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Gap Junctions in Stem Cells Provide an Essential Conduit for Cell-Cell Communication

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Abstract

Introduction

Myocardial infarction (MI) results from the death of cardiomyocytes (CM) following obstruction of blood flow and diminished oxygen supply to the tissue (hypoxia). Human adipose tissue-derived stem cells (hADSCs) used in pre-clinical models can replace damaged CM, however, this has not been replicated in human clinical trials due to early loss of hADSCs. We hypothesize that coupling of hADSCs to dying CMs may account for part of this loss. Furthermore, cell culturing is essential aspect of any in-vitro experiment. Through multiple trials we sought to maximize the efficiency of our culturing procedures in order to best facilitate the work in our lab.

Methods

Four aliquots of hADSCs were cultured and the effects of various reagents and culturing practices were investigated. To examine cell coupling, hADSCs will be cultured for different lengths of time with fluorescent dyes that are either permeable or impermeable to the cell membrane. We will assess the time course of coupling between hADSCs under both normoxic and hypoxic conditions by using fluorescent-activated cell sorting (FACS).

Results

Our previous studies demonstrate that adult mesenchymal stem cells possess membrane proteins (connexins) that contribute to cell-cell coupling. The proposed studies will address the functional significance of connexins related to hADSC coupling. The results of our hADSC culturing trials found we can optimize our cultures to facilitate these experiments through several procedural and reagent modifications. Key modifications to culturing protocols include, 1.

decreased time spent in culture, 2. utilization of pure, established cell lots, 3. using smaller flasks.

Background

Rationale

Heart disease remains the leading cause of death worldwide (6). Myocardial infarction (MI) is an especially deadly form of heart disease that leads to widespread cellular death and loss of functional contractile myocardial tissue. When a coronary artery becomes occluded the region of heart tissue it supplies experiences significant deprivation of nutrients and oxygen leading to apoptosis and oxidative damage. This infarct region often experiences significant and immediate loss of function. The surrounding boarder region is also at high risk of functionally significant apoptosis and cellular damage. This presents the perplexing challenge of containing cellular mediators of injury without further disrupting the cellular communication necessary for electrical coupling in the heart. In the emergent field of regenerative cardiology, this creates a set of paradoxical conditions known as the bystander effect (1,16,18,19).

In order to induce beneficial remodeling in the host myocardium transplanted cells must avoid coupling with damaged and dying cells while retaining their ability to eventually integrate within the viable myocardium. Regenerative therapies such as stem cell transplantation in preclinical studies have shown to be an effective means of containing the initial infarct, repairing or replacing damaged tissue, and preventing the development of deadly arrhythmias (4,7,10,12,13,17). Unfortunately, these findings have not been replicated in clinical trials, possibly due to unfavorable coupling with damaged CMs caused by the bystander effect. For these reasons, our lab seeks to further investigate the bystander effect and host-transplant coupling between CMs and hADSCs. Our work indicates that a better understanding of the

timeframe, and coupling mechanisms associated with stem cell transplantation could be used help bridge the gap between the promising pre-clinical results and the lack of beneficial outcomes seen in clinical trials.

The Bystander Effect

The cellular environment post MI is incredibly hostile to both the host myocardium and any exogenous cells that could potentially be used for regenerative purposes. The post MI infarct is characterized by a lack of blood flow and oxygen, two elements essential for proper function. Furthermore, the presence of dead cells, dying cells, and intracellular apoptotic signaling factors may induce apoptosis in newly introduced cells through the bystander effect (1,5,11,16,18). Possible molecular mediators of the bystander effect in cardiomyocytes include calcium ions, and nitrous oxide (5). Recent studies have suggested that these molecular mediators of apoptosis may be spread through gap junctions (GJ) composed of connexin proteins (5,16,19). GJs have been shown to permit the transfer of calcium ions and larger molecules such as ATP, between CMs (5). Our lab has demonstrated that connexin 43 (Cx43), in particular, is a key constituent of GJs in human mesenchymal stem cells (2,5,13). Furthermore, there is a growing body of evidence that suggests intracellular communication and coupling may be modified through manipulation of Cx43 expression (5,11,13,16,19). In order for GJs to form, Cx43 must be synthesized in the cytoplasm and then transported to the cell membrane. Connexin 43 tends to localize around the nucleus and migrate outward as the cell develops or begins to attach to another cell following transplantation (5,13,16). This suggests that Cx43 may regulate the spread of death signals through cell to cell gap junctions in a time-dependent manner. Modulation of this process may prove useful in promoting retention and survival of transplanted human mesenchymal stem cells following MI.

