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Beyond a "Pinch of this and a Handful of that:" Using Manufacturing Standards for the Isolation of Human Mesenchymal Stem Cells From Adipose Tissue- A Novel Point-of-Care Device

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Abstract

Regenerative therapies using adipose-derived stromal vascular fraction (SVF) and constituent mesenchymal stem cells (ASCs) require preparation of adipose tissue. Currently no respective preparation standard exists.Processing Quantitative Standards (PQSs) define manufacturing quantitative variables (such a as time, volume and pressure). Processing Qualitative Standards (PQLSs) define the quality of the materials and methods of manufacturing. The purpose of this study was to validate a point-of-care kit (Kit) to isolate human SVF/ASCs using PQSs and PQLSs.

PQSs included the volumes of lipoaspirate and reagents, the time/gravity of centrifugation, and the time, temperature, and tilt level/speed of incubation. PQLSs included a collagenase, processing time of 30 minutes, Kit weight of 2 lbs., transparency of Kit components, the maintenance of a closed sterile processing environment, and use of a small centrifuge and incubating rocker. The Kit isolated 5.8 x 10^4 live nucleated cells per ml of lipoaspirate with 90.7% viability. Isolation of SVF was accomplished in 30 minutes and the Kit weight was 1.63 lbs. The Critical Quality Attribute (CQA) of ASC identity was proven by consensus criteria. As new CQAs of SVF/ASCs evolve, adjustments to these benchmark PQSs and PQLs will hopefully isolate SVF/ASCs of target CQAs with greater reproducibility, quality, and safety.

Keywords: Stromal vascular fraction; Adipose-derived stem cells; Stem cell isolation; Point-of-care systems; Single-use systems; Regenerative medicine

Abbreviations: SVF= stromal vascular fraction; ASCs= adipose-derived mesenchymal stem cells; PQSs= processing quantitative standards; PQLSs= processing qualitative standards; CQA= critical quality attribute SOPs= standard operating procedures; CGMPs= current good manufacturing practices; FDA= food & drug administration

Introduction

Manipulating human tissues is the fundamental requirement in the practice of surgery. Whether dissecting, suturing, or retracting, all manipulated tissues affect the post-operative form and function respectively. Similarly, the processing of biomaterials in the operating room, such as in the preparation of lipoaspirate to be used in autologous fat grafting, requires a level of surgical skill and judgment to not only maintain Standard Operating Procedures (SOPs) but also to optimize the intended result. Accordingly, human skill and judgment must account for a seemingly countless number of patient and surgeon variables for surgical success. In this case, the SOPs of surgical technique help to limit the variables to more reliably determine the quality of the surgical result.

Regenerative Medicine & Surgery relies on the preparation of adipose tissue and the isolation of cells to be used for patient treatments instead of pharmaceuticals. However, unlike the practice of surgery, Regenerative Medicine & Surgery, at this point, does not rely on SOPs or standardized preparation procedures. There are isolation systems for Regenerative Medicine & Surgery that each have their own SOPs which may comply with Current Good Manufacturing Practices (CGMPs).However, these systems are not configured for specific protocols. CGMPs are regulations outlined by the Food & Drug Administration (FDA) which "provide for systems that assure proper design, monitoring, and control of manufacturing process and facilities which assures identity, strength, quality, and purity of drug products by requiring that manufacturers of medications adequately control manufacturing operations" [1].

As post-operative quality metrics judge surgical results, so also are the prepared tissues and cells isolated for use in Regenerative Medicine & Surgery judged for quality. The quality characteristics used to define prepared tissues and isolated cells are termed Critical Quality Attributes (CQAs). According to the FDA, a CQA is a "physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality" [2]. The FDA expects manufacturers to "identify critical parameters in the manufacturing process and critical product attributes to ensure the desired clinical effect of the final product" [3]. CQAs of clinical grade therapeutic products include cellular safety, identity, purity, and potency [2]. Processing Quantitative Standards (PQSs) and Processing Qualitative Standards (PQLSs) may help to manufacture, or prepare, tissues and cell with specific attributes.

