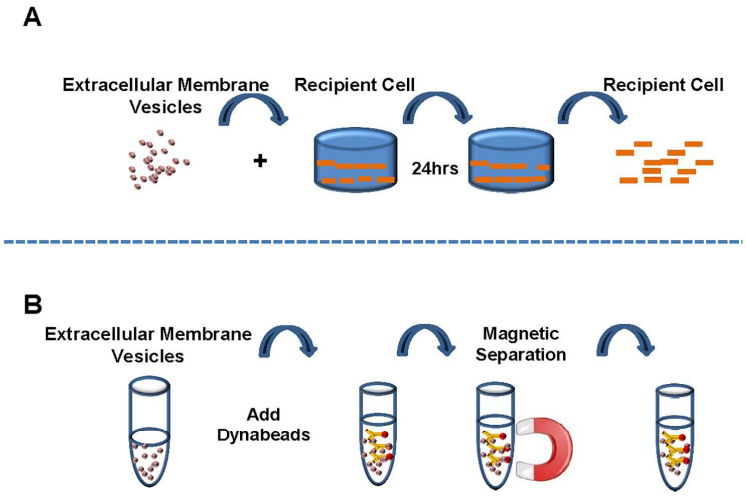
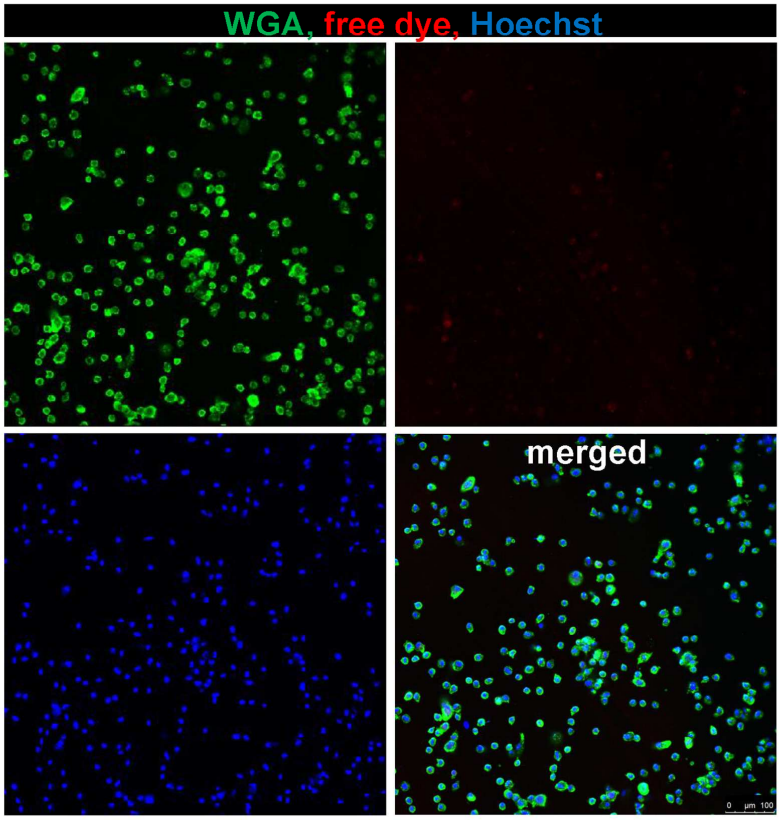
