Improved EV-Free Human Mesenchymal Stromal Cell Medium for Extracellular Vesicle Isolation and Production

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INTRODUCTION

Extracellular vesicles (EVs) produced by mesenchymal stromal cells (MSCs) can be used in cell-free therapies. A major challenge to the consistent generation and isolation of MSC-derived EVs is the presence of exogenous EVs in MSC culture media due to the addition of sera and supplements, such as human platelet lysate (hPL). EV-depleted media often fail to sustain MSC expansion and a healthy phenotype, giving users no option but to supplement culture media with hPL or serum to expand MSCs and then change to EV-depleted media prior to EV collection. In this study, we demonstrated that an improved EV-free medium (MesenCult™-ACF Plus Medium) can sustain MSC expansion and healthy phenotype, enabling the production of functional MSC-derived EVs.

METHODS

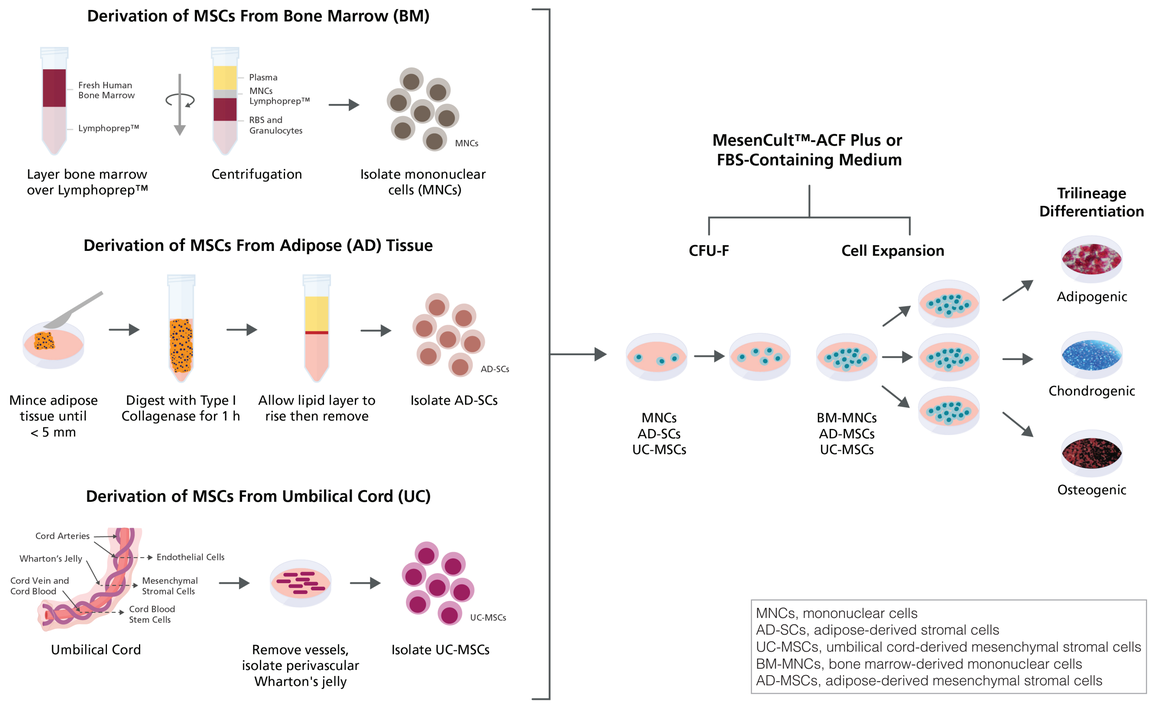

FIGURE 1. MSCs Can Be Derived From Various Human Tissues and Cultured in MesenCult™-ACF Plus Medium

