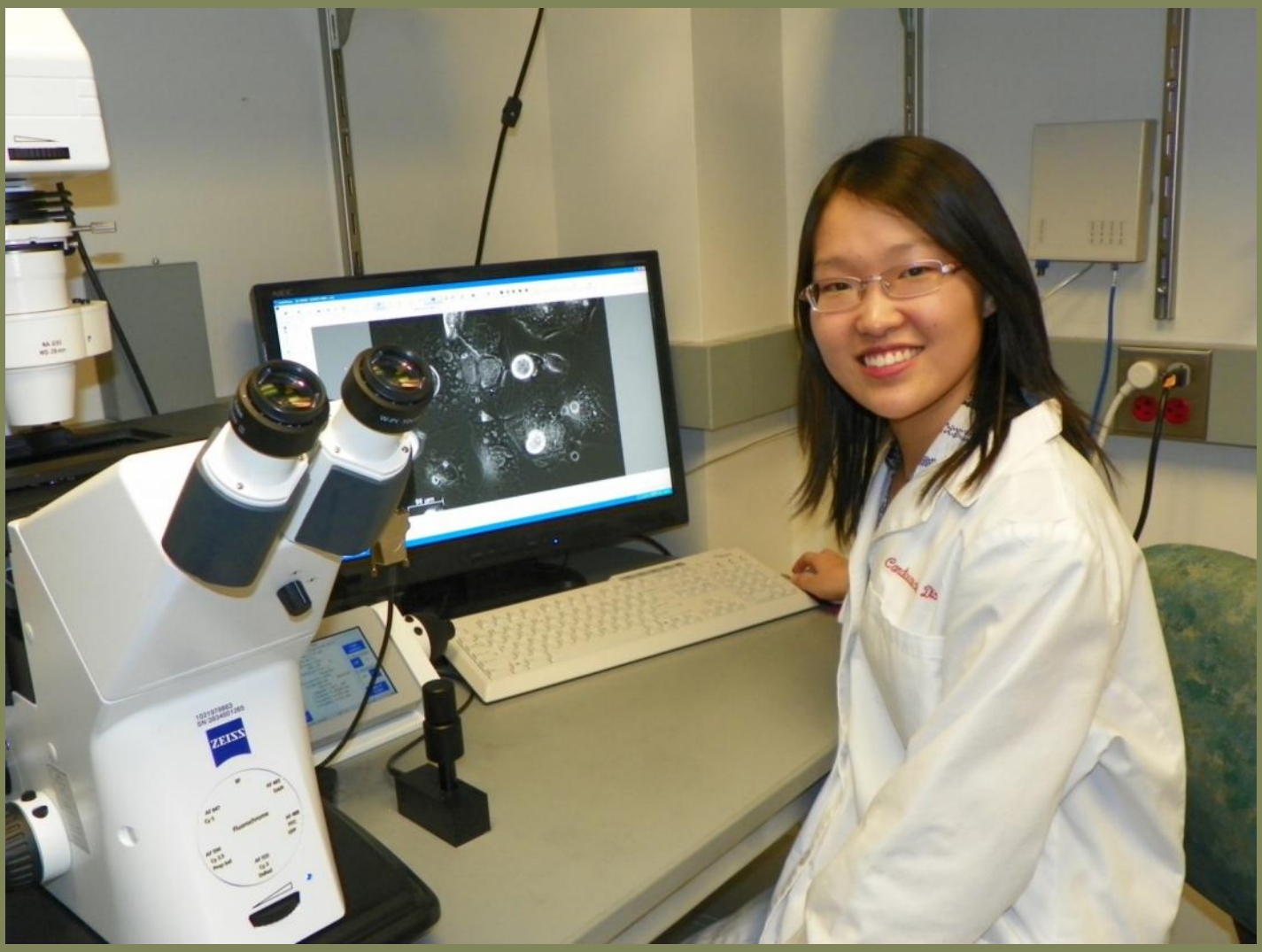
Establishment of long-term cultures of newt cardiomyocytes for use as an *in vitro* model for heart regeneration



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Introduction

Heart disease is the leading cause of death in the United States. As a consequence, intense interest has been directed toward an enhanced understanding of cardiac repair and regeneration. While the mammalian heart is capable of limited regeneration, the newt is capable of complete myocardial regeneration in response to a significant injury. In the newt, heart regeneration is believed to involve the formation of a wound epithelium after amputation, the initiation of the dedifferentiation process followed by proliferation by secreted matrix metalloproteinases. This well orchestrated process leads to the formation of a blastema, and the growth of the blastema, which eventually dedifferentiates to form a complete and functional structure (Singh, Koyano-Nakagawa, Garry, & Weaver, 2010).

In contrast, the mammalian heart has a limited ability to form new cardiac tissue. Injury in the mammalian heart model results in the loss of contractile tissue, fibrosis, and scar formation (Borchardt & Braun, 2007). This may be due to the fact that 1. Dedifferentiation in damaged tissue results in a decrease in the number of functional cells, which further impairs tissue function when dedifferentiation occurs in damaged tissue; 2. Dedifferentiation increases the possibility of generating tumorigenic cells; and 3. The regenerative process increases the mobilization of cells that are already stressed or injured (Laube, Heister, Scholz, Borchardt & Braun, 2006).

