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Cell Line-Specific Differences in Guided Differentiation of Adipose-derived Mesenchymal Stem Cells Towards Smooth Muscle Cells

Sherly Makar
University of Arkansas, Fayetteville

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Sherly Makar

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Abstract

Mesenchymal stem cells (MSCs) are a population of stromal cells found traditionally in the bone marrow and adipose tissues. They can also be found in other tissues including fallopian tube, core blood, peripheral blood, fetal liver, and lungs. Mesenchymal stem cells have profound effects in regenerative engineering, tissue repair and drug discovery owing to the excellent properties such as proliferation, self-renewal, and multipotency generating multiple cell types including adipocytes, osteocytes, cardiomyocytes (CMs), pericytes (PCs), and chondrocytes. MSCs are used as immunomodulators in generating progenitor cells to be used for transplantation, creating engineered organs, and preventing graft vs. host disease (GVHD). MSCs can differentiate into vascular lineages such as endothelial and smooth muscle cells (SMCs) which have a necessity for creating personalized cell therapies such that SMCs are considered a critical component of tissue-engineered vascular grafts. However, the use of mesenchymal stem cells is restricted by specific factors including their scarceness in tissues, donor age, culture media, origin of the cells, and gene expression profiles. For example, although adipose-derived and bone marrow-derived mesenchymal stem cells share many biological characteristics, they have some differences that affect their differentiation density, proliferation density, gene expression that results in different osteogenic and chondrogenic differentiation capacity. Consequently, these differences should be taken into consideration when planning stem cell-based therapy using MSCs. The goal of this research was to determine the effect of cell line-specific differences, specifically the donor origin, on the differentiation adipose tissue-derived mesenchymal stem cells (Ad-MSCs) towards SMCs. This goal was achieved using Ad-MSCs from a 30-year old Hispanic female (ATCC1 cell

line) as staining control, then comparing the expression of myosin heavy chain 11 (MYH11) between Ad-MSCs taken from a 24-year old Caucasian female (82726 cell line) and a 29-year old native American female (99375 cell line) after 4-day differentiation into SMCs using flow cytometer and FlowJo® software to analyze the data. The results proved that the donor's origin influences the differentiation of Ad-MSCs towards SMCs.

Introduction

MSCs are primitive multipotent progenitors present traditionally in the bone marrow and adipose tissues [1]. They can exist in peripheral blood, lungs, dermal tissues, intervertebral disc, amniotic fluid, human placenta, and cord blood [2]. In Vitro, MSCs are plastic-adherent fibroblast spindle-like shaped cells [3]. MSCs have promising future and tremendous potential in regenerative medicine and tissue engineering thanks to their capability of proliferating into daughter cells that have the same gene expression, phenotype, and stemness. MSCs can also differentiate into osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells), adipocytes (fat cells), endothelial and smooth muscle cells (SMCs) [4].

The reason why we are concerned about MSC-derived SMCs is because they proved their proliferative and impressive differentiation potential. For example, they could migrate into decellularized mouse aorta resulting in the formation of

smooth muscle layer of vascular grafts and that demonstrated they are a critical component in generating vascular grafts. They also displayed contractility *in vitro* and supported the formation of the vascular structure in the Matrigel plug assay *in vivo* [5]. Although there are surgical mainstays for peripheral vascular diseases such as synthetic grafts made of polytetrafluoroethylene (Dacron) or Teflon or autologous grafts from mammary blood vessels, these synthetic grafts are not compatible and can cause thrombosis and intimal hyperplasia. Consequently, instead of using such synthetic grafts, tissue engineering approaches like culturing SMCs on synthetic materials or natural material such as fibrin or silk to generate tubular conduits can fulfill this clinical need. However, one important factor that affects the success of these vascular grafts and their efficiency is the source of SMCs and the donor age [6].

In general, differentiation of MSCs in vitro is affected by many external factors and the culture conditions that should be controlled to direct the fate and the function of the resulted cells in clinical situations [7]. Some of these factors are the cell source, donor age, density of used cells, passage number, plastic surface quality, supplementary factors, oxygen concentration, and mechano-/electro-stimuli [8]. Geometric shape cues also affect the fate of MSCs by controlling the mechanochemical signals and paracrine/autocrine factors. When cells are exposed to a mixture of lineage cues, geometric shapes that enhance the contractility result in osteogenesis because these shapes increase the myosin that enhances the osteogenesis pathways. On the other side, geometric shapes that promote low contractility direct cells into adipocyte lineage. It was proved that

