УДК 576.54

METHOD TO GENERATE AND CULTIVATE SPHEROID CULTURE OF MSC

Рахымжанова Ақмарал Қанатқызы

akmaral.rakhymzhanova@gmail.com

Магистрант 2-курса ЕНУ им. Л.Н.Гумилева, Астана, Казахстан
Научный руководитель – Акбасова А.Ж

Multipotential stromal cells or mesenchymal stem cells (MSCs), originally isolated as single cell suspensions of bone marrow colonies of fibroblast-like cells adhering to plastic, carry multilineage differentiation potentials in vitro and in vivo after transplantation [1–6]. MSCs are relatively easy to obtain and to expand in vitro [7, 8].

Traditionally, two-dimensional (2D) adherent culture conditions have been used as a standard technique for in vitro expansion of MSCs. On the other hand, in vitro culture of

multicellular aggregates was originally described for embryonic cells 70 years ago. Because of their spherical shape, these multicellular aggregates are now called multicellular spheroids, or spheroids. Spheroids have been utilized in the field of oncology [9, 10], stem cell biology [11–14], and tissue engineering [15, 16].

Distinctive 3D constructs, as well as cell suspensions are used widely in regenerative medicine. As one of the approaches to enhance the effectiveness of cell therapy based on MMSCs, the possibility of using 3D spheroids is being considered. This allows improved viability and survival of cells during transplantation, alter the spectrum of secreted cytokines, chemokines and growth factors and also their raised production. Construction of 3D spheroids from MMSCs utilizes various techniques, varying by reproducibility expenses, automation and scale-up.

The molecular mechanisms for the forming of 3D-aggregates differ in the cell populations possessing the characteristics of MMSCs derived from multifarious tissue sources.

The aim of this research was obtaining a culture of MSCs derived from the murine compact bone and inspecting their ability to form 3D spheroids.

This article reports the methodology for rapidly and economically generating a high-quality population of 3D spheroids (mesenchymal spheres) from compact bone MSC in microwells (96 MicroWell plates, Sigma-Aldrich).

Materials and methods

Cell culture and aggregate formation

Murine compact bone-derived MSC (CBMSCs, isolated from C57BL/6 mice) were obtained from the kennel of laboratory animals named after Aikimbaev. The animals were kept in vivarium conditions including a 12-hour day/night cycle, at a temperature of 22-23ºC. All animal experiments were carried out only after the approval of the local ethics committee. The methods of isolating murine MSCs have been described previously [17]. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). Passage 3 or 4 cells were used in all experiments.

96-microwell plates (Sigma-Aldrich) were used to generate cell aggregates of various sizes (20.000, 25.000, 30.000 cells/aggregate). The probability of creating cell aggregates of assorted sizes in 96-microwell plates was appraised by varying the number of cells per well.

The 96-microwell plates were first pre-treated with 100 μL Versene solution, incubated 5-10 min at 37ºC and centrifuged at 1300g for 5 min. After centrifugation plate was repeatedly washed with DMEM. MSC monolayer was dissociated with TrypLe, counted and added to the pre-treated 96-microwell plates. Two different techniques were used for spheres generating (centrifugation and shaking). One of plates was centrifuged at 300g for 5 min, the second one was put into the shaker at 250 rpm to sediment the cells into the microwell's bottom. Overnight incubation at 37ºC under 5% CO_2 promoted aggregate formation. Cell aggregates were maintained in 96-microwell plates in regular growth medium with 10% FBS for up to 3 days in culture.

Morphological examination of cell aggregates

Phase-contrast microscopy

Phase-contrast images of cell aggregates were obtained after aggregate formation, and also at various times points during culture (Day 1, Day 2, Day 3) by using a Axio Observer A1 inverted microscope (Carl Zeiss, Germany) and image-processing software Zen 2011 (Figure 1,2).

