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Comparative Genomics of Human Mesenchymal Stem Cells and Human Mesenchymal Stem Cells Derived Vascular Smooth Muscle Cells

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Abstract:

As of 2017, vascular diseases contributed to 23.1% of all deaths in America. To address the need for more effective and sustainable treatment options for these ailments, stem cell differentiation and implantation has emerged as a viable alternative to standard bypass and graft insertions. A completely autologous treatment can be achieved by extracting adult stem cells, differentiating them into vascular smooth muscle cells (VSMCs), and then reimplanting these cells at the affected tissue site. This study aims to investigate the efficiency of the VSMC differentiation from human mesenchymal stem cells (hMSCs) by comparing 4 cell lines of untreated hMSCs with 3 cell lines of hMSCs that have been grown in two differentiation factors–platelet derived growth factor (PDGF) and transforming growth factor **β**-1 (TGF**β**-1)–in order to mature into VSMCs. The cell lines will be evaluated based on variations in RNA expression. Total RNA will be isolated from the cell lines and subsequently sequenced. Raw data will be analyzed using bioinformatic techniques to determine which genes are transcribed significantly differently between hMSCs and hMSC-derived VSMCs. Total RNA is being sequenced so that the transcription rates of all genes may be compared between cell lines; however, expected outcomes of known hMSC and VSMC markers would include increased transcription of CD 29/44/73/105 and decreased transcription of MYH11, ACTA2, and TAGLN in hMSCs. Converse results would be expected in the hMSC-derived VSMCs. Elucidating the specific variations in transcription levels between hMSCs and hMSC-derived VSMCs will lead to the development of standardized chromatin immunoprecipitation sequencing (ChIP-Seq) assays that can determine if post-treatment hMSCs have been successfully differentiated into VSMCs, leading to accurate autologous stem cell treatments to various cardiovascular diseases.

Introduction:

Background

Currently in the United States, cardiovascular diseases–which encompass both heart and blood vessel-related ailments–are the highest cause of death in the general population (Benjamin 2019). Under typical circumstances, this category of diseases contributes to approximately a quarter of all deaths; however, this percentage is only expected to rise due in part to a corresponding increase in the aging population as well as a growth in the number of people being diagnosed with diseases like diabetes that are comorbid to cardiovascular disease.

Stem cells are an invaluable component of tissue engineering based treatments for injuries and diseases affecting complex vasculature. They allow for the realm of tissue engineered vessels to be broadened by the fact that they can contribute to the angiogenesis of simple capillary vessels without scaffolds or ECM to guide this regeneration. The inverse is not true. The most effective means of involving stem cells in engineering vascular tissue is by combining cells with varying levels of potency as these different types of cells have been shown to contribute to vascular regeneration through a range of means including secreting differentiation factors to induce surrounding stem and progenitor cells to differentiate and exhibiting the ability to differentiate into cells located in all three layers of large vessels.

As various novel cellular therapies are investigated through clinical research and eventually approved for use in medical treatments, studies must be concurrently conducted to ensure that cell populations can be developed efficiently and inexpensively while maintaining uniformity and fulfilling demand (Dwarshius 2017). Most cell therapies require that patients take

several doses during the course of their treatment, and each dose typically contains a few billion cells, bringing the total number of cells needed per patient per treatment up to ~ $10^{10} - 10^{12}$ cells.

With regards to human vasculature, many varieties of stem cells have been shown to be capable of differentiating into both vascular endothelial cells (ECs) and smooth muscle cells (SMCs) which are located in the tunica intima and tunica media of larger vessels, respectively (Dimitrievska 2018; Lin 2017; Gui 2016; Patsch 2015; Guerin 2015). An example of a successful application of cell therapy is the positive effect of human mesenchymal stem cell-derived smooth muscle cells (hmSMCs) on abdominal aortic aneurysms, acute myocardial infarction, and other expressions of cardiac disease (Swaminathan 2017; Gu 2017).

