PCR using reverse primer αMyHCrev2 and forward primer αMyHCfor3. From left to right the samples are: a 1 kb+ ladder, a negative control where no DNA sample was added (H2O), a positive control of DNA containing the GCaMP gene (GCaMP+), DNA from iPS cells from one transfection (1; 7.5 μL of Lipofectamine 3000, three days of transfecting), two wells of cells from a second transfection (2, 3; 3.75 μL of Lipofectamine 3000, two days of transfecting), and two additional wells from the second transfection (4, 5; 7.5 μL of Lipofectamine 3000, two days of transfecting).

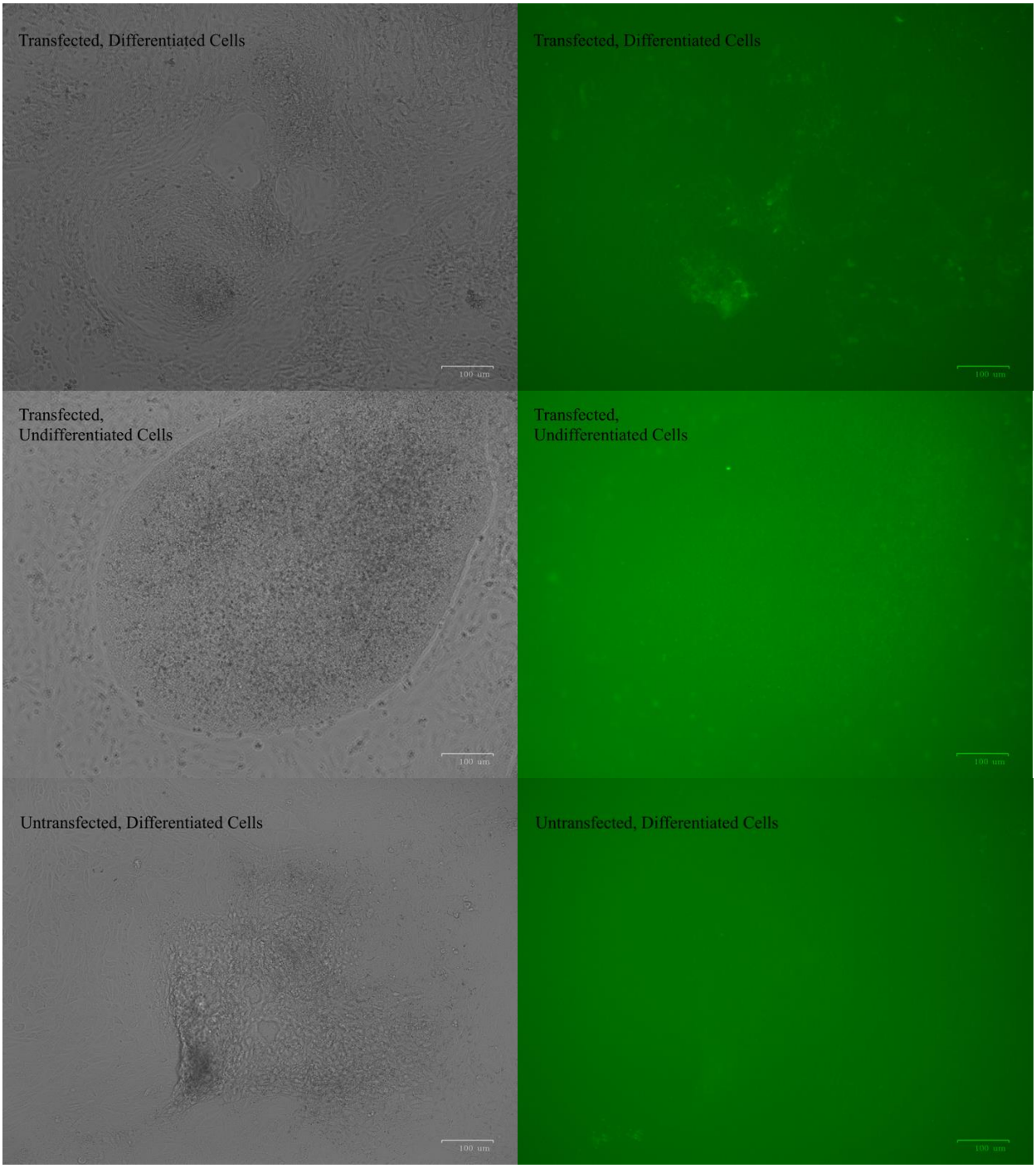

Figure 6. The top set of images depict a brightfield image (left) and green fluorescence image (right) of a section of transfected, differentiated cardiomyocytes that had displayed beating. The middle set of images depict a brightfield image (left) and green fluorescence image (right) of a section of transfected, undifferentiated iPSCs. The bottom set of images depict a brightfield image (left) and green fluorescence image (right) of a section of untransfected, differentiated cells. Images were taken with a Bio-Rad ZOE Fluorescent Cell Imager.

G418	0 μg/mL	1 μg/mL	3 μg/mL	4.5 μg/mL	6 μg/mL	7.5 μg/mL	9 μg/mL	11 μg/mL
Day 0	Cells are ~60% confluent, healthy, pink media	Cells are ~60% confluent, healthy, pink media	Cells are ~60% confluent, healthy, pink media	Cells are ~60% confluent, healthy, pink media	Cells are ~60% confluent, healthy, pink media	Cells are ~60% confluent, healthy, pink media	Cells are ~60% confluent, healthy, pink media	Cells are ~60% confluent, healthy, pink media