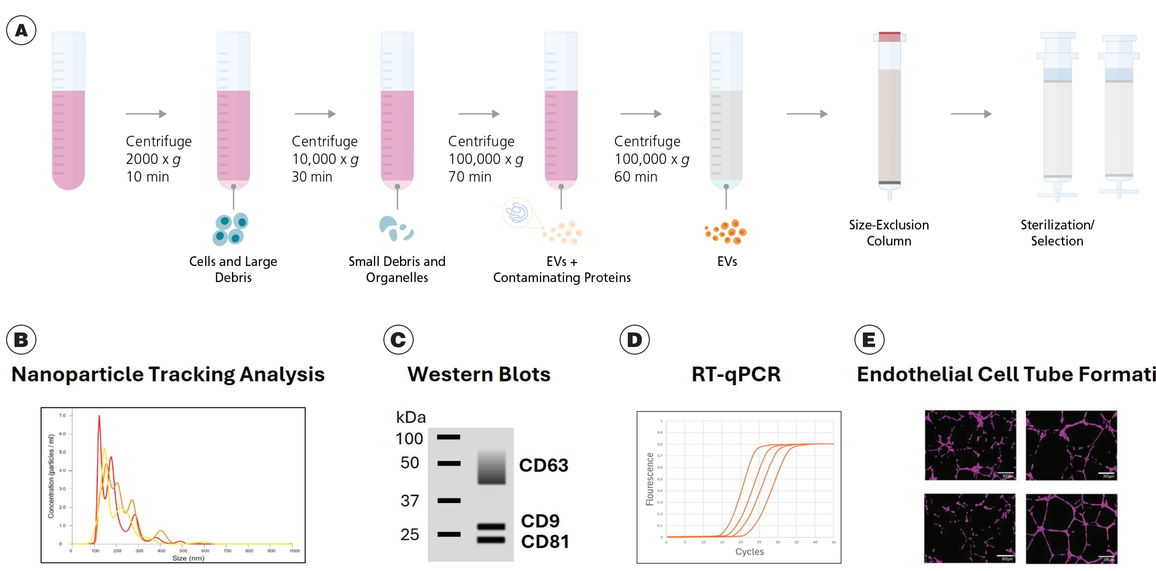

FIGURE 2. Methods of MSC EV Production, Isolation, and Analysis in MesenCult™-ACF Plus Medium

(A) EVs were produced by bone marrow (BM)-derived MSCs in 2D static cultures for 3 - 4 days. MSC-derived EVs were isolated from conditioned culture medium by differential ultracentrifugation. Size-exclusion chromatography and passage through a 0.22 µm filter are optional steps to achieve higher purity and sterility. **(B)** Samples were assessed by nanoparticle tracking analysis (NTA) for EV size distribution and concentration. **(C)** The presence of EV markers CD63, CD81, and CD9 in the samples was assessed by western blotting. **(D)** MSC EV- or serum-specific miRNA cargos were analyzed by RT-qPCR. **(E)** Endothelial cell tube formation assay was used to further evaluate the biological function of isolated MSC EVs.

RESULTS

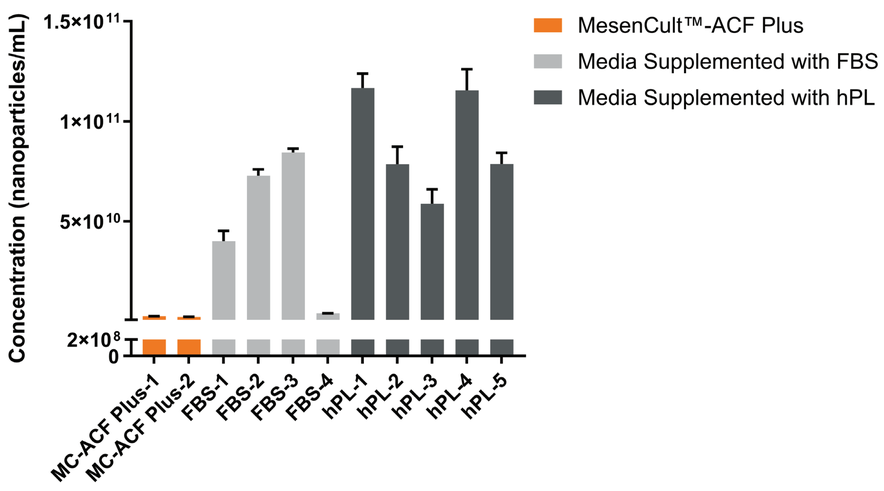

FIGURE 3. Complete MesenCult™-ACF Plus Medium Contained at Least 90% Fewer Nanoparticles Than Serum- and hPL-Supplemented Media