cells differentiate in response to shape cues in a way consistent with the native geometry of the cell lineage. For example, rounded cells with low stress enhance differentiation towards fat cells, and contractile pointed cells promote differentiation towards bone cells [9]. An example of the differences depending on the origin of the cells is that adipose and bone marrow-derived mesenchymal stem cells show differences in their immunophenotype, differentiation potential, immunomodulatory, transcriptome, proteome, and gene marker expression [10]. For example, adipose tissue-derived mesenchymal stem cells (Ad-MSCs) have higher expression of CD49d and lower expression of Stro-1 compared to bone marrow mesenchymal stem cells (BM-MSCs). In general, Ad-MSCs have higher proliferation, adipogenic capacity, more lipid vesicle formation, and higher expression of adipogenesis-related genes [11]. Ad-MSCs are also easier to be isolated, safer and give larger amounts compared to BM-MSCs. On the other hand, BM-MSCs have higher osteogenic and chondrogenic capacity compared to Ad-MSCs [10]. Consequently, BM-MSCs have higher calcium deposition and higher expression of osteogenesis and chondrogenesis-related genes [11]. Most importantly, despite the minor differences between these MSC populations, they both work effectively in clinical applications [10]. All these differences should be takes into consideration when using MSCs to generate other cell lineages to have the targeted function with the highest efficiency.

In this study, fluorescence-activated cell sorting (FACS) was used to sort heterogeneous mixtures of biological cells, one cell at a time, based on the fluorescent characteristics of each cell. FACS is based on the phenotype

differences between cells since each cell type has certain phenotype, certain extracellular and intracellular molecules, and different gene markers [12]. Typically, the cells in the suspension are tagged with fluorescent antibodies. These tags allow cells to be identified and isolated into a liquid medium for further analysis using FlowJo® software. When cell particles pass through a laser beam, they are monitored and are given positive or negative charge based on the presence or the absence of the fluorescently tagged antibody, respectively. Then these particles are separated based on their charge depending on their electric field into collection tubes with respect to the primary antibodies tagged with the fluorescent antibodies [13]. In this paper, cells were fixed, blocked, mixed with primary and secondary antibodies that attached to the extracellular gene markers. Then cells were permeabilized to provide access to intracellular antibodies, followed by mixing the cells with another primary and secondary antibodies that attached to the intracellular gene markers. The secondary antibody is a fluorescent molecule that attaches to the primary antibody specific for a particular cell surface protein after itself being attached to the extracellular or the intracellular gene markers. Figure (1) is a representation of FACS as it shows a population of mixed cells sorted into a negative and a positive sample containing cells of interest based on of the florescence dye [14]. Figure (1) also shows forward (FSC) and side scattering (SSC) gating that are commonly used when cells are distinguished based on their size and complexity, respectively, based on light reflection [14]. This gating is always used when studying cells of different sizes and complexity such as red blood cells and white blood cells but in this study the focus was on the differentiation of MSCs only, so we used the fluorophore expression.

The goal of this study was to examine the effect of cell line-specific differences, especially the donor origin as a biological variable in guiding differentiation of Ad-MSCs towards SMCs. This goal was achieved using three different cell lines from different donors: a 30-year old Hispanic female (ATCC1, which is the staining control), a 24-year old Caucasian female (82726) and a 29-year old native American female (99375). Each cell line was seeded in culture media and platelet-derived growth factor mixed with transforming growth factor beta1 (PDGF+TGFβ1), the differentiation media, for 4 days. Then Fluorescence-Activated Cell Sorting (FACS) was used to detect intracellular and the extracellular cell markers and data were analyzed using FlowJo® software.

