SUPPLEMENTAL MATERIAL

Mesenchymal Stem Cell Isolation, Harvest and Labeling

Swine MSCs were isolated and expanded from a single, healthy male Yorkshire donor as previously described\(^1\):. Briefly, bone marrow was obtained from the iliac crest, and aspirates were passed through a density gradient to eliminate undesired cell types and were plated with 25ml MEM Alpha media (Mediatech, Manassas, VA) containing 20% fetal calf Serum (Hyclone, Logan, UT) in 162 cm\(^2\) culture flasks (Fisher Scientific, Pittsburgh, PA). At 5-7 days after plating, non-adherent cells were washed away during medium changes and the remaining, plastic adherent, purified MSC population was expanded in culture. The MSC population was then harvested and transduced with green fluorescent protein (Lenti- GFP vector, Lentigen) according to manufacturers’ instructions. All used cells were harvested when they reached 80–90% confluence at passage 5. Labelled MSCs were placed in a cryopreservation solution consisting of 10% DMSO, 5% porcine serum albumin, and 85% Plasmalyte. Cells were placed in cryo bags at a concentration of 5–10 million MSCs/ml and frozen in a control-rate freezer to -180°C until the day of implantation. By using trypan blue staining, the viability of all thawed MSC lots was verified to be >85% before use in the study.

Generation of Concentrated Conditioned Medium (CCM) from MSCs.

Concentrated conditioned medium was generated according to previously described protocols\(^3\), with minor modifications. Confluent cultures of pig MSCs were charged with fresh alpha MEM plus 20% FCS and cultured at 37°C in 5% CO\(_2\) for 7 days. The media was collected and concentrated 10X using an Amicon Ultrafiltration unit with a
10,000 molecular weight retention membrane. The 10x concentrated CM was sterilized using a 0.22 um filter and frozen at -20°C in 10 ml aliquots

**Organotypic Cultures**

Cardiac biopsies were collected from the right ventricular septal wall of 7 Yorkshire swine with or without myocardial infarction. The biopsies were harvested and kept in cold Hank's Balanced Salt Solution (Lonza) containing 1% penicillin/streptomycin until processing. After washing thoroughly with DMEM (GIBCO), samples were minced in ~1mm³ cubes and digested at 37°C in a solution containing DMEM/F12 (GIBCO), 20% FBS, 1% penicillin/streptomycin and 200 units/ml Collagenase-Type II (Worthington). Following that, whole lysates were collected, washed twice with DMEM, resuspended in DMEM/F12, 20% FBS, 1% penicillin/streptomycin and plated in T-25 tissue culture flasks (Corning) that contained 2-3 x10⁵ GFP⁺ porcine MSCs or not. After 1 week, samples were collected by trypsinization and c-kit⁺ cells were purified by repeated immune panning in a Petri dish as previously described ⁴. After 2-3 days in culture, the isolated c-kit⁺ cells were trypsinized and re-plated with F12K (GIBCO), 5% FBS, 10ng/ml bFGF (peprotech), 20ng/ml LIF (Sigma) and 1% penicillin/ streptomycin where they grew for 7-10 days. Next, only the non-adherent fraction was collected and expanded as semi-adherent cells in tissue culture dishes containing DMEM/F12, 2% FBS, Insulin-Transferrin-Selenite (Sigma), 10ng/ml bFGF, 10ng/ml LIF, 20ng/ml EGF (peprotech), 100ng/ml SCF (Peprotech) and 1% penicillin/streptomycin. Subsequent immunocytochemical evaluation was performed on cytospin preparations according to manufacturers’ instructions (Sakura Finetek).

**In vitro differentiation assays**
To test the differentiation capacity of CSCs into cardiac myocytes, we performed co-cultures with neonatal rat cardiac myocytes (NRCMs). Briefly, NRCMs were isolated as previously described and plated at a density of 1x10^5 NRCMs/cm^2 in 12-well plates (Corning) containing collagen-coated glass coverslips. CSCs were then co-cultured with NRCMs in a 1/3 ratio, directly or indirectly using transwell inserts with a 0.4 μm pore size (BD). Co-cultures were maintained with NRCM medium consisting of DMEM (GIBCO), insulin-transferrin-selenite (Sigma), 2mg/ml bovine serum albumin, 20μg/ml ascorbic acid, 1% penicillin-streptomycin and incubated for up to 2 weeks in humidified incubator at 37 °C and 5% CO2. For immunocytochemical evaluation, cells were fixed in 4% paraformaldehyde for 20min at RT, 24h, 72h and 2 weeks after plating.

