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Characterization of Small Extracellular Vesicles (sEVs) Produced by Human Mesenchymal Stromal Cells in an Improved EV-Free Medium

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INTRODUCTION

- Small extracellular vesicles (sEVs), or exosomes, mediate cell-to-cell interactions via exchange of cellular constituents between cells
- Understanding how sEVs are produced and secreted, as well as the composition and function of their cargo, will facilitate their use in cell-free therapies and as diagnostic tools
- Mesenchymal stromal cell (MSC) sEVs have been shown to elicit similar effects as MSCs themselves, including increasing vascularization by promoting endothelial cell maturation and activity, inducing immunomodulatory activities, apoptosis, and cell growth and differentiation
- In vitro studies of sEVs are often hampered by the use of undefined culture media, which often contain contaminating exosomes that originate from materials present in serum or other components
- Here, we validate the use of an EV-free and animal component-free (ACF) medium for culturing MSCs and producing MSC-derived sEVs in vitro. These MSC sEVs were characterized by:
  - Expression of tetraspanin markers CD63, CD81, and CD9
  - Presence of MSC sEV-associated miRNA, including let7a, miR21, and miR26a
  - Ability to induce vascularization in a human umbilical vein endothelial cell (HUVEC) tube formation assay
- We compared the performance of this medium to media containing EV-depleted fetal bovine serum (FBS), and developed a procedure for isolating EVs by magnetic selection that we compared to ultracentrifugation and polyethylene glycol (PEG)-mediated precipitation
- Taken together, these data demonstrate that MSC sEVs can be produced with high yield in our EV-free ACF medium and that these sEVs possess similar physical, phenotypic, and functional characteristics as MSC sEVs produced in vivo

METHODS

MesenCult™-ACF Plus (MACFP) Medium is Free of sEVs and Contains No Animal-Derived Components

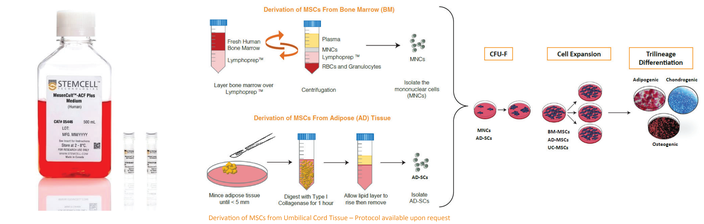
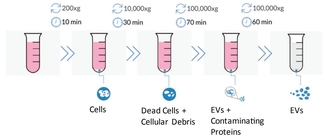


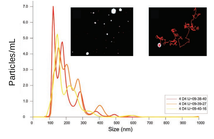
FIGURE 1. MesenCult™-ACF Plus Medium (MACFP) Supports Derivation and Culture of MSCs From Various Human Tissues.

Ⓐ MSC Static Cultures for Producing Conditioned Medium

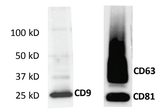
Ⓑ Differential Ultracentrifugation



Ⓒ Nanoparticle Tracking Analysis (NTA)



Ⓓ Western Blot



Ⓔ Other Assessment Assays

- HUVEC Tube Formation
- miRNA qPCR

FIGURE 2. Methods of MSC sEV Production, Isolation and Analysis in MACFP.

(A) sEVs are produced by bone marrow (BM)-derived MSCs in 2D static cultures for 3 - 4 days. **(B)** MSC sEVs are 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 (not shown). **(C)** Samples are assessed by nanoparticle tracking analysis (NTA) for sEV size distribution and concentration. **(D)** The presence of exosomal markers CD63, CD81, and CD9 in samples are identified by Western blotting. **(E)** The HUVEC tube formation assay and miRNA qPCR are used to further evaluate the biological function of isolated MSC sEVs.

RESULTS

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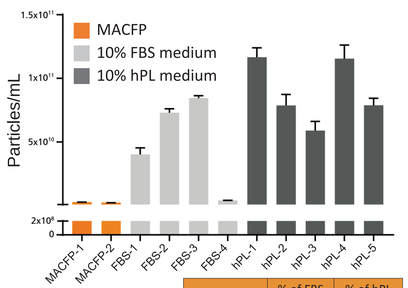

		particles/mL	% of FBS medium	% of hPL medium
MACFP	Lot 1	2.68 x 10^9	5.31	2.98
	Lot 2	2.28 x 10^9	4.53	2.55

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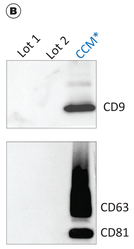

Sample	Particles Loaded
Lot 1	3.62 x 10^8
Lot 2	3.8E x 10^8
CCM	3.5 x 10^8

*CCM = MSC-Conditioned MesenCult™-ACF Plus Medium

FIGURE 3. Fresh MACFP is Free of sEVs and Contains 94% Fewer Nanoparticles Than Media Containing 10% FBS or Human Platelet Lysate (hPL).

(A) Nanoparticle tracking analysis (NTA) comparing 4 lots of FBS media, 5 lots of hPL media, and 2 lots of MACFP. **(B)** Nanoparticles identified by NTA in MACFP are not sEVs, as tetraspanin markers are undetectable by Western blotting. Conditioned culture medium (CCM) was used as a positive control and a similar number of particles were loaded per gel lane.

MSC sEVs Produced in MesenCult™-ACF Plus Express Exosomal Markers

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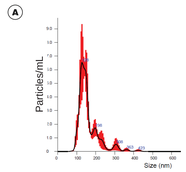

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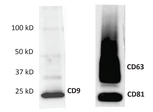

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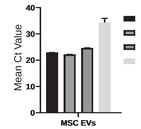

FIGURE 4. MSC sEVs Isolated From Conditioned MACFP Have Expected Size, Expression of Tetraspanin, and miRNA Markers Characteristic of In Vivo MSC sEVs.