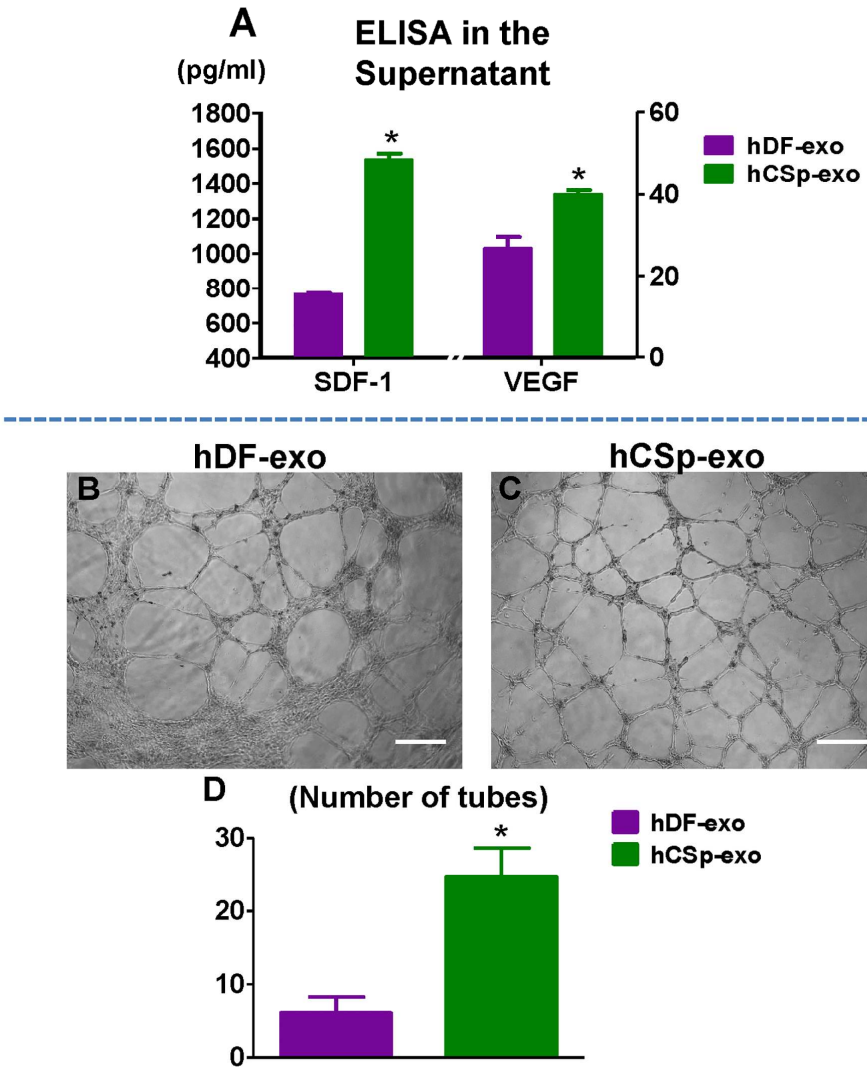
Online Figure 1