Nanoparticle tracking analysis was used to demonstrate particle concentration in 2 different lots of MesenCult™-ACF Plus Medium (orange bars), complete media supplemented with 4 different lots of fetal bovine serum (FBS; light grey bars), or complete media supplemented with 5 different lots of human platelet lysate (hPL; dark grey bars). MesenCult™-ACF Plus Medium contained substantially lower particle concentrations than media supplemented with serum or hPL. On average, MesenCult™-ACF Plus Medium contained less than 6% of the particle concentrations in serum- or hPL-containing media. Concentrations of nanoparticles in media supplemented with serum or hPL varied substantially between lots. Error bars represent standard error of mean (SEM; n = 3).

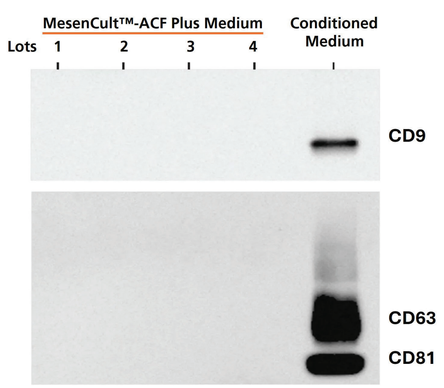

FIGURE 4. EV Markers Were Undetectable in Complete MesenCult™-ACF Plus Medium by Western Blot Analysis

The presence of EVs in 4 different lots of MesenCult™-ACF Plus Medium was investigated by western blot analysis. Conditioned MesenCult™-ACF Plus Medium was used as positive control. EV markers, CD63, CD81, and CD9 were not detected in any of the four lots of unconditioned MesenCult™-ACF Plus Medium, suggesting that this medium is EV-free. The number of particles loaded per well was 2.3 x 10^7, 1.9 x 10^8, 2.7 x 10^7, 2.5 x 10^8, and 4.6 x 10^8 per well for MesenCult™-ACF Plus lots 1, 2, 3, and 4, and the conditioned medium sample, respectively.

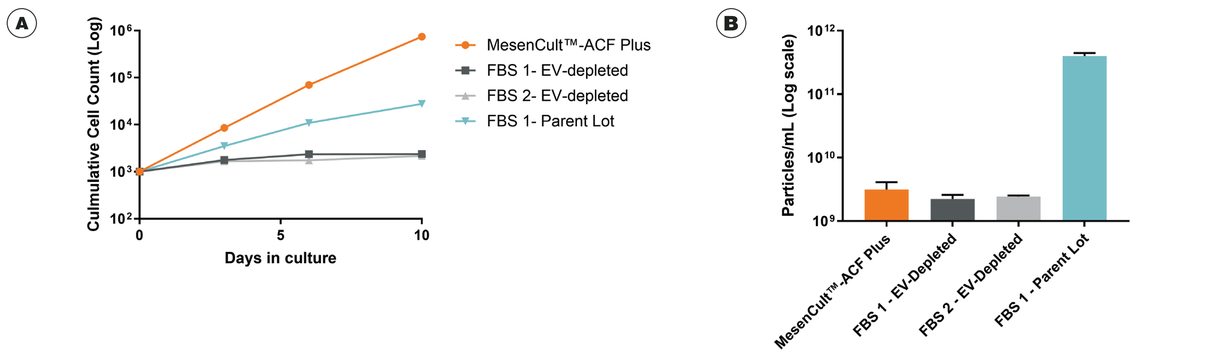

FIGURE 5. MesenCult™-ACF Plus Medium Outperformed EV-Depleted, FBS-Containing Media in Supporting MSC Expansion

The performance of MesenCult™-ACF Plus Medium in supporting MSC expansion was compared to that of media supplemented with commercially available, EV-depleted FBS (FBS 1 and FBS 2). Non-EV-depleted FBS (FBS 1 parent lot) was used as a control. **(A)** In an expansion assay, proliferation was compared when MSCs were cultured in complete MesenCult™-ACF Plus Medium or media supplemented with FBS, with or without EV depletion. MSCs cultured in complete MesenCult™-ACF Plus Medium proliferated at a faster rate than those cultured in FBS- or EV-depleted FBS-supplemented media. **(B)** Particle concentration was measured in complete MesenCult™-ACF Plus Medium and in FBS-supplemented media. MesenCult™-ACF Plus Medium had similar particle concentration to the FBS-depleted media. Error bars represent SEM (n = 3).