Human Mesenchymal Stem Cells

Human adult mesenchymal stem cells can be harvested from a variety of sources in the human body. Compared to totipotent embryonic stem cells, adult stem cells are characterized by their pluripotency, or ability to differentiate into many but not all types of cells within the body. Adult stem cells are also particularly useful in regenerative therapies because they are readily available and unlikely to trigger an immune response from their host or display teratogenesis following transplantation (4,7-10,12,13). Adipose-derived stem cells are a particularly promising source of adult stem cells due to their widespread availability and ability to form Cx43 GJs (3,8,15,21). These cells can be harvested from any source of fat tissue, which is readily available after liposuction. Furthermore, they can easily be obtained from the host for an endogenous transplant. While human trials have proven the safety of these cells, their therapeutic effects have not been shown to be clinically significant. Collectively, research from preclinical and clinical trials suggest that implantation within the first few hours results in the greatest beneficial effects (7,10,12). However, due to the aforementioned bystander effect long term retention of these cells remains low (11,12,16,). Strategies aimed at improving retention include increasing the levels of growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (8,9). While other studies have looked at cell coupling as an outcome of these forms of molecular manipulation, few investigate how coupling would vary over time following implantation. These results are necessary in order to overcome the initial loss of cells due to the bystander effect.

Investigation of Coupling through Dye Transfer

Numerous factors have been implicated in the expression of Cx43 that may provide significant gains or losses in coupling when manipulated in a time-dependent manner. One such

factor is hypoxia (low oxygen) conditions. Hypoxia has been shown to increase Cx43 and other cytokines leading to greater stem cell retention after transplantation in in vitro models (3,8,9,12). Greater retention has been shown to result in better post-MI outcomes such as decreased mortality, increased cardiac function, healthy cardiac remodeling, and protection against arrhythmia in pre-clinical models (2,4,7,10,12,13,20). A variety of other growth and transcription factors may have similar effects. This includes cytokines such as VEGF and FGF (8,9). While all of these factors have been shown to improve retention and cardiac function, little is known about how they do so, when their effects manifest, and how their effects compare to and interact with one another. For these reasons, our lab is interested in developing a time-dependent model of coupling in human adipose-derived stem cells with the intent of modeling the time course of a myocardial infarction. We will accomplish this by using dye transfer experiments.

Dye transfer experiments can be used to measure cell coupling over time. Through the selection of dyes of varying molecular sizes, it is possible to label one cell line with a fluorescent dye that will cross gap junctions and label another cell line with a fluorescent dye that will not cross gap junctions. The two cell lines are then subject to co-culture (grown together) The amount of gap junction permeable dye that crosses over into the cell line containing the impermeable dye gives a quantitative measure of cell coupling that can be taken at various points in time. Fluorescent activated cell sorting (FACS) can be used to sort cells based on which dye they contain. FACS analysis can then be used to infer the percentage of cells coupled and communicating via GJs at any given time. This is important to know and eventually manipulate as both apoptotic molecules that cause cellular death, and ions that facilitate electrical coupling can be transferred between GJs (1,5,11,13,18).

Methods

Culturing Cells

Stempro Human ADSC 1 ML; $1x10^6$ Cells/Vial (Cat #R7788115, Lot #1001001) were purchased from Life Technologies Corporation, a ThermoFisher Scientific company. Flow cytometry analysis of the surface proteins of these cells indicated greater than 95% positive events of CD29, CD44, CD73, CD90, CD105, and CD166 while less than 2% positive events were reported for CD14, CD31, CD45, and Lin1 (Gibco MSC Stem Cell Documentation). Cells arrived frozen on dry ice and were then stored in liquid nitrogen at approximately -180°C. Cells were then quickly thawed in a water bath at 37°C and were cultured in either standard T-25 or T-75 flasks at the vendor's recommended minimum 5,000 cells/cm^2. The cell media was composed of 5% Dulbecco's Modified Eagle Media (DMEM) (ThermoFischer Scientific, Cat. #11995065) and 95% MSC Qualified Fetal Bovine Serum (FBS) (ThermoFisher Scientific, Cat. #12662011, Lot #2226685P). Cells were incubated at 37°C and 5% CO_2 until they reached approximately 80-95% confluency. Media was removed and replaced every 3-4 days or if cellular waste accumulated and caused a change in media pH as indicated by a change in media color.