Immunofluoroscence cell staining

Spheroids were collected into the 15 ml tubes, centrifuged at 20g for 1 min, washed with 1ml 1^x PBS and fixed with 4% formaldehyde for 20 min at room temperature. The spheroids were permeabilized with 0.2% Triton X-100 for 30 min at 37ºC under 5% CO_2 and blocked in 1% BSA in PBS for 30 min at RT. Afterwards, the cells were incubated at 4°C overnight with the following primary antibodies: CD90 (1:50) and CD44 (1:50). The cells were washed with 0.1% Tween 20 two times and with 1^x PBS and were counterstained with DAPI («Life Technologies», UK) and

propidium iodide (PI). The cells were analyzed with Axio Observer A1 (Carl Zeiss, Germany) and confocal Imaging System (Figure 3).

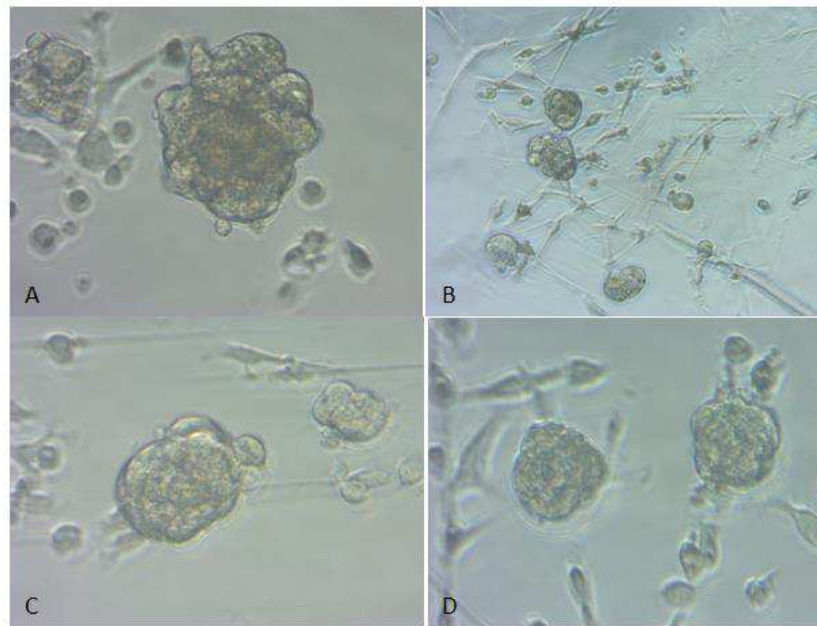

Figure 1. Sphere Culture of murine BM MSC generating by centrifugation

(A) Initial stage of formation of 3D-spheres in the culture medium, 24h (20,000 cells), (B) Spheres after the 2-day (20,000 cells), (C) Spheres after the 1-day (25,000 cells), (D) Spheres after the 2-day (25,000 cells)

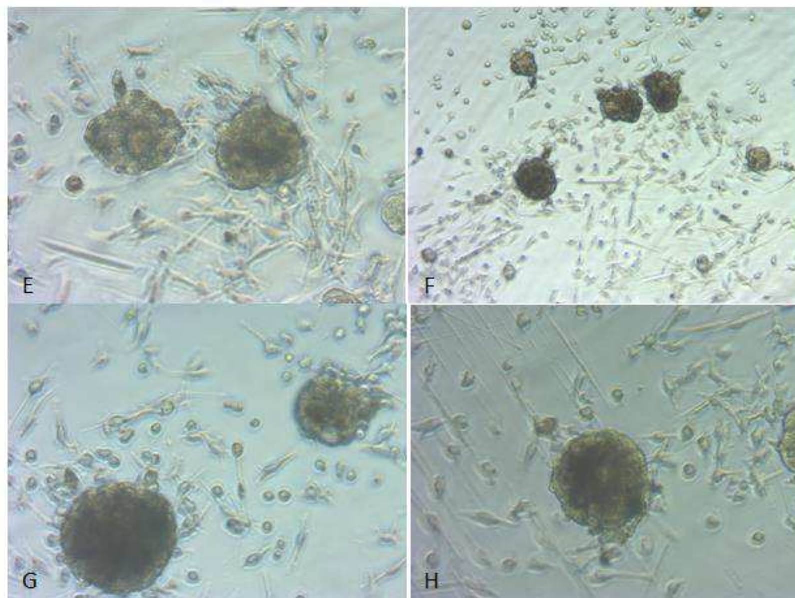

Figure 2. Sphere Culture of murine BM MSC generating by shaker

(E) Spheres after the 1-day (20,000 cells), (F) Spheres after the 2-day (20,000 cells), (G) Spheres after the 1-day (25,000 cells), (H) Spheres after the 2-day (25,000 cells).

