Supporting Information

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SI Materials and Methods

Experimental Protocol. Animal studies were approved by the University of Miami Institutional Animal Care and Use Committee [National Institutes of Health (NIH) Pub No. 80–23, revised 1985]. Ten female Gottingen pigs (20–27 kg) underwent to left anterior descending artery occlusion for 150 min followed by reperfusion. Twelve weeks later, the animals were assigned to receive injections of either male allogeneic MSCs ($n = 6$) or placebo ($n = 4$). Serial cardiac MRIs were taken at baseline, post-MI (10 days, 1, and 3 months), and following transendocardial injections (1 week and 1, 2, and 3 months).

Mesenchymal Stem Cell Harvested and Isolation. Bone marrow aspirates were collected from the iliac crest of a healthy male Landrace pig. Mononuclear cell fractionation based on Ficoll density gradient centrifugation was used to remove undesired cell types. Cells were expanded in culture based on plastic adherence (1), subsequently labeled with 5-bromodeoxiuridine at passage 3 and placed in a cryopreservation solution. All of the injected cells reached >80% of viability at the injection time. Cells were kindly provided by Osiris Therapeutics, Inc.

MI Induction. Animals underwent general anesthesia, and coronary artery instrumentation was performed using the carotid approach with a 7 French guiding catheter. Heparin (100 IU/kg) was administered to obtain 2.5-fold activated coagulation time accompanied by lidocaine infusion during the entire ischemic time. An over the wire balloon catheter (NC Raptor, 2.75 mm × 15 mm; Cordis) was inserted into the mid-LAD, inflated for 2.5 h, followed by reperfusion (2). All of the animals exhibited an antero-apical MI. The procedure-related mortality was 30%.

Transendocardial Injections Technique. Left ventriculograms were acquired in two different angiographic projections [left (−30 °) and right anterior oblique (+30 °)]. Endocardial contours from both projections were then manually delineated in diastole. A total of 2.0×10^8 cells or vehicle alone (Plasmalyte; Baxter) were injected in 0.5-mL aliquots through the pistol needle-tip injection catheter (Stiletto; Boston Scientific). Injection sites were confirmed by contrast injection before cell delivery and were distributed in a median of 15 locations within the hypokinetic and akinetic zones (2). Animals were monitored until they recovered from anesthesia.

Cardiac MRI. Images were acquired with a four-channel phase array, 1.5 T MR Scanner (Siemens Symphony) in anesthetized animals with electrocardiography gating and short breath-hold acquisition. The protocol for cine-MRI and tagging-MRI has been described before (3). Briefly, images were obtained with the following parameters: TR, 6.7; TE, 3. flip angle = 12 °; 256 × 160 matrix; 4 views/s; 4 mm slice thickness no gap; 31.25 kHz; 28 cm FOV; 1 NSA; and 6 pixels tagging space. Cine and contrast enhanced images were analyzed with research software validated by the Cardiology MR group at Lund University, Sweden (http://segment.heiberg.se). Tagged MRI images were analyzed using a commercially available software package (PLUS Diagnosoft; Diagnosoft) (4), and the peak Eulerian end systolic circumferential strain (peak Ecc) was determined from the strain map at each time point. The four most apical tagged slices were selected and overlayed with the corresponding delayed enhancement slices. Meshes were constructed with a total of 72 evenly distributed regions (24 endocardial, 24 mid-wall, and 24 epicardial) incorporating the entire myocardium. The meshes were created so that the edges of the infarct scar corresponded with the edges of mesh regions. The border zone was defined as the left- and right-most infarct-containing mesh regions, and the infarct zone encompassed all regions in between. The same slices, mesh, and defined regions were used at all time points in each pig. The values calculated were averaged between all like-defined regions and across all slices.