The research surrounding MSCs and their direct ability to differentiate into vascular cells is disputed (Patsch 2015 Ge 2018). Despite this result, one of the studies focusing on MSCs reported that, *in vivo*, undifferentiated MSCs secreted VEGF—a growth factor that can influence other stem cells and progenitor cells to further differentiate into different vascular cells (Ge 2018). These results may indicate that a combination of stem cells with varying levels of potency could be utilized for optimal *in vivo* angiogenesis.

For the purposes of this project, focus is placed on the ability of hMSCs derived from human adipose tissue to differentiate into smooth muscle cells. It is the long-term goal of this research that healthcare providers can begin conducting individual cardiac patient-centered care by treating them with hmSMCs derived from their own stem cells and expanded to a viable dose using human fibronectin-containing media. By obtaining stem cells from the patients themselves, the risk of immunologically rejected VSMC implantation is greatly reduced as there are no foreign biological or synthetic materials being introduced to the body.

In order to confirm that smooth muscle cells created under various culture conditions have characteristics consistent with native smooth muscle cells, multiple methods may be employed to compare cellular genomes, proteomes, and metabolomes. By using total RNA sequencing, it is possible to both ensure that cells differentiated from hMSCs are no longer expressing typical hMSC gene products as well as affirming that they are expressing various SMC gene markers. Additionally, this method can be used to verify that there is no significant difference between hMSC-derived SMCs cultured in either fetal bovine serum (FBS) or serum-free media (SFM).

Typically, in order to establish the control presence of hMSCs, cells are tested for the upregulation of markers such as CD73 and CD105 (Singh 2016; Lemcke 2017). In the past, the Rao lab has also used the presence of CD29 and CD44 as additional hMSC markers.

In contrast, typical SMC markers that have been used for genomic confirmation include: ACTA2, TAGLN, and MYH11 (Brun 2015; Ji 2016). The Rao lab has also used SM22$\boldsymbol{\alpha}$ in previous proteomic studies to indicate the successful differentiation of SMCs from hMSCs.

Objectives

The main objective of this study is to use total RNA sequencing to confirm that post-differentiation cells exhibiting changes in phenotype that are characteristic with smooth muscle cells are also up- or down-regulating specific SMC gene markers.

Expected Outcomes

It is expected that there will be a significant difference in the expression and transcription levels of various genes between the MSC samples and SMC samples. If this is not the case, then that implies that although there may have been a discrepancy between the phenotype of these cell populations and their genotype. However, if there are specific gene products that are found to be transcribed at a higher rate in one of the cells types than the other, then these findings can be used to further confirm differentiation of SMCs from hMSCs in future research.

Among the known gene products that are expected to be relatively upregulated in hMSCs are those associated with protein markers CD 29/44/73/105. Conversely, gene products expected to be upregulated relatively higher in the SMCs compared to the hMSCs in this study are those corresponding to protein markers MYH11, ACTA2, and TAGLN

Materials and Methods:

Cell Culture

For this experiment, undifferentiated and differentiated cells from four cell lines were used to compare the variations in genetic transcription between human stem cells and stem cell-derived smooth muscle cells. **Table 1** in the Appendix specifies the cell lines used, their origins, the differentiation treatment used, and the sample number assigned to each cell type.

All cells were seeded in T-75 flasks and incubated at 37℃ with 5% O_2. Media for all flasks was changed every 2-3 days, and flasks were passaged once they reached approximately 80% confluency or greater. Undifferentiated samples 2, 4, and 6 were fed using human fibroblast-containing SFM, and differentiated samples 3, 5, and 7 were fed using serum-free

differentiation media to maintain their differentiated state. Flasks containing ATCC3 cells were fed using a specific ATCC3 differentiation media.

Cell Count

In order to collect enough genetic material to yield substantial RNA analysis, each sample must have between $10^5 - 10^7$ cells, which is approximately the number of cells that a T-75 flask holds once the culture has reached total confluency. To confirm that the number of cells was in the range needed to continue to RNA isolation, cell counting was performed.