Unlike mammals, certain amphibians and teleost fish have the capacity to regenerate functional myocardium. It has been shown that newt cardiomyocytes undergo a downregulation of MyHC, cTnT, cTnI, cTnC and cardiac actin seven days after injury. At approximately fifteen and twenty-eight days after implantation of cardiomyocytes, a re-expression of MyHC was

observed (Laube et al., 2006). This result suggests that newt cardiomyocytes dedifferentiate in order for the proliferation of new cardiomyocytes to occur. This project focused on the adult Eastern Red-spotted newt (*Notophthalmus viridescens*). This amphibian has the ability to survive after a large section (30-40%) of its ventricle is amputated and completely regenerates the myocardium within a two month period (Singh et al., 2010). Although previous studies have demonstrated that the cardiomyocytes were able to re-enter the cell cycle (S phase) and some cells were able to undergo one or more complete cycles of cell division (Bettencourt-Dias, Mittnacht & Brockes, 2003), the molecular regulation of the dedifferentiated and differentiated states of the cardiomyocytes remain unclear.

Previous studies have indicated that the newt may have an increased proliferative capacity. To date, there are no newt cardiomyocyte cell lines to test this hypothesis. **The purpose of the project is to establish a newt cell line as a model to further determine the factors involved in the regeneration process.** To achieve this, I have outlined three specific aims: 1. Determine the best method to isolate live cardiomyocytes from whole newt ventricles; 2. Establish the culture conditions for the long-term culture of newt cardiomyocytes; and 3. Identify characteristics of long-term cell cultures with respect to cell morphology, proliferative capacity, and cell type-specific markers. **The hypothesis of this research is that a successful newt cardiomyocyte cell line as a model for heart regeneration will be established.** Using the suggested methodologies, this research will: 1. Successfully isolate newt cardiomyocytes from whole ventricles; 2. Fully define the culture conditions to culture newt cardiomyocytes long-term; and 3. Successfully identify characteristics of the long-term culture. The generation of an *in vitro* model for heart regeneration through the culture of newt cardiomyocytes will allow us to modify and control conditions responsible for dedifferentiation and proliferation. This study has the potential to provide insights into possible mechanisms for enhancing mammalian heart regeneration.

Materials and Methods

Newt maintenance

Adult Eastern Red-spotted newts (*Notophthalmus viridescens*) were obtained from Charles Sullivan & Co. (Nashville, TN). Newts were kept in aquariums with water maintained at 20°C and fed with frozen bloodworms twice a week. Before all organ harvests, newts were anesthetized in 0.1% Tricaine (ethyl 3-aminobenzoate methanesulfonate salt; Sigma) for 10-15 minutes.

Enzymatic isolation of adult newt cardiomyocytes

The concentration of collagenase, cell collection cycles, and centrifuge speed were modified from Laube et al., 2006. Four ventricles were obtained from adult newts. Under aseptic conditions, the bulbus cordis (aorta) and atrium were resected in 70% Leibovitz's L-15 medium supplemented with 1% penicillin-streptomycin. Ventricles were mechanically minced in enzyme solution: 0.5% trypsin, 3800 U/ml collagenase (Collagenase Type 2; Worthington), 0.15% bovine serum albumin, 50 µg/ml gentamicin, and 0.3% glucose in aPBS (amphibian PBS; 70% PBS in water) to loosen the tissue. The minced tissue was mixed at 350 rpm, 27°C for two hours. The supernatant was then discarded since this sample consisted primarily of blood cells. Additional enzyme solution was added to the remaining tissue and the tissue continued to mix for one hour cycles. After each cycle, the cell suspension was removed to complete L-15 medium (70% L-15, 10% FBS, 1% penicillin-streptomycin dissolved in water) and new enzyme solution was added. After six cycles, the collected cells were centrifuged at 1000 rpm, 4°C for 10 minutes. The cell pellet was resuspended in complete L-15 medium. Cells were plated on glass cover slips with approximately 5×10^4 cells per cover slip for 48 hours at 25°C, 0% CO_2. During this step, many of the fibroblasts and remaining blood cells attached to the plastic dish. After 48 hours, the cell suspension was collected and centrifuged. Cells were plated onto

laminin-coated cover slips with approximately 1.38 x 10^4 cells per cover slip. Cultures were incubated at 25°C, 0% CO_2 (Figure 1).

Immunohistochemistry

Cells were trypsinized onto poly-D-lysine and laminin cover slips for immunohistochemical analysis. Cover slips were fixed in 4% paraformaldehyde for 10 minutes and stored at 4°C in PBS. Fixation was repeated with 70% MeOH/30% Acetone for 20 minutes at -20°C. Cells were permeabilized with 0.1% Triton X-100 for 20 minutes at room temperature. Blocking was performed with 1% NDS, 1% BSA, 0.3% Triton, 0.02% NaN_3 for 2 hours at room temperature in a humid chamber. Cover slips were incubated with anti-MyHC (clone A4.1025; Millipore; 1:100 dilution) overnight at 4°C. Cover slips were then incubated with secondary antibodies (Jackson IR; 1:200 dilution) conjugated with FITC or Cy-3 for 2 hours at room temperature. Counterstaining was performed with DAPI or propidium iodide (PI).

Imaging

All images of cell cultures were photographed with an Axio Observer Z1 inverted microscope with LD Plan-Neofluar/Korr Ph 2 M27 lenses. Immunohistochemical images were taken on an Axio Imager M1 fluorescence microscope with Plan-Neofluar lenses.