Counting Cells

Cells were counted before being placed in culture, either following thawing or a passage. Cells were counted using a Sigma-AldrichBright-Line™ Hemocytometer (StemCell Technologies, Cat #Z359629). A 10µL aliquot of cells suspended in media was pipetted into a microcentrifuge tube along with 10µL of Corning® Trypan Blue Solution, 0.4% (w/v) in PBS, pH 7.5 ± 0.5 (Corning, Cat #25-900-Cl) for a 1:1 dilution. Cells were counted under an inverted light microscope; dead cells were identified as they took up Trypan Blue Solution. Estimated total cell count and percent viability were determined using a publicly available software application.

Passaging Cells

Cells were passaged when they reached approximately 80-95% confluence, in an effort to capture and transfer them while they were in a log stage of growth. The culture media was aspirated, and cells were rinsed with Dulbecco's Phosphate-Buffered Saline (DPBS) (ThermoFisher Scientific, Cat. #14190144). The DPBS was then discarded and cells were detached from the bottom of the flask using TrypLE Express with Phenol Red (ThermoFisher Scientific Cat. #12605010). After exposure to this trypsin enzyme solution, cells were placed in the incubator for approximately five minutes or until approximately 90% of the cells were detached from the flask. The suspended cells were then diluted with 2 parts media and centrifuged in 15-mL conical tubes at 210 x gravity for 5 minutes at room temperature. The media was then aspirated leaving a dense pellet of cells at the bottom of the conical tube. The pellet was then resuspended in a minimal amount of media that was prewarmed to 37°C. A count of the cells were taken and the media was diluted to facilitate a seeding density of 5,000 cells/cm^2. Flasks were then returned to the incubator to facilitate attachment.

Freezing Cells

In order to preserve isolated cell lines for extended periods of time cells were frozen and stored in liquid nitrogen at approximately -180°C. A standard passaging procedure was followed however, rather than diluting cells to a 5,000 cells/cm^2 seeding density, cells were resuspended in Synth-a-Freeze™ Cryopreservation Medium (ThermoFisher Scientific, Cat. #A1254201) at a density of 1-2 x 10^6 cells/mL. Cells were then slowly cooled at a rate of 1°C/hour in an Mr. Frosty™ Freezing Container (ThermoFisher Scientific, Cat. # 5100-0001) containing isopropanol. Cells were then placed in the -180°C storage container.

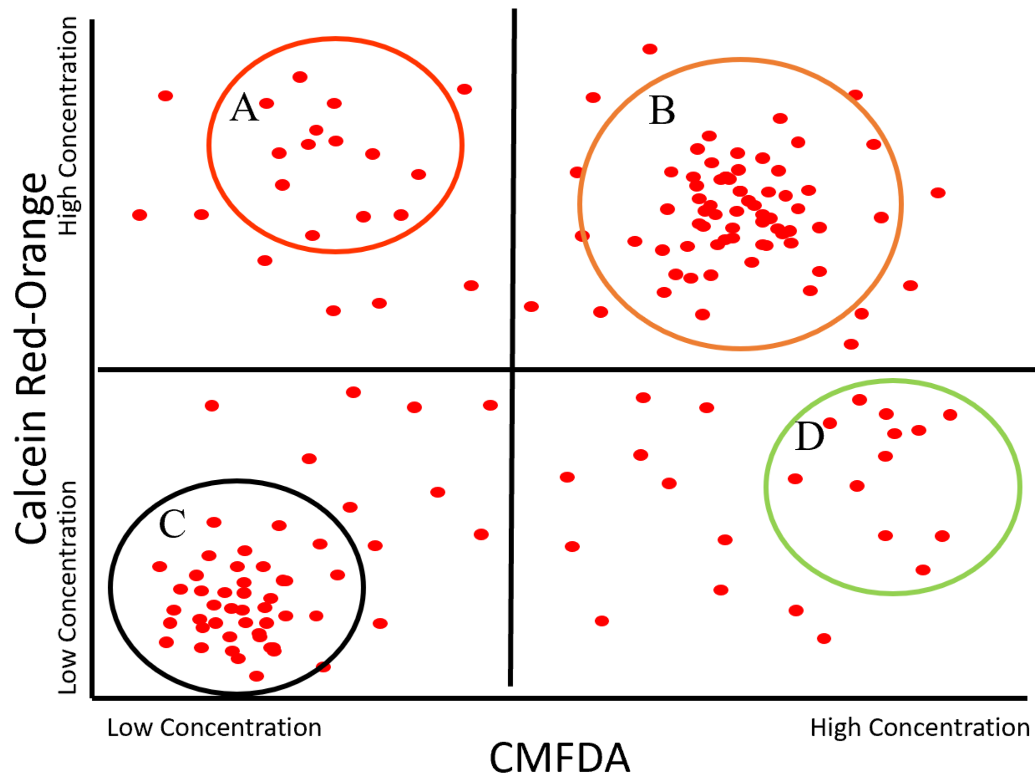
Human Adipose-Derived Stem Cell (hADSC) Labeling with Fluorescent Dyes