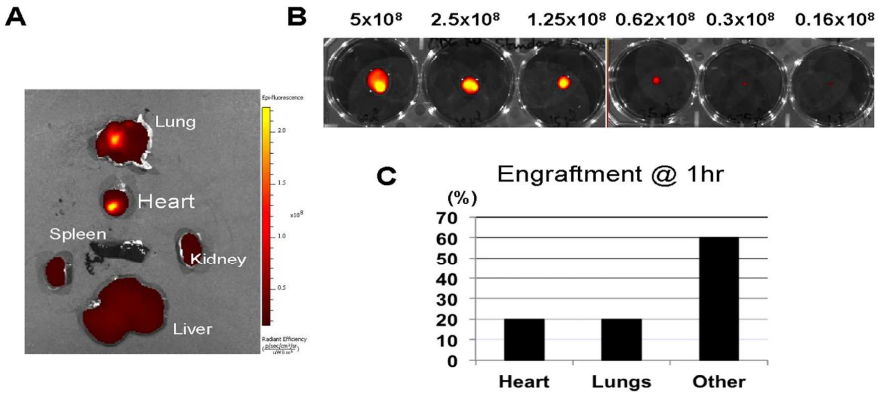
Online Figure 2



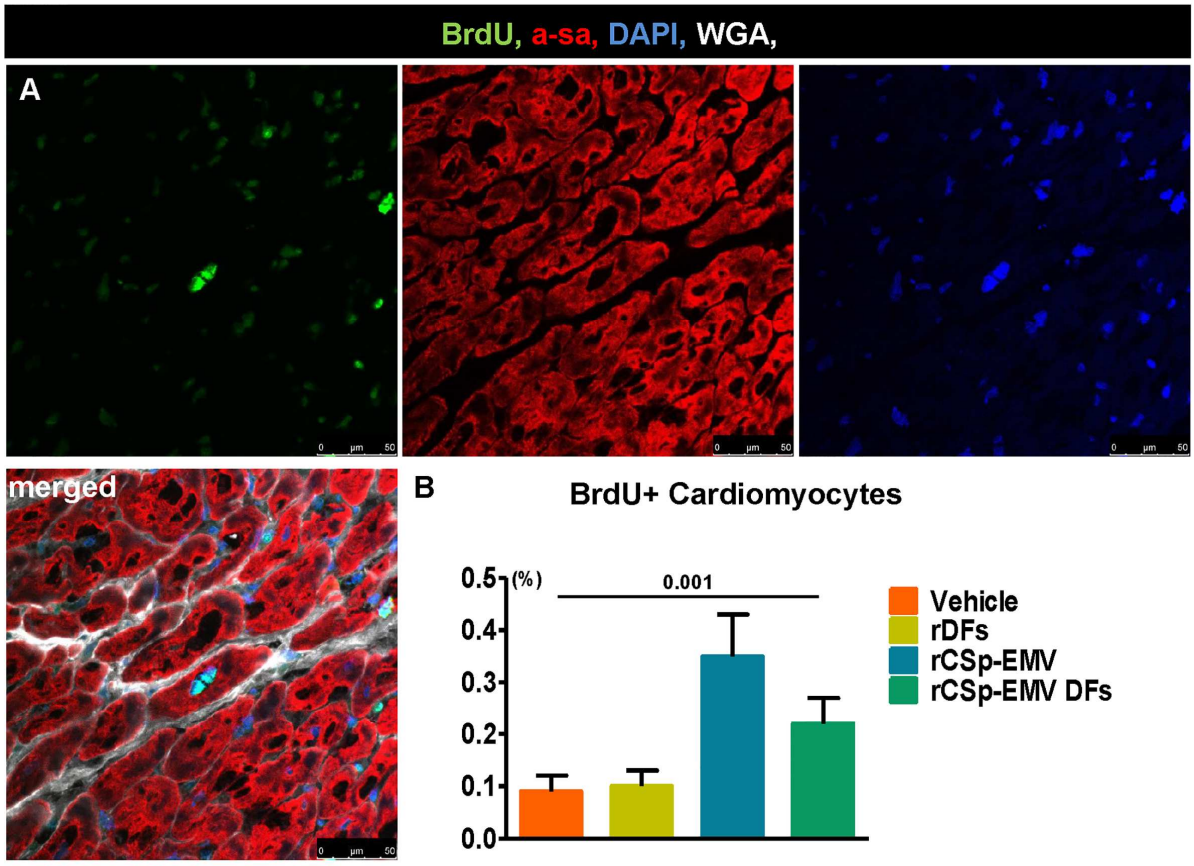
Online Figure 3



Online Figure 4



Online Figure 5



Fibroblasts rendered antifibrotic, anti-apoptotic and angiogenic by priming with cardiosphere-derived extracellular membrane vesicles

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Supplementary Data

Animals

Female Wistar Kyoto rats (n=54) 5-6 weeks of age were used for *in vivo* experiments. Animal care followed guidelines established by the NIH Guide for the Care and Use of Experimental Animals and were approved by the institutional Animal Care and Use Committee. To create MI, animals underwent permanent LAD ligation as described[1]. Four weeks later they underwent a second survival thoracotomy with animals randomly assigned to intramyocardial border zone injection of one of four treatments: 1) r CSp-EMV derived from 2M cells (n=16); 2) 2M rDFs (n=12); 3) 2M rDFs which had been incubated overnight with r CSp-EMV, then washed (r CSp-EMV DF, n=16); or 4) vehicle (phosphate-buffered saline [PBS]; n=10). The animals were followed for an additional 4 weeks before endpoint functional and histological studies as described below.