Results

Specific Aim 1: Determine the best method to isolate live cardiomyocytes from whole newt ventricles. Three pilot studies were conducted to maximize the number and quality of the cells obtained from the isolation procedure. The quality was assessed based on the length of time the cells could be kept in culture and their contractility. In the first pilot study, after the aorta and atrium were dissected from the newt hearts, 4 newt ventricles were placed in an

enzyme isolation containing 50 µg/mL gentamicin, 0.5% trypsin, 380 U/mL collagenase, 0.15% bovine serum albumin, and 0.3% glucose for an initial 1 hour incubation. At 15 minute intervals after the initial incubation, the supernatant was removed into complete L-15 medium and new enzyme solution was added. The ventricles underwent a total of 3.5 hours of enzymatic dissociation. These cells were healthy and continued to have contractile activity at 9 days after plating. Only individual cells were contracting since there were not enough cells obtained from the isolation for them to attach at a high confluency. After 19 days in culture, many of the cells formed numerous vacuoles near the center of the cells, suggesting that the cells were undergoing autophagy. At this point, the cells were fixed for immunohistochemical analysis.

In the second pilot study, in order to attempt to maximize the number of cells obtained from the isolation, the enzymatic dissociation times were decreased to a total of 2 hours – 1 hour initial incubation and two 30-minute cell collections. Most of the cells obtained from the digestion were elongated, blood cells that did not attach during the plating process. At the conclusion of the two hour time period, the ventricles were not completely digested. Approximately 4×10^3 cells were obtained at the end of the two hour digestion period. Seven days after plating, the cells in the culture remained unattached and the cultures showed signs of cell death, so the cultures were discarded. Hematoxylin and eosin staining of the remaining tissue confirmed the presence of numerous, undigested cells, indicating the enzyme dissociation period in Pilot 2 was not adequate.

In the third pilot study, the enzymatic dissociation time was increased to a total of 8 hours – 2 hours initial incubation and six 1-hour cell collection cycles. Pilot 3 was designed to decrease the number of blood cells in the primary culture since blood cells were noted to be the first cells released during the enzymatic dissociation. In Pilot 3, the concentration of collagenase was also increased to 3800 U/mL to completely digest the heart tissue. Approximately 2.55×10^5 cells were obtained at the end of the enzymatic digestion and 1.65×10^5 cells were obtained after the pre-plate process. At 5 days after plating, the cells in Pilot 3 were individually

contracting. At 6 days after plating, the cells were contracting synchronously with neighboring cells. As many as 20-30 cells were observed to be contracting at the same time. These cells have undergone seven passages with the last passage being 90 days after plating.

Specific Aim 2: Establish the culture conditions for the long-term culture of newt cardiomyocytes. Four different substrates were tested to determine which substrate was best for attachment of isolated newt cardiomyocytes. Because cardiomyocytes were observed to attach within 96 hours, cell suspensions from Pilot 1 were observed on each substrate over a period of 96 hours. Initially, regular 6-well tissue culture treated plastic dishes were used. Cells that were round in suspension attached after the first 24 hours. However, the majority of the cells with the characteristics of unattached cardiomyocytes was observed to remain in suspension after 48 hours. To test the second substrate, glass, suspended cells were added on top of sterile glass cover slips. After 48 hours, the majority of the cells were observed to have remained in suspension. Subsequently, after 96 hours, there was an increase in the amount of debris in the cultures, which was consistent with cell death. The third substrate was poly-D-lysine-coated glass cover slips. Results were similar to the tissue culture treated plastic. The cells with the morphology of cardiomyocytes in suspension did not attach after 96 hours of incubation. The last substrate tested was mouse laminin and this was done in the form of glass cover slips coated overnight with poly-D-lysine and then mouse laminin. The majority of cells in culture (~80%) was observed to be attached after 48 hours. Only a few cells with the familiar cardiomyocyte shape were observed to remain unattached after 96 hours.

In addition to the types of substrates, the frequency of medium changes was also examined. Using the cultures from Pilot 1, cultures were changed both daily with a wash of PBS and every other day. The cultures with daily medium changes contained less debris than the cultures with every other day medium changes, but an increasing number of cells were observed with numerous vacuoles in the centers of the cells in these cultures. This suggests the

daily medium changes were unnecessary and even harmful due to the frequent washes with PBS.

Cultures were passaged when they reached 80-90% confluency because crowding (i.e. increased cellular density) was observed at confluencies higher than 70%. Thus far, cultures have been passaged seven times with an increase in the number of cells at each passage (Figure 2). This suggests the cells in culture are still highly proliferative.

Specific Aim 3: Identify characteristics of the long-term cell cultures with respect to cell morphology, proliferative capacity, and cell type-specific markers. The cardiomyocytes in suspension have a thin, elongated cell body with cytoplasmic protrusions. Striations are visible along the cell body (Figure 3 A and B). These cells were found in the pre-plate cultures and in the first three days after cultures were plated onto laminin-coated dishes. After 48 hours, once the cells attach, cardiomyocytes are morphologically round with a spread-out cell body and a large nucleus. Multiple nuclei were seen in some cardiomyocytes (Figure 3 C and D). After five days in culture, contractility was observed in the cells on the plates. Cells which were closely attached were synchronously contracting. The rates of contractions ranged from 5 bpm to 32 bpm depending on the confluency of the attached cells. Individual cells were contracting at a lower rate while numerous cells contracting synchronously were contracting at a higher rate.

