SOP: **Generation and Propagation of Human Dedifferentiated Amniotic Fluid Mesenchymal Stem/Stromal Cells (DAF-MSCs)**

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Cell Information

Human AF-MSCs are isolated from second trimester amniotic fluid samples, obtained during routine amniocenteses for prenatal diagnosis (see SOP for *Human AF-MSCs*).

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. (see SOP for AL cells).

Briefly, for adipogenic induction: AF-MSCs are plated at $5x10^4$ cells/well density and cultured in Growth Medium for AF-MSCs for 2-3 days in 6 well culture plates. Then, Medium for Adipogenic induction is added for 3 weeks.

Dedifferentiated AF-MSCs (DAF-MSCs): To induce dedifferentiation process, after 2 weeks in culture the adipogenic medium is removed from the AL cells and it is replaced by Growth Medium for AF-MSCs for another 2 weeks. After 2 weeks in Growth Medium for AF-MSCs, cells exhibit a phenotype similar to that of AF-MSCs, and are termed as DAF-MSCs (http://www.nature.com/cddis/journal/v4/n4/pdf/cddis201393a.pdf).

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 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

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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 ^{o}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 4 ml of Medium for Adipogenic induction for 2 weeks.

5. Medium changes are 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. Dedifferentiation procedure

1. AL cells are cultured in Medium for Adipogenic induction for 2 weeks in a 6 well culture plate.
2. Aspirate medium.
3. Wash cells twice with 1X D-PBS.
4. Add 4 ml of Growth Medium for AF-MSCs for 2 weeks.
5. Sub-culture if necessary.
6. Examine cells under the microscope.

E. Harvest

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 publication:

1. 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