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Appendix E1: Supplemental Methods

Culture of MSC and MSC-TF

To avoid variations in rat MSC isolation, all reagents, including MSC, culture medium, and serum supplement were purchased (Cell Applications, #R492–05). Cells at passage 2 were received, thawed, washed, and cultured in tissue culture–treated T225 flasks (Corning) in a volume of 25 mL medium with 1% penicillin/streptomycin (Life Technologies [Invitrogen]), and 10 mg/mL gentamycin (Life Technologies [Gibco], #15710–064). MSC (or MSC-TF) between passages 2 and 15 were maintained in a 5% CO_2 incubator at 37°C and were plated at a density of 5×10^5 to 1×10^6 cells, with the culture medium changed every other day. Cells were passaged at 80% confluence with 0.05% trypsin-EDTA (Life Technologies).

Lentiviral Vector Production and Calculation of Virus Titer

High-titer lentivirus was engineered with a second-generation lentiviral vector system, as described previously (16). A 4-day procedure was used for high-titer lentivirus production. On day 1, 10–12 million 293FT cells (Life Technologies [Invitrogen]) between passage 3 and passage 10 were plated in 15-cm cell culture dishes in 18 mL of complete Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, #11965–092) containing DMEM, 10% fetal bovine serum (Life Technologies [Gibco]), 1% penicillin-streptomycin-glutamine (Life Technologies, #10378–016), and 1% nonessential amino acids (Life Technologies [Gibco) #11140–050). On day 2, a calcium phosphate transfection procedure was used. Per 15-cm cell culture dish, a DNA mixture containing 37.5 μg of the viral vector, 25 μg of pCMV D 8.74 (1), and 12.5 μg of pMD2.G (2) (kindly provided by Karl Diesseroth, MD, PhD, Stanford, Calif) was resuspended in a total of 1062 μL sterile H_2O to which 188 μL of 2M $CaCl_2$ (Quality Biologicals, Gaithersburg, Md) was added. The DNA/$CaCl_2$ solution was quickly mixed with 1250 μL of 2× HBS with 50 mmol/L HEPES (Life Technologies [Invitrogen]; 1.5 mmol/L Na_2HPO_4; 180 mmol/L NaCl; pH, 7.12) by means of vigorous mixing. After 20 minutes of incubation at room temperature, the DNA/$CaCl_2$/HBS solution was added to 15.5 mL of room-temperature complete DMEM, and chloroquine (Sigma-Aldrich, St Louis, Mo, #1118000) was added to make a final concentration of 25 μM. The media in the dish were aspirated and replaced with the transfection solution. On day 3, after 8–16 hours, the transfection solution was removed and the cells were washed once with room-temperature phosphate-buffered saline (PBS). The media were replaced with 18 mL of fresh complete DMEM with 10 mmol/L HEPES. On day 4, 48 hours from the time of transfection, the media from all 12 dishes were collected and centrifuged at 300 g for 5 minutes to precipitate large cell debris. The viral supernatants were filtered by using a 0.45-μm low-protein-binding filter flask (Millipore, Billerica, Mass) and were centrifuged by using a SW-28 rotor (Beckman Coulter, Brea, Calif) for 2 hours at 50 000 g. The viral pellet was resuspended in 50 μL OptiMEM (Life Technologies [Invitrogen], #31985070) with 1% fetal bovine serum, and aliquots were stored at –80°C for future use. Titration of lentiviruses was performed on the Jurkat cell line (ATCC, Mansassas, Va, #TIB152) (an immortalized T lymphocyte cell line) as follows: A total of 100 000 Jurkat cells were transduced

in 96-well flat-bottomed plates in a total of 100 μl OptiMEM with 8 μg/mL polybrene (Sigma-Aldrich, Milwaukee, Wis, #AL-118). Transduction was performed for 3–4 hours in 37°C at 5% CO_2, after which the cells were transferred to 24-well plates with 500 μL RPMI, 10% fetal bovine serum, 1% penicillin/streptomycin and 10 mmol/L HEPES and were cultured overnight. Cells were then washed once and cultured in RPMI medium (Life Technologies [Invitrogen], #11875–093) for at least 48 hours before the assay for reporter gene activity.