In order to determine the baseline for proliferation, a time lapse microscopy experiment was conducted. Two cells were photographed over a period of 66 hours at each hour. The cells were contracting at the beginning of the experiment, confirming they were indeed cardiomyocytes. Two types of divisions were captured in the time lapse (Figure 4). After 8 hours from the start of the experiment, the cell on the right of the experiment started detaching. After 18 hours, the cells completely detached, and after 19 hours, they had undergone cytokinesis. After 20 hours, the divided cells started reattaching and an attached binucleated cardiomyocyte was clearly visible after 38 hours (Figure 4A). The cell on the left started detaching after 29

hours and after 32 hours, two daughter cells were visible. At 36 hours, the cells were observed to reattach onto the laminin-coated cover slip and two daughter cells were visible after 42 hours (Figure 4B). When the experiment ended, the cells had begun to detach again for a second division, suggesting the cardiomyocytes in culture undergo cell division every 50 hours.

In order to further confirm the identity of the cells in culture, cells were fixed and stained for immunohistochemical analysis (Figure 5). Eleven days after plating, the majority of the cells, once plated onto laminin, stained positive for myosin heavy chain (MyHC), a protein expressed in cardiac muscle. The cells 48 days after plating, which were subject to four passages, continued to express MyHC. Similarily, the cells 112 days after plating, which were subject to seven passages, also stained positive for MyHC. These results indicate after four and seven passages, respectively, cells were still exhibiting cardiac-specific markers.

Illustrations

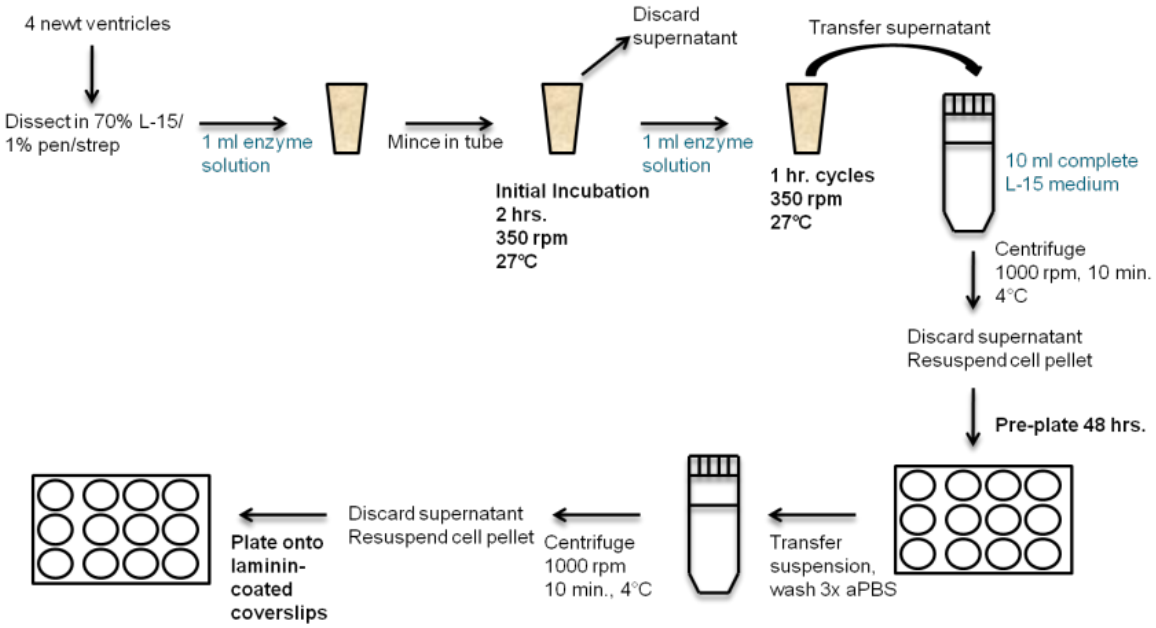

Figure 1. Isolation of cardiomyocytes from whole ventricles.

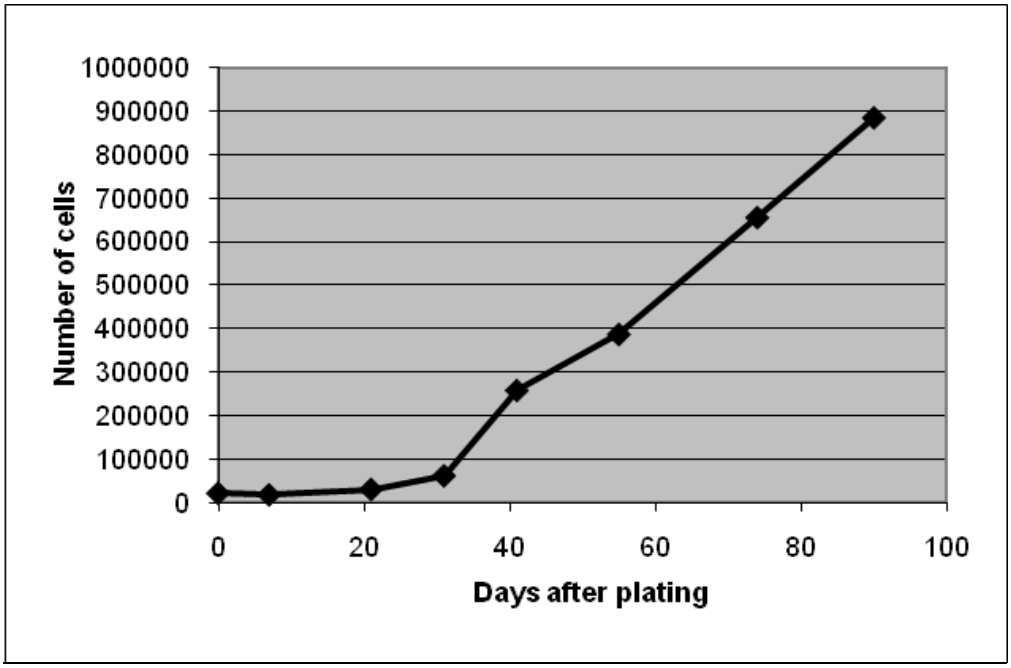

Figure 2. Cells in culture proliferate. The number of cells slightly decreased from the plating to the first passage, which may be due to limited cell death from the initial plating. Starting from the first passage, the number of cells increased at an exponential rate. Each marker denotes when the cultures were passaged.

