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Stirred Suspension Bioreactor Differentiation of Human Mesenchymal Stem Cells into Smooth Muscle Cells

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by

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Table of contents

Abstract

Human mesenchymal stem cells (hMSCs) are a promising candidate for cellular therapies due to their multipotency, self-renewal capacity, and immunomodulatory properties. However, their isolation is a difficult and potentially painful process with very low yield, and traditional static mammalian cell culture techniques are too slow and expensive for large scale growth and differentiation of stem cells to be practical. Current research is focused on improving methods for cultivating hMSCs in stirred suspension bioreactors (SSBs), but little work has been done with regard to their differentiation in dynamic conditions such as those in SSBs. Differentiation at a large scale would increase the amount of paracrine factors released that can potentially be isolated and purified for further use. The main goal of this research is to establish how effectively hMSCs differentiate into SMCs in stirred suspension bioreactor conditions. The initial findings suggest that the cells were successfully growing inside the bioreactor, but the differentiation results were inconclusive due to very low expression of the examined markers. Issues with the staining and flow cytometry procedures need to be fixed before more samples are tested.

Introduction

Mesenchymal stem cells (MSCs) are a type of adult stem cell that have traditionally been extracted from bone marrow, but are present in many different tissues in the human body. They have the capability to differentiate into many different cell types, such as the ones present in bone, cartilage, muscle, fat, etc (6). While they don't have the same differentiation capability as embryonic stem cells and induced pluripotent stem cells, MSCs also don't have some of their disadvantages. Both ESCs and iPSCs have a risk of forming teratomas after transplantation and there is ongoing debate about the ethics of ESC use (7). Due to the multipotecy, self-renewal capacity and immunomodulatory properties of MSCs, they have become a standard for research in the field of regenerative medicine (5). While mesenchymal stem cells can be obtained from several areas in the body, there are advantages and disadvantages to each source since they don't yield identical MSCs (6). Out of all MSCs, bone marrow MSCs are the most widely studied and clinical trials have confirmed their safety and effectiveness (9). hBM-MSCs have extensive differentiation capability, but they are difficult to retrieve and the process can be very painful for the donor. Billions of cells need to be used in order for stem cell therapies to be effective (1), but it is not feasible to extract a substantial amount as MSCs are about 0.001% of cells in the bone marrow (8). Traditional static methods of mammalian cell culture are expensive and slow to upscale, so another method needs to be used to grow the necessary amount of cells for clinical purposes.

Over the past decade many advances have been made to improve the efficiency of culturing stem cells in dynamic conditions. There have been effective protocols developed for growing different types of stem cells in stirred suspension bioreactors, including pluripotent and mesenchymal stem cells (2-4). There has not, however, been much development in establishing meth-

ods for stem cell differentiation while they are still in suspension in a bioreactor. Successful differentiation in SSB culture would result in an increased amount of paracrine factors being released which could then be purified and processed for further use.

This study examines the proliferation and differentiation of of hB-MSCs into smooth-muscle-like cells in a stirred suspension bioreactor. The MSCs were first attached to microcarriers and allowed to grow in the bioreactor for two days. Afterwards, seven day differentiation process was carried out using PDGF and TGF-β1 to induce differentiation in the stem cells.

Materials and Methods

Spinner Flask Differentiation of hBM-MSCs

1 vial of hBM-MSCs, 1 vial of hAoSMCs, and 1 vial of hIAoSMCs were seeded onto respective T-75 flasks and cultured until 90% confluence was reached, after which each cell type was passaged to a T-175 flask. hBM-MSCs for static differentiation were seeded onto a human fibronectin coated (20 µg/ml sterile PBS) T-175 flask and cultured until >90% confluence was reached while hAoSMCs and hIAoSMCs were further passaged for analysis. Passaged hBM-MSCs were counted and evaluated for viability via hemacytometer and trypan blue staining. Before the seeding procedure, 2.5 grams of plastic plus (SoloHill) microcarriers were incubated in a 50 ml conical tube (0.5 g/ 50 ml medium) with human fibronectin (0.17 µg/cm^2) in 50 mL sterile PBS for 2 hours at room temperature. The fibronectin solution was removed and the microcarriers were resuspended in 50 mL of hBM-MSC medium. The microcarriers were added to 200 ml hBM-MSC growth medium in spinner flask. hBM-MSCs were seeded onto hBM-MSC growth medium conditioned and human fibronectin coated polystyrene microcarriers (1.5 x 10^7 cells/spinner flask). The cell-microcarrier mixture was agitated via gentle stirring (25 rpm) every 15 minutes for the first hour and then allowed to sit static overnight at 37ºC and 5% CO2 to fa-

