Assessing Biological Mechanisms of Action of Soluble Paracrine Factors and Extracellular Vesicles Secreted by Human Cardiosphere-Derived Cells

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Introduction

Cardiosphere-derived cells (CDCs) are a regenerative cell product that is being tested clinically for numerous disease indications including Duchenne muscular dystrophy, heart failure with preserved ejection fraction, and pulmonary arterial hypertension. The mechanism of action is understood to be paracrine in nature, through reduction of inflammation and fibrosis, and enhancement of viability and angiogenesis. CDCs have a complex secretome that includes extracellular vesicles (EVs), several growth factors, and many other proteins. While the EVs are believed to mediate many of the CDCs' effects, the soluble growth factors might play a role in the activities mediated by the cells.

Objectives

(1) The goal of this work was to develop assays to quantify CDCs' bioactivities and to distinguish between effects mediated by growth factors secreted by CDCs and CDC-EVs.

(2) To examine the effect of UFC molecular weight cutoff (MWCO) used to concentrate EVs upon the different activities mediated by CDC paracrine factors (CDC-EVs and secreted growth factors).

(3) To compare two production conditions: 15 day serum starvation and 24h hypoxia.

Methods

- **EV isolation** was performed using ultrafiltration by centrifugation (UFC) in 10, 30, 100, and 1000 kD filter units.
- **EV concentration** was determined using a Nanosight 300.
- **Protein concentration** was determined using Bio-Rad's DC protein assay.
- **Flow cytometry for EV markers** Streptavidin-conjugated magnetic beads were mixed with biotinylated Tim4 molecules to capture CD81 labeled EVs from suspension via Tim4-phosphatidylserine interactions. Bead-bound EVs were analyzed by flow cytometry.
- **Immunomodulatory effects in peritoneal macrophages** Rats were injected IP with 3% Brewer's Thioglycolate, and 3 days later, macrophages were isolated from peritoneal lavage fluid. Macrophages were treated with 2000 EVs/cell for 6 hours and the expression of pro- and anti-inflammatory genes analyzed by qPCR.
- **Anti-inflammatory effects in HUVECs** HUVECs were pre-treated with $5x10^8$ EVs/mL overnight followed by stimulation with 5 ng/mL TNF. After 1 h, RNA was extracted and expression of VCAM, ICAM and E-Selectin was determined by qPCR. HPRT housekeeping gene served as loading control for normalization.
- **qPCR** RNA from EVs was extracted using Qiagen miRNEasy Blood and Serum kit or using RNEasy Mini kit for macrophages or HUVECs. cDNA was synthesized and real time qPCR was performed using TaqMan probes.
- **Western blot for phospho-ERK in HUVECs** Serum starved HUVECs were treated with concentrated conditioned medium (CCM) from different preps containing $5x10^8$ EVs/mL, or an equal volume of flow-through (FT). Lysates were probed with phospho- and total antibodies to ERK1/2 and Akt. Actin was used as the loading control.
- **Mass Spec** LC/MS/MS analysis was performed using an Ultimate 3000 LC connected to an Orbitrap Elite mass spectrometer. Raw data was searched using the most recent version of the Uniprot Rat Unreviewed database with the Sorcerer Sequest search engine (Sagen), and processed using Scaffold 4.

Results

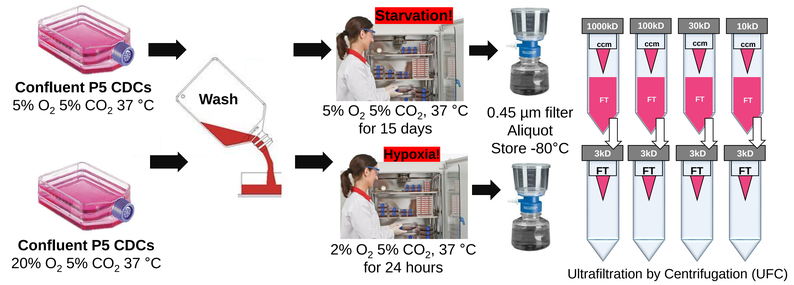

Figure 1. Production of Conditioned Medium and Concentration of EVs. CDCs were grown to confluence and then subjected to serum starvation (SS) for 15 d or hypoxia and SS for 24 h. Conditioned media were concentrated using 10, 30, 100 or 1000 kD MWCO UFC filters. For some experiments, the flow-through was then concentrated using a 3 kD filter.