Gross Pathology and Histology. Animals were humanely killed at the completion of the study, and the heart was arrested with a bolus of saturated potassium chloride. The hearts were harvested, perfused, and fixed in 10% formalin. Next, hearts were filled with addition silicone impression material to avoid tissue shrinkage and, then, sectioned in 4-mm-thick short axis slices. Each slice was digitally photographed. Myocardial samples were then taken from three regions: Infarct, border, and remote zones. Formalin-fixed paraffin-embedded (FFPE) tissue slides were prepared from each section. Each section was also stained with hematoxilyn & eosin and Masson's trichrome to assess the tissue structure.

Inmunohistochemistry and Morphometric Analysis. Deparaffinization of FFPE tissue slides was performed by heating the slides at 65 °C for 45 min with subsequent alcohol-graded baths immersion followed by microwave heating for 20 min in citrate buffer pH 6.0 (Dako). Next, slides were blocked with 10% normal donkey serum (Chemicon) for 1 h at room temperature. Both groups were studied by FISH for the porcine Y chromosome and co-localization with anti-BrdU (1:100 overnight incubation at 4 °C, rat monoclonal, AbD; Serotec). FISH were performed according to manufacturer's instructions (STARFISH; Cambio). Anti-cianine-HRP conjugated antibody (1:300 for 1 h at 37 °C, sheep polyclonal; Abcam) in combination with tyramide signal amplification (Perkin-Elmer) was used to enhance the signal. Quenched of the endogenous peroxidase was performed with 3% H_2O_2 (Sigma) in PBS (Gibco) as needed for HRP-conjugated antibodies. A suspected Y chromosome positive cell (Y^{pos}) was examined in a laser confocal microscopy (LSM, 510 META; Zeiss), and serial z-stacks were acquired to confirm the nuclear co-localization. The Y chromosome FISH is known to have a sensitivity of 50% or less (5). The results of the FISH analysis were confirmed with co-stained for anti-BrdU.

Transplanted cells were quantitated based on the Y chromosome/100 mm^2 (6) and then phenotypically characterized by double inmunofluorescence with the following primary antibodies: Anti-α-sarcomeric actinin, smooth muscle actinin (both 1:100 overnight incubation at 4 °C, mouse monoclonal), connexin-43 (1:1500, rabbit polyclonal) (all from Sigma), tropomyosin (1:50 overnight incubation at 4 °C, mouse monoclonal), laminin (1:25, rabbit polyclonal), SM22-α (1:1000, rabbit polyclonal), calponin (1:200, rabbit polyclonal) (all from Abcam), GATA-4, Nkx2.5 (both 1:50, goat polyclonal; R&D Systems), and factor VIII-related antigen/VWF (1:100, rabbit polyclonal; Biocare Medical), followed by fluorophore-conjugated secondary antibodies raised in donkey (1:100 for 1 h at 37 °C; Jackson Inmunoresearch). All primary antibodies were diluted in a background reducing solution (Dako) and incubated for 1 h at 37 °C, unless different conditions were specified. Nuclei were counterstained with DAPI (1:1,000 for 15 min at room temperature; Invitrogen).

New vessel formation was assessed for the presence of Y^{pos}

inserted into blood vessels and co-localization with smooth muscle proteins or endothelial markers. Capillaries were considered as a single endothelial layer with diameter <20 μm. Medium and large vessels were considered as composed for a coating of smooth muscle layers.

Myocyte diameter was assessed in both MSCs and placebo hearts stained with α-sarcomeric actinin (or tropomyosin) and DAPI. Morphometric sampling consisted of measuring by confocal microscopy the profiles per unit area in which the cardiac muscle cells were cut perpendicularly. At least 100 myocytes were assessed per tissue sections from different regions (infarct, border, and remote myocardium; two sections per each area, respectively) were stained per animal (MSCs = 6, placebo = 4). Morphometric analysis was performed by using Image J (NIH) and Zeiss LSM image browser for confocal imaging analysis. Adobe Photoshop CS 2 was used to adjust brightness, contrasts, and assembly of the multipanel images.

Statistical Analysis. All values are expressed in means ± SEM. Two-way ANOVA with one-factor repetition was used to test for significance between variables using commercially available software (Stata; StataCorp LP). A level of $P < 0.05$ was considered statistically significant. Pearson correlation was applied to find a relation between functional parameters (peak Ecc, myocardial perfusion, and infarct size) and cell engraftment.