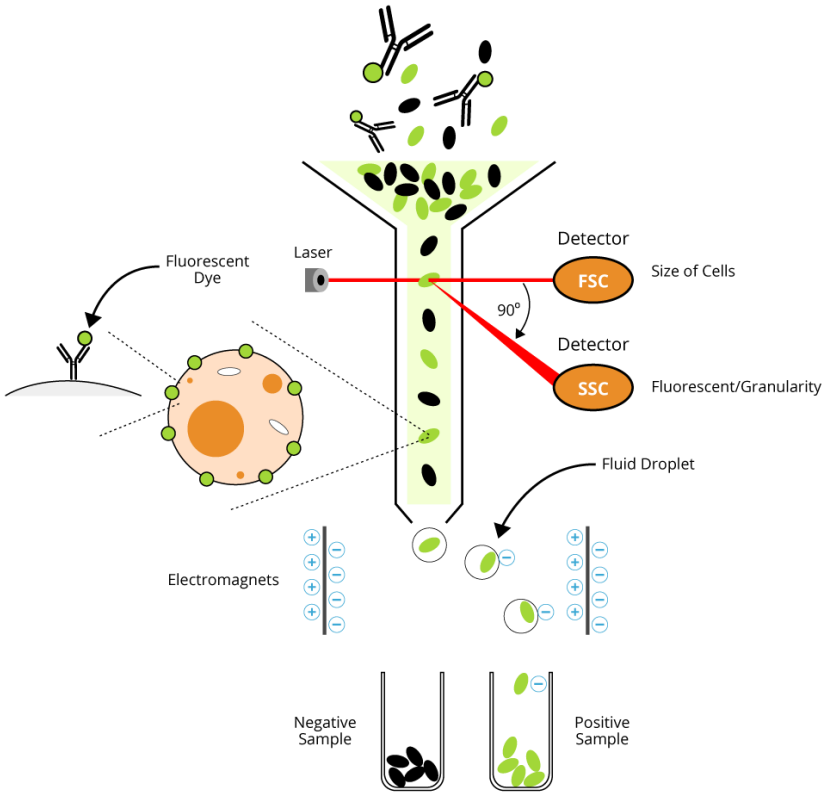


Figure (1) represents fluorescence-activated cell sorting (FACS). FSC is forward scattering & SSC is side scattering. Obtained from https://www.bosterbio.com/protocol-and-troubleshooting/flow-cytometry-principle#flow_principle [14].

Methods

Thawing and Passaging Cell Lines

Thawing and passaging is done in the laminar flow and before using the hood it should be sterilized by sparing 70% ethanol. Also, everything that enters the hood should be sprayed first to avoid contamination. The three cell lines that were used are from a 30-year old female Hispanic (ATCC1), a 24-year old Caucasian female (82726) and a 29-year old native American female (99375). The passage number of these cell lines was between 4-15 and they were proliferated in the lab for almost a year. Before getting the cell ampoules out of the liquid nitrogen, the culture media (low-glucose DMEM medium (Gibco, MA), supplemented with 10% v/v MSC qualified FBS (Gibco, MA) and 1% v/v AB/AM (Gibco, MA)) was warmed in the water bath for at least 15 minutes. Three $15ml$ centrifuge tubes were prepared by labeling each one with the cell line name and adding 8 ml of pre-warmed culture media in each tube. The cell ampoules were taken out of the liquid nitrogen storage wearing the personal protective equipment. Then these ampoules were rubbed gently for 1 minute and placed in the water bath for 1 minute. Thawing should be done rapidly to minimize any damage to the cell membranes. To avoid contamination, the ampoules were not immersed completely in the water bath, only the bottom part was immersed. The cell solution from each vial was added in its corresponding centrifuge tube. To make sure that there were no cells left on the walls of the ampoules, $1ml$ of the culture media was added to each ampoules and pipetted two times then the solution was added to its corresponding tube. The centrifuge tubes were then centrifuged at $1200\ RPM$ at $40℃$ for $3\ minutes$. During the centrifugation, three

T-175 flasks were prepared by adding $8\ ml$ of culture media and labeling each flask with the cell line name, passage number, and the date. When centrifugation was done, the supernatant was discarded carefully without disrupting the pellet leaving $1\ ml$ of the supernatant in each tube. Then the bullet was disrupted in that $1ml$ by pipetting almost 10 times carefully to avoid having air bubbles. After the bullet was completely dissolved in the $1ml$ solution, each cell solution was added to its corresponding flask. After that, rock each flask back and forth several times to make sure that the cells would evenly grow without forming any clusters. Finally, the flasks were checked under the microscope to make sure that the media was spread evenly and then were placed in the incubator.

To proliferate cells, passaging was done once every two days over a year. Trypsin EDTA which is the dissociation reagent, trypsin neutralizing solution (TNS) that neutralizes the trypsin EDTA, culture media, and phosphate buffered saline (PBS) that lacks Ca^{2+}/Mg^{2+} and is used to wash the cells from their wastes were put in the water bath for at least 15 minutes before being used. Before starting passaging, the flasks were checked under the microscope to make sure there was no contamination or deterioration. Under microscope, alive cells were kept adherent to the flask bottom while dead cells would be floating in the culture media. Spent medium was removed using sterile pipette and the cells were washed twice using $10\ ml$ of the pre-warmed PBS for each flask. Each time after adding the PBS, flasks were shaken in the four directions to get complete coverage of the cell layer. Followed by aspirating the PBS and adding $10\ ml$ of pre-warmed trypsin EDTA to each flask then the flasks were shaken again to