hADSCs were labeled with various fluorescent dyes prior to coculture. Cells were washed 2 times in PBS to remove serum and then incubated with the dye in DMEM containing fetal bovine serum (FBS). Following dye labeling, cells were washed in PBS. Cells were subsequently labeled with 2.5 μM of the cytoplasmic gap junction permeant calcein red-orange AM (Cell Trace; Molecular Probes/Invitrogen, C34851) for 1 hour or 5 μM of the cytoplasmic gap junction impermeant chloromethyl fluorescein diacetate (Cell Tracker Green CMFDA; Molecular Probes/Invitrogen, C7025) for 1 hour with a media replacement after 30 minutes.

After labeling, hADSCs were cocultured for 4 or 20 hours. hADSCs were resuspended in DMEM and FBS media and 0.25×10^6 of the hADSCs labeled with the calcein red-orange AM were layered onto 0.25×10^6 confluent hADSCs labeled with the Cell Tracker Green in 6-well plates. Following coculture, cells were washed in PBS, lifted with 0.25% trypsin, centrifuged, resuspended in 3 mL PBS/BSA/EDTA, passed through a 100 μM filter, centrifuged, and resuspended in 100 μL PBS/BSA/EDTA for analysis by flow cytometry (FACS). FACS analysis enabled us to count those cells expressing the combined green and red fluorescent signals and compare this number to those cells only expressing the green signal. This analysis gave us the percentage of cells that exhibited dye transfer and coupling within the co-culture. FACS was performed under the supervision of Dr. Kristen Renkema, Assistant Professor in Biomedical Sciences at GVSU using a Beckman CytoFlex flow cytometer.

Figure 1

Example FACS Analysis by Flow Cytometry



Note. Following exposure to fluorescent dyes and co-culture, hADSCs will be sorted by fluorescent excitation activity by a flow cytometer. (A) hADSCs containing high concentrations of calcein red-orange. These cells are representative of host cells that did not couple with transplanted cells. (B) hADSCs containing high concentrations of both calcein red-orange and CMFDA. These cells are representative of transplanted cells that coupled with and received dye from the host cells. This is the population of cells our lab seeks to quantify as a function of time. (C) hADSCs that did not take up either dye. (D) hADSCs containing high concentrations of

CMFDA. These cells are representative of transplanted cells that either have not or will not couple with the host cells within the observed timeframe.

Results

hADSCs were cultured under the aforementioned conditions and imaged daily. Over the course of the experiment several issues involving the growth, attachment, and sterility of the hADSCs were observed and issues were investigated. After a through investigation of all lab materials and supplies, we concluded this most significant source error was likely present in the particular cell lot supplying the individual aliquots placed in culture. The particular lot supplied by the vendor resulted in three cultures that showed signs of contamination and one culture that failed to attach to the T-25 flask. After ruling out the individual components of the cell media, the laboratory equipment, and other reagents, it was determined that the cell lot needed to be replaced. Further trials on blank media confirmed the sterility of the laboratory setting and practices further implicating the particular cell lot. Current efforts are underway to culture hADSCs from a lot confirmed to proliferate at the expected rate.