1. Pittenger MF, et al. (1999) Multilineage potential of adult human mesenchymal stem cells, *Science* 284:143–147.
2. Schuleri KH, et al. (2008) Early improvement in cardiac tissue perfusion due to mesenchymal stem cells. *Am J Physiol Heart Circ Physiol* 294:H2002–H2011.
3. Amado LC, et al. (2006) Multimodality noninvasive imaging demonstrates in vivo cardiac regeneration after mesenchymal stem cell therapy. *J Am Coll Cardiol* 48:2116–2124.
4. Garot J, et al. (2000) Fast determination of regional myocardial strain fields from tagged cardiac images using harmonic phase MRI. *Circulation* 101:981–988.
5. Anversa P, Nadal-Ginard B (2002) Cardiac chimerism: Methods matter. *Circulation* 106:e129–e131.
6. Quaini F, et al. (2002) Chimerism of the transplanted heart. *N Engl J Med* 346:5–15.

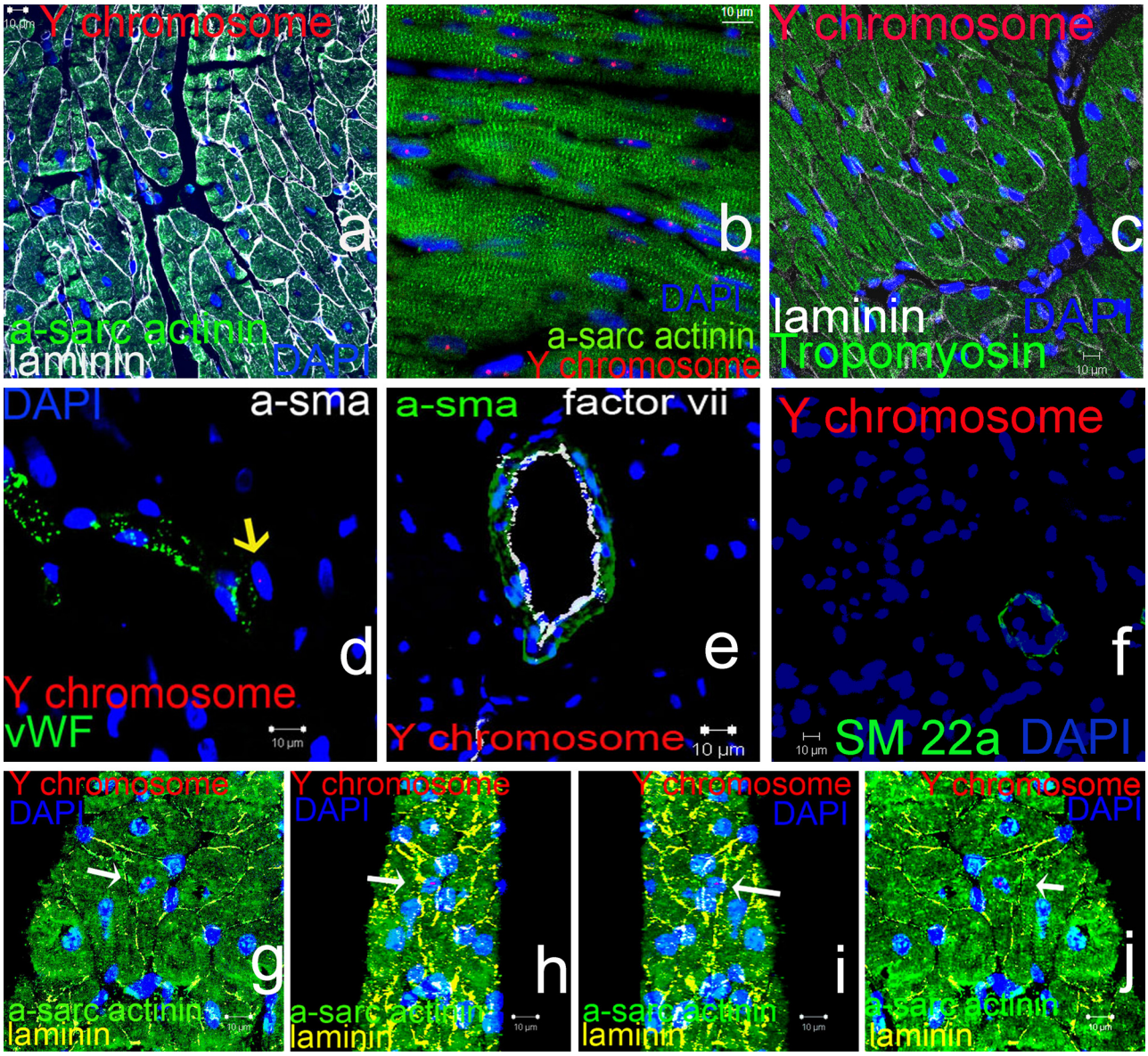

Fig. S1. Confocal imaging analysis of transplanted cells. (*a–c*) FISH for the Y chromosome in placebo-treated heart with no evidence of nuclear co-localization (*a*), male swine heart (*b*, positive control), and female infarcted nontreated heart (*c*, negative control). (*d–f*) Y^{pos} cell (red, arrow) were incorporated into capillaries of MSC-treated heart (*d*, arrow), whereas placebo hearts did not show evidence of signal for the Y chromosome in the vessel composition (*e*). Female infarcted nontreated heart was used as negative control (*f*), respectively. (*g–j*) Triple immunofluorescence staining of a tissue section in the border zone of MSC-treated hearts. A reconstructed image rotated around the y-axis depicts the Y^{pos} cell (red, arrow) co-localized with α-sarcomeric actinin (green) and laminin (yellow). Nuclei were counterstained with DAPI. MSCs-treated hearts ($n = 4$), placebo hearts ($n = 4$), controls ($n = 2$ for positive and negative respectively). Factor viii/vWFm, factor VIII related antigen/von Willebrand Factor; SM 22 a, smooth muscle protein 22 alpha.

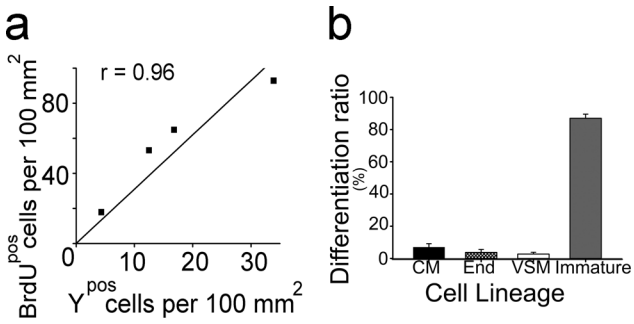

Fig. S2. Cardiac commitment of transplanted BrdU^{pos} cells. (*a*) At 12 weeks posttransplantation, BrdU^{pos} cells survive and cell quantitation per unit area correlate with the Y chromosome in the border zone of MSC-treated animals. (*b*) Although the BrdU^{pos} cell population accounted 3× the Y^{pos} count, the proportions as what they differentiate were consistent between the two cell tracking methods. ($P < 0.05$ for Pearson correlation, $n = 4$ for both BrdU and Y chromosome hearts). At least four tissue sections per animal were evaluated comprising a total area of 1,742.55 mm^2. CM, cardiomyocyte; End, endothelial cells; VSM, vascular smooth muscle.