cilitate cell attachment to polystyrene microcarriers. The cell-microcarriers suspension was then stirred at 60 rpm for 24 hrs at 37ºC and 5% CO2, after which the hBM-MSC growth medium was aseptically removed from the spinner flask and replaced with hBM-MSC SMC differentiation medium. hBM-MSCs were cultured in differentiation medium containing PDGF (5 ng/mL) and TGF-β1 (2.5 ng/mL) for 7 days. There was a 50% medium change after 4 days. Daily 1 ml medium samples were taken and stored for metabolic analysis, and stored at -20 ºC. The differentiation medium was aseptically removed from the spinner flask, and 100 ml of sterile DPBS was added to the flask The microcarriers were gently stirred at 40 rpm for 10 minutes and allowed to settle before the DPBS was aseptically removed. This step was repeated once to remove any residual medium. The PBS was aseptically removed and 100 ml of TrypLE Express (Gibco) was added to the microcarriers and stirred at 150 rpm for 7 minutes in 37ºC and 5% CO2s. The cells were then filtered through a 40 μm cell strainer immediately after dissociation. Equal volumes of trypsin neutralizing solution were added to the centrifuge tubes containing the filtered single cell-suspensions. 100 μL of cell suspension sample was used for cell counting and viability analysis via trypan blue staining and for FACS analysis.

FACS

Immediately following cellular harvest, the cells were stained for expression of CD44 (MSC marker) and myosin heavy chain 11 (SMC marker). Following a viable cell count, cell suspensions were centrifuged at 300 x g for 10 minutes and re-suspended in 1 X sterile PBS with 1% w/v bovine serum albumin. 1×10^5 cell aliquots were added to tubes and centrifuged at 10,000 x g for 15 s and washed twice with FACS buffer (1 X sterile PBS, 25 mM HEPES, 1% w/v BSA, 0.02% w/V NaN_3, 0.5 mM EDTA). Cells were then fixed in the dark for 20 min using IC fixation buffer, then permeabilized with 1 X permeabilization buffer Cells were then incubat-

ed in the dark for 15 minutes with SM-22$^+$/MHC$^+$ cells with PE-Cy7-conjugated goat polyclonal

SM22α antibody and AlexFlour 647-conjugated anti-smooth muscle myosin heavy chain (SM-

MHC) mouse IgG1 antibody. Cells were permeabilizaed, centrifuged at 10,000 x g for 15 s,

washed twice with permeabilization and FACS buffers separately, and re-suspended in FACS

buffer before being filtered through a 40 μm cell strainer. Cells were analyzed using a BD flow

cytometer, and 20,000 events were collected from each experimental group. The data was ana-

lyzed using FlowJo.

Ammonia Assay (Sigma Aldrich®)

The ammonia assay reagent was reconstituted in 10 mL of sterile DI water, and then ali-

quoted and stored at -20 ℃. The following solutions were pipetted into marked cuvettes: reagent

blank (1.0 ammonia assay reagent, 100 μL), test (1.0 ammonia assay reagent, 100 μL), and

standard (1.0 ammonia assay reagent, 0.05 mL). A spectrophotometer was set to 340 nm and the

absorbance to zero using water as the reference. The contents in each cuvette were mixed and in-

cubated for 5 minutes at room temperature, after which the absorbance at 340 nm was measured.

10 μL of L-glutamate dehydrogenase solution were added to each cuvette, mixed and incubated

for 5 minutes at room temperature. The absorbance was measured again at 340 nm.

Calculation:

Determining ΔA_{340} for the reagent blank, test, and standard.