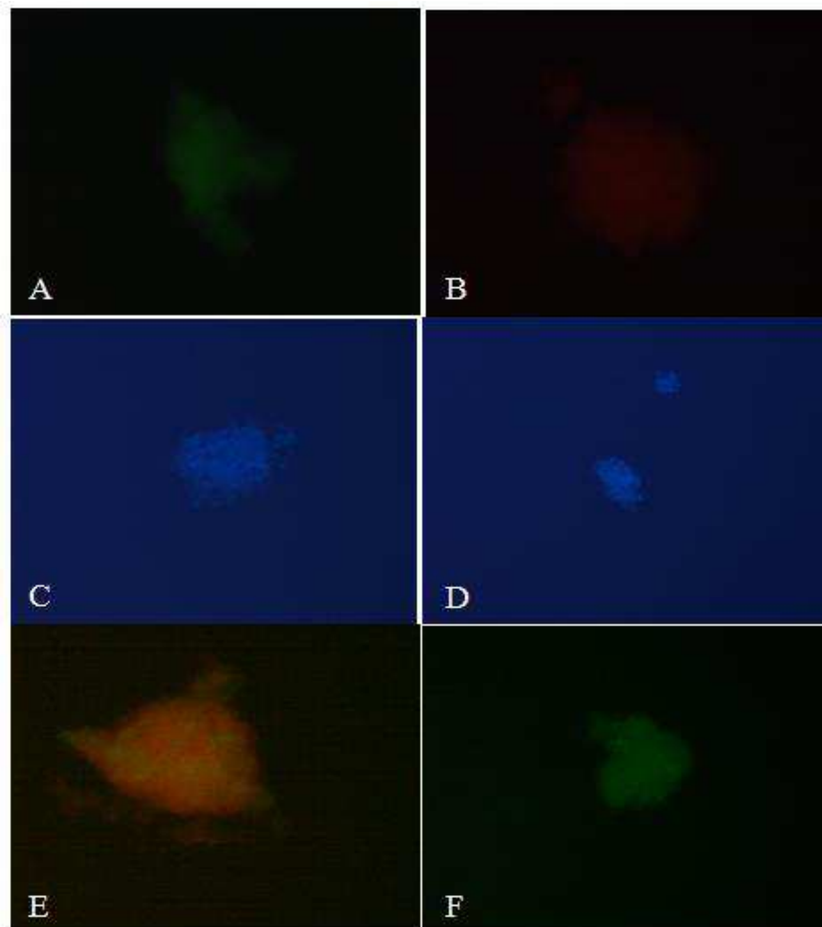

Figure 3. Immunofluorescense cell staining

(A) Cells generated by shaker and stained with PI (CD90) and (B) with PI (CD44); (C) Cells generated by centrifugation and stained with DAPI, CD44 and (D) CD90; (E) Merge CD90, PI; (F) Merge CD44, DAPI.

Results and conclusion

In the current work, 2D cultures of murine compact bone mesenchymal stem cells were employed to generate compact spherical micro-tissues or 'spheroids'. The investigational roadmap in Figure 1 depicts that MSCs are encouraged to self-assemble into spheroids in microwells for 24-48 hr, after which the spheroids can be collected and potentially utilized in both research and clinical applications. A large number of cells needed for sphere production can be acquired within 1-3 days by seeding the MSCs at low density, typically 100-200 cells per cm^2.

Following expansion and harvest, the MSCs are suspended at high cell concentration to generate spheroids, typically 30-40 aggregates per well. MSCs first assemble into small aggregates that eventually coalesce to generate a single compact sphere at 48 hr on the bottom of the microwell. During sphere assembly, MSC phenotype changes radically.