ensure the coverage of all cells with the dissociation reagent. The flasks were incubated for 5 minutes. In the meantime, prepare three $15ml$ centrifuge tubes by labeling them with the names of the cell lines and prepare four T-175 flasks, two for each cell line, by labeling them with the date, the name of the cell line, the passage number, and by adding $14\ ml$ of culture media. After the 5 minutes of incubation, the flasks were tapped from the four directions to expedite cell detachment then checked under microscope ensuring that the cells were floating as single circles or clusters. This process should not take long as the more the cells left in the trypsin EDTA, the more damage will happen to the cell membranes and cells may die. Thus, equal amount of TNS was added to each flask ending with ratio of 1:1 of trypsin EDTA: TNS. Then each cell solution was transferred to the pre-prepared centrifuge tubes and centrifuged at $1200\ RPM$ at $40℃$ for $3\ minutes$ to remove any residual dissociation reagent. When centrifugation finished, the supernatant was discarded using a pipette without disrupting the pellet leaving $2\ ml$ of the solution in each centrifuge tube to resuspend the pellet by pipetting carefully without forming air bubbles in order not to waste cells and to ensure homogenous solution for single cells. Then $1\ ml$ from each centrifuge tube was pipetted into its corresponding pre-prepared flask. Finally, rock each flask back and forth several times to make sure that the cells covered the flask and would grow evenly, not in clusters, and incubate them for another two days.

Coating, Counting Cells Using Hemocytometer and Differentiating the cells

After 4 weeks of culturing and passaging the cells and before starting counting the cells, six T-25 flasks were coated to be prepared for differentiation. This was done my mixing $3.43\ ml$ of Hanks' Balanced Salt Solution (HBSS) with $0.34\mu l$ of fibronectin then $0.86\ ml$ of this solution was added for $1\ hour$ into each opened T-25 flask in the laminar flow cabinet. HBSS is used as a transport media to preserve cells and minimize bacterial overgrowth [15] and fibronectin is important for cell differentiation, adhesion, and growth [16]. In the meanwhile, the differentiation media was prepared by mixing Platelet-derived growth factor of concentration $10\ ng/ml$ and transforming growth factor β1 of concentration $2.5\ ng/ml$ (PDGF+TGFβ1) .

To count cells, the passaging steps were followed till the step of resuspending the cells in the $2\ ml$ of the solution. To achieve single cell suspension and accurate cell count, the cell solution in each centrifuge tube was repeatedly pipetted without forming any air bubbles. After disrupting the cell pellet, small portion of the cell solution ($10\ \mu l$) was taken from each centrifuge tube and put into two microcentrifuge tubes after labeling them with the cell name. An equal amount of trypan blue dye was added to each microcentrifuge tube ($10\ \mu l$) so the cell solution: trypan blue dye was 1:1. The trypan blue is used to determine the ratio of alive to dead cells by staining dead cells blue while alive cells with intact cell membrane are kept white or colorless with a small dot inside which is the nucleus. In each microcentrifuge tube, the solution was mixed well by pipetting 5 times without forming air bubbles and allowed to stand for 3-5 minutes. For then, the cover slip and the hemocytometer were cleaned using 70% ethanol and kimwipes. Then $10\ \mu l$ of blue-cell suspension from each microcentrifuge tube

were transferred to one of the two chambers of the hemocytometer without overfilling or underfilling the chamber. Then cells were viewed under microscope at $100 \times$ magnification and the alive cells in the lateral four squares were counted. The following equation was used to count cells:

$$average\ of\ the\ cells\ in\ the\ four\ squares \times 2 \times 10^4$$

The 2 represents the ratio of the cell solution: trypan blue and the 10^4 is the area of the four squares.

Number of cells of ATCC1: 187500 $cells/ml$

Number of cells of 82726: $280000\ cells/ml$

Number of cells of 99375: $560000\ cells/ml$

There should be $2000\ cells/cm^2$

Then, there should be $\frac{2000\ cells \times 25 cm^2}{cm^2} = 50000\ cells$ in each T-25 flask.

Two flasks for each cell line were used: one that had culture media (low-glucose DMEM medium (Gibco, MA), supplemented with 10% v/v MSC qualified FBS (Gibco, MA) and 1% v/v AB/AM (Gibco, MA)) and the other one had PDGF+TGFβ1, the differentiation media. After two days the culture media was changed completely, and half of the differentiation was changed (semi-batch culture).

