Cell Culture Techniques For Human Mesenchymal Stem Cells (Poietics- from Lonza)

References:

- T Guo, L Yu, CG Lim, AS Goodley, X Xiao, JK Placone, KM Ferlin, BNB Nguyen, AH Hsieh, and JP Fisher. Effect of Dynamic Culture and Periodic Compression on Human Mesenchymal Stem Cell Proliferation and Chondrogenesis. Annals of Biomedical Engineering. 44: 2103-2113 (2016). (Pubmed)
- BNB Nguyen, RA Moriarty, T Kamalitdinov, JM Etheridge, and JP Fisher. Collagen Hydrogel Scaffold Promotes Mesenchymal Stem Cell and Endothelial Cell Coculture for Bone Tissue Engineering. Journal of Biomedical Materials Research, Part A. (2017) (Pubmed)

Preparing Gibco Culture Media

1. 860 mL high glucose DMEM (11960044)
2. 100 mL FBS (12662-011)
3. 10 mL Pen/Strep (100 units/mL each)
4. 10 mL nonessential amino acids (0.1 mM) (11140050)
5. 20 mL L-glutamine (4 mM) (25030081)
6. For Osteogenic Media, Add:
7. 1 mL dexamethasone soln (0.0392g/L to give 100 nM)
8. 10 mL β-glycerophosphate soln (2.1604g/10 mL to give 10 mM)
9. 1 mL ascorbic acid solution (51.22g/L to give 50 mg/L)
10. When using media, prepare with 10% FBS (12662-011)

Thawing cells/ initiating culture

1. Recommended density for hMSCs is 5000-6000 cells/cm2
    a. T75: 3.75-4.5x10^5; T150: 7.5 -9x10^5
2. Add media to flasks and allow to equilibrate in the incubator for at least 30 min: 0.2-0.4 mL/cm^2
    a. T75: 15-30 mL; T150: 30-60 mL
3. Prepare a volume of 5 mL of temperature equilibrated medium
4. Wipe cryovial with ethanol before opening. In biohood, briefly twist the cap a quarter turn to relieve pressure, then retighten.
5. Quickly thaw cryovial in 37°C water bath, do not submerge completely- WATCH CLOSELY. When the last sliver of ice melts, remove it. Thawling for longer than 1 ½ minutes results in less than optimal results
6. Wipe the outside of the cryovial to dry it, sterilize with ethanol, and wipe to remove excess.
7. Using a micropipette, gently add the thawed cells to the 5 mLs of temperature equilibrated medium
8. Centrifuge at 500 xg for 5 minutes
9. Resuspend the pellet in a minimum volume of temperature equilibrated medium by gently pipetting, count the total number of viable cells
10. Add the calculated volume of cell suspension to each prepared flask, and gently rock to disperse the cell suspension over the growth surface
11. Place in the incubator

Maintenance

1. hMSC cultures should be fed 3-4 days after plating
2. Remove flask of cells from incubator and check them under the inverted microscope at 10x magnification.
3. Place media in the 37°C water bath. Remove media after it is warm, wipe off water and spray with 70% ethanol. Place solutions and flasks in the biohood.
4. Unscrew the cap of the flask and place aside.
5. Move the media to the corner top of flask – no cell area. Aspirate off the media using a Pasteur pipet attached to a vacuum line. Be careful not to disturb the cells in the flask as well as avoiding the neck of the flask.
6. Pipet necessary volume of media into the flask.
7. Make sure that the cell plated side is covered with media by rocking the flask.
8. Place cap back onto the flask and put it into the incubator.

** The color change of the media from red to orange to yellow (i.e., decreasing in pH) is an indication that the cells are consuming the nutrients. Changing the media before it turns to a yellow color will aid the cells in growing more efficiently (low pH values is detrimental for cell proliferation).

Passaging Cells

** Some steps are similar to the changing media procedure.

1. hMSCs should be near confluence after 6-7 days of culture.
2. Remove flask of cells from incubator and check under the inverted microscope at 10x magnification.
3. If cells are 90% confluent (90% of flask contains cells and 10% is open space), cells are ready to be passaged. Put the flask back into the incubator.
4. Place trypsin and media in the 37°C water bath. Remove media after it is warm and remove other solutions after they have been thawed. Place into the biohood, with the flasks.
5. Move the media to the corner top of the flask – no cell area. Aspirate off the media.
6. Pipet PBS w/o $CaCl_2$ and $MgCl_2$ or DPBS. Rinse cells (face the pipette tip on the cell area side being careful not to disrupt the cells).
7. Rock flask to coat surface. Aspirate off PBS.
8. Pipet trypsin into flask 0.05mL/cm^2 (T75: 3.75mL, T150: 7.5mL). Rock flask to coat surface.
9. Incubate at room temperature for 5 minutes, check under microscope to see if cells are 90% detached. If not, check every 3 minutes up to 15 minutes. Note: can tap the edge of flask to expedite cell detachment.
10. Once detached, stand flasks on end for a minimal length of time to allow cells to drain, add an equal volume of temperature equilibrated MSCGM to each vessel. Disperse the solution by pipetting over the cell layer surface several times.

*Remember the total volume of the cell solution, this will be required to calculate the total number of cells.

11. Pipet the cell solution from the flask into a Falcon tube, remove 100 uL of the cell solution into a microcentrifuge tube for the cell count.
12. Centrifuge Falcon tube at 600 g for 5 minutes. During this time count the cells.
13. Carefully remove Falcon tube from centrifuge, spray and put into the biohood, with new flasks.
14. Aspirate off supernatant with Pasteur pipet. Resuspend in MSCGM
15. Pipet calculated volume from cell count into new flask to acquire necessary cell density. (5000-6000 cells/cm^2)

- Place flasks in incubator.

Counting Cells

1. Obtain 100 uL of cell sample.
2. Dilute sample according by adding trypan blue.
3. Pipet 10 uL of sample into hemacytometer. Avoid air bubbles.
4. Count cells in four corners of the grid.

**The average count in a square should be ~50. Do not count cells in all four edges of the corner squares (only count for 2). If cell count is too high, dilute further.

5. When using trypan blue, cells with a ring are considered viable while blue dead cells are dead. For cell viability studies, count total number of cells that are alive and dead.

Cell viability calculation: $\frac{\text{total \# of alive cells}}{\text{total \# of cells}} \times 100 = \%$ viability

Calculate total number of cells: $\frac{\text{Cell count}}{\text{\# of squares}} \times \text{Dilution} \times 10^4 = \text{\# of cells/mL}$

Total number of cells in Falcon tube = # from cells/mL x total volume

Calculate total number of flasks: # of flasks = $\frac{\text{total number of viable cells}}{\text{growth area x seeding density}}$

Cryopreservation

1. Prepare freezing media: 70 % MSCBM, 10% DMSO, 20 % Human Serum Albumin 25% solution, sterile filter
2. Harvest cells and centrifuge to collect into a pellet
3. Resuspend cells in cold freezing solution at 500,000 to 2,000,000 cells/mL. WORK QUICKLY!! Once exposed to DMSO cells become fragile.
4. Pipet 1 mL aliquots into freezing vials.
5. Insulate aliquots in propanol freezing canister. Store cells at -70^{o}C overnight.
6. Within 12-24 hours, place in liquid Nitrogen for long-term storage