1. For each: $\Delta A_{340} = A_{initial} - A_{final}$

2. $\Delta(\Delta A_{340})$Test or Standard $= \Delta A_{340}$(Test or Standard) $- \Delta A_{340}$(Blank)

Determining mg of NH_3/mL of original sample

1. C = (A*TV*MW of Ammonia)(F)/(ε*d*SV*Converson Factor μg to mg)

2. C = (A*TV*17*F)/(6.22*1*SV*1,000)

3. C = ((A*TV*F)/SV)*0.00273

A: absorbance of test or standard

TV: total assay volume in mL

SV: sample volume in mL

F: dilution factor from sample preparation

ε: millimolar extinction coefficient for NADPH at 340 nm

d: light path = 1 cm

C: concentration in mg/mL

Lactate Assay (Sigma Aldrich®)

 Serum samples (0.5–10 µL/assay) were assayed directly by adding in duplicate to 96-well plate. If there is lactate dehydrogenase activity, samples should be deproteinized with a 10 kDa MWCO spin filter. Samples were brought to final a volume of 50 µL/well with Lactate Assay Buffer. For unknown samples, several sample volumes should be tested to make sure the readings are within the standard curve range. It should be noted that Lactate Dehydrogenase (LDH) degrades lactate. Samples that contain LDH (such as culture medium or tissue lysate) should be kept –80 °C for storage, and filtered through a 10 kDa cut-off spin filter. Complete medium containing FBS should be deproteinized due to high LDH content. The Reaction Mixes shown in the table were set up. 50 µL of the appropriate Reaction Mix is required for each reaction (well).

Reagent	Samples and Standards	Blank Sample
Lactate Assay Buffer	46 µL	48 µL
Lactate Enzyme Mix	2 µL	-
Lactate Substrate Mix	2 µL	2 µL

NADH or NADPH from cell or tissue extracts generates background for the lactate assay. To remove the effect of NADH or NADPH background, a blank sample may be set up for each sample by omitting the Lactate Enzyme Mix. The blank readings can then be subtracted from the

sample readings. 50 µL of the appropriate Reaction Mix was added to each of the wells. The solutions were mixed by pipetting, and incubated for 30 minutes at room temperature. During this time, the plate was protected from light. Afterwards, the absorbance at 450 nm (A450) was measured.

Calculations

1. The background for the assay is the value obtained for the 0 (blank) lactate standard. Correct for the background by subtracting the blank value from all readings. Background values can be significant and must be subtracted from all readings. Use the values obtained from the appropriate lactate standards to plot a standard curve.

2. Subtract the blank sample readings from the sample readings. The amount of lactate present in the samples may be determined from the standard curve.

Note: A new standard curve must be set up each time the assay is run.

3. Concentration of Lactate

a.   Sa/Sv = C

b.   Sa = Amount of lactate acid in unknown sample (nmole) from standard curve

c.   Sv = Sample volume (µL) added into the wells.

d.   C = Concentration of lactate acid in sample

e.   Lactate molecular weight: 89.07 g/mole

Results and Discussion

Ammonia Assay (Sigma Aldrich®)

All of the absorbance were measured at a wavelength of 340 nm. Ammonia is a metabolic by-product that is generally considered to be harmful to cell growth. An increase in concentration is expected during cell growth. The sharp drop at day 7 is due to the 50% media change. The data

for the first two days showed negative concentration, so those results were left out of the table

and graph.