**Induction of Myocardial Infarction and transendocardial Injections**

Thirty one healthy female Yorkshire swine weighed 25-35 kg, were included in this study. Experimental myocardial infarction was generated according to our previously described protocols. Briefly, the right common carotid artery was canulated under anesthesia induced with ketamine (33 mg/kg, IM) and maintained with isoflurane (1.5-2.0%). MI was induced by accessing the Left Anterior Descending (LAD) coronary artery and occluding it after the first diagonal branch by inflating a coronary angioplasty balloon (2.75x15mm) for 60min followed by reperfusion. All animals were adequately heparinized during the procedure. The study was conducted in 2-phases. In the first phase, we sought to explore the mechanisms underlying MSCs-based cardiac repair, therefore animals received intramyocardial injections of allogeneic GFP labelled porcine MSCs (75x 10^6 cells) or Placebo (Plasmalyte alone, Baxter Edwards Critical Care, Deerfield, IL), three days after MI. The second phase was
designed to address whether MSCs-implantation is necessary for successful cardiac repair or their secreted factors alone could exert similar effects; therefore animals were randomized to receive intramyocardial injections of allogeneic GFP labelled porcine MSCs (100x 10^6 cells) or the rich in secreted factors conditioned medium were the MSCs had been expanded into, concentrated 10x. All investigators involved in this study were blinded. All injections were performed under fluoroscopy, with a pistol-needle tip injection catheter advanced to the LV through a steerable guide catheter (Stiletto, Boston Scientific, Natick, Massachusetts). Hypokinetic, akinetic, and dyskinetic areas were identified during contrast ventriculography, and injections were performed within and at the borders of the dysfunctional area, as defined by bi-plane ventriculography. A total of 15 injections were performed in each animal, with each injection containing 0.5 ml of the injectate. Each injection was fluoroscopically guided to distribute cells evenly throughout the entire infarct and border zones.

**Cardiac MRI**

For the second phase, therapeutic effect on cardiac function was assessed by Cardiac MR imaging (cMRI), as previously described. Cardiac structure and function were monitored at baseline, 1 day prior to injections, 4 days, 2 weeks and 8 weeks post-injections. Serial cMRI images were acquired with a four channel phase array, 1.5T MR Scanner (Siemens Symphony, Erlangen, Germany) in anesthetized animals with electrocardiography gating and short breath-hold acquisition. The protocol for cine-cMRI has been described before. Briefly, LV Global function was assessed in steady state free precession with a number of slices to cover the entire LV from apex to base. Imaging parameters were as follow: Echo delay time(TE)=1.9 ms, repetition time(TR)= 4.2 ms; flip angle 45°; 256 x 160 matrix; 8 mm slice
thickness/ no gap; 28 cm field of view (FOV) and 1 number of signal average (NSA). Cine images were analyzed with research comprehensive software validated by the Cardiology MR group at Lund University, Sweden (http://segment.heiberg.se).

The protocol included an intravenous bolus of Gadolinium-DTPA (0.1 mmol/kg, 5 m/s; Magnevist™, Berlex, Wayne) through a peripheral intravenous line. Images were acquired 15 minutes later at the same location as the short axis cine-images. Imaging parameters were TR=7.3, TE=3.3, TI=200 ms; flip angle=25°, 256x 196 matrix; 8 mm slice thickness gap 31.2 kHz, 28 cm FOV and 2 NSA. The infarction zone was delineated using an automatic edge detection algorithm, as described.