(A) Isolated MSC sEVs have expected size between 80 -150 nm with a modal size of 128 nm as measured by NTA. **(B)** Western blot analysis of these sEVs demonstrated high expression of CD9, CD63, and CD81 markers. **(C)** High expression of let7a, miR21, and miR26a was demonstrated by qPCR analysis. An miRNA that is mostly expressed in serum, miR451a, was used as a negative control with an amplification Ct (threshold cycle) value of ~35, which is considered background noise.

MSC sEVs Produced in MesenCult™-ACF Plus are Biologically Functional

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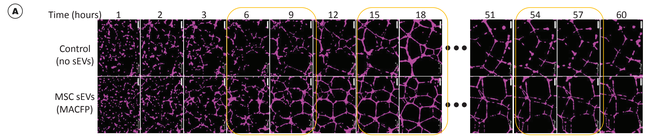

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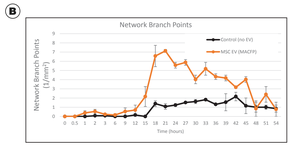

FIGURE 5. MSC sEVs Isolated From Conditioned MACFP Improve Endothelial Vascular Tube Formation.

(A) Addition of MSC sEVs to HUVEC cultures accelerated and extended tube formation in vitro. In HUVEC cultures containing MSC sEVs, tube formation was observed as early as 6 - 9 hours after cells were seeded and lasted for over 54 hours. In control cultures without MSC sEVs, tube formation was only observed 15 - 18 hours after cells were seeded and lasted less than 50 hours. **(B)** Quantification of network branch points showed that tube formation activity was 7-fold higher at 18 hours post seeding in cultures containing MSC sEVs and remained superior to control cultures for the duration of the assay.

EV-Free MesenCult™-ACF Plus Medium Supports Robust Expansion of MSCs

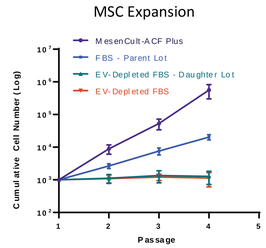

FIGURE 6. EV-Free MACFP Medium Supports Consistent and Robust MSC Expansion

MSC expansion was greatest when cultured in MACFP, followed by FBS-based medium. Depletion of sEVs from FBS led to a further reduction in the MSC expansion rate.

MSC sEVs Produced in MesenCult™-ACF Plus are Stable Long-Term

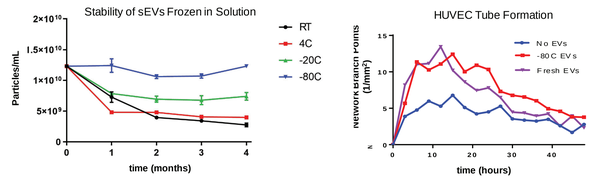

FIGURE 7. MSC sEVs Isolated From Conditioned MACFP Are Stable in Liquid Form When Stored at -80°C.

MSC sEVs isolated from conditioned medium were stored in liquid form at room temperature (RT), 4°C, -20°C, and -80°C. sEV concentration was assessed at 1 month intervals by NTA. After 4 months, only the sEVs stored at -80°C were maintained at similar concentration to that at time 0 and performed as well as freshly isolated sEVs in a HUVEC tube formation assay.

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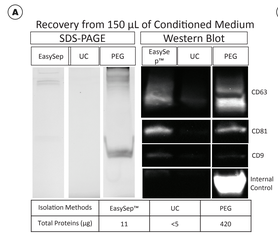

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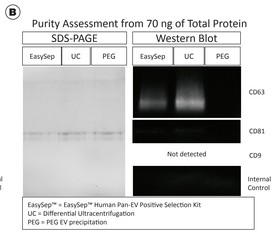

FIGURE 8. Isolation of MSC sEVs Isolated Using EasySep™ Human Pan-Extracellular Vesicle Positive Selection Kit Achieved Good Recovery and Purity Compared to Differential Ultracentrifugation and Polymer-Based (PEG) EV Precipitation Techniques.

(A) sEVs were isolated from 5 mL of MSC-conditioned MACFP using EasySep™ Human Pan-Extracellular Vesicle Positive Selection Kit, differential ultracentrifugation (UC), and a polymer-based (PEG) EV precipitation kit. Isolated EVs were resuspended in 1 mL PBS and subjected to bicinchoninic acid assay (BCA) to determine protein content. EV recovery was assessed by subjecting 150 µL from each isolation to SDS-PAGE and Western blotting. **(B)** sEV purity was assessed by subjecting 70 ng of protein from each isolation to SDS-PAGE and Western blotting. CD9, CD63, and CD81 were used as EV markers and a representative contaminant protein was used as an internal control.

Summary

- Fresh MesenCult-ACF Plus Medium is free of sEVs as demonstrated by:
  - Western blot analysis
  - RNA analysis
- MesenCult™-ACF Plus Medium supports robust expansion of MSCs and their production of sEVs at high yield.
- sEVs produced in vitro by MSCs in MesenCult™-ACF Plus Medium are similar to sEVs present in vivo, and are biologically functional as assessed by:
  - NTA
  - HUVEC tube formation assays
  - miRNA analysis
  - Western blot analysis
- Isolated intact MSC sEVs can be stored long-term
- EasySep™ Human Pan-Extracellular Vesicle Positive Selection Kit:
  - Is compatible with several downstream assays
  - Provides better recovery of EVs than ultracentrifugation