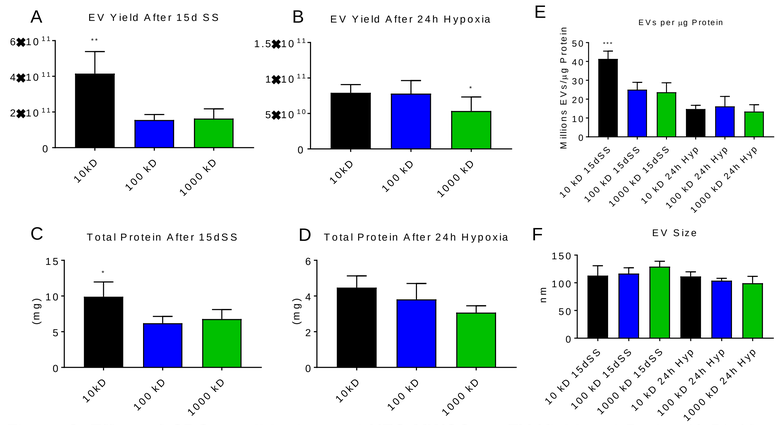

Figure 2. Effect of CDC conditioning and UFC MWCO on EV Yield and Purity. (A-D) After UFC, EV and protein concentrations of each preparation were determined, and based on the volume, the total yields of EVs and protein were calculated. (E) As a measure of purity of the EVs, the ratio of EVs per µg protein was determined. (F) Average size of the EVs; n=4 donors with 1-3 experimental replicates; *P<0.05, **P<0.01, ***P<0.001.

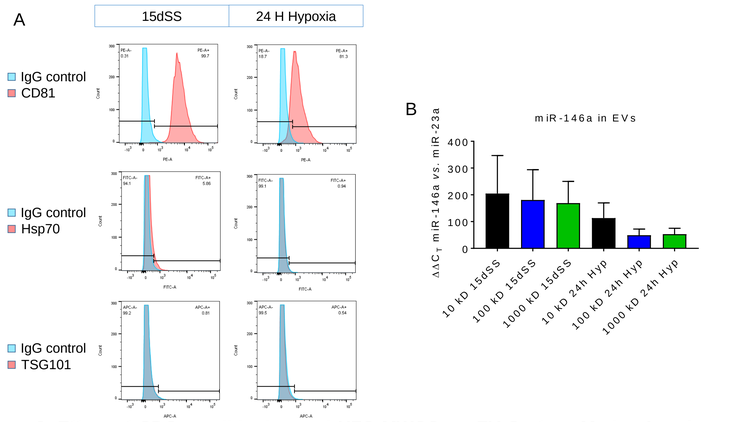

Figure 3. Effect of CDC conditioning and UFC MWCO on EV Surface Marker Levels and Levels of miR-146a. (A) CDC-EVs were analyzed by bead-based flow cytometry for CD81, Hsp70, and Tsg101 (red area) or for isotype control (blue area) and % of positive cells calculated. (B) Expression of miR-146a was normalized to that of miR-23a, a housekeeping gene that is consistent in all CDC lines (Gouin, K., et al 2017). The expression in CCM of 4 CDC cell lines was averaged for each different UFC MWCO.