First, media containing cell waste and dead cells was aspirated from the flasks under a hood and disposed of in a sterile container. 2 mL PBS was added to each flask and aspirated to wash the cells. This step was then repeated. 3.5-5 mL of trypsin was added to each flask to detach and lift the cells from the bottom of the flasks. The flasks were then incubated at 37℃ at 5% O_2 for 5 minutes. During this time, one 10 mL centrifuge tube and one microcentrifuge tube were labelled to correspond with each flask. 10 µL of trypan blue was added to each microcentrifuge tube.

After 5 minutes, the flasks were physically agitated, observed under a microscope to ensure that the cells have detached, and then placed back under the hood. At this time, the trypsin/cell solution was removed from each flask and placed in an appropriately labelled 10 mL centrifuge tube. An equal amount of trypsin neutralizing solution (3.5-5 mL) was added to each flask to collect any stray cells, and then added to the corresponding 10 mL centrifuge tube. At this time, the flasks were inverted several times to distribute the cells equally in the ~7mL-10mL of solution. Then, 10 µL of the cell solution was removed from each 10 mL centrifuge tube and

added to the corresponding microcentrifuge tube. The 10 mL centrifuge tubes were then centrifuged for 4 minutes at 12,000 RPM or ~16,000 G.

While the cell solutions were being centrifuged, a pipette was used to mix the contents of each microcentrifuge tube. For each sample, 1 µL of the trypan blue cell solution was added to each side of a hemocytometer that was then loaded into an automated cell counter, which provided a cell count once the user input the total volume of the cell solution.

Sample Preparation

Once cells were counted and all of the samples were centrifuged, the solution was removed from the centrifuge tubes, taking care not to disturb the cell pellet at the bottom of the 10 mL centrifuge tube. 1 mL of trizol was added to each tube, cells were resuspended, and samples were moved to microcentrifuge tubes before being incubated for 5 minutes at room temperature.

Following room temperature incubation, 200 µl of chloroform was added to each tube and tubes were then vortexed for 15 seconds. Samples were again incubated at room temperature for 2-3 minutes before being centrifuged at 12,000 RPM, or ~16,000 G, for 15 minutes at 2-8°C. After this phase separation sequence, the supernatant from each sample was transferred to autoclaved centrifuge tubes before proceeding to the Qiagen RNeasy RNA Isolation protocol.

RNA Isolation

Once isolated, the aqueous samples from the previous step were centrifuged at 23°C for 5 min at 5000 G, or ~6500 RPM. Following centrifugation, the supernatants were discarded, and

350 µL of RLT buffer was added per tube. The tubes were then vortexed on auto for 2-5 minutes to incorporate the RLT buffer with the cells. The mixtures were then transferred to purple Qia Shredder Spin Columns and centrifuged for 2 minutes at 21,000 G, or ~13,500 RPM.

350 µL of supernatant was collected from the Spin Columns following centrifugation and pipetted into clear gDNA eliminator columns. These samples in these columns were then centrifuged again at 10,000 G, or ~9,500 RPM, for 30 seconds. 350 µL of 70% ethanol was then added to flow-through the gDNA eliminator column and mixed with the existing buffer solution. Then, 700 µL from each sample was added to pink RNeasy columns before discarding the flow-through from each of these columns. The filters were saved and placed back into their respective columns before 700 µL of RW1 buffer was added. Following this addition, the columns were centrifuged again at 10,000 G, or ~9,500 RPM, for only 15 seconds.

After centrifugation the flow-through was discarded once more, the filter was replaced back in the column, and 500 µL of RPE buffer was added to each column. The samples were again centrifuged at 10,000 G, or ~9,500 RPM, for 15 seconds, and again the flow-through was discarded.

500 µL of RPE buffer was added to each sample once more before samples were centrifuged at 10,000 G, or ~9,500 RPM, for 2 minutes and flow-through was subsequently discarded. The filters were then transferred to collection tubes and centrifuged for a penultimate time at 21,000 G, or ~13,500 RPM, for 1 minutes. The filters were again removed from the collection tubes and placed in Eppendorf tubes. It is important to note that this set-up is intentional despite the inability to fully close the Eppendorf tubes when the filters are present.