PQSs are metrics used to standardize manufacturing quantitative variables, such as time, volume and pressure. PQLSs are metrics used to standardize the quality of the materials and methods of manufacturing. PQSs and PQLSs help determine the quantity and quality of manufactured products while improving manufacturing reproducibility.

The purpose of this proof-of-concept study was to validate a novel collagenase-based technique using PQSs and PQLSs to isolate stromal vascular fraction (SVF) and its adipose-derived stem cell (ASC) subpopulation from freshly harvested adipose tissue. To control for the manufacturing PQSs and PQLSs, the technique employs a closed preparation system (the "System") in the form of a kit (the "Kit"). The targeted CQA of the ASCs was identity alone. The Preparation System includes a kit (the "Kit") as well as ancillary devices to include a small centrifuge and incubating rocker. Targeted PQSs included the volumes of lipoaspirate and reagents, the time and gravity of centrifugation, and the time, temperature, tilt level, and tilt speed of incubation. Targeted PQLSs included the use of a CGMP collagenase, processing time of 30 minutes or less, Kit content weight of 2 lbs. or less, transparency of Kit components, modular design of Kit components, the maintenance of a continuously closed sterile processing environment, and use of a small centrifuge and incubating rocker configured by the manufacturers for use with the Kit. This study is not a comparative study to any other isolation method.

Materials and Methods

Overview

Fresh human waste lipoaspirate from three informed and consented healthy females was used for this University of Florida Jacksonville Institutional Review Board approved study (IRB# 201601520). The entire SVF isolation and evaluation sequence was performed on each of the three different lipoaspirate samples. This entire sequence was performed within the closed environment inherent to the Kit. The transfers described below were made via standard transfer hubs, vial-to-syringe adapters, standard 60cc syringes, and patented syringes with removable plunger rods (all of which were configured as a part of the Kit). Kit components are itemized in Table 1. The SVF isolation, ASC culturing, and data acquisition were performed by the second author, to reduce bias. This study incorporated triple biological replicates with double technical replicate testing for cell quantity (average taken), immunophenotyping, and differentiation.

Table 1. Kit contents

1. Sterile Work-area Mat (1)
2. Biohazard Bag (1) (Biohazard cartridge in Kit)
3. Transfer Hubs (4) (standard)
4. Alcohol Wipe (1)
5. Vial-to-Syringe Adapters (8)
6. 60mL Trituration Syringes (2) (standard and pre-labeled)
7. 35mL Transfer Syringes (4) (patented and pre-labeled)
8. 20mL Injectable Normal Saline (8) (pre-measured and pre-labeled)
9. 35mL Concentrate Syringe (1) (standard and pre-labeled)
10. 10 ML PBS (1) (pre-measured and pre-labeled)
11. Corase® Aliquot (1) (prepared during a pre-operative protocol)
12. 35mL Corase® Syringe (1) (standard and pre-labeled)