**Histology**

For the first phase of the study, microscopic evaluation between the treated (n=3), placebo (n=3) and control (n=3) Yorkshire pigs was performed at 2 weeks after the intramyocardial injections. Moreover, in order to assess the time course of MSCs engraftment and differentiation, 8 more animals were sacrificed at 24h (n=2 placebo and n=2 MSCs treated) and 72h (n=3 placebo and n=3 MSCs treated) post-injections. For the second phase of the study, microscopic evaluation between the MSCs and CCM-treated pigs was performed at 2 weeks (n=3 each) and 8 weeks (n=3 each) after TEIs. All animals were humanely euthanized through intravenous infusion with KCL to arrest the hearts in diastole. The explanted hearts were then washed in ice-cold phosphate buffer saline (PBS) to remove any residual blood, followed by perfusions through the left and right coronary arteries with 10% buffered formalin. Heart chambers were then filled with dental impression material (Imprint, 3M ESPE) to preserve heart’s shape during fixation. The hearts were then fixed for 24h in 10% buffered formalin and sliced transversely into seven to eight -4mm thick
slices using a commercial meat cutter, weighted and digitally photographed. Representative samples were selected from the infarcted (IZ), border (BZ) and remote areas (RZ) of each slice, and embedded in paraffin (FFPE) for immunohistochemical evaluation. These regions of interest were selected based on CMRI and gross pathology findings; samples were harvested for each of the transverse ventricular sections as follows: (i). One sample from the middle of the scarred, infarcted tissue. (ii) One sample containing the left border of the infarct along with non-scarred tissue; one sample containing the right border of the infarct along with non-scarred tissue. The last two were defined as the border zones macroscopically. Microscopically, within these samples, border zones were defined as the areas that were 1-1 ½ high power fields distant from scarred zones. (iii) One sample from the posterior non-infarcted LV wall. Hematoxylin and Eosin (H&E), as well as Masson’s Trichrome staining were used for the primary histological examination. For confocal immunofluorescence quantification, 4-5μm thick FFPE slides from each region (IZ, BZ, RZ) were evaluated. The total numbers of positively-stained cells were quantified per slide to calculate the number of cells per unit volume (cm³) on each sample. Morphometric analysis was performed by using a custom research package (Image J, NIH, Bethesda, Maryland).

**Immunofluorescence Confocal Microscopy**

Immunofluorescence studies were carried out on 4μm-thick paraffin sections, according to previously described protocols. Briefly, after deparaffinizing and rehydrating the tissue sections, antigen unmasking was performed by microwaving the slides for 20min in citrate buffer Solution, pH=6 (Dako, Carpenteria, CA). The sections were blocked for 1h at RT with 10% normal donkey serum (Chemicon
International Inc, Temecula, CA), followed by 1h incubation at 37°C with the primary antibody. The following antibodies were used: C-kit (kindly provided by Dr. Revilla⁹), α-sarcomeric actinin, α-smooth muscle actin, α-smooth muscle myosin heavy chain, Connexin-43 (Sigma, Saint Louis, MO), N-cadherin, anti-GFP, Laminin, Phospho-Histone H3, cardiac troponin-I (Abcam, Cambridge, MA), GATA-4, MDR1, VE-cadherin, CD3, CD14, CD68 (Santa Cruz Biotechnologies, Santa Cruz, CA), activated Caspase-3 (BD Biosciences, San Jose, CA), Nkx2.5 (R&D systems Inc, Minneapolis, MN), Factor VIII-related antigen (Biocare Medical, Concord, CA), cardiac myosin light chain-2 (Novus Biologicals, Littleton, CO) and KDR (Cell Signaling, Boston, MA). Consequently, the antibodies were visualized by incubating the sections for 1h at 37°C with FITC, Cy3 and Cy5- conjugated F(ab’)₂ fragments of affinity-purified secondary antibodies (Jackson Immunoresearch, West Grove, PA). Slides were counterstained with DAPI, mounted with ProLong Antifade Gold reagent (Invitrogen, Carlsbad, California) and stored at 4°C until further examination. Microscopic evaluations and image acquisitions were performed with a Zeiss LSM-510 Confocal Microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY). The Zeiss Axiovision software (release 4.7.1.0, Carl Zeiss Imaging Solutions, GmbH) was used for 3D rendering of the confocal Z-stack images.