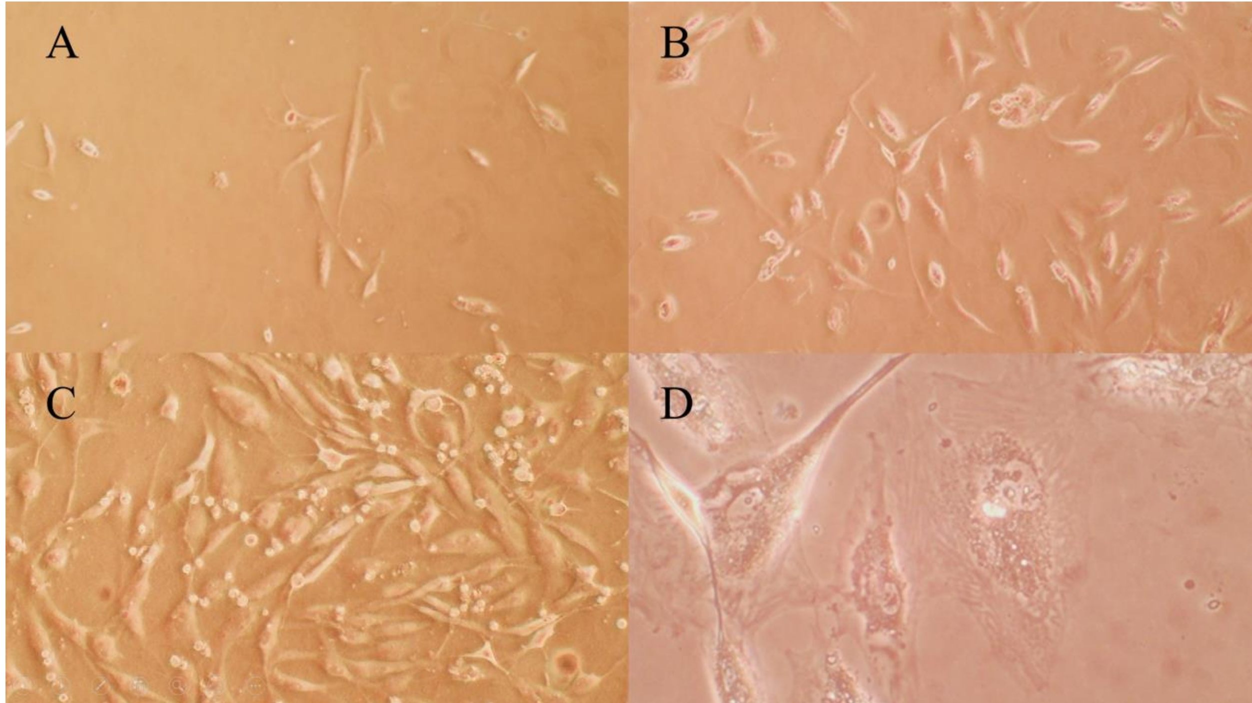
Growth

Once placed in culture hADSCs were observed to proliferate with a doubling time ranging from five to nine days. Based on prior work and information provided by the cell vender this was inconsistent with the expected doubling time of approximately three days. Additionally, cells proliferated at inconstant rates with flasks of the same passage number reaching 90% confluency at differing times. Variation between aliquots was also observed as one reached 90% confluency in approximately three days while another failed to reach 90% confluency after ten days cultures. Within the individual flasks variations in density were observed with hADSCs aggregating at along the edges of the flasks or in smaller clusters distributed throughout the flask.

These high-density regions would reach 90% confluency before the remainder of the flask became confluent if at all. Attempts to increase the rate of growth included increasing the seeding density to 20,000 cells/cm^2 and using smaller flasks. Another notable technique attempted was capturing cells in the log phase for passaging. The aim of this method was to lift the cells from the flask just before they reached 90% confluency in order to minimize cell contact inhibited growth suppression in attempt to preserve the cells in a constant phase of logarithmic growth. Qualitative observations suggested these methods improved cell density and proliferation. However following a successful passage, the hADSCs quickly experienced attachment or sterility issues suggesting the problem with the cell line extended beyond cell proliferation and culturing practices.

Figure 2

Proliferation and Density



Note. A. 10x magnification of hADSCs demonstrating inconsistent density at four days of culturing. B. 10x magnification of a region selected for density displaying approximately 40-50% confluency at seven days in cultures. C. 10x magnification of hADSCs from a different aliquot demonstrating approximately 90% confluency and relatively consistent density, representing ideal conditions to passage the cells. D. hADSCs from image B at 30x magnification.