Method of SVF isolation and ASC expansion

Method of SVF isolation

The lipoaspirate for this study was harvested using standard operative tumescent techniques using manual (not machine-assisted) syringe-based liposuction. The tumescent solution included injectable normal saline, lidocaine, and epinephrine. Shortly after harvest, the lipoaspirate was processed using the Kit which was configured for this study to correspond to a proprietary 10-step protocol. In brief, shortly after the lipoaspirate was obtained, 70mLs of unconcentrated lipoaspirate was evenly transferred into two Trituration Syringes (standard 60mL syringes labeled as such and organized within the Kit). Next the lipoaspirate was transferred back-and-forth between the Trituration Syringes for a total of five transfers. Then, 17.5mLs of lipoaspirate was transferred from the Trituration Syringes into each of four 35mL patented Transfer Syringes (produced for Reviticell by Cardinal Health, Dublin, OH, labeled as such, and organized within the Kit). Subsequently, injectable normal saline was transferred into each Transfer Syringe (until filled) from prefilled 20mL vials (pre-labeled and filled, labeled as such, and organized within the Kit) to create a homogenous suspension. Then, the plunger rods of the Transfer Syringes were removed, and all four Transfer Syringes were centrifuged for 3 minutes at 340 x g (Drucker Diagnostics, 755VES, State College, PA, configured for Reviticell to spin up to 8 Transfer Syringes horizontally in this small bench-top unit). What is unique about the Transfer Syringe is that the plunger rod can be removed without having to remove the plunger which, thereby, preserves the integrity of the closed system. The resulting concentrated pellet at the bottom of each of the four Transfer Syringes was then transferred into the Concentrate Syringe (standard 35mL syringe labeled as such and organized within the Kit). After discarding the aqueous infranatant from two of the Transfer Syringes, the 7.5mLs of lipoaspirate lowest in the adipose column of each of the two remaining Transfer Syringes was transferred to the Concentrate Syringe. Then, 10mLs of phosphate buffered saline (PBS) (pre-measured, labeled as such and organized in the Kit) and an aliquot of Corase® were transferred into the Corase® Syringe (standard 35mL syringe labeled as such and organized in the Kit). Aliquots of Corase® (0.5mL frozen sterile water with 1 mg of Corase®) were prepared preoperatively via a pre-op module of the Kit (not described as a part of this study). Corase® is identical to Liberase™ MNP-S (Roche, Basel, Switzerland), which is a highly purified blend of three enzymes which meet CGMP guidelines. These enzymes include: collagenase I, collagenase II, and thermolysin. Corase® specific activity is 3.8U/mg, or activity of 19-32 Wunsch Units. Corase® is produced exclusively for these Kits by Roche Custom Biotech (a business unit of Roche, Basel, Switzerland). The contents of the Concentrate Syringe were then transferred to the Corase® Syringe (thus resulting in 15mLs of concentrated lipoaspirate, the centrifuged pellets from all four Transfer Syringes, PBS and the Corase® aliquot). The Corase® Syringe was then placed into an incubating rocking platform shaker (VWR, Radnor, PA) at 37^{0}C for 20 minutes at tilt level of 6 and speed of 30. Subsequently, half of the contents of the Corase® Syringe was transferred to each of the two remaining Transfer Syringes (after proper disposal of any remaining contents). Then, injectable normal saline was transferred into each Transfer Syringe (until filled) from prefilled 20mL vials (pre-labeled and filled, labeled as such, and organized within the Kit), after which

both syringes were again centrifuged for 3 minutes. The resultant SVF pellet at the bottom of each syringe was then collected and combined for analysis and culture.

Method of ASC expansion

SVF was added to 6mLs of animal-component-free defined- media (MesenCult-ACF basal medium #05451 and 5X supplement #05452, Stemcell Technologies, Vancouver, Canada) in a T-25 culture flask (pre-treated with attachment substrate, Stemcell Technologies, #05444. Flask: VWR, Nunclon tissue culture flask #470174-450) and incubated at humidified 5% carbon dioxide. Half media change was performed at day 6 with then complete media change at day 10 and M/W/F thereafter). The plastic adherent monolayer of ASCs was then expanded to 80% confluence. At this stage (non-passaged stage), ASCs were harvested for differentiation stimulation as well as immunophenotyping analysis.

Methods of SVF and ASC analyses

Method of SVF analyses

Directly after isolation from the adipose tissue, the SVF underwent standard cell counting and viability testing using an automated cell counter (Beckman Coulter Vi-Cell XR, Beckman Coulter, Brea, CA) with trypan blue exclusion. Two runs were performed with cellular averages calculated.