Fluorescence-Activated Cell Sorting

MSC-TF were harvested by using 0.05% trypsin-EDTA (Gibco, #23500), were washed in PBS three times, and were then transferred to fluorescence-activated cell sorting buffer (PBS with 1% fetal bovine serum). Cells were then counted by using a cell counter (Cellometer Auto T4; Nexcelom Bioscience, Lawrence, Mass). Fluorescence-activated cell sorting was performed by using the Stanford University shared fluorescence-activated cell sorting facility. Nontransfected cells were used as a negative control to set compensation. Gates were drawn on the phycoerythrin-against-EGFP (fluorescein isothiocyanate) plot, and cells that were sorted were typically 10^3 to 10^4 in arbitrary fluorescence level and at least 100× above background. After sorting (approximately 1%–10% of total cells), cells were expanded for 2 weeks and then resorted. The sorting procedure was as follows: The cells from the first sort were sorted for high-EGFP–expressing cells. These cells were then expanded in culture for 2 weeks. Some of these high-EGFP cells then remained in culture, while others were set aside for sorting. For sorting, the cells were sorted a second time for EGFP, and this population was called the second sort. After the second sort, these high-EGFP–expressing cells were again expanded in culture, and a third sort was performed on a fraction of the cells for the high-EGFP population. A total of three sorts, after expansion of sorted populations, were performed. After the third sort, cells were expanded and frozen in cell aliquots. These aliquots were used to expand MSC-TF prior to transplantation in the heart.

In Vitro Bioluminescence Imaging of MSC-TF

To determine levels of reporter gene activity with respect to FLUC2, MSC-TF populations were assessed for FLUC2 activity. MSC-TF were serially expanded in culture and serially sorted for high or low expression of the reporter gene and then expanded in culture and either stored or recultured for 2 weeks and resorted. The following three populations were analyzed with bioluminescence imaging: *(a)* high-expressing MSC-TF from the first sort, *(b)* high-expressing MSC-TF from the second sort, and *(c)* high-expressing MSC-TF from the third sort. Cells were plated in 48-well plates at 10 000 cells per well in 96-well plates with black walls. D-Luciferin (Caliper Life Sciences, #119222) was added at 20 μg/mL per well, and imaging was performed by using a IVIS 50 cooled charge-coupled device camera system (Caliper; Caliper Life Sciences). Imaging parameters were as follows: medium binning; field of view, 12 cm; f-stop, 1; and time, 2 minutes. Analysis of five samples for each set of cells was performed. For analysis, regions of interest were drawn over each well and were quantified by using Living Image Software, version 2.5. The average radiance (in photons per second per square centimeter per steridian) was calculated and compared between the high-expressing cells from the first, second, and third sorts.

Cell Culture Uptake Studies of MSC-TF Populations for Thymidine Kinase Activity

So that we could determine levels of reporter gene activity of SR39TTK, MSC-TF that had already been serially sorted three times were assessed for thymidine kinase activity compared with MSC alone. MSC and MSC-TF were cultured at a seeding density of 10.4×10^3 cells per square centimeter overnight and were incubated with 8–3H-penciclovir (Moravek Biochemicals, Brea, Calif) at a concentration of 0.82 uCi/mL (1.44×10^5 mg/mL) in culture medium. The wells were incubated at 37°C for 60, 120, 180, and 300 minutes. At the end of each incubation period, the radioactive medium was removed and radioactivity was determined. The collected medium, washing buffer, and cell lysates were analyzed in a gamma counter (Cobra II; Packard, Meriden, Conn). The wells were washed with cold PBS, the cells were harvested by trypsinization, and the cell-associated radioactivity also was determined. Triplicate samples were performed at each time point. The same wells also were used for determining total protein content for each infected population by using a Bradford protein assay (Biorad, Hercules, Calif). Data are expressed as the net accumulation of probe in [disintegrations per minute of cells/(disintegrations per minute of medium at the start of exposure)/microgram protein] ± standard deviations.

