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Differentiation of Mesenchymal Stromal Cells to Endothelial-like cells in Spheroidal Culture

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We use this protocol and it's working

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Abstract

In this protocol, a easy and cost-friendly method to form mesenchymal stromal cell (MSC) spheroids was specified. The MSC spheroids would form in 1-3 days and are suitable for endothelial differentiation. Accutase treatment can be used to disassociate the spheroids into single cells, for further analysis.
Multiple growth factors were used to differentiate MSC Spheroids into endothelial-like spheroids, which express endothelial markers such as von willebrand factors (vWF) and CD31 detected by immunofluorescence.

Materials

	A	B	C
	Reagent Name	Company/ Brand	Catalogue number
	0.05% Trypsin-EDTA (1x)	Gibco	25300-054
	Trypsin Neutralizing Solution	Lonza	CC-5002
	Recombinant human epidermal growth factor/EGF	PELOBiotech	C029-B
	Recombinant human fibroblast growth factor (bFGF)	PELOBiotech	C046-A
	Recombinant human vegf-a/vegf165	PELOBiotech	C083-A
	Propidium Iodide Solution	Sigma-Aldrich	P4864
	Mesencult-ACF plus culture kit	Stemcell Technologies	5448
	L-Glutamine (100x)	Gibco	25030-024
	IBIDI Angiogenesis Slides	ibidi	81506
	Cell culture plate, 96 wells, round bottom, untreated, individually wrapped	VWR	392-0291
	Dulbecco PBS, w/o Ca++/ Mg++	Promocell	C-40232
	Human serum from human male ab plasma	Sigma-Aldrich	H4522
	ibidi mounting medium	ibidi	50001
	Trypan Blue Solution, 0.4%	Gibco	15250061
	PBS, pH 7.2	Gibco	20012027
	TWEEN 20	Sigma-Aldrich	P1379
	Tris Buffered Saline (TBS), 10x	Sigma-Aldrich	T5912

	A	B	C
	Paraformaldehyde (PFA), 16% w/v, methanol free	Thermo Scientific Chemicals	043368-9M
	Bovine Serum Albumin (BSA)	Sigma-Aldrich	A3294
	Fetal Bovine Serum (FBS)	Sigma-Aldrich	F7524
	Triton X-100	Sigma-Aldrich	T8787
	Olympus FV1000 confocal microscope	Olympus	/
	Accuri C6 flow cytometer	BD Biosciences	/

Mesenchymal stromal cells (MSC) culture

1 Coat culture flasks with Animal Component-Free Cell Attachment Substrate prior to cell seeding. Dilute Animal Component-Free Cell Attachment Substrate 1:15 in D-PBS (without Ca++ and Mg++) and coat the culturewares 2 hours at room temperature (15-25 °C), using the sufficient volume to cover the culture surface. Alternatively, coat flasks overnight at 2-8 °C with flask lid sealed by parafilm.

2 Bring flasks with attachment substrate to room temperature if flasks were coated at 2-8 °C. Remove attachment substrate completely by tilting the flask and gathering the substrate to the edge of the flask.

3 Wash flasks once with D-PBS. Then the flasks are ready to use.

4 Complete 500 mL MesenCult-ACF Plus Medium by adding 1 mL of MesenCult-ACF Plus 500X Supplement and 2 mM of L-glutamine.

5 Trypsinze MSCs with controlled treatment time. Normally 30s-1min is sufficient to detach MSC from flasks. Use Trypsin neutralizing solution to neutralize trypsin since there is no serum in the cell culture medium.

6 Count cells with Trypan Blue exclusion and seed MSC in fully-supplemented MesenCult-ACF Plus Medium.

Mesenchymal stromal cell spheroids formation

7 Split and resuspend MSC in fully-supplemented MesenCult-ACF plus medium. 5m

8 Count cells with Trypan blue and dilute cell to 3x10e5/ml or 12x10e5/ml. Cells need to be over 95% viable in order to form spheroids. 10m

9 Seed 50uL of cell suspension to each well of a 96-well round-bottom plate. There will be 15,000 cells per well. 5m

10 Keep MSC in incubator for 1-3 days until one spheroid is formed in each well. Depending on the size of the spheroids, they can be observed either with eyes or underneath a microscope. Culture condition is 37 degree, 5% CO_2 and 80% humidity.

