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Intercellular Communication via Gap Junctions Influences Cell Survival During Hypoxia

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Abstract

Stem cell therapy can be beneficial following myocardial infarction. However, when murine bone marrow-derived mesenchymal stem cells (mBM-MSCs) are injected into the ischemic area, a large percentage of these cells undergo apoptosis resulting in decreased therapeutic benefits. We hypothesize that the loss of these mBM-MSCs is regulated by intercellular channels or gap junctions (GJs) that provide apoptotic signals passed between ischemic cardiomyocytes and mBM-MSCs. Our research aims to attenuate these GJs by suppressing Connexin-43 (Cx43) expression, the predominant channel-forming protein. We will accomplish this by transiently transfecting a Cx43 siRNA into mBM-MSCs. Our data demonstrate that intracellular fluorescent dyes and FACS analysis can quantify cell-cell coupling between mBM-MSCs in co-culture. Disrupting Cx43 expression will identify a potential therapeutic target for increasing the retention of mBM-MSCs following myocardial infarction.

Introduction

Stem cell therapy is a vast topic that has been investigated and used in many different therapeutic approaches. Specifically, previous studies have shown that injection of stem cells can prevent the loss of function post-myocardial infarction (Boomsma & Geenen, 2014). While these cells provide beneficial factors to the ischemic tissue, many of the cells do not survive after implantation and injection. Many the mechanisms of this action are unknown, however, many predict that this relationship is regulated by gap junctions (GJs). Gap junctions are intercellular membrane channels that facilitate intercellular transfer of small molecules. These channels conduct ions, amino acids, and drugs among many other things. However, they cannot transmit RNA, proteins, and other large molecules (Boengler & Schulz, 2017). GJs are also comprised of various connexin proteins. Connexins proteins are widely expressed in tissues and have been identified based on their molecular weight. Heart tissue expresses a range of connexins, but connexin-43 shows the most abundance (Boengler & Schulz, 2017). This high Cx43 concentration can serve as a target to attenuate the function of a gap junction to prevent the loss of transplanted stem cells.

While these channels are vital for coupling between cells, we predict that they are communicating apoptotic signals from nearby dying cardiomyocytes. When these stem cells enter the hypoxic environment, they couple with the ischemic tissue. Gap junction intercellular communication (GJIC) is established which allows for metabolic and electrical communication (Totland et al., 2020). In the present study, we aimed to attenuate GJIC by targeting Cx43. Doing so allows us to increase viability of mBM-MSCs and model the transfer of materials through GJs.

Methods

mBM-MSCs/ cell culture:

Murine mesenchymal stem cells (mMSCs) were isolated from FVBN/J mice (Phinney Lab) as previously described. Bone marrow was harvested from the femur of mice at 4-6 weeks old. The mice were euthanized by carbon dioxide gas and rinsed with 70% ethanol. The muscle was dissected from the femur and tibia and remaining bone was placed in harvest buffer on ice. The epiphyseal ends of the bones were cut just below the marrow cavity and a 22-gauge needle was inserted into the spongy bone. The bone marrow cells were collected and placed into single cell suspension by three passages through an 18-gauge needle and ran through a 70 μM filter. Once extracted from the femur and tibia, the bone marrow cells are suspended in complete media.

Fluorescently tagged cells indicated greater than 90% positive events of CD105, CD73, CD44, CD29, and SCA1 and less than 0.5% of cells were positive for CD106, SSEA1, CD45, CD34, and CD11b. $1x10^6$ cells were suspended in 1mL 1x PBS and transported on dry ice and stored with liquid nitrogen at -180 degrees C. Cells were thawed and resuspended in complete culture media (CCM) containing 90% MEM-a (ThermoFisher Scientific, Cat. No. 12561056), 10% fetal bovine serum (ThermoFisher Scientific, Cat. No. 16000069), and 1% penicillin/streptomycin (ThermoFisher Scientific, Cat. No. 15070063). Cells were plated on Nunc™ Cell-Culture Treated Multidishes (ThermoFisher Scientific, Cat. No. 140675) at a density of 2,500 cells/cm^2. Dishes were then placed in an incubator with 5% oxygen, 5% carbon dioxide, and 90% nitrogen. 24 hours after initial incubation, half of the CCM was removed and replaced with 2mL new CCM. Cells were placed back in the incubator. After 3 days, all CCM

was removed, cells were washed with 37°C PBS and replaced with 2mL of new CCM. Once cells reached 80-95% confluence, they were lifted off the wells and removed from the culture dish.