For the qualitative assessment of EMV engraftment and biodistribution, particles labeled with a far-red highly lipophilic dye (DiIC18(7) or 1,1'-dioctadecyltetramethyl indotricarbocyanine Iodide; Xenolight; Perkin Elmer) were injected in the myocardium as described (2) and euthanized one hour post-injection, for imaging of the lungs, heart, kidneys, liver and spleen using IVIS (Perkin Elmer). To avoid fluorescence of the dye we injected animals only with dye at the concentration used for EMV labeling. For

studies of cardiomyocyte proliferation, 5-bromo-2'-deoxyuridine (BrdU) was intraperitoneally injected as described (3). Briefly, BrdU (50mg/kg) was injected every 48hrs starting on the day of the second surgery. The animals received a total of 3 doses and were sacrificed 48hrs after the last BrdU injection.

Echocardiography measurements

Baseline transthoracic echocardiography was performed 28 days post-MI (2 days before the second thoracotomy) as described (1). Briefly, long-axis images were used to measure left ventricular end-systolic and end-diastolic volumes and ejection fraction. Short-axis M-mode images at the level of the papillary muscle were used to measure end-systolic diameter. The follow-up echocardiographic analysis was performed 4 weeks post injections followed by euthanasia.

Cell culture/ Conditioned media preparation

For *in vivo* experiments, CDCs were isolated from male Sprague Dawley and Brown Norway rats and cultured in IMDM (Gibco) supplemented with 20% FBS and antibiotics. Following cell expansion, rCSp-EMV were isolated as described below. For *in vitro* experiments, hCSps and hCSp-EMV from 2 different donors (OD220 and OD211) were used as described (4). Normal hDFs and rDFs were purchased from ATCC. All cells were incubated at 37℃ in 5% CO_2 (4).

To form cardiospheres, 15M CDCs were incubated with IMDM supplemented with penicillin/streptomycin and 0%FBS in ultra-low attachment dishes (5). Three days later, the conditioned medium was collected and processed for EMV isolation as depicted in

Figure 1A. For DF-secreted EMV (DF-EMV) isolation, cells were seeded in fibronectin-coated flasks with serum-free media for 3 days.

Isolation and characterization of extracellular membrane vesicles

CSp-conditioned medium was subjected to two successive centrifugation steps: 2000rps for 20 min and 10000g for 30min. The resulting supernatant was precipitated by polyethylene glycol (ExoQuick^{TC}) followed by overnight incubation at 4^0C. The next day, vesicles including exosomes, larger ectosomes or apoptosomes were isolated by centrifugation at 1500g for 30 min (6, 7). For functional analysis of CSp-EMV *in vivo* we used the dose equivalent of 2M rCDCs (~250µg evaluated by BCA [Thermo Scientific] quantification protocol, in 120µl of Phosphate Buffered Saline (PBS; Gibco) in each animal. rDFs for *in vivo* experiments were seeded on fibronectin-coated dishes in serum-free medium for 24hrs before assay. rCSp-EMV were added for an additional 24hrs (dose equivalent of 1:1 ratio of CDC: fibroblast (170µg)) followed by washing and trypsinization. A total of 2M rCSp-EMV primed or unprimed rDFs were intramyocardially injected into four sites in the peri-infarct zone, similar to rCSp-EMV.

A second method of isolation was also added to verify the exosomal component of our extracellular vesicles (Online Fig. 3). Here, the conditioned media was filtered with a 0.2µm filter and vesicles were separated based on size and density. Two serial centrifugations at 2000g and 10000g removed media and large particles. The last centrifugation at 10000g pelleted the exosomes (7).

Internalization assay

To test the hypothesis that EMVs are efficiently taken up by recipient cells, hDFs were seeded on fibronectin-coated dishes and cocultured with CSp-EMV (Online Figure 1A). In order to visualize the EMVs, a highly lipophilic dye (DiIC18(7) or 1,1'-dioctadecyltetramethyl indotricarbocyanine Iodide; Xenolight; Perkin Elmer, 250µg/ml), was added in the CSp conditioned media followed by the addition of polyethylene glycol and overnight incubation at 4^0C. Centrifugation as described was performed to isolate the EMVs followed by dilution in serum-free media in a 1:1 CDC: fibroblast ratio. 2cc's were added per well. The hCSp-EMV-fibroblast incubation was performed for 24hrs (37^0C, 5%CO_2) and stopped by extensive washing in cold PBS. Two different staining protocols were applied. For cells in culture we used phalloidin to visualize the cytoskeleton (Invitrogen) followed by DAPI (4',6-diamidino-2-phenylindole). For assessment of internalization, live cells were trypsinized and stained with wheat germ agglutinin (WGA, Invitrogen) and Hoechst (Invitrogen). To evaluate dose- and time-dependent internalization of EMVs, we incubated with one of 3 different nanoparticle concentrations (5Billion, 20Billion and 40Billion particles/2 ml) and performed confocal microscopy (Zeiss axiovert 200) 6, 12 and 24 hrs post hCSp-EMV addition to the culture. EMV internalization and intensity per cell were evaluated by Image J (NIH) from n=3-5 high-power (20x) images per group. To avoid free-dye fluorescence we used a control group. DiIC18(7) was added in media at the same concentration as described above (250µg/ml) followed by addition of polyethylene glycol and overnight incubation at 4^0C. After centrifugation, the media was further diluted 1:1000 and 2 cc were added per