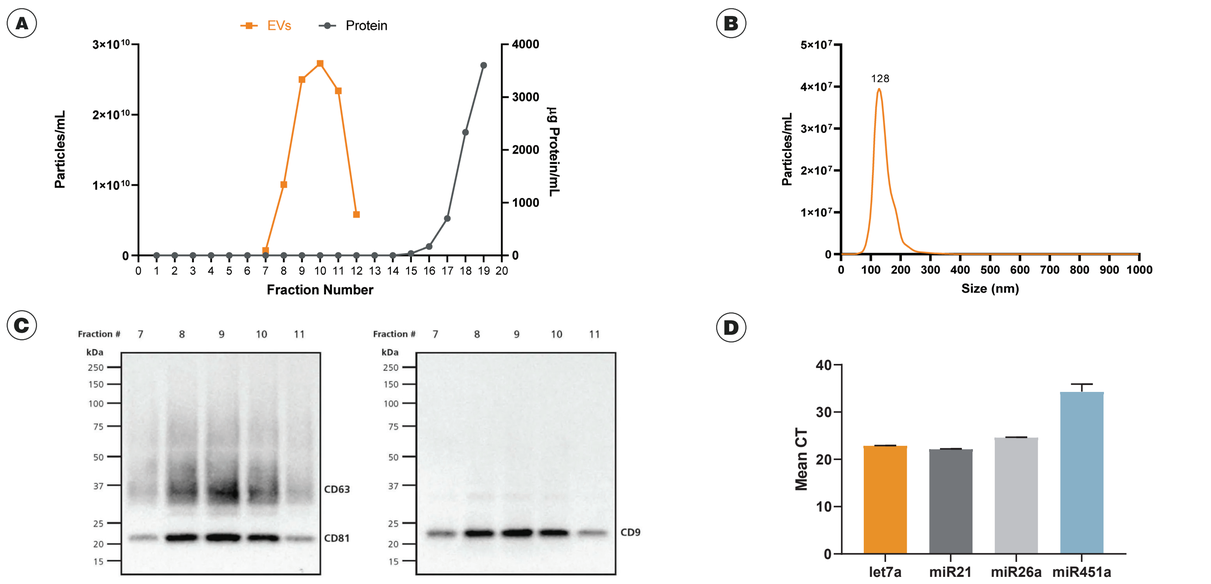

FIGURE 6. MSC-Derived EVs Isolated From Conditioned MesenCult™-ACF Plus Medium Demonstrated Size, Protein Markers, and miRNA Markers Characteristic of In Vivo MSC-derived EVs

MSC-derived EVs were isolated by differential ultracentrifugation of conditioned culture medium following 3 - 4 days in culture and were further purified using size-exclusion chromatography. **(A)** Fractions highly enriched in EVs can be selected and pooled to achieve EV samples with **(B)** characteristic EV size and **(C)** EV markers CD9, CD63, and CD81. **(D)** High levels of let7a, miR21, and miR26a were measured in MSC-derived EVs, as indicated by low threshold cycle (Ct) values in RT-qPCR analysis. miR451a is mostly expressed in serum; it was used as a negative control with a Ct value of ~35, which is considered background noise. Error bars represent SD (n = 3 for let7a, mir21, mirR26a and n = 2 for miR451a).