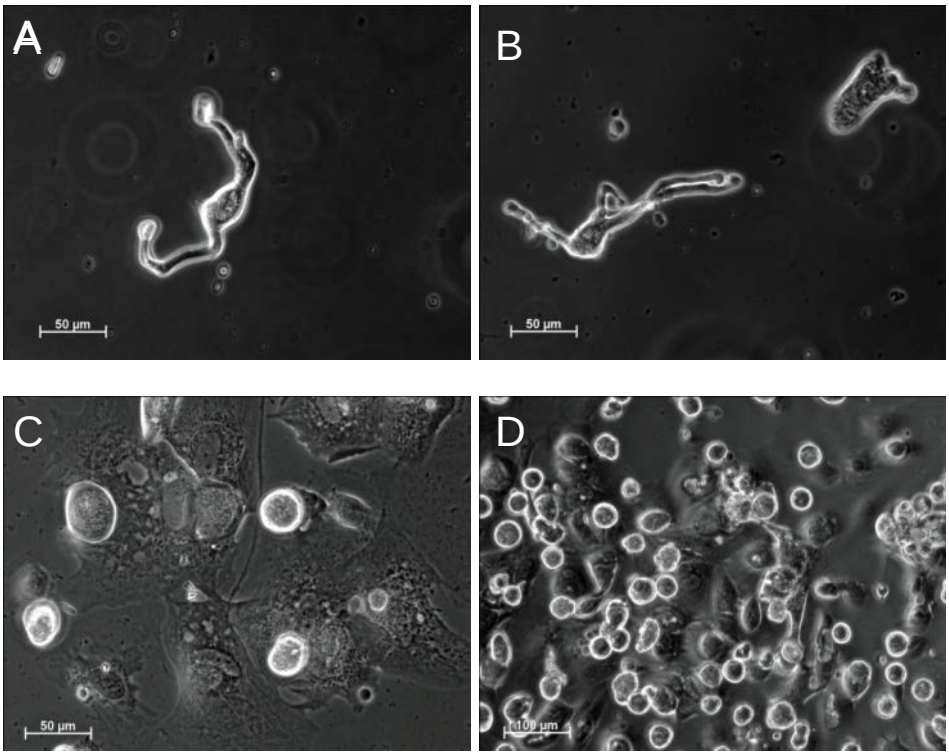

Figure 3. Primary cultures of newt cardiomyocytes. Panels A and B depict the 3-day-old culture of cells in suspension. Cardiomyocytes in suspension have an elongated, thin cytoplasmic morphology with striations visible in the cell body. Panels C and D show the 6-day-old cultures of attached cells. Once attached, cells exhibited synchronous contractions.

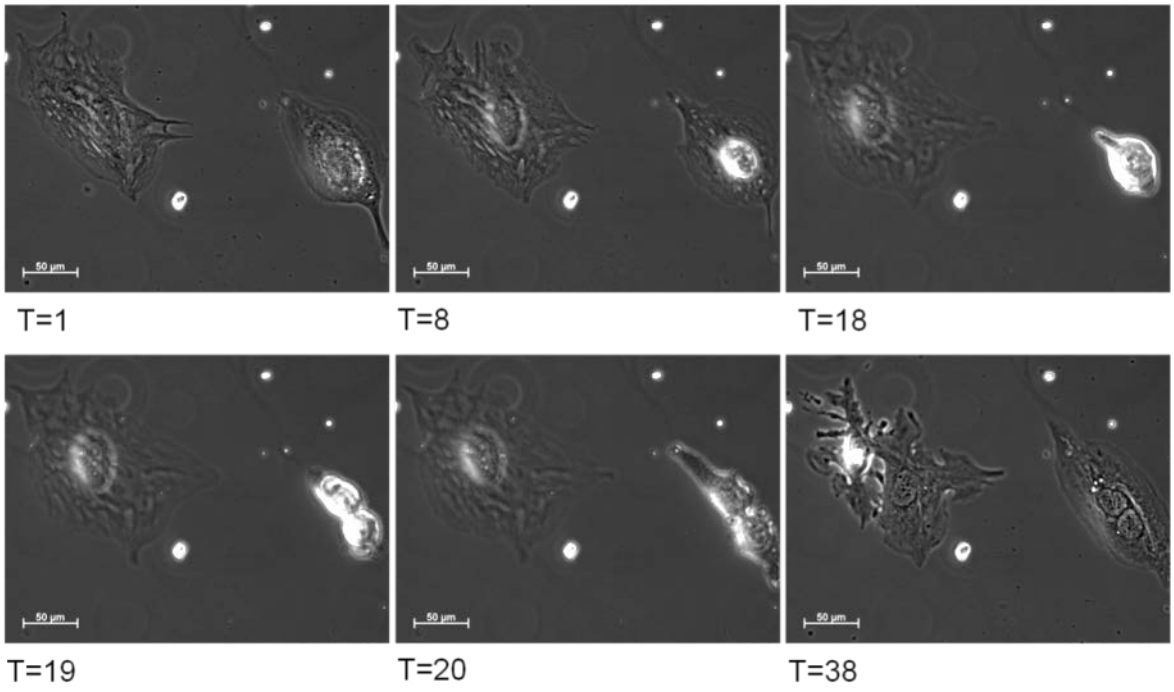

Figure 4A. Newt cardiomyocyte division into binucleated cell. The cell on the right was contracting at 1 hour after the start of the experiment but stopped when it started detaching at 8 hours. At 19 hours, the cell underwent cytokinesis. After division, the cell reattached into a binucleated cardiomyocyte.