well. The fluorescence detected per cell was subtracted from the above groups analyzed.

Western blots and antibodies

DFs 24hrs post hCSp-EMV priming were lysed in RIPA (Thermo) buffer, centrifuged for 20min at 15000rpm and the soluble fraction was resuspended in SDS loading buffer with a protease inhibitor cocktail. Equal amounts of protein from cell lysate were separated by SDS-PAGE and blotted onto transfer membranes followed by blocking overnight at 4 ℃ with gentle agitation using 5% mi lk in TBS-T buffer. Following incubation with primary antibody, blots were washed three times for 5 min each with TBS-T buffer and incubated with appropriate horseradish peroxidase-labeled secondary antibodies in 5% milk in TBS-T buffer for 1h at room temperature. ECL was used for protein detection. Quantification of bands was performed using Image J (NIH). Primary antibodies used for western blot analysis were rabbit anti-human smad2/3 antibody (Thermo, 1:500), rabbit anti-smad4 antibody (Thermo, 1:500), anti-mouse GAPDH (Life Technologies) and anti-human snai1 (Sigma Aldrich,1:1000).

Flow cytometry

CSp-EMV were analyzed for tetraspanin CD63, CD81 and CD9 expression by flow cytometry as described (6). Briefly, CSp-EMV were incubated overnight with CD63, or CD81 or CD9 coated dynabeads at 4^0C, followed by washing with 0.1% FBS and magnetic separation per manufacturer's instructions. The beads were then incubated with primary anti CD63, CD9 and CD81 antibodies (System Biosciences; Rabbit IgG 1:1000 dilution) for 1hr at room temperature, followed by extensive washing and

appropriate secondaries for an additional 1hr at room temperature before analysis (Invitrogen; anti –Rb 488 1:600 dilution). This method allows us to characterize the expression of the aforementioned tetraspanins but not to quantify them. As control groups we used beads incubated with the primary followed by the secondary antibody and beads incubated with the secondary antibody only at the same concentration as the CSp-EMV groups and analyzed contemporaneously.

For the phenotypic alterations in the human DFs post CSp-EMV treatment we used cells 24 hrs post CSp-EMV priming. Cells were trypsinized, rinsed with PBS, incubated with primary antibodies in 0.1%BSA for 45 min at 4^0C: rabbit anti-FSP1 (Novus), rabbit anti-DDR2 (Abcam), mouse anti-CD105 PE conjugated (BD), mouse anti-CD90 conjugated FITC (BD). Appropriate secondaries and isotypes were used in parallel. Cell populations were analyzed on an LSRII flow cytometer (Beckton Dickinson). Flow cytometry data were analyzed using FlowJo.

Nanoparticle Tracking Analysis (NTA)

CSp-EMVs or CSp-exosomes resuspended in PBS at a concentration of ~200 μg of protein/ml were further diluted 100- to 500fold for analysis using the LM10-HS system (NanoSight), which focuses a laser beam through a suspension of the particles of interest for visualization by light scattering. A 60 s video records all events for further analysis by NTA software. The mean size and SD values obtained by the NTA software correspond to the arithmetic values calculated with the sizes of all the particles analyzed by the software.