Day 1	Cells are ~75% confluent, yellow-orange media	Cells are ~75% confluent, yellow-orange media	Cells are ~75% confluent, yellow-orange media	Cells are ~75% confluent, yellow-orange media	Cells are ~75% confluent, yellow-orange media	Cells are ~75% confluent, yellow-orange media	Cells are ~75% confluent, yellow-orange media	Cells are ~75% confluent, yellow-orange media
Day 2	Cells are ~80%, yellow media	Cells are ~80%, yellow media	Cells are ~80%, yellow media	Cells are ~80%, yellow media	Cells are just under 80%, yellow media	Cells are just under 80%, yellow media	Cells are just under 80%, yellow media	Cells are just under 80%, yellow media
Day 3	Cells are ~90%, yellow media	Cells are just under 90%, yellow media	Cells are just under 90%, yellow media	Cells are just under 90%, yellow media	Cells are ~85%, yellow media	Cells are ~85%, yellow media	Cells are ~85%, yellow media	Cells are ~85%, yellow media
Day 4	Cells are ~95%, yellow media	Cells are just under 95%, yellow media	Cells are ~90%, darker yellow media	Cells are just under 90%, less than yesterday, darker yellow media	Cells are ~80%, orange-yellow media	Cells are ~80%, orange media	Cells are ~75%, orange media	Cells are ~70%, pink-orange media
Day 5	Cells are ~95%, yellow media	Cells are just under 95%, yellow media	Cells are ~90%, yellow-orange media	Cells are ~85%, orange media	Cells are ~80%, orange media	Cells are ~75%, orange media	Cells are ~70%, pink-orange media	Cells are ~65%, pink media
Day 6	Cells are ~100%	Cells are just under	Cells are ~80% yellow-	Cells are ~80%	Cells are ~70%	Cells are ~65%	Cells are ~60% pink-	Cells are ~50% pink-