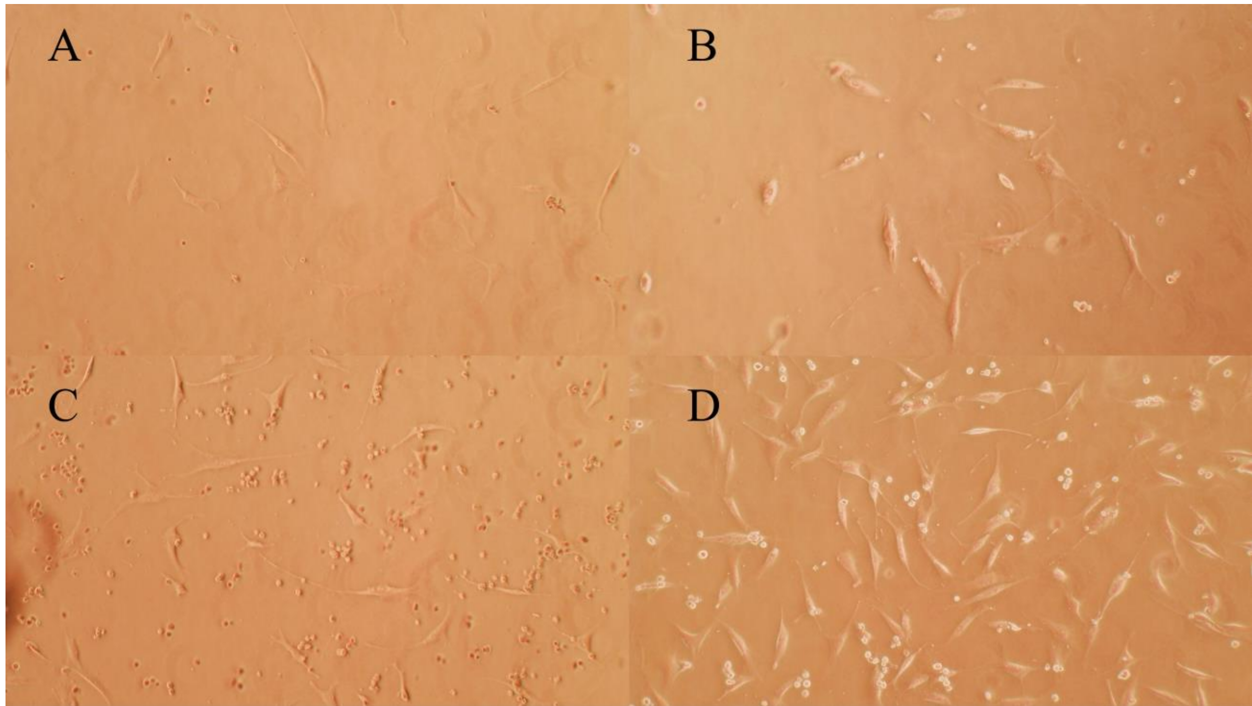
Attachment

Once placed in culture, cells must form a secure attachment to the bottom of their flask in order to grow and proliferate. Throughout these cell culture trials cells were observed failing to attach to culture treated plasticware and becoming disconnected after initial attachments were

formed. In particular, one aliquot failed to attach to the flask at any point of the trail. The variety of flasks tested suggests that the issue was likely with the hADSCs rather than the flasks.

Figure 3

Cell Attachment



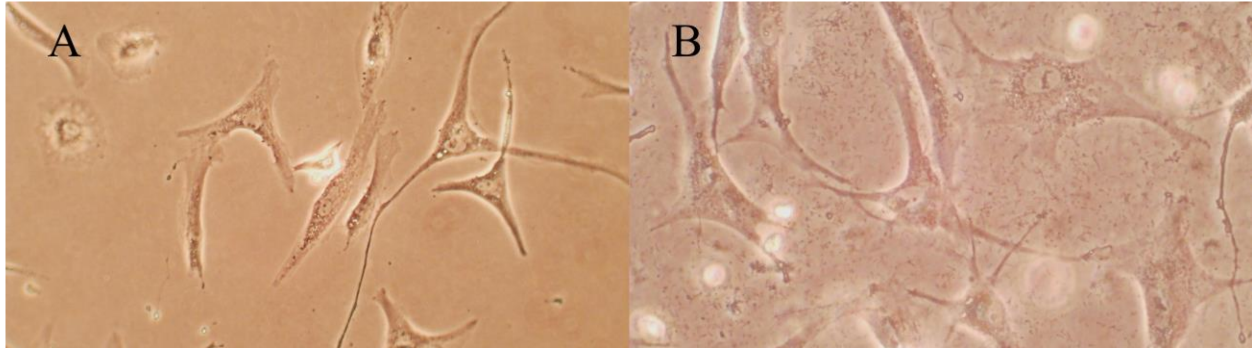
Note. 10x magnification of hADSCs at various stages of growth and confluency. Healthy hADSCs display an elongated spindle shaped morphology, these cells are able to form secure attachments to the flasks and other cells. Detached cells display a smaller circular morphology and float in the media, these cells are either dead or have failed to form the necessary attachments to the flask. B. hADSC displaying an abnormal oval shaped morphology, indicating poor attachment and risk of death.