ELISA

Secretion of SDF-1 and VEGF from primed or unprimed hDFs was measured in conditioned culture media by ELISA (R&D, per manufacturer's protocol). Briefly, 24hrs post hCSp-EMVs or CSp-exosome priming of hDFs, the cells were washed twice with PBS and new serum-free medium was added for an additional 24hrs. Unprimed hDFs or NHDF-exo primed hDFs were similarly treated and observed contemporaneously.

Immunohistochemistry

Unless otherwise noted, animals were euthanized 4 weeks after injection. Cardiac sections (8μm) were cut with a cryostat for the histopathological assessment as described (1). Briefly, slides were washed with PBS and fixed with 4% paraformaldehyde followed by blocking solution (Dako). Slices were incubated overnight at 4 ℃ with the primary antibody; mouse anti-smoot h muscle actinin (sma, 1:500, Sigma), rabbit anti-von Willebrand factor (vWf) (1:250, Abcam), mouse anti-α sarcomeric actinin (1:400, Sigma), WGA (1:500, Invitrogen), mouse BrdU (1:200, Abcam). Slices were washed three times with PBS and were incubated with a secondary antibody of the appropriate species; Alexa 547 goat anti-mouse (1:200, Invitrogen) and Alexa 488 goat anti-rabbit. The microscope analyses were performed using an epifluorescence microscope (Zeiss).

To evaluate scar mass and infarct wall thickness, we stained serial slides cut from the base to the apex with Masson's trichrome (Sigma) as described (1). Image J was used for wall thickness and scar size evaluation, and scar mass was calculated according to

the heart weight. For histological evaluation, n=5 different heart samples were included in each group.

For fibroblast immunofluorescence staining, the following primary antibodies were used: rabbit anti-FSP1 (Novus), mouse anti-sma (Sigma), followed by appropriate secondaries.

Neonatal rat ventricular cardiomyocyte viability assay

Neonatal ventricular cardiomyocytes (NRVMs) were obtained from Sprague Dawley rats, plated in 6-well dishes at a density of 2M/well and grown in DMEM containing 10% FBS under 37^0C, 5%CO_2 for 24hrs. To minimize basal apoptosis we switched to 2%FBS for an additional 48hrs. 3 days post plating the media was removed, the cells were washed with PBS and new serum free media with CSp-EMVs, or conditioned media from CSp-EMVs primed DFs, or DF-EMVs was added for 24hrs. For cell stressing, 100μmol H_2O_2 was added and the cells were followed for an additional 24hrs. At the end of the incubation period cells were trypsinized, washed in PBS and viability was evaluated for Annexin V staining (BD) as per manufacturer's instructions. Cell populations were analyzed on an LSRII flow cytometer (Beckton Dickinson). Flow cytometry data were analyzed using FlowJo.

Tube formation assay

HUVECs (PromoCell) were expanded *in vitro* according to manufacturer's instructions. To evaluate the effects of CSp-EMV and CSp-EMV primed human DFs on angiogenesis, HUVECs were plated on Matrigel coated 96-well plates at a density of

40.000 cells per well. CSp-EMV, or CSp-EMV primed DF-conditioned media or DF-EMV was added in triplicate. 5-10 high-power (10x) images were obtained under light microscopy 4 hrs post cell and media plating. Quantification of tube number was performed using Image J (NIH). To validate that the exosomal component of the CSp-EMV contributed to this effect we also treated HUVECs with $5x10^9$ particles per well CSp-exosomes and hDF-exosomes isolated by ultracentrifugation and analyzed the number of tubes formed at the same time point (Online Fig. 3).

MicroRNA array analysis

To characterize the miR cargo of the CSp-EMV and to evaluate potential alterations in the miR signature of DF-secreted vesicles before and after priming with CSp-EMV, we used microarrays according to manufacturer's instructions (SA Biosciences). Briefly vesicles from the conditioned media were extracted as above described. EMV pellet was resuspended in Trizol and miR was extracted using miRNeasy kit (Qiagen). A total of 100ng of total RNA from each sample was first reverse transcribed and MiScript arrays with total 88 genes (Qiagen) were used for loading. Analysis was performed according to manufacturer's instructions. For the miR profile of the primed and unprimed DFs, a similar protocol was followed. n=3 per group.

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