| ΔA340	Replicate 1	Replicate 2	Change in Test		Average Absobance	Concentra-tion (mg/ml)
Blank	0.027	0.018				
Day 3	0.068	0.059	0.041	0.041	0.041	0.00123
Day 4	0.071	0.061	0.044	0.043	0.0435	0.00131
Day 5	0.056	0.067	0.029	0.049	0.039	0.00117
Day 6	0.078	0.14	0.051	0.122	0.0865	0.0026
Day 7	0.051	0.044	0.024	0.026	0.025	0.00075
Day 8	0.162	0.088	0.135	0.07	0.1025	0.00308
Day 9	0.076	0.118	0.049	0.1	0.0745	0.00224
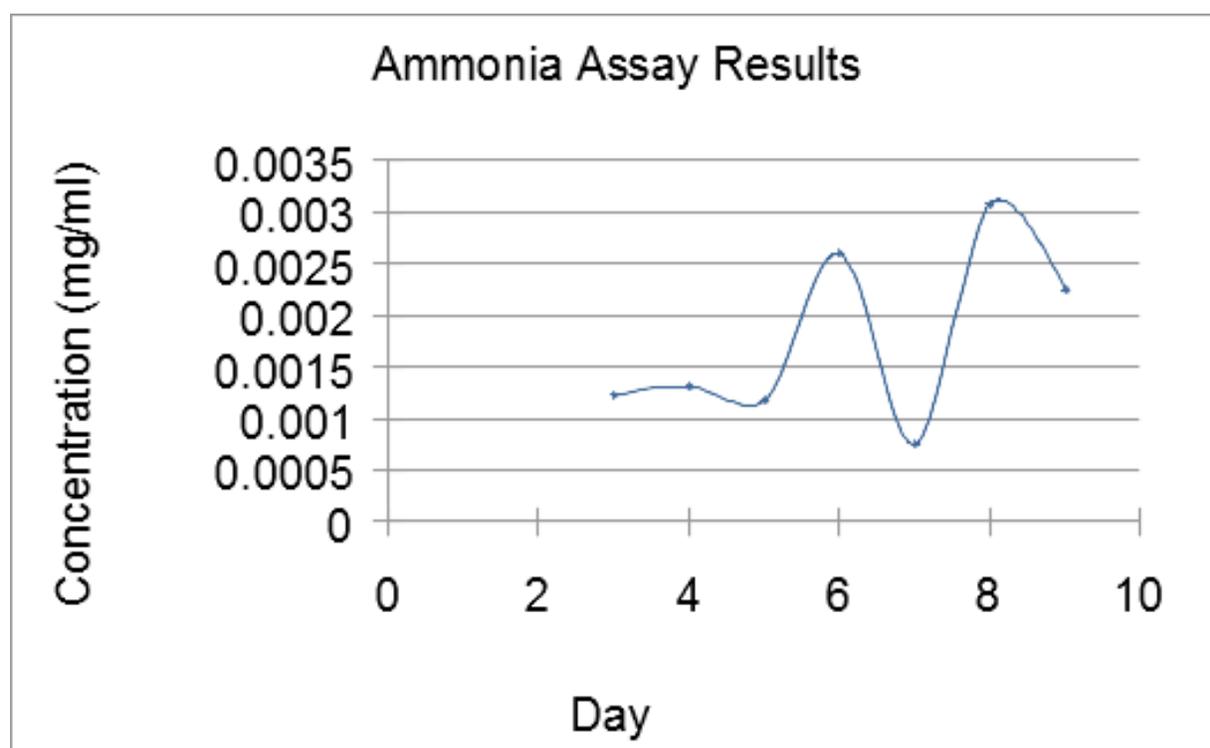
Lactate Assay (Sigma Aldrich®)

All readings were taken at a wavelength of 450 nm. Just like ammonia, lactate is a metabolic by-product that is produced by cells. After the standard stem cell growth media was completely re-placed with differentiation media on day 2, lactate concentration rapidly increased up until day 6, which suggests that the cells were growing during this time period. On day 7 the 50% media change resulted in a large concentration drop.

Standard concentration (nmole/well)	Average absorbance
0	0.0
2	0.2155
4	0.3820
6	0.4845
8	0.6535
10	0.8270