Intracellular and Extracellular marker staining of cells

To dissociate cells, $2ml$ of TrypLE™ Express enzyme was added to each T-25 flask after taking the media out of the flask. TrypLE™ Express enzyme is used instead of trypsin in harvesting cells because of its lower cell toxicity and higher stability for more than 12 months at $4℃$ and at room temperature. In addition to that, enzyme assay results proved that at $37℃$, TrypLE™ Express enzyme maintains 85% activity for 8 days [17]. After dissociation, six $15ml$ tubes were prepared by labeling them with the cell line name and the media they were seeded in. Cells were then transferred to the corresponding tube, followed by washing the cells twice by $3ml$ of PBS each time and centrifugation after each wash for 4 minutes at $200 \times g$. Then cells were fixed by adding $1\ ml$ of 4% paraformaldehyde (PFA) for 10 minutes in each flask. Cell fixation prevents autolysis and necrosis of excised tissues and maintains cells morphology and antigenicity during IHC [18]. Cells were then washed with $3ml$ of PBS twice and centrifuged after each wash at $400 \times g$ for $4\ minutes$. In order to block non-specific binding, $3\ ml$ of blocking solution (6% goat serum/ 94% PBS) was added for $45\ minutes$ to each tube. After that the primary antibodies CD29/44/105 were added and the cells were incubated for $1\ hour$ at room temperature. CD29/44/105 are primary mouse anti-human antibodies that attach to the MSCs gene markers CD29, CD44, and CD105, respectively. Figure 2 shows more details about these antibodies. The cells were subsequently washed in $3\ ml$ of blocking solution twice followed by centrifugation at $400 \times g$ for $4\ minutes$ after each wash. Then the secondary antibody Alexa Fluor® 488 (AF488) was added to the cells for $1\ hour$. Alexa Fluor® 488 was added at a 1:2000 ratio. Thus, we added $0.25\ \mu l$ per $500\mu l$ of blocking solution per centrifuge tube, followed by washing the cells using $3\ ml$ of

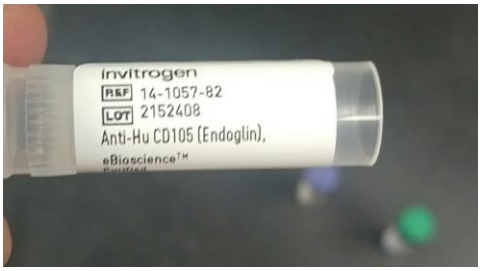
blocking solution twice and centrifugation after each wash at $400 \times g$ for $4\ minutes$. AF488 antibody is a green-fluorescent dye that binds to the primary antibodies CD44/29/105. The cells were permeabilized by adding $3\ ml$ of permeabilization solution ($50\ ml$ of high salt buffer with $25\ \mu l$ of Tween 20) thrice and centrifugated after each time at $400 \times g$ for $4\ minutes$ to allow the second primary and secondary antibodies enter through the cell membrane. $2.5ml$ of the permeabilizing solution was removed followed by adding $0.5ml$ of the second primary antibody, Myosin 11 (MYH 11, 6% goat serum) and incubation for $1\ hour$ at room temperature. Figure 3 shows more details about MYH11. The cells were then washed in $3\ ml$ of blocking solution twice and centrifugated after each wash at $400 \times g$ for $4\ minutes$. Subsequently, $2.5ml$ of the blocking solution was removed after washing the cells followed by adding $0.5ml$ of the second secondary antibody CF647 which is a red- fluorescent dye that binds to the primary antibody MYH11. Then cells were incubated for $1\ hour$ at room temperature. Finally, the cells were washed in $3\ ml$ of blocking solution twice and centrifuged after each wash at $400 \times g$ for $4\ minutes$. Cells were then stored in $4℃$ until FACS was done.

Fluorescence-Activated Cell Sorting (FACS)

$2\ ml$ of the supernatant was removed from each tube to have more concentrated marker treated cells and the remaining $1\ ml$ from each tube were transferred into a glass vial. Then the expression level of MSC and SMC-specific markers were analyzed using flow cytometer that detects the intracellular and the extracellular cell markers. This was done using a BDFACSCanto™ II flow cytometer (BD); 20 000

events were collected from each experimental group and the data were analyzed using FlowJo® software.

A



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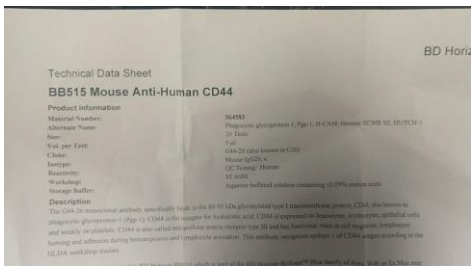


C

Figure 2: Primary antibodies that attach to CD105/44/29 MSCs gene markers (A)Mouse anti-Hu CD105. (B) Mouse anti-human CD44. (C)Mouse anti-human CD29

