SOP:	**Generation and Propagation of Human Adipocyte-Like Cells (AL) Derived from Human Amniotic Fluid Mesenchymal Stem/Stromal Cells (AF-MSCs)**
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Cell Information

Human Adipocyte-Like cells (AL) are derived from a human Amniotic Fluid Mesenchymal Stem/Stromal Cells (AF-MSCs) after in vitro adipogenic differentiation at the University of Athens, Medical School, Athens, Greece. AF-MSCs are isolated from second trimester amniotic fluid samples, obtained during routine amniocenteses for prenatal diagnosis (see SOP for *AF-MSCs*)**.**

Materials List

1. Dulbecco's Modified Eagle Medium (DMEM) (1X), liquid (high glucose), with L-glutamine (Life Technologies, Cat# 41966-029)
2. Fetal Bovine Serum (Life Technologies, Cat# 10500-064)
3. Penicillin/Streptomycin, liquid (Life Technologies, Cat# 15140-122)
4. Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X), liquid (Lonza, Cat# LONZ17-516Q)
5. Ham's F-12 Nutrient Mix Media (1X), liquid (Life Technologies, Cat# 21765-029)
6. Rabbit serum, (Life Technologies, Cat# 16120-099)
7. Human insulin (Sigma-Aldrich, Cat# I9278)
8. Dexamethasone (Applichem Cat# A2153,0001)
9. $75cm^2$ and $25cm^2$ Tissue Culture Flasks and 6 well culture plates.
10. Conical Polypropylene Centrifuge Tubes (15mL and 50mL)
11. Graduated Serological Pipets (1, 5, 10, 25, 50mL)
12. Trypsin-EDTA (10X), (Life Technologies, Cat# 15400-054)
13. Accutase Enzyme Cell Cell Dissociation Reagent (Life Technologies, Cat# A11105-01)
14. DMSO, (Sigma-Aldrich, Cat# D2650)
15. Freezing Medium (90% FBS, 10% DMSO)
16. Freezing cryotubes (Corning, Cat# 430489)
17. Beckman Coulter Centrifuge
18. -80 Freezer (Thermo Scientific)
19. Mr. Frosty™ Freezing Container (Thermo Scientific)
20. Neubauer counting chamber
21. Trypan blue (BIOCHROM, Cat#L6323)
22. Microscope

Growth Medium for AF-MSCs

400mL DMEM high Glucose Medium
100mL FBS
5mL Penicillin/Streptomycin

Freezing Medium for AF-MSCs Cells

9mL FBS
1mL DMSO

Medium for Adipogenic induction

200mL DMEM high Glucose Medium
250mL F-12 Medium
50mL Rabbit Serum
0.5µg/mL human insulin
10^{-7}M Dexamethasone

Procedure

A. Thawing Frozen AF-MSCs and Starting Cell Culture

1. When ready to start cell culture, quickly thaw 1 vial in a small container of room temperature water or a in a 37°C waterbath.
2. After about 10-15 seconds, dispense contents of vial into a 15mL conical and centrifuge tube containing 8mL complete culture medium.
3. Pellet cells at 1200 rpm x g for 10 minutes.
4. Re-suspend cell pellet in 5mL complete culture medium and dispense into one 25cm^2 tissue culture flask (1.5 x 10^5 AF-MSCs/25cm^2 tissue culture flask)
5. To culture, place the flask in a 37°C, 5% CO_2 humidified incubator.

B. Sub-culture

1. Propagate cells for 3-4 days, changing medium every 1-2 days.
2. Aspirate medium.
3. Wash cells with 1X D-PBS.
4. Add 1mL of Trypsin to 25cm^2 tissue culture flask (2mL for 75cm^2 tissue culture flask) and let sit at room temperature for 5-10 minutes, or until cells detach.
5. Immediately remove cells, rinse tissue culture plate with equal amount of growth medium to collect residual cells, and pellet at 1200rpm x g for 5 minutes.
6. Gently re-suspend cell pellet in growth medium.
7. Perform 1:2 to 1:4 split every 3-4 days.

C. Adipocyte differentiation

1. Fifth to thirteenth-passage AF-MSCs, grown as described above, are plated in 6 well culture plates at a density of 5x10^4 cells/well in Growth Medium for AF-MSCs for 2-3 days.
2. Aspirate medium.
3. Wash cells twice with 1X D-PBS
4. Add 4mL of Medium for Adipogenic induction for 3 weeks
5. Medium changes were carried out twice weekly

For the determination of adipogenic differentiation, formation of intracellular lipid droplets was minitored under microscope and was confirmed by Oil Red O staining.

D. Harvest, Cryopreservation, Nuclei

1. At time of harvest, rinse plates with PBS.
2. Add 3mL Accutase and incubate for 10-15 minutes at 37°C.
3. Remove cells to 15mL conical centrifuge tube and rinse dish with culture medium to collect residual cells.
4. Pellet cells at 300 x g for 5 minutes.
5. Wash pellet in PBS
6. Count number of cells and proceed to SOP for cultured cells: crypreservation of adherent mammalian tissue culture cells, nuclei isolation, and DNaseI treatment.

Key publications:

1. Roubelakis MG, Pappa KI, Bitsika V, Zagoura D, Vlahou A, Papadaki HA, Antsaklis A, Anagnou NP. Molecular and proteomic characterization of human mesenchymal stem cells derived from amniotic fluid: comparison to bone marrow mesenchymal stem cells. Stem Cells Dev. 2007 Dec;16(6):931-52.
   http://www.ncbi.nlm.nih.gov/pubmed/18047393

2. Roubelakis MG, Bitsika V, Zagoura D, Trohatou O, Pappa KI, Makridakis M, Antsaklis A, Vlahou A, Anagnou NP.
   In vitro and in vivo properties of distinct populations of amniotic fluid mesenchymal progenitor cells. J Cell Mol Med. 2011 Sep;15(9):1896-913. doi: 10.1111/j.1582-4934.2010.01180.x.
   http://www.ncbi.nlm.nih.gov/pubmed/21166769

3. Zagoura DS, Trohatou O, Bitsika V, Makridakis M, Pappa KI, Vlahou A, Roubelakis MG, Anagnou NP. AF-MSCs fate can be regulated by culture conditions.Cell Death Dis. 2013 Apr 4;4:e571. doi: 10.1038/cddis.2013.93.
   http://www.ncbi.nlm.nih.gov/pubmed/23559005