Radioactivity Counting

All 3H counting was performed by using a liquid scintillation counter (LS-9000; Beckman, Indianapolis, Ind) with Biosafe II scintillation fluid (Research Products International, Costa Mesa, Calif). Corrections for background activity and efficiency (96.1% for 14C and 51.4% for 3H) based on calibrated standards (Beckman) also were performed to obtain disintegrations per minute. Tritium counts also were corrected for quenching effects. Corrections for the efficiency of tissue sample counting were performed by sending random tissue samples (PTRL, Richmond, Calif) for counting by a combustion method.

Immunocytochemistry of MSC

We characterized rat MSC and MSC-TF for established cell surface markers CD45 (Biolegend, #202201), CD90 (Biolegend, #202501), and CD105 (Santa Cruz, #SC-19793) as done previously. Cells cultured for 48 hours in 12-well plates at a density of 50 000 cells per well were washed three times with PBS and were fixed in 4% electron microscopy–grade paraformaldehyde for 10 minutes at room temperature. Samples were then washed with PBS and blocked for 30 minutes with 1% bovine serum albumin and 5% donkey serum at room temperature, followed by staining with primary antibodies overnight at 4°C. The dilutions of primary antibodies used were as follows: CD45, 1:100; CD90, 1:100; and CD105, 1:100. After additional washes with PBS, samples were stained with fluorescently tagged secondary antibodies at a dilution of 1:500 for 45 minutes at room temperature and were washed three times with PBS. Cells were then imaged by using phase-contrast and fluorescent microscopy (Carl Zeiss, Jena, Germany) and were captured by using software (AxioVision, version 3.1; Carl Zeiss).

MTT Assay of MSC-TF

MTT assay was performed by using the Vybrant MTT cell proliferation kit (Life Technologies, #V13154) according to the manufacturer's instructions. Briefly, MSC and MSC-TF were seeded

in 24-well plates (5000 cells per well) overnight. Cell proliferation was evaluated by using the 3-(4, 5-dimethylthiazo-2-yl)-2, 5-diphenyltetrazolium bromide reagent on days 1, 2, 3, and 4, after which absorbance was measured at 540 nm by using a microplate spectrophotometer system. Data were normalized to day 1.

Luminometry Assay for Luciferase Activity in Ex Vivo Heart Tissue

Two separate groups of mice—eight with MI and five without MI—were analyzed for ex vivo luciferase activity by sacrificing the mice on days 4, 8, and 14 and using the Luciferase Assay System (Promega, #E1500). Transfected cells were lysed in 200 μL of ice-cold 1× passive lysis buffer and were shaken for 15 minutes on ice. Cell lysates were centrifuged for 5 minutes at 1.3×10^4 g at 4°C to remove cell debris. To determine FLUC activity, 20 μL of supernatant, in duplicate, was assayed by addition of 100 μL of LAR II (Promega, Madison, Wis) in 100 μL of 0.05 M PBS (pH, 7.0), followed by photon counting in a luminometer (model T 20/20; Turner Designs, Sunnyvale, Calif) for 10 seconds in duplicate. Protein concentrations in cell lysates were determined by using a Bradford assay (Bio-Rad Laboratories, Hercules, Calif). FLUC activities, in duplicate, were normalized for protein content and were expressed as relative light units per microgram protein per minute of counting.

Induction of MI in Mice and Intracardiac MSC Injection

All surgical procedures were performed in 8–10-week-old female nude mice (Charles River Laboratories, Davis, Calif) and were performed by three microsurgeons with extensive experience (including J.E.C. and A.B.G.). Nude mice were intubated and ventilated, and anesthesia was maintained with inhaled isoflurane (1%–2.5%). A median sternotomy was performed, and the pericardium was opened. The left anterior descending artery was identified and was permanently ligated with a 9-0 Ethilon suture (Ethicon, Somerville, New Jersey) just distal to the level of the left atrium to induce MI. MI was visually confirmed by the observation of blanching of the anterior region of the left ventricle along with dyskinesis. A total of 5×10^5 MSC-TF were injected intramyocardially superiorly in the peri-infarct zone in a total volume of 20 μL with 50% Matrigel in each animal. Control animals had MSC-TF injection without MI. Other control animals received PBS with 50% Matrigel only, and the skin of sham-operated animals was opened and closed without injection. A thoracotomy tube was placed, and lungs were reexpanded by using positive-pressure ventilation. The chest cavity was closed in layers with 5-0 Vicryl suture (Ethicon, Johnson and Johnson, New Brunswick, NJ), and the animal was gradually weaned from the respirator. Once spontaneous respiration resumed, the endotracheal and thoracotomy tubes were removed, and the animals were placed in a temperature-controlled chamber until they resumed full alertness and mobility. Mice were then imaged serially by using bioluminescence imaging as described above.