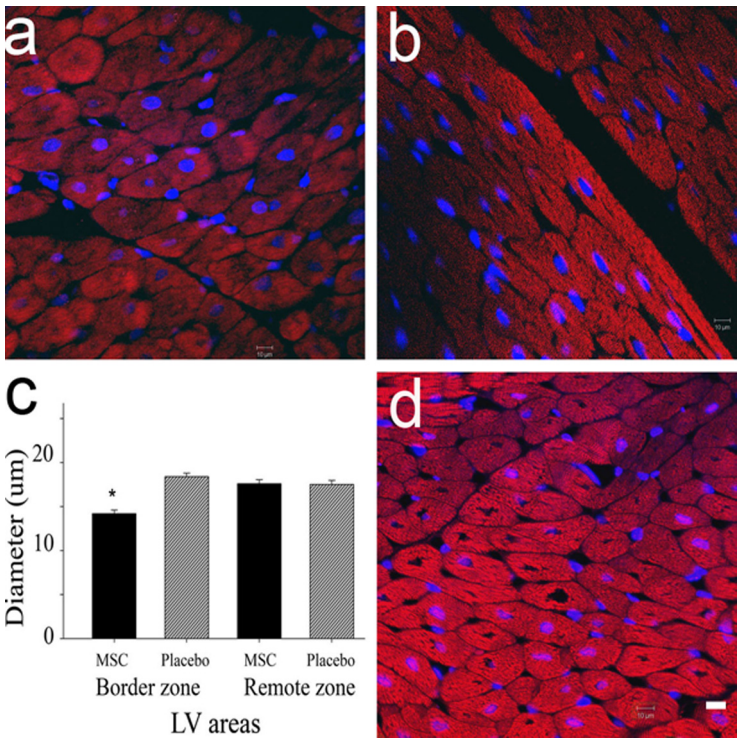

Fig. S3. Myocyte diameter. (*a* and *b*) To determine the transversal myocyte diameter, cross-section of heart tissue from border zone of MSC-treated (*a*) and placebo (*b*) groups were co-stained for tropomyosin (red) and DAPI (blue). Myocytes diameters across the nucleus were included and measured. (*c*) Bar graph distribution of myocyte diameters according to left ventricle areas. Myocyte from border zone of MSC-treated group were found with smaller diameter than placebo hearts, indicating one the benefits of the therapy rely in the reverse remodeling. (*d*) Section of myocytes in remote myocardium of placebo group was not different from the corresponding section in MSC-treated group (*$P < 0.05$; scale bar, 10 μm). Cardiac myocytes in cross-section were randomly evaluated in the border zone of MSC-treated ($n = 6$) and placebo ($n = 4$) hearts (169.2 $\pm$ 18.6 myocytes in cross-section per animal).

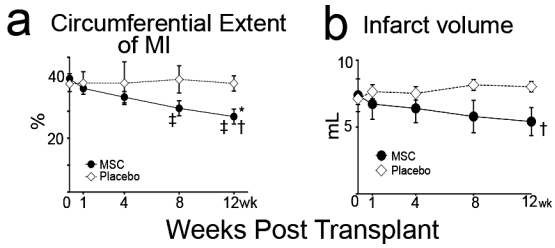

Fig. S4. Scar size reduction evaluated by delayed contrast-enhancement MRI. (*a*) Circumferential extent of MI decreased in MSC-treated hearts by week 8 after treatment and continued to the end of follow-up period (*$P < 0.001$ ANOVA within MSC group, $^{\dagger}P < 0.001$ vs. placebo, $^{\ddagger}P < 0.001$ vs. preinjection status by Student-Newman-Keuls posthoc test). (*b*) Infarct volume assessment showed a tendency to decrease following MSC-therapy ($^{\dagger}P < 0.001$ vs. placebo). At least five time points in MSCs-treated ($n = 6$) and placebo ($n = 4$) hearts.