Methods of ASC analysis

Immunophenotyping

Expanded cells at initial 80% confluence were dissociated from the culture flask (Mesencult-ACF Dissociation Kit #05426, Stemcell™ Technologies). A Becton-Dickenson Stemflow™ human MSC analysis kit (cat#562245) was then used for flow cytometric analysis of the ASCs using the instructions for use for the kit. The samples were run on a BD LSR II (BD Biosciences, San Jose, CA) flow cytometer with accompanying analytical software (BD FACSDiva™). In brief, preparation of the cell suspensions were as follows: cells were detached from the flask using the MesenCult™-ACF Dissociation Kit (cat#05426). Cells were then suspended at a concentration of 5 x 10^6 in BD Pharmingen™ Stain Buffer (cat#554656). In accordance with the Becton-Dickenson Stemflow™ kit, 100 µl of the prepared suspension was added equally to all analysis tubes to include FITC mouse anti-human CD90, PE mouse anti-human CD44, PerCP-Cy™5.5 mouse anti-human CD105, and APC mouse anti-human CD73 with positive and negative controls and positive and negative cocktails. After the cell suspensions were added, the tubes were incubated in the dark for 30 minutes and the cells were then washed twice with BD Pharmingen™ Stain Buffer and resuspended to 500 µl in BD Pharmingen™ Stain Buffer. Cells were then kept in the dark and on ice until analysis later that same day.

Tri-lineage differentiation

Expanded cells at initial 80% confluence were dissociated from the culture flask (Mesencult-ACF Dissociation Kit #05426, Stemcell™ Technologies). ASCs were first passaged and seeded on six-well culture plates at approximately 100,000 cells per well. After attachment, cells were grown to 80% confluence. Each well received a different respective differentiation medium.

For osteogenic differentiation, basal growth media was exchanged for conditioned osteogenic differentiation medium (Stemcell™ Technologies, Mesencult™ Osteogenic Stimulatory Kit #05404). Alizarin Red (Sigma-Aldrich, St. Louis, MO) staining was performed (to specifically stain alkaline phosphatase deposits) on day 14 and photomicrographs were obtained using whole field bright-light microscopy captured at 15x.

For adipogenic differentiation, basal growth media was exchanged for conditioned adipogenic differentiation medium (Stemcell™ Technologies, Mesencult™ Adipogenic Differentiation Medium #05412). Oil red O (Sigma-Aldrich) staining was performed (to specifically stain the lipid droplets) on day 14 and microphotographs were obtained using whole field bright-light microscopy captured at 30x.

For chondrogenic differentiation, basal growth media was exchanged for conditioned chondrogenic differentiation medium (Stemcell™ Technologies, Mesencult™ Chondrogenic Differentiation Medium #05455). Alcian Blue / Nuclear Fast Red (Sigma-Aldrich) staining was performed (to specifically stain sulfated proteoglycans) on day 14 and photomicrographs were obtained. The micromass sphere was photographed by indirect microscopy.

Results

PQSs of the Kit with respective results are reviewed in Table 2 and PQLSs with respective results are reviewed in Table 3. The minimum CQAs targets and respective results of isolation of the SVF and ASCs are reviewed in Table 4. The average nucleated cell viability of the SVF was 90.7% with an average viable total cell count of 8.7 x 10^5 cells. The average diameter of the nucleated cells was 15.59 µm. An average of 5.8 x 10^4 nucleated

cells per ml of concentrated lipoaspirate processed (15mLs of lipoaspirate with pellets from all four lipoaspirate syringes before enzymatic digestion of the 15mLs of concentrated lipoaspirate) was harvested. Immunophenotype analysis by flow cytometry revealed strongly positive markers for CD73, CD90, CD105 and some dimly positive CD44 staining (see figure 1) and negative markers for CD11b, CD 19, CD34, CD45, and HLA-DR. The CD44 staining was much less than the other three markers, but data demonstrated some CD44 staining was present. The CD90 and CD105 showed the highest amount of antigen staining compared to the isotype controls and the CD73 and CD44 markers. Cellular phenotypic ontology revealed plastic adherent cells with colony formation (see figure 2). Differentiation to adipocytes, osteoblasts, and chondroblasts was confirmed by appropriate lineage staining (see Figures 3). Endotoxin level was <0.004 EU/ml (tested by Associates of Cape Cod, East Falmouth, MA).