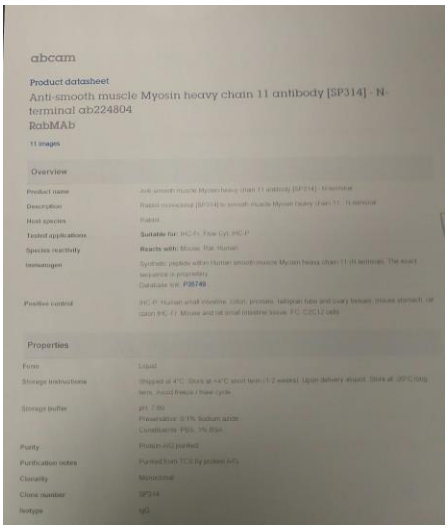


Figure 3: Details about the second primary antibody, MYH11 that binds with SMCs gene marker.

Results

Figure 4 represents the FACS results of ATCC1 cell line that was used as a negative control to determine the gate. It shows no specific binding because no primary antibodies were added, only secondary antibody that resulted in the autofluorescence (background signal). From figure 4a, the gate of the Ad-MSCs was determined to be 10^3 because AF488 is the secondary antibody that attaches to CD105/44/29 which is the primary antibody specific for MSCs gene markers. Before 10^3, there is non-specific binding, so it resulted in negative expression of AF488 ($AF488^-$). After 10^3, it showed the specific binding which results in the positive expression of AF488 ($AF488^+$). In other words, $AF488^-$ means that particles detected in this region are not mesenchymal stem cells while $AF488^+$ means that particles detected in this region are mesenchymal stem cells. Similarly, for figure 4b, the gate of SMCs was determined to be 10^3 because CF647 is the secondary antibody that attaches to MYH11 which is the primary antibody specific for SMCs gene markers.

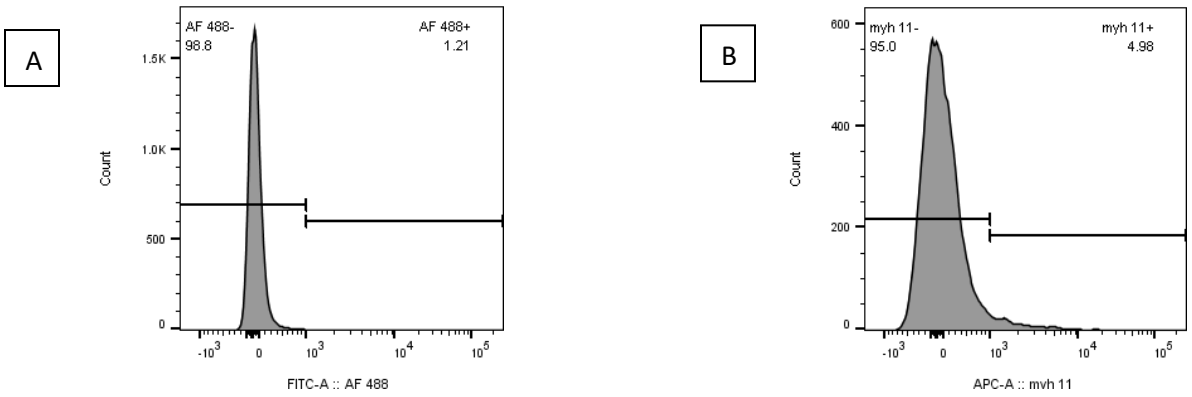


Figure 4: FACS results of the ATCC1 cell line, the staining control, after being subjected to secondary antibodies only showing the gate to be 10^3. (A) represents the percentage of AF488. (B) represents the percentage of MYH11.

Figure 5 represents the percentage of the Ad-MSCs and SMCs of 82726 cell line after being grown in culture media for 4 days. In Figure 5a, 17% of the events showed positive expression for CD44 ($CD44^{+}$) which means that 16.9% of the cells had Ad-MSCs phenotype. On the other hand, in figure 5b, 5.92% of the cells expressed $MYH11^{+}$ which means that these particles had the SMCs phenotype.