Endothelial differentiation of MSC spheroids

11 Make endothelial differentiation medium by adding 10ng/ml (final concentration) VEGF, EGF, bFGF and 2% (v/v) human serum (HS) into fully supplemented MesenCult ACF-plus medium. 5m

STEP CASE

MSC spheroids formation after 3 days in culture 20 steps

Primary umbilical cord MSC and hTERT BMMSC (an immortalized bone marrow MSC cell line) were used in the experiment. MSC were seeded at 60,000 or 15,000 per well.
Visible spheroids formed in each well after 3 days of culture. However, more than one spheroids were formed in some wells, especially in the wells with 60,000 MSC seeded. This observation suggested 15,000 cells per well is a good starting concentration to form MSC spheroids.

PDF
Step 10 case.pdf
137KB

12 Carefully remove the media from each well, avoid the spheroid stucking at the end of the tips. 10m

13 Add 50ul of differentiation medium into each well. Keep some spheroids as untreated control by adding normal culture medium. 5m

14 Differentiation duration can be between 5-11 days. It can be determined by how advanced the differentiated cells need to be.

Immunofluorescence to check endothelial markers of differentiated spheroids 1h

15 Remove medium in each well carefully. Before fixation and permeabilization, wash spheroids three times with PBS, 5 min each.

16 The same 96-well round-bottom plate was used for fixation. Fixation was done with 4% PFA for 1 hour at room temperature (RT). Fixation time varies depending on the size of spheroids. 1h

17 Wash spheroids with 100uL PBS per well three times, 5 min each. 15m

18 Block spheroids using blocking buffer (PBS with 3% FBS, 1% BSA, 0.5% Triton X-100 and 0.5% Tween) for 2 hours at RT on a shaker. 2h

19 Dilute primary antibodies 1:100–1:200 (or according to manufacturor's instruction) in the blocking buffer. Add 50ul of primary antibody to each well and incubated overnight at 2-8 °C. Primary antibodies produced from different species can be used together.

20 Remove solution from each well and wash Spheroids with PBST (0.05% Tween® 20 detergent in PBS) for 10 min at RT, three times. 30m

21 Add corresponding secondary antibodies at 1:250 in blocking buffer for 2 h at RT. 2h

22 Remove solution from each well and wash spheroids with TBST (0.1% Tween® 20 detergent in Tris-buffered saline), 20 min each time, 3 times. 1h

23 Stain Spheroids with 300nM DAPI for 20 min at 37 degree. Then wash the spheroids once with 50 uL PBST for 5 min. 25m

24 Use a 1 ml pipette to take the spheroid up together with the solution from each well. Transfer the spheroid to a well of ibidi µ-Slide 15 Well 3D. 10m

25 Remove PBST from each well of ibidi µ-Slide, add a drop of ibidi mounting medium to mount the spheroids. Control the amount of mounting medium added, so that the spheroids will be located at the bottom of each well. 20m

26 Image the spheroids with a Olympus FV1000 confocal microscope (Olympus Lifescience), or other suitable confocal microscope.

Disassociation of MSC from Spheroids for flow cytometry analysis

27 Harvest 10 spheroids from each condition into one 1.5 mL Eppendorf tubes. Centrifuge at 300g, 4 min. Then remove and discard the supernatant.

STEP CASE

Immunofluorescence images of MSC spheroids 4 steps

After 5 days of spheroids formation, hTERT-BMMSC spheroids were differentiated for 11 days before harvested for immunofluorescence analysis.
Endothelial differentiation of MSC spheroids expressed vWF and CD31, which the CD31 expression hadn't been observed in 2-D MSC endothelial differentiation assay in the lab, even after 23 days of endothelial differentiation medium treatment.

PDF

Step 26
case.pdf
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28 Add 400 uL accutase solution (Sigma/ Cat: 6964) to each tube and incubate the tube on a Thermomixer comfort (Eppendorf, Hamburg, Germany) at 37 °C, 1400 rpm for 10min. Use a 200uL pipette to pipette up and down for ten times to further disassociate the spheroids. This is considered as one disassociation cycle.

29 Repeat the disassociation cycle 1-3 times more until no visible cell clumps remaining in the tube.

30 Wash cells once with 1 mL PBS. Centrifuge at 300g for 5 min and then cells can be resuspend in flow cytometry buffer for antibody labelling.

Protocol references

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