Table 2. Processing Quantitative Standards

Processing Quantity	Processing Quantitative Standard
Volume of unconcentrated lipoaspirate	70 mls
Volume of saline	160 mls
Mass / activity of Corase® (collagenase)	1 mg / 19-32 Wunsch Units
Centrifugation, time	3 minutes
Centrifugal force (x g)	340
Incubation, time	20 minutes
Incubation, temperature	37°C
Incubation, tilt level	6
Incubation, tilt speed	30

Table 3. Processing Qualitative Standards

Processing Quality	Processing Qualitative Standard
Collagenase: CGMP grade	Confirmed
Processing time of ≤30 minutes	30 minutes
Collective weight of kit contents ≤2 lbs.	1.63 lbs.
Transparency of kit components	Confirmed
Modular design of kit to allow configuration for a variety of protocols	Confirmed
Maintains continuously closed sterile processing environment	Confirmed

Table 4. The minimum Critical Quality Attributes (CQA) targets and results of isolation of Stromal Vascular Fraction and Adipose-Derived Stem Cells from lipoaspirate.

Minimum CQA targets	Results
SVF with >70% viability of nucleated cells	90.7%
SVF with >10,000 live nucleated cells per ml of concentrated lipoaspirate enzymatically processed	58,000 live nucleated cells per ml of concentrated lipoaspirate enzymatically processed
ASC plastic adherence	ASC plastic adherence: confirmed
ASC tri-lineage differentiation • Adipogenic • Chondrogenic • Osteogenic	ASC tri-lineage differentiation • Adipogenic: confirmed • Chondrogenic: confirmed • Osteogenic: confirmed
ASC Immunophenotype • +: CD90, CD73,CD105,CD44 • -: CD11b,CD19,CD34,CD45,HLA-DR	ASC Immunophenotype • +: CD90, CD73: confirmed • +CD105: confirmed • +CD44: confirmed • -: CD11b,CD19,CD34,CD45,HLA-DR: all confirmed
Endotoxin level: <0.01 EU/ml	Confirmed: <0.004 EU/ml

Discussion

Processing biological materials at the point-of-care for therapeutic use will become more prevalent as regenerative therapies using prepared tissues and isolated cells achieve greater clinical success. Currently, the processing of lipoaspirate for conventional fat grafting remains one of the most common autologous techniques for improvement of human form or function.The SVF is a population of cells isolated from the reticular network of adipose tissue.SVF bears a wide cellular constituency which includes putative ASCs. Though certainly far from completely understood, the ASC remains the only known cell that may differentiate along several tissue lineages; as such, it remains a cell of great importance in the development of regenerative cell therapies and tissue engineering [4-8].

In Regenerative Medicine & Surgery, the surgeon or practitioner using a system to prepare tissue and isolate cells is viewed by the FDA as an unregulated manufacturer of a biologic. Even when preparing such treatments within the same point-of-care setting, the perspective of the FDA does not change because the same-surgical exemption no longer applies when surgeons or practitioners are “more than minimally manipulating” biomaterials. Therefore, the process of isolating and preparing cells or tissues for Regenerative Medicine & Surgery must rely on systems that have been cleared or approved by the FDA for a specific use, not only surgical skill and judgment (i.e., devices cannot be used “off-label”).