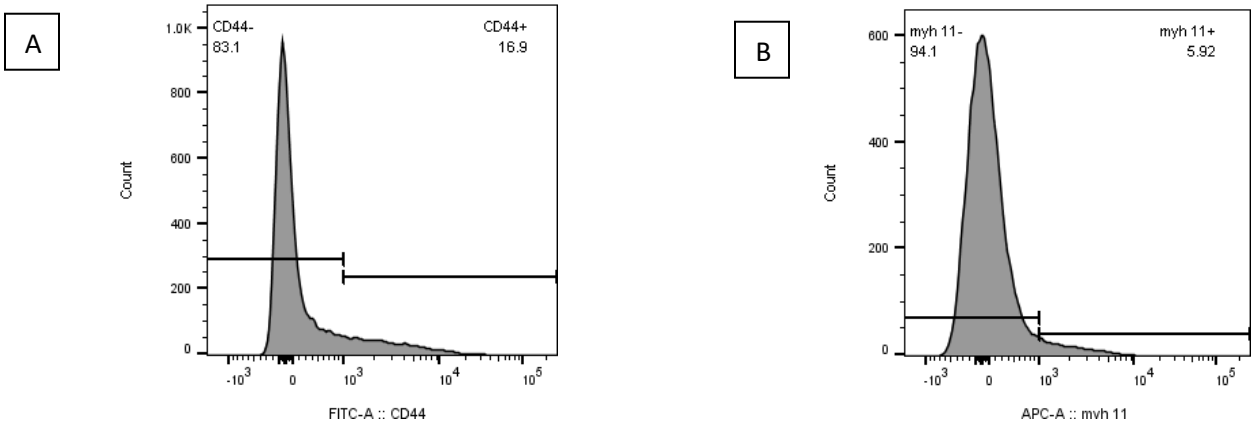
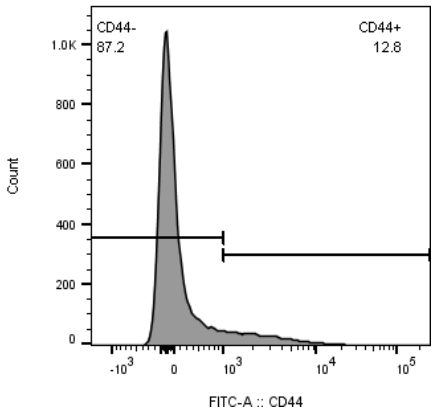


Figure 5: FACS results of 82726 cell line after being seeded in culture media for 4 days. (A) represents the percentage of Ad-MSCs. (B) represents the percentage of SMCs.

Figure 6 shows the percentage of MSCs and SMCs of 82726 cell line after being grown for 4 days in the differentiation media $PDGF + TGF\beta 1$. From figure 6a, 12.8% of the cells expressed $CD44^{+}$ which means that they have Ad-MSCs phenotype. From figure 6b, 6.25% of the particles expressed $MYH11^{+}$ which indicates they have SMCs phenotype.

A



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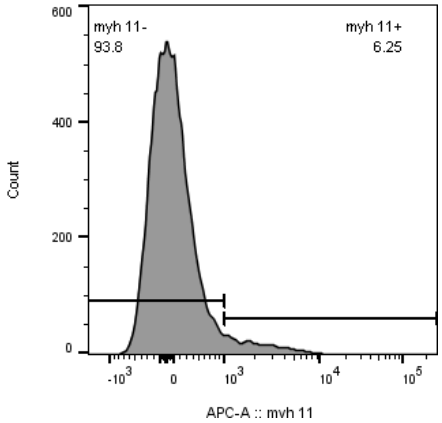
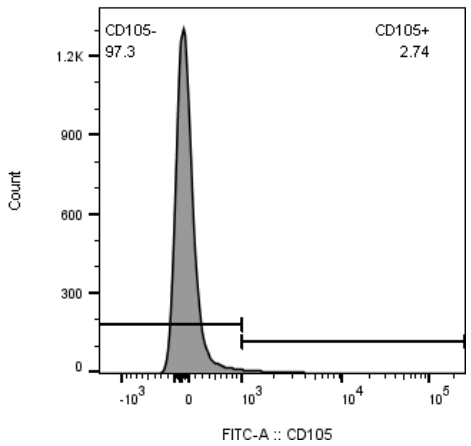


Figure 6: FACS results of 82726 cell line after being seeded for 4-day in the media $PDGF + TGF\beta 1$. (A) represents the percentage of Ad-MSCs. (B) represents the percentage of SMCs.

Figure 7 represents the percentage of MSCs and SMCs of 99375 cell line after growing in culture media for 4 days. Figure 7a shows that 2.74% of cells expressed $CD105^{+}$, so they have the Ad-MSCs phenotype. Figure 7b shows that 7.9% of the particles expressed $MYH11^{+}$, so they have SMCs phenotype.

A



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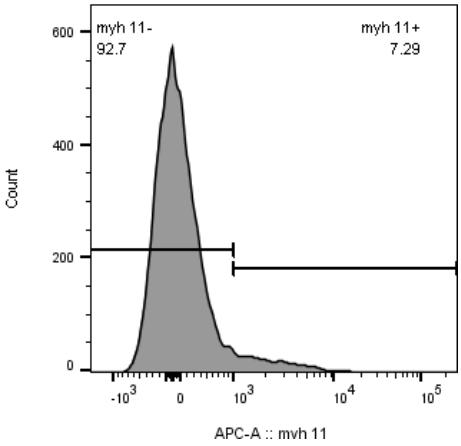

Figure 7: FACS results of 99375 cell line after being seeded for 4-days in culture media. (A) represents the percentage of MSCs. (B) represents the percentage of SMCs.