Sterility

Based on our current observations, the sterility of the aliquot likely played the greatest role in the inability of the hADSC to survive in culture for an extended period of time or proliferate at the expected rate. Cells obtain from lot 1001001 invariably exhibited signs of either bacterial infection or buildup of microscopic cellular debris, provide the cells did not experience any issues with attachment first. Per the request of the vendor, a new lot of FBS (12662002) was used to culture successive cell lines after issues were experienced with the initial aliquot. This was originally intended to reduce the presence of endotoxin that was reported in the initial lot of FBS. This indirectly confirmed that the FBS was likely not the source of contamination as switching lots did not improve outcomes. In order to test other reagents in the lab for possible sources of contamination 12.5 mL of combined media was subject standard laboratory procedures, including pipetting, transferring between flask, and exposure to an unventilated microscope bench. A control flask containing 12.5 mL of combined media was cultured under identical conditions, however it received no manipulation following the initial transfer to its flask. This suggests that the hADSCs themselves were most likely the source of contamination. Additionally, all instrumentation in the lab was regularly autoclaved. After the initial aliquot experienced contamination, only disposable pipettes were used to transfer the hADSCs and the media. The most convincing observation that suggests the cells themselves the source of contamination was the fact that any given flask of hADSCs would invariably show signs of contamination if left in culture long enough. This occurred regardless of passage number, number of freeze-thaw cycles, or any other protocol the cells were subject to. Cells left unperturbed in culture would also show signs of contamination.

Figure 4

Cell Contamination



Note. A. 30x magnification of healthy hADSCs showing no visible contamination. B. 30x magnification of hADSCs showing clear signs of contamination in the surrounding media. Contamination presented as microscopic impurities surrounding the cells, possibly bacteria, fungi, or other exogenous impurities likely originating from the source of the cells.

Discussion

Recent evidence suggests that early coupling between transplanted and host cells plays an essential role in the development of sustainable engraftment of adult stem cells (2,12,13). Despite the clear need to modulate early cell coupling few studies have characterized the timeframe of gap junction formation immediately following transplantation of human adult stem cells. Our work seeks to provide insight into the functional changes in early hADSC coupling. Knowledge of this timeframe could be used to better assess current strategies aimed at increasing the retention of transplanted stem cells. The addition of growth factors and genetic modification have been shown to increase the short-term functional benefits of adult stem cell therapies (8,9,12,13). However, a better understanding of how these changes affect early communication

and attachment of transplanted cells could be used to attenuate early cell death and increase long term retention.