	yellow media	100% yellow media	orange media	yellow-orange media	orange media	orange media	orange media	orange media
Day 7	Cells are ~100% yellow media	Cells are ~85% yellow media	Cells are ~80% orange media	Cells are ~75% orange media	Cells are ~65% orange media	Cells are ~60% pink media	Cells are ~55% pink media	Cells are ~10% pink media
Day 8	Cells are ~95% yellow media	Cells are ~80% yellow-orange media	Cells are ~50% orange media	Cells are ~40% orange media	Cells are ~33% orange media	Cells are ~25% pink media	Cells are ~20% pink media	Cells are ~10% pink media
Day 9	Cells are ~80% yellow media	Cells are ~80% yellow-orange media	Cells are ~40% orange media	Cells are ~50% orange media	Cells are ~40% orange media	Cells are ~30% pink media	Cells are ~15% pink media	Cells are ~5% pink media
Day 10	Cells are ~75% yellow media	Cells are ~50% orange media	Cells are ~40% orange media	Cells are ~30% orange media	Cells are ~25% pink media	Cells are ~15% pink media	Cells are ~10% pink media	Cells are ~5%, pink media
Day 12	Cells are ~75% yellow media	Cells are ~50% orange media	Cells are ~30% orange media	Cells are ~20% pink media	Cells are ~20% pink media	Cells are ~15% pink media	Cells are ~10%, pink media	Cells are ~5%, pink media

Table 1. Kill curve observations after exposing untransfected human induced pluripotent stem cells (iPSCs) to varying concentrations of the antibiotic G418.

	GAPDH C_T	**GFP C_T**	**dC_T** ($GFP\ C_T - GAPDH\ C_T$)	**No RT GFP C_T**	**ddC_T** ($tCM\ dC_T - tiPSC\ dC_T$)	**Expression Fold Change** (2^{-ddCT})
Transfected iPSCs (tiPSC)	19.2	31.2				
	19.1	30.7				
	19	30.9				
Average	**19.1**	**30.9**	11.8	32.9		
Transfected Cardiomyocytes (tCM)	22	33.8				
	22	33.5				
	21.9	33.1				
Average	**22**	**33.5**	11.5	36.7		
Untransfected iPSCs	18.9	N/A				
	18.9	N/A				
	18.8	N/A				
Average	18.9	N/A				
					-0.3	1.23

Table 2. A table of RT-qPCR data for a series of different cDNA samples, each with triplicates: transfected iPSCs are the top row, transfected and differentiated cells are the middle row, and untransfected iPSCs are the bottom row. The GFP TaqMan assay was used to measure GCaMP expression and GAPDH was measured as well, along with a control that was not exposed to the reverse transcriptase enzyme. The first column shows triplicate values and averages for GAPDH C_T, the second column shows triplicate values and averages for GFP C_T, and the third column shows ΔC_T values, which is the GAPDH C_T value subtracted from the GFP C_T value. The next column displays the C_T of the control that was exposed to the GFP TaqMan assay but not the reverse transcriptase enzyme. The next column shows ddC_T, which is the transfected and differentiated dC_T minus the transfected and undifferentiated dC_T. The final column shows the expression fold change, which is calculated by 2^{-ddCT}.

	GAPDH C_T	**GFP C_T**	**dC_T** ($GFP\ C_T - GAPDH\ C_T$)	**No RT GFP C_T**
Transfected iPSCs (tiPSC)	19	31.6		
	19	31.4		
	18.9	31.6		
Average	**19.0**	**31.5**	12.6	31

Table 3. A table of data of a single RT-qPCR of undifferentiated but transfected iPSCs. The GFP TaqMan assay was used to measure GCaMP expression. GAPDH was measured as well, and a control that was not exposed to the reverse transcriptase enzyme was included.

Acknowledgements

I would like to thank the Kotlikoff Lab and Dr. Michael Kotlikoff for providing me with the support and resources necessary to conduct this project. I would also like to thank Jane Lee of the Kotlikoff Lab for her continual guidance and support throughout this project, and Frank Lee for his help and support as well. I would like to thank Frank Lee, Shaun Reining, and Marco Hiller for their help with fluorescent imaging. I would like to thank Bayan Yazdi and Joseph Neumeyer for their initial work in creating the αMHC-GCaMP8 construct and the αMHC-GCaMP8-iCAG insertion plasmid as well. Without this team of excellent researchers and their insights and expertise, this project would not have been possible.

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