Figure 8 presents the percentage of MSCs and SMCs of 99375 cell line after being seeded in $PDGF + TGF\beta 1$ for 4 days. Figure 8a shows that 1.63% of the particles expressed $CD105^{+}$, so they have MSCs phenotype. Figure 8b shows that 6.17% of the particles expressed $MYH11^{+}$, so they have SMCs phenotype.

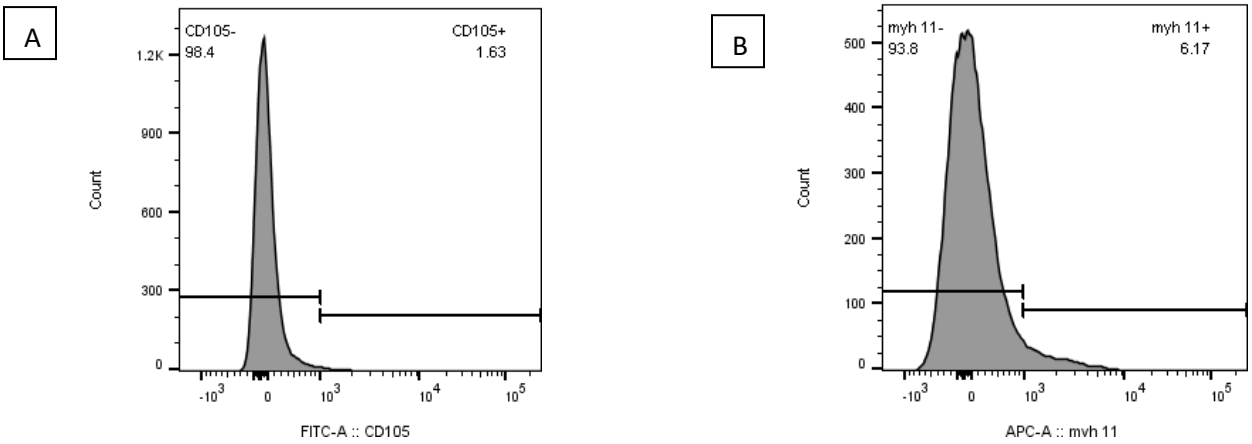

Figure 8: FACS results of 99375 cell line after 4-day differentiation in $PDGF + TGF\beta 1$. (A) represents the percentage of MSCs. (B) represents the percentage of SMCs.

Discussion

For 82726 cell line, when comparing the results from figures 5b and 6b, it is concluded that the percentage of SMCs increased when $PDGF + TGF\beta1$ was used and that indicates the differentiation media $PDGF + TGF\beta1$ consists of important growth factors that help in the differentiation of Ad-MSCs derived from a 24-year old Caucasian female. In addition to that, 4 days is enough for these growth factors to differentiate this cell line into SMCs.

Regarding cell line 99375, by comparing percentages in figures 7b and 8b, we conclude that the percentage of SMCs in $PDGF + TGF\beta1$ was less than that in culture media. This indicates the growth factors in the differentiation media $PDGF + TGF\beta1$ needs more than 4 days to differentiate cells derived from a 29-year old native American female. In other words, the older the donor is, the more time the cells need to differentiate form Ad-MSCs towards SMCs.

Comparing the percentages of $MYH11^{+}$ from figures 6b and 8b, it is concluded that 82727 cell line expressed more SMCs when cultured in $PDGF + TGF\beta1$ than 99375 cell line. This conclusion proves the donor origin has effect on the differentiation of Ad-MSCs towards SMCs.

Conclusion

Differentiation of Ad-MSCs towards SMCs depends on a lot of external factors: the origin of the cell, the culture media, age of the donor, passage number, plastic surface quality, supplementary factors, oxygen concentration, and mechano-/electro-stimuli. In addition to the factors, this study proved that the origin of the donor is considered one of the factors that should be taken into consideration when generating SMCs form Ad-MSCs. Controlling these factors will result in increasing the efficiency and productivity of SMCs related clinical applications such as synthetic vascular graft since SMCs are considered one of its main components. In the future, triplicates should be done on each cell line (82726, 99375) for each media (culture media, $PDGF + TGF\beta 1$) to ensure the statistical significance of the results.

Acknowledgments

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