In the presented work, the possibility of forming 3D-aggregates by murine BM MSCs was demonstrated using one of the standard approaches. The obtained results can be further used to improve the technology of directed formation of 3D-spheres.

References

1. A.J. Friedenstein, U. F. Gorskaja, and N. N. Kulagina, “Fibroblast precursors in normal and irradiated mouse hematopoietic organs” Experimental Hematology, vol. 4, no.5, pp.267–274, 1976.

2. M. Owen and A.J. Friedenstein, “Stromal stem cells: marrow-derived osteogenic precursors” Ciba Foundation Symposium, vol. 136, pp. 42–60, 1988.

3. M.F. Pittenger, A.M. Mackay, S.C.Beck et al., “Multilineage potential of adult human mesenchymal stem cells” Science,vol. 284, no. 5411, pp. 143–147, 1999.

4. D.J. Prockop, “Marrow stromal cells as stem cells for non-hematopoietic tissues” Science, vol.276, no. 5309, pp.71–74, 1997.

5. K. Tamama, V.H.Fan, L.G.Griffith, H.C.Blair, and A.Wells, “Epidermal growth factor as a candidate for ex vivo expansion of bone marrow-derived mesenchymal stem cells” Stem Cells, vol. 24, no. 3, pp. 686–695, 2006.

6. K. Tamama, C.K.Sen, and A.Wells, “Differentiation of bone marrow mesenchymal stem cells into the smooth muscle lineage by blocking ERK/MAPK signaling pathway” Stem Cells and Development,vol.17,no.5,pp. 897–908, 2008.

7. K. Tamama and D.J. Barbeau, “Early growth response genes signaling supports strong paracrine capability of mesenchymal stem cells” Stem Cells International, vol.2012, Article ID 428403, 7 pages, 2012.

8. K. Tamama, H. Kawasaki, and A. Wells, “Epidermal growth factor (EGF) treatment on multipotential stromal cells (MSCs). Possible enhancement of therapeutic potential of MSC” Journal of Biomedicine and Biotechnology, vol. 2010, Article ID 795385, 10 pages, 2010.

9. W. Mueller-Klieser, “Multicellular spheroids—a review on cellular aggregates in cancer research” Journal of Cancer Research and Clinical Oncology, vol. 113, no.2, pp.101–122,1987.

10. W. Mueller-Klieser, “Three-dimensional cell cultures: from molecular mechanisms to clinical applications” American Journal of Physiology—Cell Physiology,vol.273,no.4,pp.C1109–Cl123, 1997.

11. Y. Sasai, “Next-generation regenerative medicine: organogenesis from stem cells in 3D culture” Cell Stem Cell,vol.12, no.5, pp. 520–530, 2013.

12. Y. Sasai, “Cytosystems dynamics in self-organization of tissue architecture” Nature, vol.493, no.7432, pp.318–326, 2013.

13. S. Sart, A.C.Tsai, Y.Li, and T.Ma, “Three-dimensional aggregates of mesenchymal stem cells: cellular mechanisms, biological properties, and applications” Tissue Engineering Part B: Reviews, vol. 20, no.5,pp. 365–380, 2014.

14. L. Vallier and R.A.Pedersen, “Human embryonic stem cells: an in vitro model to study mechanisms controlling pluripotency in early mammalian development” Stem Cell Reviews, vol.1, no. 2, pp. 119–130, 2005.

15. R.Z. Lin and H.Y.Chang, “Recent advances in three-dimensional multi-cellular spheroid culture for biomedical research” Biotechnology Journal, vol. 3, no.9-10, pp. 1172–1184, 2008.

16. T.M. Achilli, J.Meyer, and J.R.Morgan, “Advances in the formation, use and understanding of multi-cellular spheroids” Expert Opinion on Biological Therapy, vol.12, no.10, pp.1347– 1360, 2012.

17. Heng Zhu, Zi-Kuan Guo, Xiao-Xia Jiang, “A protocol for isolation and culture of mesenchymal stem cells from mouse compact bone”