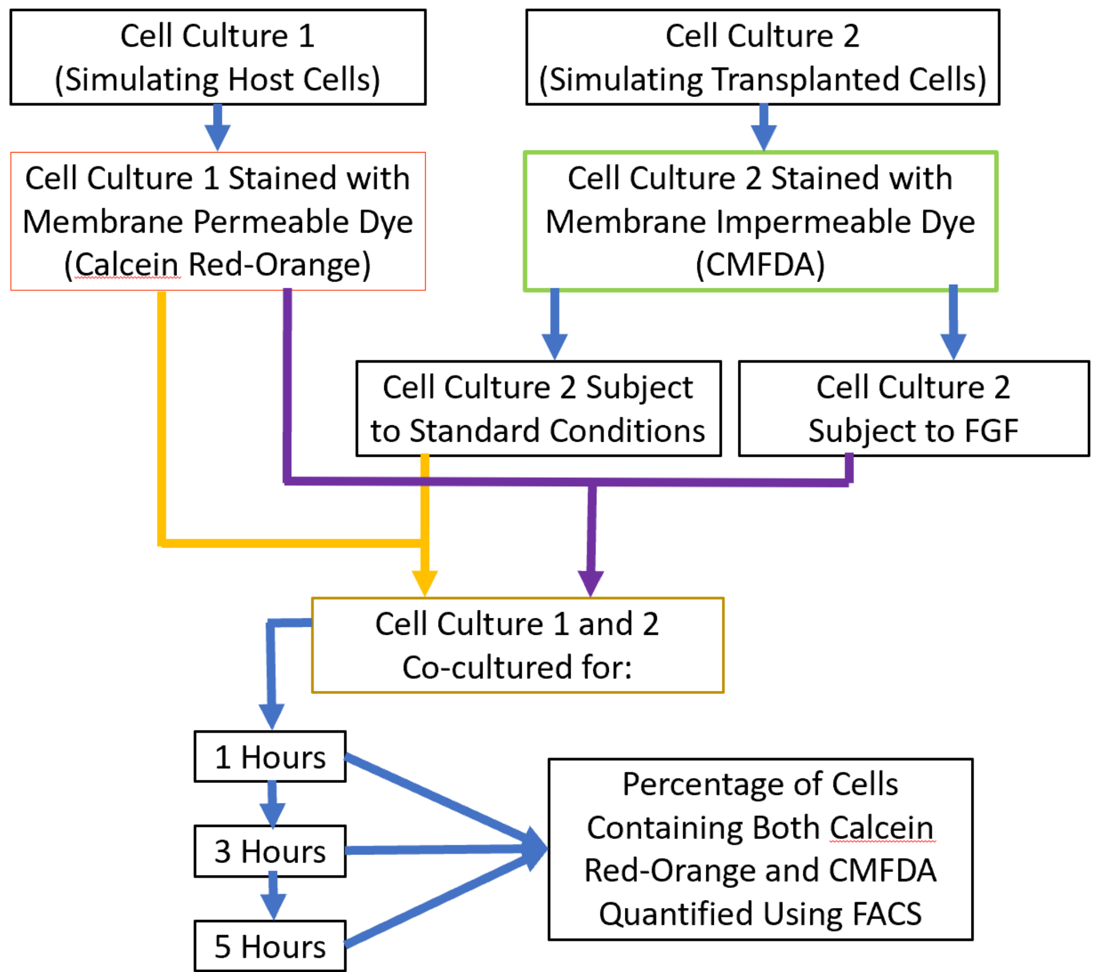
Viability of commercially available hADSCs is a key factor in producing consistent, and reliable results. Our trials using different reagents, techniques, and aliquots of hADSCs provided our lab with valuable insights that will inform our future work. Most notably, how issues with cell contamination impacted proliferation and attachment. A thorough investigation of culturing practices and laboratory equipment and reagents revealed several key findings that will streamline future work. Our trials demonstrated that seeding the hADSCs at an initial density above the recommended 5,000 cells/cm^2 resulted in increased proliferation and a shorter time to reach approximately 90% confluency. Additionally, using more smaller flasks instead of fewer larger flasks (three T-25 flasks vs one T-75) resulted in qualitatively healthier cells. This was likely due to maximized contact with flask edges, allowing for two dimensions of cellular attachment, and minimized instances of isolated individual cells in the center of the flasks.

Antibiotics such as penicillin and streptomycin were useful in preventing or suppressing short term contamination, however these reagents halted cell proliferation. This has been observed in other human cell lines and is likely due to aminoglycoside induced inhibition of protein synthesis at crucial periods of cell attachment (14). There is reason to believe that hADSCs are especially sensitive to this as we have shown attachment is a delicate and essential process for the immediate and long-term survivability of these cells. Keeping the cells in culture for a shorter period of time is likely to reduce levels of contamination, although we did not always observe this to be the case. However, performing experiments as soon as sufficient numbers of cells are reached is likely to produce more favorable outcomes. For this reason, our lab recommends that future experimental designs utilize less conditions and more directly

investigate key variables. A modified study flow diagram based on these findings is shown below.

Figure 5.

Modified Study Design for Future Work



Note. A study flow diagram illustrating how the findings of our culture trials can be used to design a more efficient experiment. Notably less time conditions are analyzed. Cells will also be subject to FGF rather than hypoxia in order minimize variability in cell culture conditions. FGF can readily be pipetted into any flask containing cells, while hypoxia would require more manipulation of the culture environment.

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