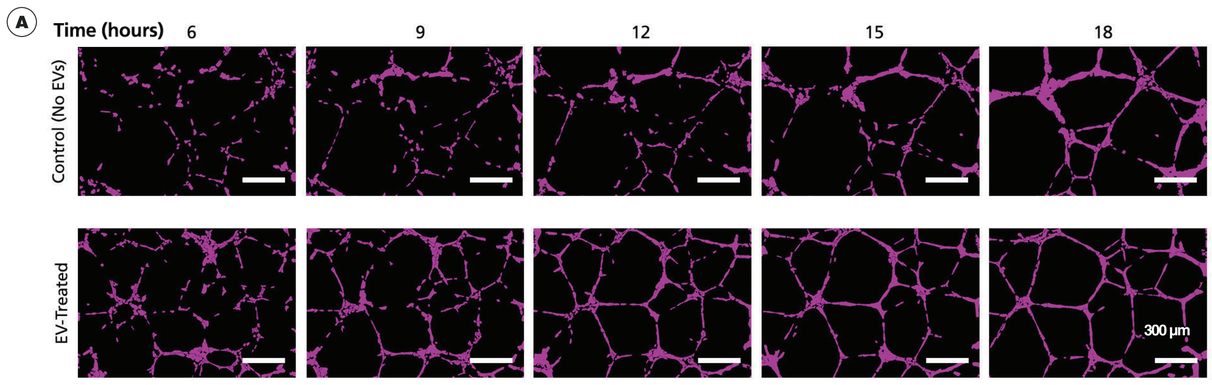

FIGURE 7. MSC-Derived EVs Enhanced Angiogenesis of Endothelial Cells

MSCs were cultured in complete MesenCult™-ACF Plus Medium, and EVs were subsequently isolated from the conditioned culture medium by differential ultracentrifugation then further purified by size-exclusion chromatography. Human umbilical vein endothelial cells (HUVECs) were plated at 10,000 cells/well (Matrigel®-coated 96-well plate) in EC-Cult™-XF Culture Medium and treated with 2.6 x 10^9 EVs/well or without EVs (control). **(A)** Cells were imaged by IncuCyte® live-cell imaging (Angiogenesis Software module) over 54 hours. **(B)** Quantification of tube formation showed that EV-treated cells formed a more extensive number of tubular networks compared to the untreated control group. Error bars represent SEM (n = 3).

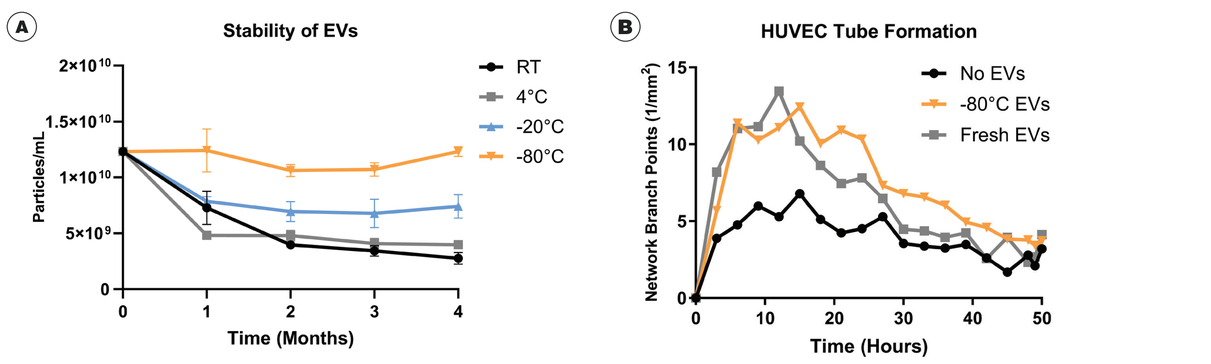

FIGURE 8. MSC-Derived EVs Isolated From Conditioned MesenCult™-ACF Were Stable in Liquid Form After Storage at -80°C

MSC-derived EVs isolated from conditioned medium were stored at room temperature (RT), 4°C, -20°C, or -80°C. **(A)** EV concentration was assessed at monthly intervals by NTA. The concentration of EVs stored at -80°C remained stable for 4 months, while the concentrations of EVs stored at other temperatures declined substantially. **(B)** EVs stored at -80°C for 4 months performed similarly to freshly isolated EVs in promoting tube formation of HUVECs.

Summary

- MesenCult™-ACF Plus Medium is free of detectable EVs, as demonstrated by western blot.
- EV-Free MesenCult™-ACF Plus Medium supports robust expansion of MSCs and their production of EVs better than other EV-depleted supplemented media.
- EVs produced in vitro by MSCs cultured in MesenCult™-ACF Plus Medium demonstrated typical MSC-derived EV characteristics and were biologically functional as assessed by NTA, western blot analysis, miRNA analysis, and endothelial cell tube formation assay.
- Isolated, intact MSC-derived EVs can be stored at -80°C and remain functional.

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