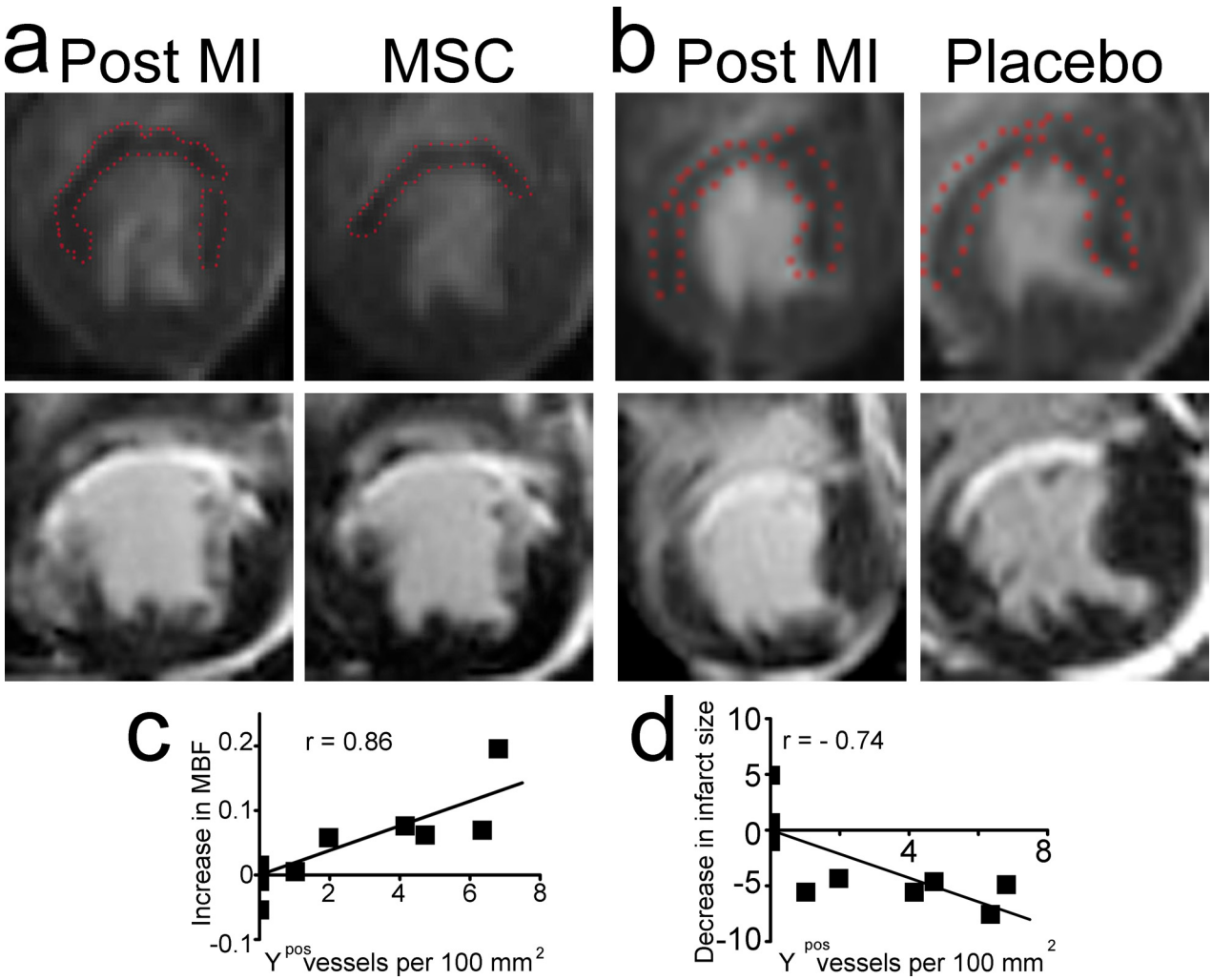

Fig. S5. Cardiac tissue perfusion analysis. (*a* and *b*) Comparative short-axis heart images of the first-pass perfusion MRI (top) before and 12 weeks post therapy. Regions with impaired perfusion remain dark (red dotted contours) due to impaired gadolinium (white) distribution. Equivalent delayed contrast-enhancement MRI images (bottom) of the same hearts to assess the infarct extension prior and 12 weeks following transplantation. (*a*) MSC-treated heart showed correspondence between infarct reduction and recovery of myocardial perfusion, while (*b*) placebo-treated hearts remained with hypoperfused segments, and the area of infarcted myocardium expanded. (*c* and *d*) New vessels quantitation per unit area correlate with the increase in myocardial perfusion (*c*) and the decrease in infarct size (*d*). At least five time points in MSCs-treated ($n = 6$) and placebo ($n = 4$) hearts. $P < 0.05$ for Pearson correlation.