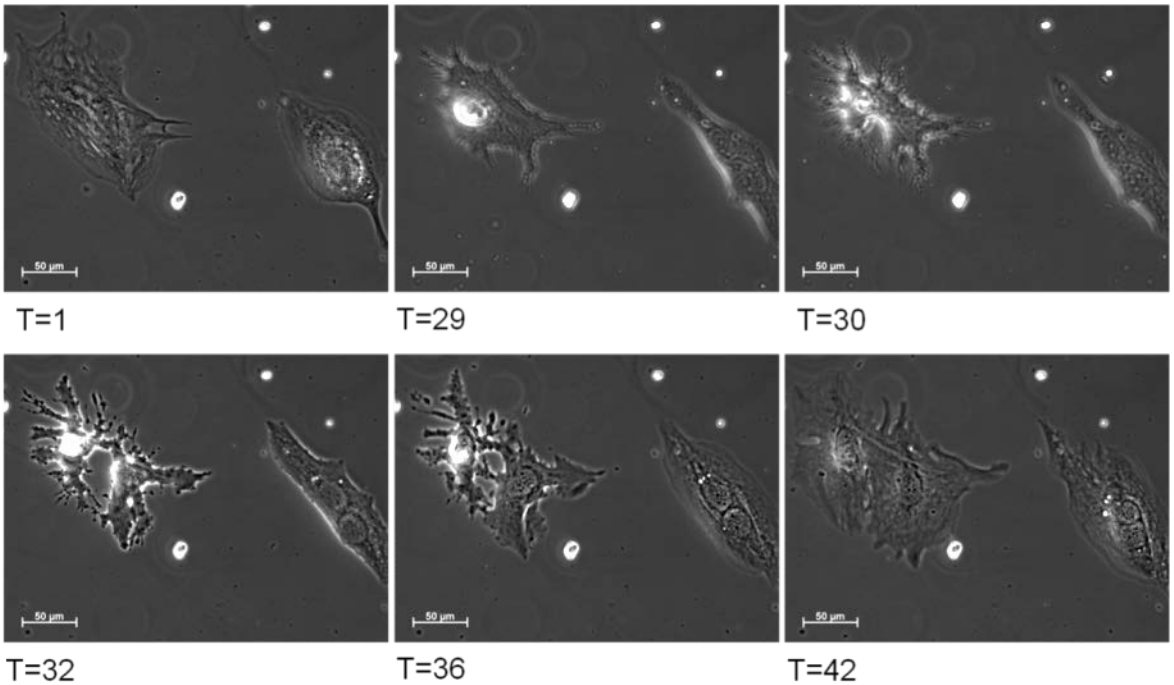

Figure 4B. Newt cardiomyocyte divides into two daughter cells. The cell on the left was contracting at the start of the time lapse experiment. At 29 hours, the cell started to detach from the cover slip. At 32 hours, the cell divided into two daughter cells. After the cells reattached, two cells are clearly visible.