**Fluorescence in Situ Hybridization**

Fluorescence in Situ Hybridization (FISH) was employed to detect the Y-chromosome of the sex-mismatched transplanted allogeneic MSCs in the female porcine hearts. The Y-chromosome containing cells were detected by hybridizing the tissue samples with Cy3-conjugated porcine Y chromosome paints (StarFISH, Cambio Ltd, Cambridge, UK) according to manufacturers’ instructions. Briefly,
following deparaffinization and rehydration, the samples were microwaved for 20min in citrate Buffer, pH=6 (Dako). After cooling for 30min at RT, tissues were digested for 3 min at 37°C with pepsin, washed with 2X SSC buffer (Invitrogen) and dehydrated through serial ethanol washing steps. The samples were air-dried and the probe was applied. After covering the samples with a coverslip and sealing them with rubber cement, the samples were placed in the hybridizer (Dako) for denaturation (10min at 80°C) followed by overnight hybridization at 37°C. The next day, samples were washed with 2X SSC, mounted with DAPI and covered as previously described.
Reference List


Legends for supplementary video files

**Online Video I.** A 3D reconstructed z-stack confocal series, documenting Y-chromosome (yellow) containing cardiomyocytes (cardiac Troponin-I in red) in the border zone of an MSCs-treated heart 2 months after transplantation.

**Online Video II.** A 3D reconstructed confocal Z-stack series of a differentiated c-Kit⁺ CSCs expressing cardiac Troponin I (red). The CSCs were co-cultured with NRCMs in transwell inserts; the striated pattern of cTnI illustrates their full differentiation capacity into mature cardiomyocytes.
Supplementary Figures

Online Figure I

Online figure I. A schematic of the study sequence and timing of procedures
Online Figure II. Confocal evaluation of FISH controls. Representative negative (A) and positive (B) controls of FISH for the porcine Y-chromosome probes. Negative control is from a female and positive control from a male porcine myocardium. Y-chromosome probes are Cy-3 conjugated (red). Green fluorescence corresponds to the level of autofluorescence. False positive signals in negative controls, show up as yellow fluorescence after overlapping the emission signals following by laser light excitation at 543nm and 488nm (LP560 and BP505-530 filter sets respectively). No Y-chromosome positive cells could be detected in the control female hearts. The sensitivity of FISH in male controls is 45.5±2.1%.
Online figure III. Phenotype of adult porcine c-kit+ CSCs. (A-C) C-kit+ CSCs detected in the infarcted myocardium of MSCs-treated swine co-express MDR-1 (green, A), GATA-4 (white, A), N-cadherin (white, B) and Connexin-43 (C, yellow). (D-G) Isolation and expansion of c-kit+ CSCs from organotypic cultures of porcine myocardial biopsies supported by MSCs. The c-kit+ CSCs are more than 90% MDR-1+ (green, D), GATA-4+ (white, F) and Nkx2-5+ (white, G), while also expressing connexin-43 (green, E).
Online figure IV. Dual labelling confocal analysis of the allografts fate. (A), Bargraphs illustrating the cardiovascular fate of MSCs during the first 3 days post-transplantation. Red bars correspond to myocytic (GATA-4+) and yellow bars to vascular (Factor-VIII related antigen+) commitment of the detected GFP+ cells. (B), Dual labelling confocal analysis demonstrating the successful co-localization of GFP (green) with Y-chromosome (red) cells. (C), A male porcine heart that has not received GFP+ cells, documenting the successful detection of Y-chromosome (red), and no GFP signal.(D) A panoramic stitched confocal image illustrating a GFP+ (green) cell stream in the infarcted porcine heart. Yellow arrows indicate co-expression of GATA-4 (white). The insets highlight one of these cells [left inset depicts DAPI (blue) and GATA-4(white) stains; GFP (green) is added in the right inset].
Online Figure V. Cardiomyocyte differentiation of MSCs. Chimeric cardiomyocytes were detected by Confocal Immunofluorescence analysis. Co-localization of α-sarcomeric actinin (red, A) with Y-chromosome (green, B) were used to identify mature cardiomyocytes of exogenous origin. Laminin (white, C) was also used to illustrate morphology of adult myocytes. DAPI (blue, D) was used to stain nuclei. (E) Merged image of panels A-D. Yellow arrows indicate Y-chromosome+ chimeric cardiomyocytes.
Online Figure VI. Cardiomyocytic commitment of MSCs 72h after transplantation. (A) A GFP+ MSC (green) co-expressing the cardiomyocyte-specific homeobox transcription factor Nkx2-5 (white, arrow). (B), A GFP+ MSC (green) co-expressing the cardiac muscle specific isoform of troponin-I (red).
Online Figure VII

(A) GFP⁺ MSCs (green) co-expressing the endothelial cells-specific VE-cadherin (red).