Counting Cells

Samples of cells were counted prior to being placed in culture. A vial of cells was thawed at room temperature for 10-20 minutes. A 10 μL aliquot of cells suspended in media and 10 μL of Corning® Trypan Blue Solution, 0.4% (w/v) in PBS, pH 7.5 ± 0.5 (Corning, Cat #25-900-Cl) were pipetted into a microcentrifuge tube in a 1:1 dilution. Cells were placed under a microscope and were counted. Those that took up the Corning® Trypan Blue Solution were dead and unstained cells were alive. Percent viability and cell count was calculated for each vial of cells.

Fluorescent Dyes:

mBM-MSCs were labeled with 1 μM cytoplasmic gap junction impermeant fluorescein diacetate (CellTracker™ Green CMFDA, ThermoFisher Scientific, Cat. No. C7025) for 45 minutes or 2.5 μM cytoplasmic gap junction permeant CellTrace™ calcein red-orange AM (ThermoFisher Scientific, Cat. No. C34851) for 1.5 hours with media replacement after 1 hour. Fluorescence dyes represent transfer through gap junctions. Once labeled with dyes, CellTracker™ Green CMFDA is impermeant to cytoplasmic gap junctions, while CellTrace™ calcein red-orange AM is permeant.

After dye labelling, mBM-MSCs were co-cultured for 1 hour, 5 hours, or 21 hours. FVBN/J murine bone marrow mesenchymal stem cells were seeded at 150,000 cells per 6-well plate and placed in CCM in a sealed incubator (5% O_2, 5% CO_2, balance N_2) for 8-9 days. Cells were cultured under previous descriptions and lifted from the wells after reaching 80-95% confluence. Cells containing CellTrace™ calcein red-orange AM were washed with 2mL warmed 1x PBS and then subjected to 1mL Trypsin-EDTA (0.25%), phenol red (ThermoFisher Scientific, Cat. No 25200056) and placed into the incubator for 3-4 minutes. Once all cells had lifted from the bottom of the plate, 2mL warm CCM was added. All cells and media were transferred to 15mL conical tubes and placed in a centrifuge to spin at 400g for 5 minutes. Cells were resuspended in 1mL CCM and dispersed into wells containing CellTracker™ Green CMFDA labeled cells. After co-cultures began, plates were contained in a sealed incubator (5% O_2 5% CO_2, balance N_2). Then, co-cultured cells were washed with 1mL warm 1x PBS and subjected to 1mL 37-degree C trypsin. After 3-4 minutes in the incubator, 2mL warm CCM was added to each well and cells/media were spun in the centrifuge at 400g for 5 minutes. Co-cultured cells were resuspended in 500 µL or 750 µL for analysis by flow cytometry (FACS). Finally, co-cultured cells were resuspended in 0.5mL cold 1x PBS. LIVE/DEAD™ Fixable Green Dead Cell Stain (ThermoFisher Scientific, Cat. No L34969) was added to samples containing only single layers of mBM-MSCs. LIVE/DEAD™ Fixable Green Dead Cell Stain was brought to room temperature and mixed with 50uL DMSO. Then, 0.5 µL of the fixable stain was added to cell samples containing only a single layer of mBM-MSCs for 30 minutes on ice before being resuspended in 1mL ice-cold 1x PBS and analyzed by flow cytometry. FACS analysis was performed using a Beckman CytoFlex flow cytometer under support by Dr. Kristen Renkema, Assistant Professor at Grand Valley State University.