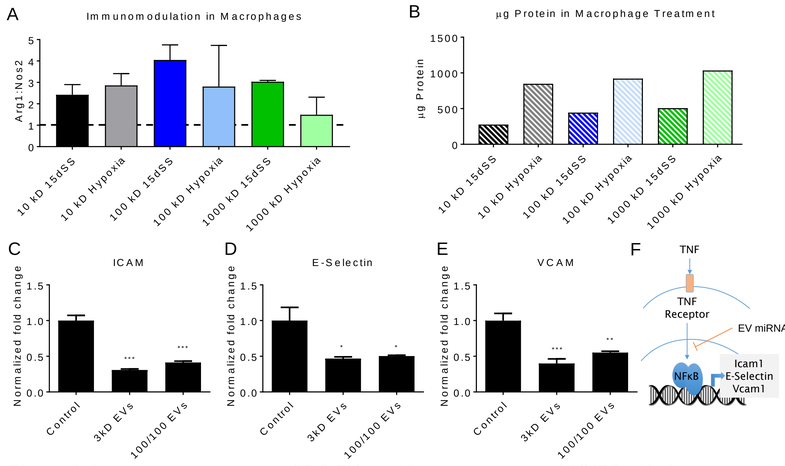

Figure 4. Immunomodulation by CDC-EVs in Macrophages and HUVECs (A) Incubation of freshly isolated rat peritoneal macrophages with EVs resulted in increased expression of Arg1 relative to Nos2, compared to untreated macrophages (average shown by dashed line), indicative of polarization to an anti-inflammatory M2-like phenotype. This effect was independent of UFC MWCO and (B) total protein in the treatment, suggesting that it is mediated by EVs and not secreted proteins. (C-E) HUVECs were pre-treated with 15dSS EVs obtained by 3 kD UFC or 100 kD UFC with two 100 kD washes ("100/100 EVs"). Upon stimulation with TNF-α, both EVs blunted the expression of ICAM-1, E-Selectin, and VCAM-1 to a comparable extent; n=4, *P<0.05, **P<0.01, ***P<0.001. (F) Schematic of potential anti-inflammatory mechanism.

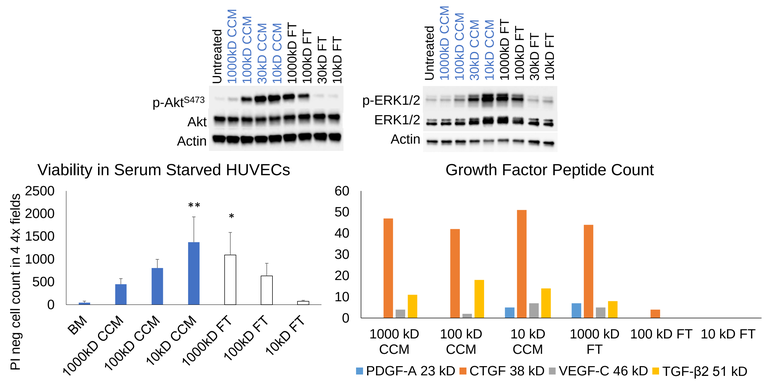

Figure 5. Effects of Extravesicular Factors in Endothelial Cells (A) Treatment of HUVECs with CDC-EVs resulted in phosphorylation of Akt and ERK1/2, consistent with activation of growth factor receptors. (B) These treatments increased viability of serum starved HUVECs, n=3, *P<0.05, **P<0.01. These effects were dependent on the UFC MWCO, and thus are most likely mediated by secreted proteins. (C) Proteomics on the CCM and FT revealed the presence of variable amounts of growth factors which correlated with the pro-survival effects.

Conclusions and Future Studies

15dSS yields more EVs than 24h hypoxia, but also more secreted proteins. The EVs produced by 15dSS are more strongly positive for CD81 and tended to have more miR-146a than those produced by 24h hypoxia. Regardless of the UFC MWCO and preparation method, the EVs had comparable anti-inflammatory effects in macrophages and HUVECs. However, the MWCO of the filter had a significant effect on AKT and ERK1/2 signaling and viability in HUVECs, which resulted from the presence of growth factors, rather than EVs.