Finally, 40 μL RNAse-free water was pipetted straight onto each filter and the tubes with filters attached were centrifuged at 10,000 G, or ~9,500 RPM, for 1 minutes. Filters could then be discarded, and the Eppendorf tubes were stored in a -80°C freezer until RNA quality could be assessed.

RNA Concentration and Quality Determination

Samples were removed from the -80°C freezer and placed immediately on ice with a bottle of RNAse-free water in a large beaker before being transported to a plate reader. The plate itself was prepared by being sprayed with RNAse Away and wiped down with sterile Kimwipes. Before loading samples in the plate reader, the Gen 5 program was opened and parameters were selected to determine the concentration and quality of RNA samples. The final step in preparing the program was selecting the A1 and A2 squares on the grid and marking them as blanks to calibrate the plate reader.

Once the program had been set up, 2 μL of RNAse-free water was pipetted using a filtered PCR pipette in each of the sample positions that corresponded with the grid locations selected during the previous step. The option to "Read Blanks" was selected before carefully closing and placing the plate inside the plate reader and pressing "OK." If the A1 and A2 spots on the program's grid displayed green dots, then the plate reader had been calibrated appropriately and sample concentrations and quality could be determined. However, if the A1 and A2 spots displayed red dots, then the machine had not been calibrated correctly, and the plate needed to be cleaned before loading another set of blanks and repeating the above steps.

Following calibration, the 14 grid spots on the program needed to be labelled with the names of the samples that would correspond to their positions on the plate, keeping in mind that duplicate trials are to be performed on each sample. After preparing the computer program, 2 µL per sample per trial were loaded in their predetermined position on the plate again using filtered PCR pipettes. The plate was then closed carefully and placed inside the plate reader before selecting "Read Sample" on the program. The subsequent data was then exported to an Excel spreadsheet and emailed to the researcher after which the plate was cleaned with RNAse Away and Kimwipes before replacing it to its storage case.

Concentrations were reported in ng/µL and the 260/280 value, which indicates sample purity, was reported as a unitless number. Ideal 260/280 numbers should be in the range of 1.9-2.1.

In addition to checking the sample concentration and quality using a plate reader available for use by the researcher, samples were also delivered to another on-campus facility that assessed these characteristics as well using a nucleic acid tapestation. Concentrations were reported in ng/µL like the plate reader; however, the quality was reported in the form of an RNA Integrity Number (RIN) where good quality samples are considered to have a RIN that is greater than 7.

Sample Transportation

In order to sequence the total RNA, samples needed to be shipped to the Novogene genomic sequencing lab in Davis, California on the University of California at Davis campus. The seven microcentrifuge tubes containing the seven individual samples were packed in a large

conical tube with Kimwipes to ensure that the microcentrifuge tubes would not be displaced during travel. This large conical tube was then placed in ~5 lbs of dry ice packed in a styrofoam box that was taped shut and shipped in a cardboard container.

Results:

Unfortunately, due to delays caused by the ongoing COVID-19 pandemic, there have been some delays retrieving total RNA sequencing data from Novogene. This has not, however, interfered with the concentration and quality data collected from two separate sources that will be outlined below.

Based on the results gathered from the plate reader, which can be found in **Table 2** in the Appendix, only samples 1 and 3 had an average 260/280 number that was not within the accepted range of 1.9-2.1. Interestingly, there was a clear pattern between the concentrations of undifferentiated cells and differentiated cells of each cell line that demonstrated much higher concentrations of RNA amongst the undifferentiated samples, samples 2, 4, and 6, that ranged from 28.9 ng/μL to 99.7 ng/μL. Conversely, the differentiated samples 1, 3, 5, and 7 all had an RNA concentration between 5-6 ng/μL with only one outlier that was 15.7 ng/μL.