Calcein Trials

Repeated experiments of calcein red-orange trials were conducted including various dye incubation and esterase removal times. Experimental groups included (1) 1 hour dye incubation/30-minute esterase removal, (2) 1 hour dye incubation/ 1 hour esterase removal, (3) 3-hour 40 minute dye incubation/ 30-minute esterase removal, and (4) 3 hour 40 minute dye incubation/ 1 hour esterase removal. Groups were run through flow cytometer and revealed that Group (1) showed the highest fluorescence.

Cx43 Transfection:

A fluorescently tagged Oligo (BLOCKIT Alexa Fluor Red Oligo; Cat. No. 14750100) was used to determine transfection efficiency in the mBM-MSCs . In 6-well plates, 90,000-120,000 cells were plated in 2.5 mL growth media (90% MEM-a, 10% FBS) without antibiotics and placed in each well. 3 µL of the fluorescent oligo was diluted in 150 µL MEM-a without serum. 9 µL of Lipofectamine™ RNAiMAX was diluted in 150 µL MEM-a and mixed. The diluted fluorescent oligo was combined with the diluted Lipofectamine™ RNAiMAX and incubated for 5 minutes at room temperature. The combined oligo-Lipofectamine™ RNAiMAX complex was added to each well containing cells to get a final oligo concentration of 10 nM. Cells were incubated 24-48 hours at 37- degrees C in CO_2 incubator. Cells are then subjected to flow cytometry and FACS analysis.

Fluorescent Imaging:

mBM-MSCs were cultured on coverslips until 80% confluence. Cells were then fluorescently labeled with 2.5 µM CellTrace™ calcein red-orange AM or 1 µM CellTracker™ Green CMFDA based on previously described protocol. Cells were flooded with ice cold methanol for 10 minutes until being washed three times with 1x PBS. Prior to mounting on the glass slides, VECTASHIELD Antifade Mounting Medium with DAPI was added to each coverslip. Fluorescence images were collected using a fluorescence microscope.