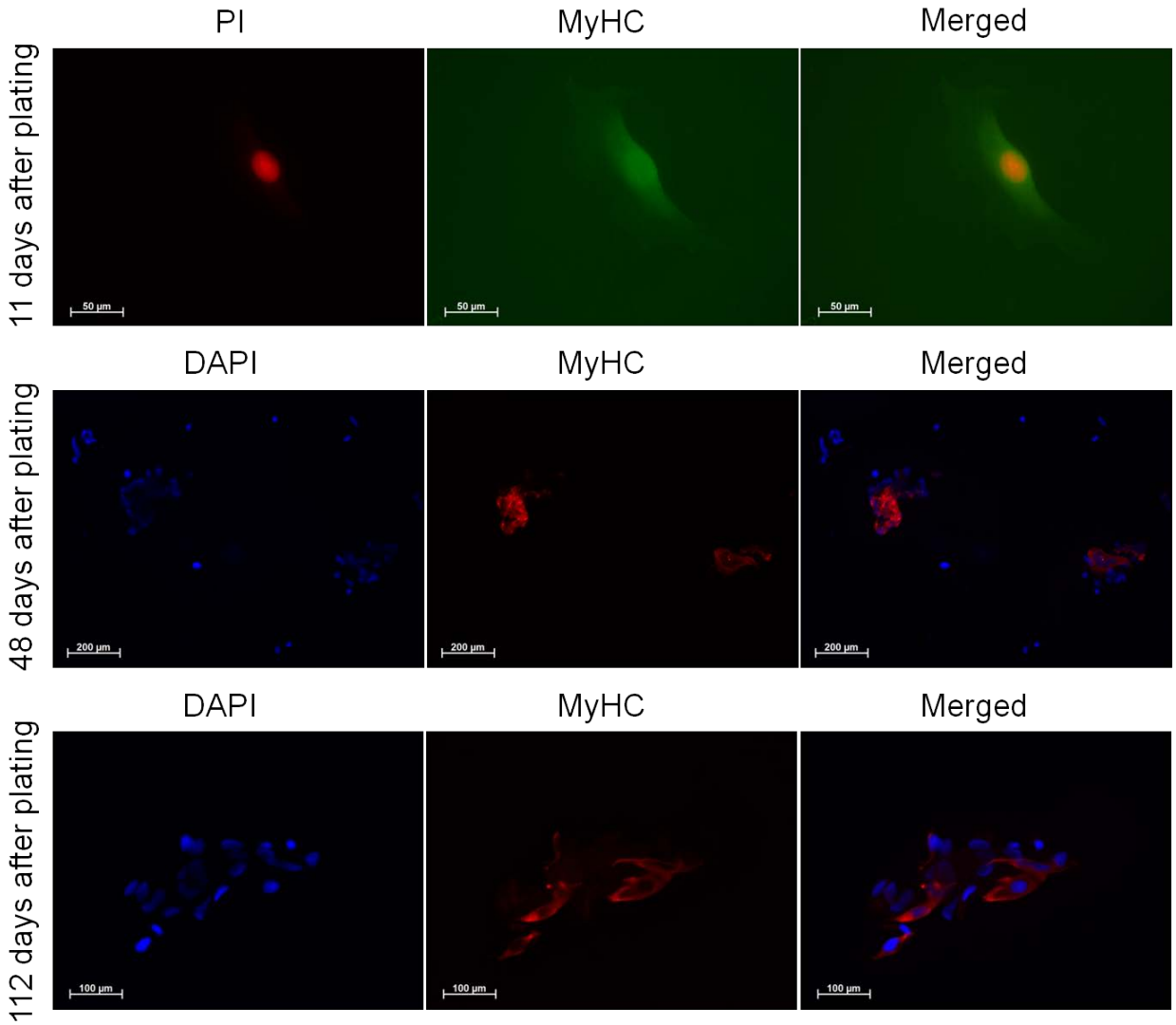

Figure 5. Cells in culture exhibit cardiac markers. In cultures 11 days after plating, cells stained positive for myosin heavy chain (MyHC), a protein expressed in cardiac muscle. Cells were counterstained with propidium iodide (red), an agent that stains DNA. Cells continued to express MyHC (red) in culture after four passages (48 days after plating) and seven passages (112 days after plating). These cells were counterstained with DAPI (blue) to label the DNA.