(B), A cluster of GFP⁺ MSCs (green) co-expressing the vascular smooth muscle cell specific protein, smooth muscle myosin heavy chain (red).

(C), A cluster of GFP⁺ cells (green) co-localized with a vascular smooth muscle cell specific protein, α-smooth muscle actin (red).

Online Figure VII. Vascular commitment of MSCs 72h after transplantation. (A) GFP⁺ MSCs (green) co-expressing the endothelial cells-specific VE-cadherin (red). (B), A cluster of GFP⁺ MSCs (green) co-expressing the vascular smooth muscle cell specific protein, smooth muscle myosin heavy chain (red). (C), A cluster of GFP⁺ cells (green) co-localized with a vascular smooth muscle cell specific protein, α-smooth muscle actin (red).
Online Figure VIII

Online Figure VIII. Putative electrical and mechanical coupling of MSCs. (A,B), Within the first 3 days after implantation, GFP+ MSCs (green) could be detected to co-express the gap-junctional protein Connexin-43 (red, A) indicating putative electrical coupling with the host myocardium, and the adherence glycoprotein N-cadherin (red, B), indicating putative mechanical coupling with the host myocardium.
Online Figure IX. Karyo and cytokinesis of mitotic cardiomyocytes.
A mitotic cardiomyocyte in the MSCs-treated hearts expressing ser-10 phosphorylated Histone-H3 (white, inset). The laminin staining (green) highlights the separation of the cardiomyocyte into two daughter cells, implying that cell division – rather than cell ploidy- occurs.
Online Figure X. Chimeric myocardium exhibits potential electrical coupling. Co-localization of a sarcomeric-actinin (red, A) with Y-chromosome containing cardiomyocytes (white, B) and connexin-43 (green, C) illustrates the potential of the newly formed, chimeric tissue to couple electrically to the host myocardium. DAPI (blue, D) was used to stain nuclei. (E) Merged image of panels A-D. Yellow arrows indicate Y-chromosome\(^*\) chimeric cardiomyocytes.
Online Figure XI. Potential Mechanical coupling of MSCs to the host myocardium. (A), expression of α-sarcomeric actinin (red) illustrates a surviving rim of myocardial tissue within the infarcted myocardium. (B) Expression of the adherence protein N-cadherin (white) between surviving cardiomyocytes and non-cardiomyocytes. (C) A cluster of GFP+ MSCs cells is present within the infarcted tissue. (D) DAPI staining illustrates the nuclear content in the area. (E,E1) The transplanted GFP+ MSCs express N-cadherin indicating their potential to develop adherence connections with the host tissue.
Online Figure XII. Cell-Cell interactions between endogenous and exogenous stem cells. (A,B) Immature MSCs (green) are found within the host myocardium to interact with resident c-kit⁺ CSCs (red) by connexin-43 (A, white) and N-cadherin (B, white, arrow) connections, closely resembling cardiac stem cell niches.
Online figure XIII. Organotypic Cultures of porcine heart biopsies with porcine MSCs. (A,B) A small number of cells outgrow from the biopsies cultured alone (A). Organotypic cultures with MSCs have become confluent while, some GFP+ MSCs have infiltrated the heart samples (B). Panels A, B were obtained at 5 days in organotypic cultures. (C) Immunostaining on cytospins from the primary co-cultures illustrating large cell clusters containing c-kit+ and GFP+ cells in proximity. These clusters resemble stem cell niches. (D-D3), Cytospins of purified c-kit+ CSCs, illustrating co-localization with MDR1, while lack of the surface marker CD68 from the vast majority of them excludes an inflammatory or mast cell phenotype (E-E2) Lack of KDR from c-kit+ porcine CSCs. (F-F1) Nkx2-5, is expressed in more than 90% of the CSCs.