While several techniques to isolate SVF from adipose tissue have been developed over the last two decades, no standard exists. Manual manipulation is one such technique but, even when following a precise protocol, outcomes are largely dependent on personal skill and the equipment being used which makes it difficult to isolate cells of specific CQAs with consistency. Without a standardized method and means for isolating a SVF subpopulation with a specific CQA, reproducibly isolating SVF for targeted regenerative therapies would be difficult if not simply unreliable. A modular system that can be configured in the form of a variety of point-of-care single-use kits, each of

Figure 1. IMMUNOPHENOTYPING OF CULTURED ADIPOSE-DERIVED STEM CELLS (ASCS) BY FLOW CYTOMETRY, DIAGNOSTIC FOR ASCS

Row A: Scatterplots displaying respective gating to singlets.

Row B: Scatterplots and histograms displaying positive immunophenotypes (in the "Cocktail"): CD44 (row B), CD73 (row C), CD 90 (row D), and CD105 (row E).-

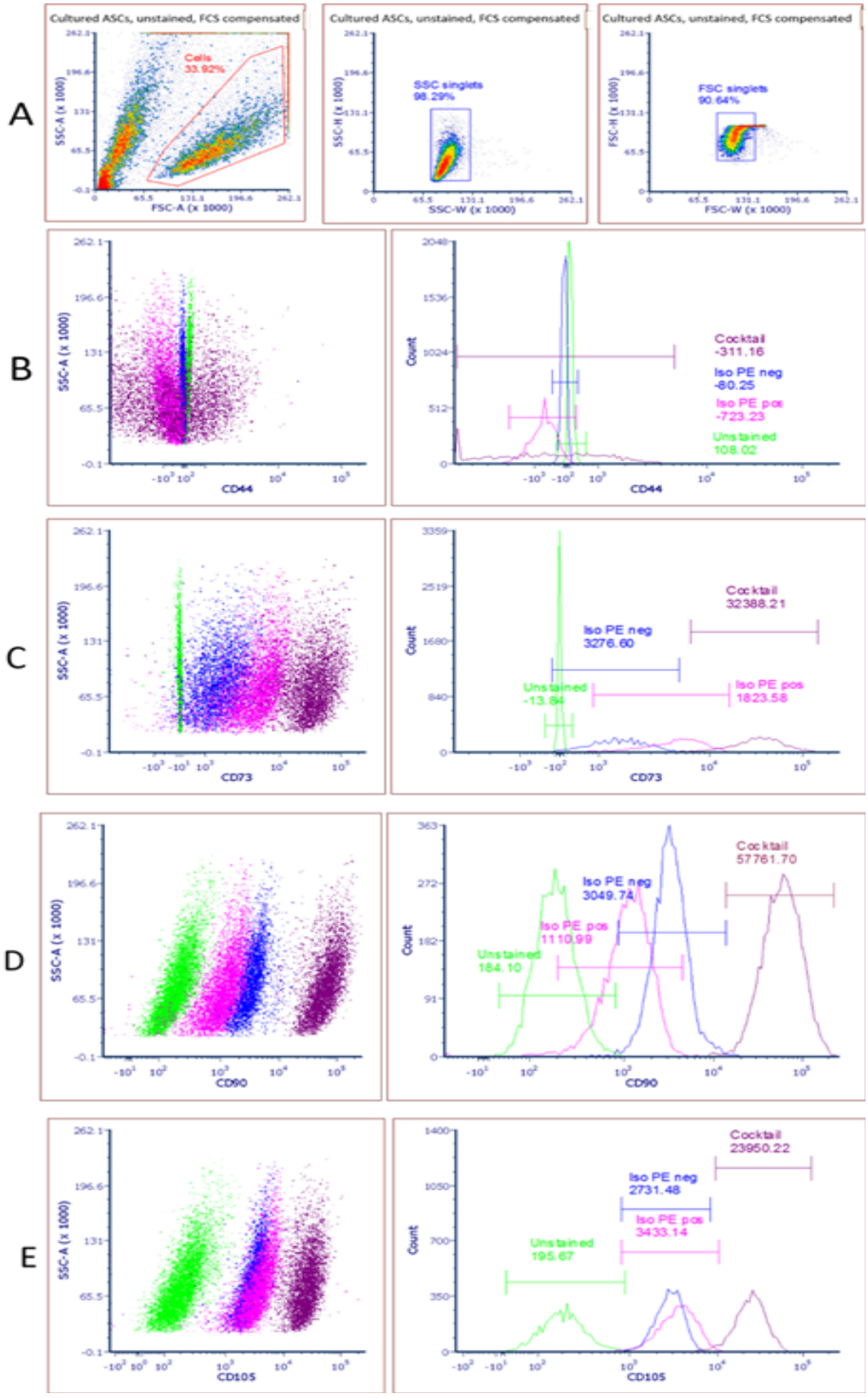


Figure 2. GROWTH OF ADIPOSE-DERIVED STEM CELLS
Representative bright-field 10x micrograph illustrating plate adherence and colony formation of adipose-derived stem cells.

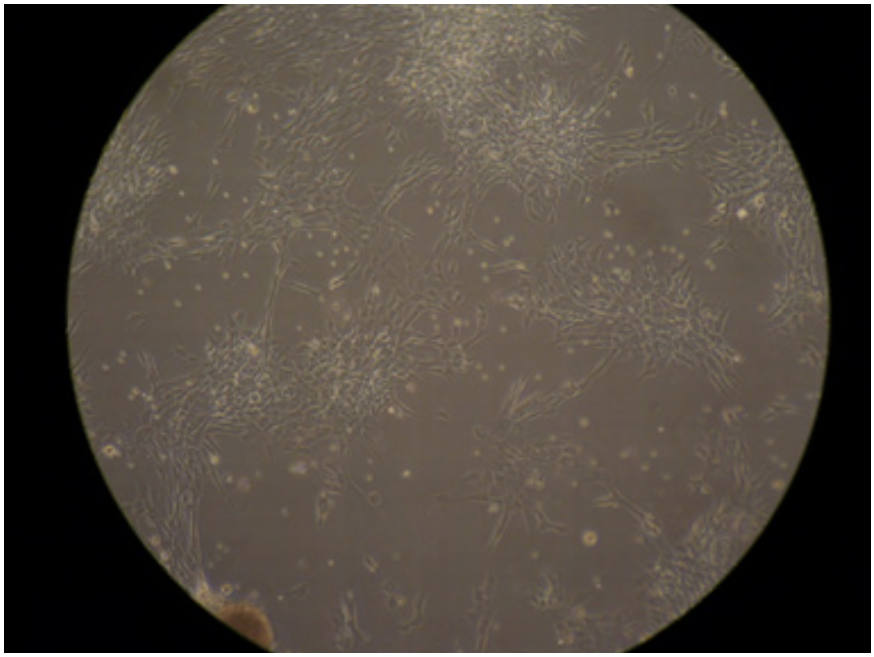
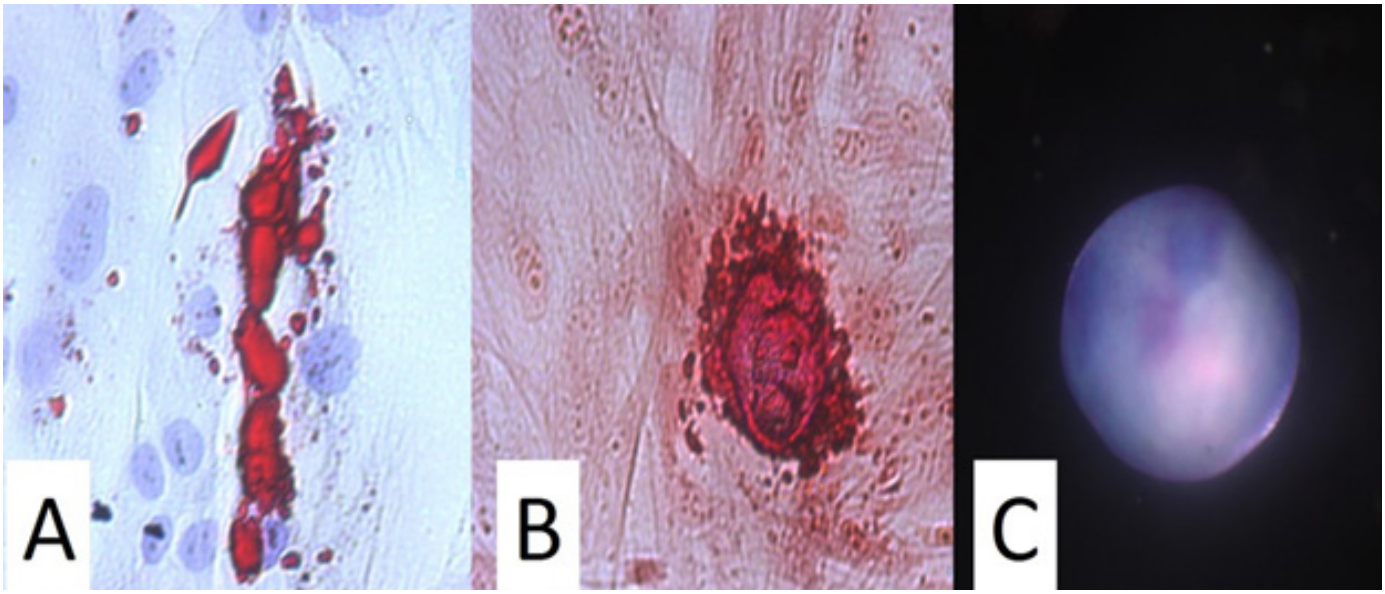