Standard Lactate Concentration Curve

$y = 0.079307x + 0.030548$

$R2 = 0.99229$
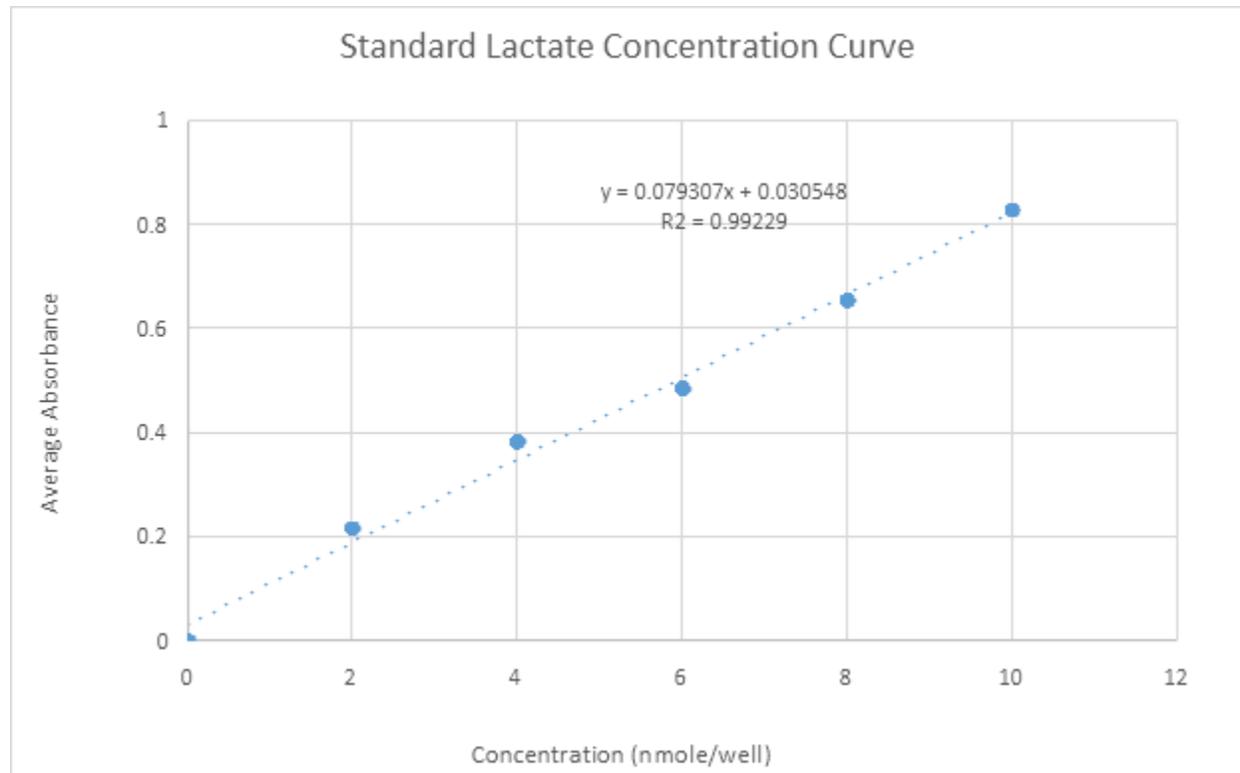
	Average Absorbance	Amount of Lactate (nmol)	Concentration (mg/mL)
Day 1	1.164	14.30	0.025
Day 2	1.244	15.31	0.027
Day 2.1	0.172	1.78	0.003
Day 3	0.351	4.05	0.007
Day 4	0.715	8.63	0.015
Day 5	0.592	7.08	0.013
Day 6	1.523	18.82	0.034
Day 7	0.921	11.22	0.020
Day 8	1.564	19.33	0.034
Day 9	1.175	14.43	0.026