The tapestation delivered somewhat similar results to the ones outlined above. Exact values for the tapestation tests are located in **Table 3**. With regards to the quality, the RIN for all of the samples was reported to be exactly 10.0, well above the 7.0 threshold needed to proceed with further testing. These results contradict with the results from the plate reader that determined based on the 260/280 that samples 1 and 3 were not of a high enough standard to move forward with future testing. Concentration values derived from the tapestation test were

remarkably similar to those received from the plate reader. Again, undifferentiated samples 2, 4, and 6 exhibited RNA concentrations in the range of 41.2 ng/μL to 121 ng/μL, whereas differentiated samples 1, 3, 5, and 7 all reported RNA concentrations between 5-6 ng/μL with the same outlier at 18.1 ng/μL.

Analysis and Discussion:

Conclusion

As mentioned above in the Results section of this paper, due to delays related to the COVID-19 pandemic, RNA sequencing data for this project has been delayed and cannot be discussed at the present moment.

However, the concentration data alone indicates that there are significant differences between the overall transcription levels of the undifferentiated and differentiated cell samples. When the concentration data derived from the plate reader was subject to an independent one-tailed equal variance t-test comparing the average concentration of the undifferentiated samples 2, 4, and 6 with the differentiated samples 1, 3, 5, and 7, a p-value 0.017 was returned. The same test administered on the same sample groups using data from the tapestation also yielded a p-value of 0.011. Both of these values are well under the threshold of $p<0.05$ that indicates statistical significance. This confirms that there is a quantifiable difference in the total RNA concentration levels of these two samples. Because all procedures for this project were performed on all samples simultaneously through the period of this research, it is most likely that these differences are due to variations in DNA transcription that would lead to an overall lower level of RNA present in differentiated SMC samples than in undifferentiated MSC samples.

Future Directions

The first and foremost step that will be taken in this project is to incorporate the delayed RNA sequencing data to complete this honors thesis. This will include bioinformatic analysis to determine if there are specific genes that are more highly expressed in either differentiated or undifferentiated samples. It is expected that among the more highly transcribed genes in undifferentiated MSCs will be genes associated with CD 29/44/73/105 proteins, and in differentiated SMCs, genes corresponding to MYH11, ACTA2, SM22α and TAGLN proteins will be more highly transcribed.

Beyond this research project, the information derived from the RNA sequencing data obtained through these samples may be able to help create a standardized chromatin immunoprecipitation sequencing (ChIP-Seq) assay that could be used to confidently assess whether SMCs differentiated from MSCs reflect genotypic as well as phenotypic characteristics of native smooth muscle cells.

Eventually, it is the long-term goal of this lab to create stem cell-based treatments for various cardiovascular ailments and diseases that are safe for patient use, consistent between treatment doses, and easily producible by clinicians and healthcare providers.

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Appendix:

Table 1: Sample numbers, cell lines, differentiation status, and origin tissue

Sample Number	Cell Line	Cell Type	Origin
1	ATCC3	Differentiated with PDGF+TGF**β**-1	hMSCs derived from 21-year old vietnamese male
2	41035	Undifferentiated	Human adipose tissue
3	41035	Differentiated with PDGF+TGF**β**-1	Human adipose tissue
4	82726	Undifferentiated	Human adipose tissue
5	82726	Differentiated with PDGF+TGF**β**-1	Human adipose tissue
6	99375	Undifferentiated	Human adipose tissue
7	99375	Differentiated with PDGF+TGF**β**-1	Human adipose tissue

Table 2: Sample characteristics derived from the plate reader. Concentrations reported in ng/µL and quality reported in unitless 260/280 numbers.

Sample Number	Concentration (ng/µL)	260/280
1	6.6	1.75
2	28.9	1.94
3	6.6	1.77
4	99.7	2.02
5	15.7	1.96
6	50.2	2.03
7	5.3	1.91

Table 3: Sample characteristics derived from the on-campus tapestation. Concentrations reported in ng/μL and quality reported in unitless RIN.

Sample Number	Concentration (ng/μL)	RIN
1	5.1	10.0
2	41.2	10.0
3	6.1	10.0
4	121.0	10.0
5	18.1	10.0
6	63.0	10.0
7	5.1	10.0