Induction of MI in Rats and Intracardiac MSC Injection

All animal procedures were approved by the Institutional Administrative Panel on Laboratory Animal Care at Stanford University. All surgical procedures were performed in adult female Sprague-Dawley rats (weight, 200–300 g; Charles River Laboratories, Wilmington, Mass) by a single experienced microsurgeon. Rats were intubated and ventilated, and anesthesia was maintained with inhaled isoflurane (1%–2.5%). A lateral thoracotomy was performed, and the pericardium was opened. The left anterior descending artery was identified and was permanently

ligated with a 6-0 polypropylene suture just 2–3 mm from the tip of the left auricle. Infarction was visually confirmed by the observation of blanching of the anterior region of the left ventricle along with dyskinesis. A total of 5×10^6 MSC-TF were injected intramyocardially superiorly in the peri-infarct zone a total volume of 50 µL with 50% Matrigel in each animal. Control animals received an injection of MSC-TF in the same location and with the same volume, but without infarction. Control animals received a PBS injection only, and the skin of sham-operated animals was opened and closed without injection. A thoracotomy tube was placed, and lungs were reexpanded by using positive-pressure ventilation. The chest cavity was closed in layers with 5-0 Vicryl suture (Ethicon, Johnson and Johnson, New Brunswick, NJ), and the animal was gradually weaned from the respirator. Once spontaneous respiration resumed, the endotracheal and thoracotomy tubes were removed, and the animals were placed in a temperature-controlled chamber until they resumed full alertness and mobility. Rats were imaged serially by using bioluminescence imaging as described above.

In Vivo Bioluminescence Imaging of MSC-TF

Animal procedures were approved as stated above. Mice or rats were anesthetized at 2% isoflurane and were placed into the IVIS 200 (Caliper Life Sciences) in a state of continuous anesthesia for bioluminescence imaging. D-Luciferin was injected intraperitoneally at a concentration of 5 µL per gram of body weight. For injection of MSC-TF subcutaneously, the imaging parameters were as follows: medium binning; field of view, 12 cm; f-stop, 1; and time, 20 seconds. For imaging of MSC-TF after CCT, all parameters were the same, except the imaging time was 1 minute. For analysis, serial imaging was performed for at least 25 minutes after luciferase injection, and regions of interest were drawn over the cell implant at the maximum signal for each animal and were quantified by using Living Image Software, version 2.5. The maximum radiance or average radiance (in photons per second per square centimeter per steridian) was calculated and compared between conditions. For the CCT studies, animals were imaged on days 2, 4, 8, and 14. The four groups evaluated were MSC-TF with no MI ($n = 5$), MSC-TF with MI ($n = 8$), mock injection ($n = 3$), and MSC with no reporter gene ($n = 3$). Similar studies were performed in rats.

Animal Imaging of MSC-TF

All animal procedures were approved by the Institutional Administrative Panel on Laboratory Animal Care at Stanford University. Mice were anesthetized at 2% isoflurane and were placed into the IVIS 200 (Caliper Life Sciences) in a state of continuous anesthesia. D-Luciferin was injected intraperitoneally at a concentration of 5 µL per gram of body weight. For injection of MSC-TF subcutaneously, the imaging parameters were as follows: medium binning; field of view, 12 cm; f-stop, 1; and time, 20 seconds. For analysis, regions of interest were drawn over the cell implant and were quantified by using Living Image Software, version 2.5. The average radiance (in photons per second per square centimeter per steridian) was calculated and compared between conditions.

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