Lactate Assay Results
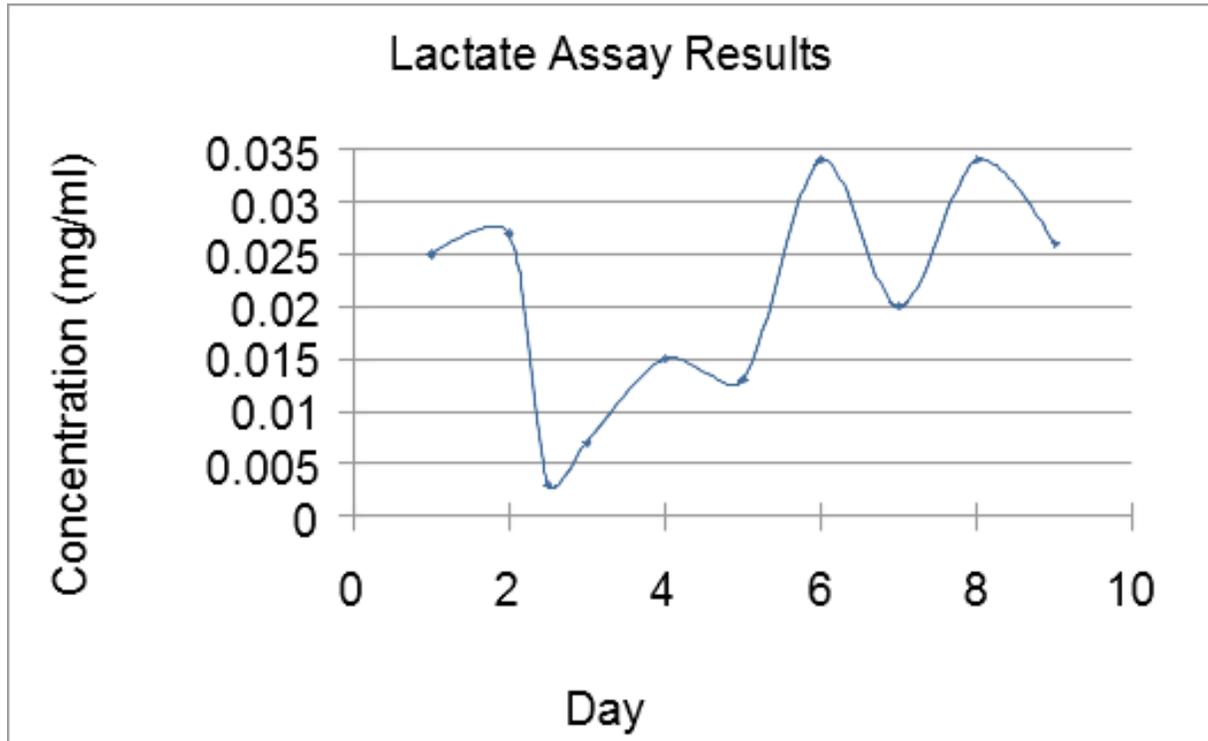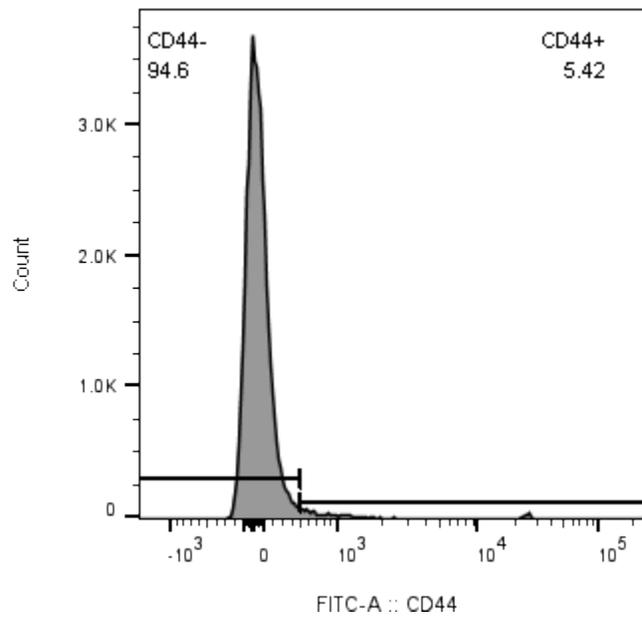
FACS

Positive Control (HASMC):