Figure 3. TRILINEAGE DIFFERENTIATION

A. ADIPOCYTE DIFFERENTIATION: Representative bright-field 30x micrograph illustrating lipid droplet staining of adipose-derived stem cells differentiated to adipocytes. B. OSTEOBLAST DIFFERENTIATION: Representative bright-field 15x micrograph illustrating alkaline phosphatase staining of adipose-derived stem cells differentiated to osteoblasts. C. CHONDROBLAST DIFFERENTIATION: Representative indirect micrograph illustrating a sulfated proteoglycan sphere stained with alcian blue & nuclear fast red indicating adipose-derived stem cells differentiated to chrondroblasts. Sphere diameter 2mm.



which contains the exact components necessary to conduct a specific protocol, may be best able to provide the standardization that is needed when isolating SVF and a targeted population of ASCs. Further, with such a system also being modular, it would be possible for it to rapidly accommodate newly discovered CQAs of the ASC target population. Additionally, components of such kits, organized to correlate with each particular protocol, with imposed PQSs and PQLSs, may improve the reproducibility of SVF and ASC isolation as well as make possible the standardization of processing biological materials [9].

This study establishes the Kit's functionality and efficacy as designed with PQSs and PQLSs as indicated by the resulting data presented herein. As end-users (physicians, clinicians and researchers) possess differing skill-set levels, the simple and straightforward use of the Kit with inherent PQSs and PQLSs may help reduce variability in the isolation of SVF and its ASC constituency.

Conclusions

Using the Kit with the PQSs and PQLSs described in this study enables the isolation of ASCs which meet consensus identity criteria. As the discovery of new CQAs of ASCs evolve, such as with respect to purity and potency, adjustments to these benchmark PQSs and PQLs will hopefully isolate ASCs of various CQAs with greater reproducibility, quality, and safety. Confirming studies will need to be completed.

Acknowledgements

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Disclosure of potential conflicts of interest

J.D.M. is a founder, Board Member, and an owner of common shares of Reviticell Holdings, Inc. stock; however, J.D.M. acted independently in his capacity as a physician at UF Health Jacksonville and Reviticell did not have any oversight with respect to the cellular study design. J.D.M. did not solicit nor consent patients, nor obtain or interpret data for this study.

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