Results

Calcein Trials

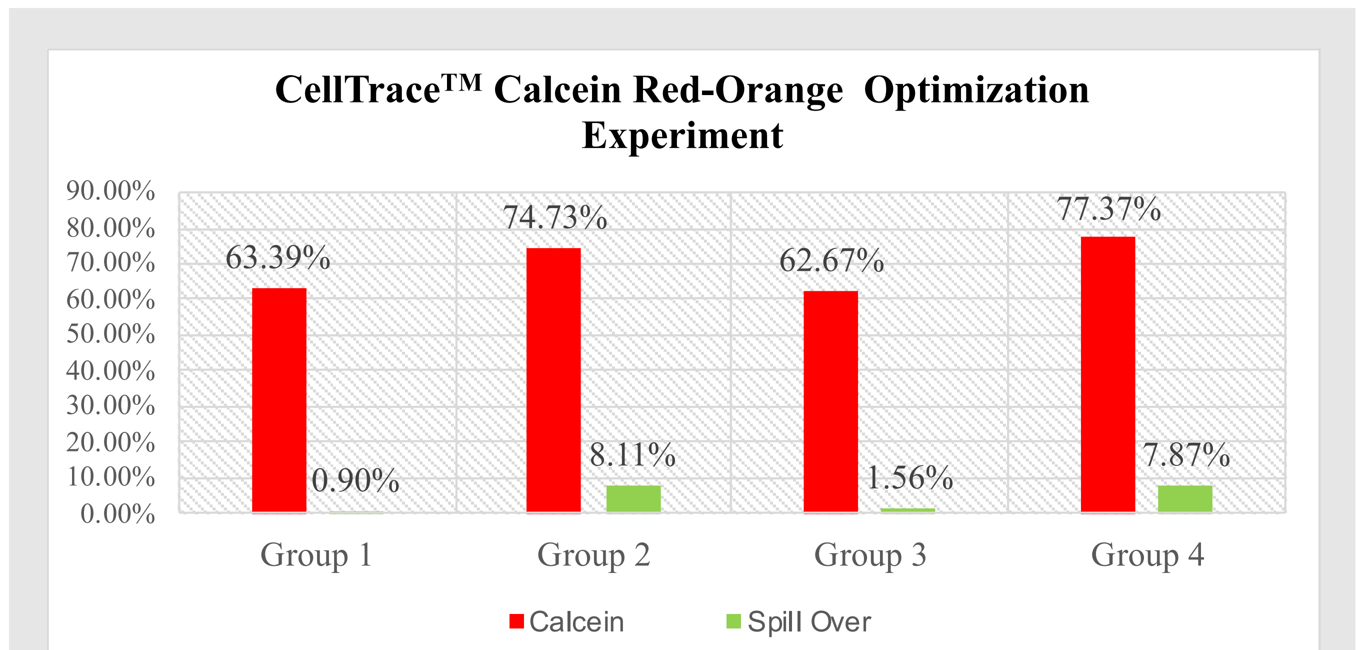

Figure 1. Calcein-AM Optimization Results. Cells were incubated with the dye according to their group designation (see Methods). The red bars depict the percent of cells that expressed CellTraceTM calcein fluorescence. Green bars depict red fluorescence spill-over into the FITC channel in each group. Group 1 incubation and AM ester removal times achieved the greatest calcein expression with the least amount of spill-over (spectral overlap).

Once cells had been prepared with their designated conditions (see methods), they were then subjected to FACS analysis. This quantified the number of cells in each condition that contained the CellTrace^{TM} calcein red-orange AM fluorescent dye (Figure 1). Group one revealed an average of 63.39:0.90 (% of sample with calcein fluorescent dye: % of sample being recognized in the incorrect flow cytometer channel, Figure 1). This group became the standard for future fluorescent labeling because of the small sample of cells being identified incorrectly. Group two revealed 74.73:8.11 (Figure 1). While this group had the highest intensity of calcein labeled cells, the percent of spillover also increased. Group three revealed 62.67:1.56 and had the lowest amount of calcein labeled cells (Figure 1). Finally, group three revealed 77.37:7.87 and was not used as the standard for future fluorescent labeling because of the large amount of spillover between channels (Figure 1).

Co-Culture/ Double Positive Population

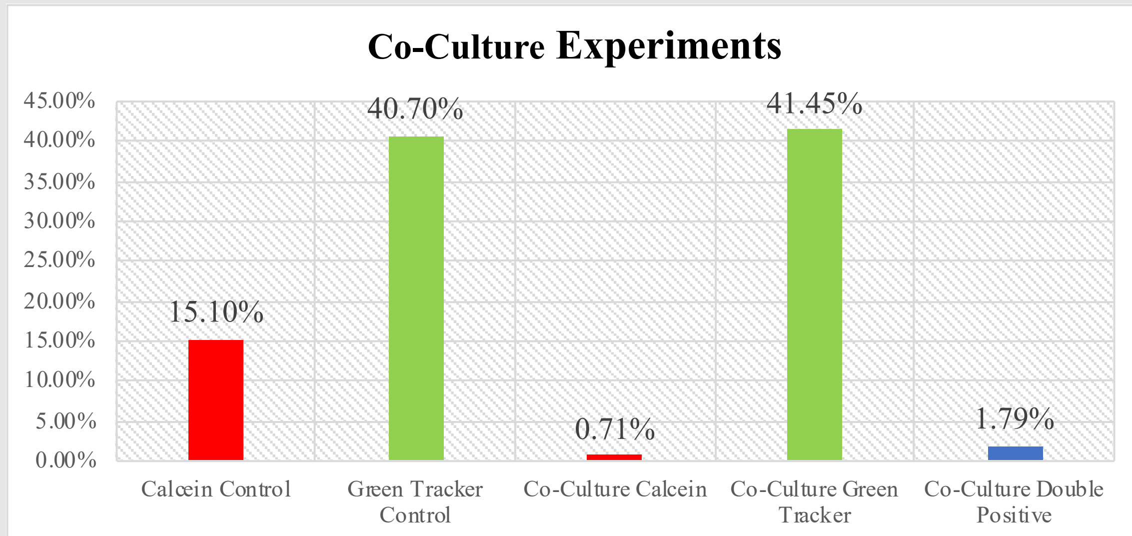

Figure 2 Co-Culture Fluorescence. 'Calcein Control' and 'Green Tracker Control' bars illustrate the percent of monolayered cells retaining their respective dyes after 21 hours of culture. 'Co-Culture' describes double layered cells labeled with their respective dyes and the percent of cells that fluoresce Calcein alone (red bar), Green tracker alone (green bar) or cells containing both dyes (blue bar) representing coupled cells (GJIC) 21 hours after co-culture.