The FACS results showed that 9.73% of the smooth muscle cells expressed CD105 (stem cell marker) and only 2.55% expressed myh11 (smooth muscle cell marker).
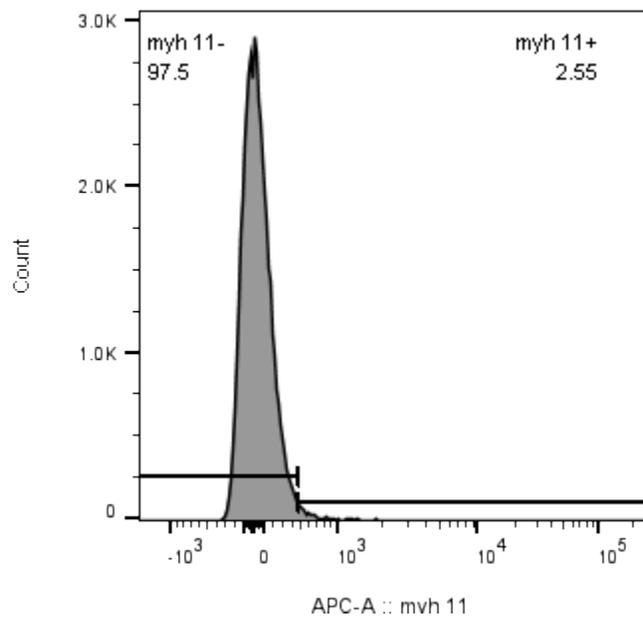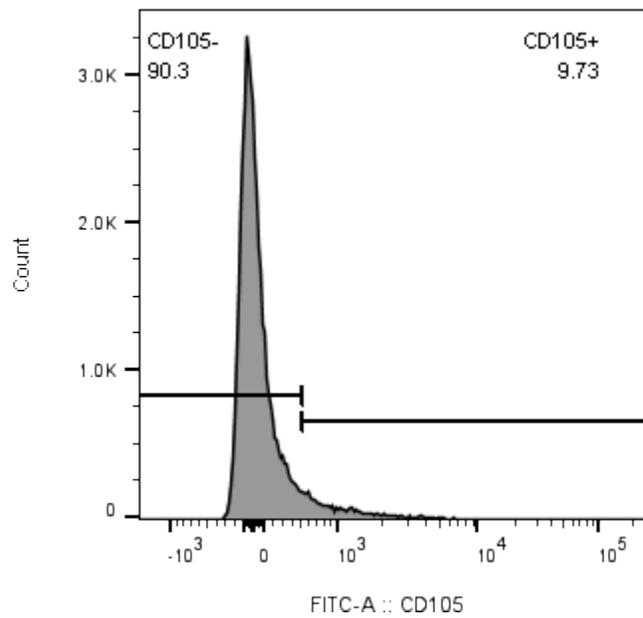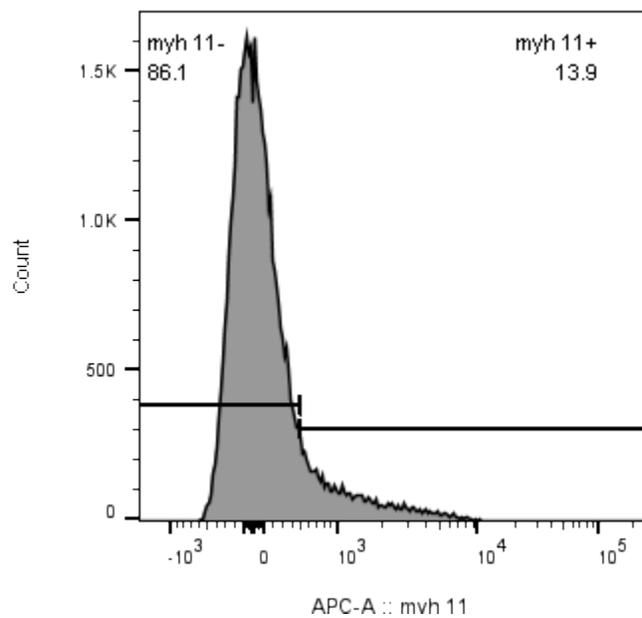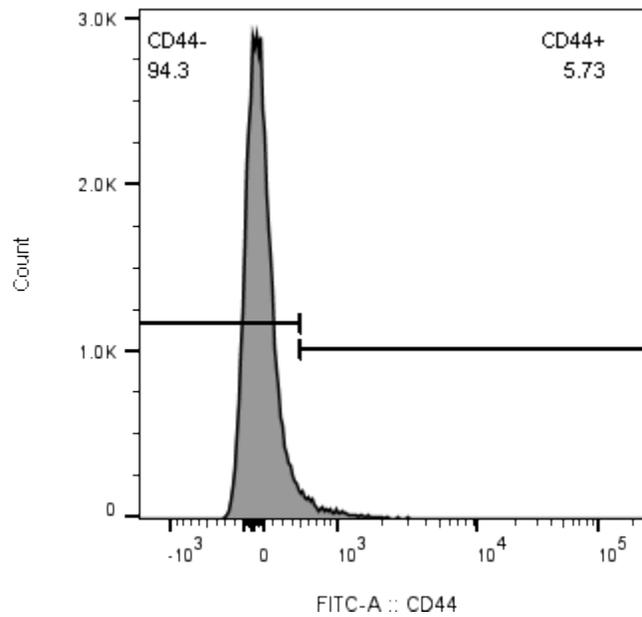
Negative Control (ATCC1 hBM-MSC):

The MSCs had higher expression of the SMC marker (7.57%) than the MSC marker (5.42%).