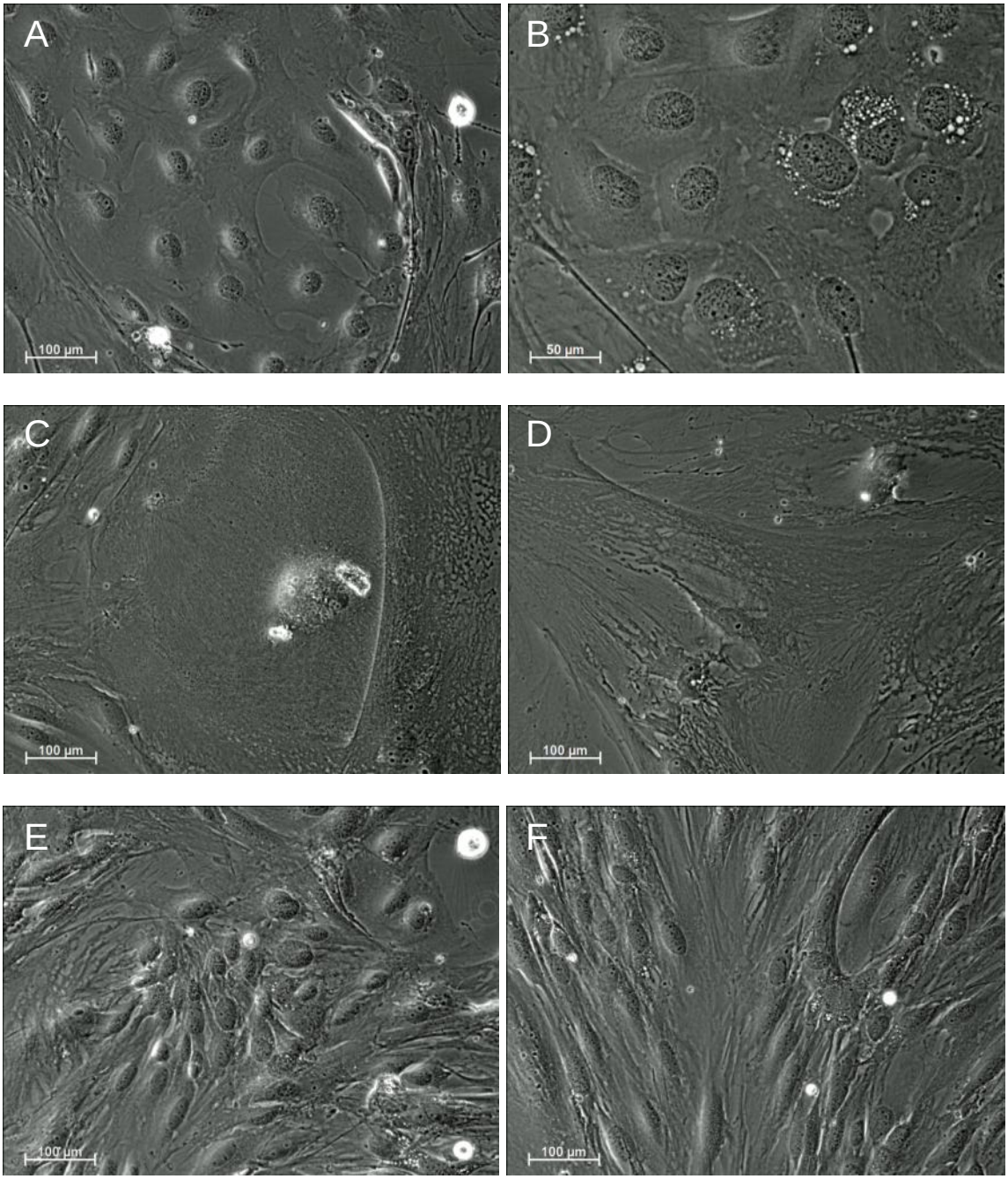

Figure 6. Cultures contain three different cell morphologies 54 days after plating. Panels A and B depict cells with large nuclei, and round, flat cytoplasm. Panels C and D show relatively large cells with flat cytoplasm. These cells often had multiple nuclei. Panels E and F depict cells with long, thin cell bodies and small nuclei. These cells often have protruded cytoplasm extending to neighboring cells.

Discussion

In order to study the process of heart regeneration in the model of the newt, primary cultures must be obtained through the isolation of cardiomyocytes from newt ventricles. This project is a first but significant step in generating a proliferating cell line to further study the factors involved in the regeneration process. At the time of the writing of this report, the long-term cultures have been maintained for 139 days and have small cell populations that are still expressing cardiac-specific markers. This research is unique because this is the longest *in vitro* populations of proliferative adult newt cardiomyocytes have been maintained. Previous studies followed individual cell divisions for 18 days and showed only three successive cell divisions (Bettencourt-Dias et al., 2003). This study has established culture conditions in which cells are able to survive and actively divide at an exponential rate for up to seven passages.

A second implication for these studies is the common mechanisms that govern heart regeneration between the newt and mammalian models. The methods established in this research may provide pertinent knowledge when mouse cardiomyocytes are used to establish a continuous cell line. Application of this model for heart regeneration to mice brings the current understanding closer to the factors involved in the regeneration of human hearts.

Additionally, after a continuous cell line is established, this *in vitro* model can be used to study innumerous factors involved in heart regeneration. One of the mechanisms that can be studied is the signaling pathways during regeneration. By studying the signaling pathways involved in adult newt regeneration, the hope is to provide further insight into which factors are involved in the newt heart's ability to regenerate. Previous studies suggest that the Wnt and Notch signaling pathways play important roles in enhancing the proliferative capacity of newt cardiomyocytes *in vitro*. In the adult zebrafish, preceding *Msx* activation, there is a marked increase in the expression of *notch1b* and *deltaC*, and a similar up-regulation of these factors

during fin regeneration (Raya, Koth, Buscher, Kawakami, Itoh, Raya, Sternik, Tsai, Rodriguez-Esteban, & Izpisua-Belmonte, 2003). With the availability of a continuously propagating cardiac cell line, the role of signaling pathways will be evaluated with the addition of various drugs to activate or inhibit certain pathways.

Conclusions and Future Work

In this study, the best method to isolate live cardiomyocytes from whole newt ventricles was determined from three pilot studies. The necessary culture conditions to culture the isolated newt cardiomyocytes long-term were established. Additionally, the long-term cultures were characterized by cell morphology, active proliferative capacity, and the positive immunohistochemical staining of cardiac-specific markers.

Because this study's current culture of newt cardiomyocytes has only been completed once, it is important to note that these observations are limited by the possibility that this is a singular occurrence. Since it is the longest that newt cardiomyocytes have been kept in culture, there is no previous basis for these cells to survive this long. Repeated isolations will confirm the validity of the enzymatic isolation protocol and culture conditions. Additional immunohistochemical analysis with other cardiac-specific markers will confirm the presence of newt cardiomyocytes.

Ongoing research

After 54 days, three different types of morphologies can be observed in the cultures, suggesting a heterogeneous culture (Figure 6). In order to isolate only the cardiomyocytes, single cell cloning by limiting dilution was performed. **The purpose of these experiments is to obtain clonal cell cultures that will proliferate, creating individual populations of cells and isolate the populations of newt cardiomyocytes.** Cell suspensions were diluted to a

concentration of one cell per 100 µL of complete Leibovitz L-15 medium in each well of 96-well laminin-coated plates. Currently, four different clonal populations have been established. 139 days have elapsed since the initial plating after isolation. Once more cells have proliferated, the identity of these cultures will be confirmed with ELISA assays, thus allowing us to focus only on those cells expressing cardiac-specific markers.

Next steps

The effect of signaling pathways will be tested in both the primary culture of newt cardiomyocytes and its cell line. This will be achieved with the addition of drugs like BIO, a Wnt signaling activator, and IWR-1-Endo, a Wnt signaling inhibitor, to study the Wnt and Notch signaling pathways. Cells will be pulsed with BrdU (to label the S phase of the cell cycle and permanently label cells that underwent a proliferative event) to establish a baseline for proliferation that can be used to compare with the increased or decreased ability of the cells to proliferate due to the effect of the drugs.

A second aspect to study is the methods to enhance the proliferative capacity of cardiomyocytes *in vitro*. With the addition of FGF and an increase in serum in the media, it is possible the rate of proliferation may be increased and cells may be maintained in a dedifferentiated state.

Finally, much remains unknown about the exact factors involved in heart regeneration and the factors that promote a dedifferentiated state in cardiomyocytes. Though this study's model has yet to be tested, progress has been made in generating an *in vitro* model of heart regeneration. This advancement in knowledge of the continuous cell line has the potential to significantly impact the scientific community's understanding of heart regeneration.

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