Imaging Cells

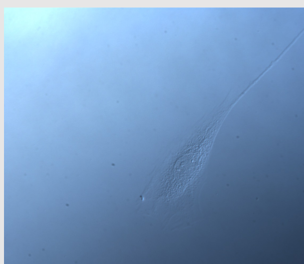

Figure 3a. Brightfield Image of mBM-MSC

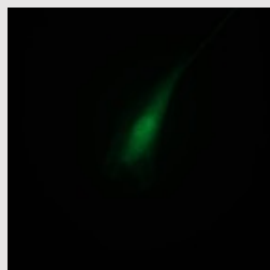

Figure 3b. CellTracker Green fluorescence image

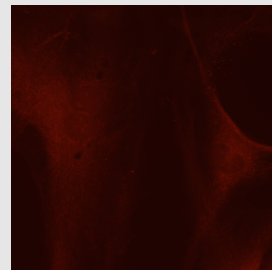

Figure 3c. Cell Trace Calcein Red-Orange fluorescence image

Images were collected by use of fluorescence microscopy to represent cell structure and fluorescent dyes.

Discussion

Previous studies show that cells can communicate through gap junction channels (Boomsma & Geenen, 2014; Czyż et al., 2000; Juul et al., 2000; Warawdekar, 2019). These channels, while ultimately vital to the survival of the cell, are hypothesized to send apoptotic signals. As a result of this, the stem cells often experience cell death post-transplantation into the ischemic cardiac tissue. Our lab seeks to identify the function of this gap junction and the role of intercellular communication during hypoxia in the heart. Connexin-43, the predominant protein in this channel, has previously been shown to be associated with many other heart conditions such as hypertrophic cardiomyopathy, heart failure, and ischemia (Michela et al., 2015). Due to this, we investigated the role of connexin-43 during cell-cell communication. Our results showed the majority of the mBM-MSCs were successfully labeled with the CellTrace™ calcein red-orange AM or CellTracker™ Green CMFDA during single layer culture. However, FACS analysis showed very small amounts of both fluorescent dyes during co-culture.

Based on these findings there is likely another factor affecting the formation and communication of gap junctions between mBM-MSCs. Culture conditions and lifting the adherent cells using trypsin, may affect gap junction formation. To avoid this, creating lab protocols with varying fluorescence dye concentrations may better represent the gap junction formation. In addition, the use of antibiotics such as penicillin and streptomycin were included in cell cultures. This is a prophylactic treatment to avoid contamination of cell cultures. However, one study showed that antibiotic use in human mesenchymal stem cells (hMSCs) resulted in poor

cell proliferation when used in high concentrations (Chang et al., 2006). In future studies, avoiding the addition of penicillin/streptomycin may benefit the cell culture conditions and viability. This may increase cell density and allow for increased gap junction intercellular communication to occur.

Previous studies have also demonstrated similar methods to represent gap junction communication. The scrape loading method includes creating a scrape line by a surgical blade on a monolayer of cells. This allows two fluorescent dyes with distinct molecular weights to enter the injured cells. The high molecular weight dye stains the cells as the low molecular weight dye crosses into the adjacent cell through gap junctions (Hervé & Derangeon, 2013). The adaptation of this labeling method may allow for better representation of gap junction intercellular communication. Another study revealed their use of the scrape loading method. This protocol was shown to be applicable with several different types of cells including epithelial, neural, somatic testicular, skin, and intestinal (Sovadinová et al., 2021). This method has been recently modified to further evaluate parameters and increase use amongst multiple cell lines. In future studies, using this method to label cells with fluorescent dyes may reveal a greater population representing mBM-MSC GJIC.

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