Just like the other control, results are the opposite of what is expected.
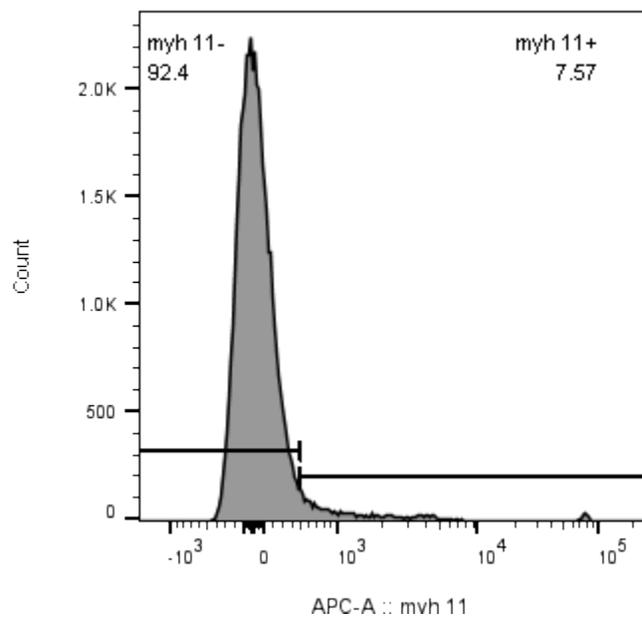
ATTC1 SMLC:

The cells taken from the bioreactor had 2.4 times higher expression of myh11 than CD44, but the

level of expression of both was still low.

Conclusion

The goal of this study was to examine how effectively hB-MSCs differentiate in stirred suspension bioreactors as well as how well they and the resulting smooth muscle like cells proliferate in those same conditions. Daily media samples were used to create a concentration profile for lactate and ammonia, byproducts whose production is associated with cell growth. With the exception of the sharp drops due to media change, the concentration of both increased steadily throughout the duration of the experiment. This data suggests that the mesenchymal stem cells were proliferating inside the bioreactor, but the FACS results were inconclusive and it cannot be said that differentiation took place with any certainty. While the harvested cells did have higher expression of the smooth muscle cell marker, there were large issues with the controls and the low level of expression of each marker. FACS analysis should be repeated in order to confirm the results. A possible cause of the low levels of expression is the method of staining. The same sample underwent extracellular staining for CD44 or CD105, then membrane permeabilization and intracellular staining for myh11. The test needs to be redone using separate samples in order to see if the issue lies in the staining process. After the issues present in the current protocol are solved, the next step in the project is to find methods of enhancing the efficiency of the process. Using SMC ECM particles as a microcarriers could be beneficial for the differentiation process and result in higher expression of smooth muscle cell markers.

Aknowledgments

I would like to pay my special regards to my mentors Christofer Baldwin, Dr. Raj R. Rao and Dr. Srikanth Sivaraman for taking me in and teaching me how to work with cell cultures before I had any on hand experience. Christofer Baldwin assisted me through every step of this project and I deeply appreciate everything he's done.

References

1. Berebichez-Fridman R, Montero-Olvera PR. Sources and Clinical Applications of Mesenchy-mal Stem Cells: State-of-the-art review. Sultan Qaboos Univ. Med. J. 2018;18:e264–e277

2. Kehoe DE, Jing D, et al. Scalable stirred-suspension bioreactor culture of human pluripotent stem cells. Tissue Eng Part A. 2010;16(2):405–421

3. Yuan Y., Kallos M. S., Hunter C., Sen A. Improved expansion of human bone marrow-derived mesenchymal stem cells in microcarrier-based suspension culture. *Journal of Tissue Engineering and Regenerative Medicine*. 2014;8(3):210–225

4. Stephenson M., Grayson W. Recent advances in bioreactors for cell-based therapies. F1000 Res. 2018;7:517

5. Shi M, Liu ZW, Wang FS. Immunomodulatory properties and therapeutic application of mes-enchymal stem cells. Clin Exp Immunol. 2011;164(1):1–8.

6. Murray I. R., Peault B. (2015). What is a mesenchymal stem cell, and why is it impor-tant? *BMC Biol.* 13:99. 10.1186/s12915-015-0212-7

7. Lukomska B., Stanaszek L., Zuba-Surma E., Legosz P., Sarzynska S., Drela K. Challenges and controversies in human mesenchymal stem cell therapy. *Stem Cells International*. 2019;2019:10. doi: 10.1155/2019/9628536.9628536

8. Matsiko A, Levingstone T, O'Brien F. Advanced strategies for articular cartilage defect repair. Materials 2013;6:637–68.

9. Kim H. J., Park J. S. (2017). Usage of human mesenchymal stem cells in cell-based therapy: advantages and disadvantages. Dev. Reprod. 21 (1), 1–10. 10.12717/DR.2017.21.1.001