Comparison of Various Kinds of Bone Marrow Stem Cells for the Repair of Infarcted Myocardium: Single Clonally Purified Non-Hematopoietic Mesenchymal Stem Cells Serve as a Superior Source

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Abstract   A variety of adult stem cells have been used to transplant into the infarcted (MI) heart, however, comparative studies are lacking to show more suitable source of cells for transplantation. We have identified a single non-hematopoietic mesenchymal stem cell subpopulation (snMSCs) isolated from human bone marrow and clonally purified, that over 99% of them expressed MSC marker proteins and cardiomyocyte marker proteins when induction in vitro. We also compared the effects of the snMSCs with unpurified MSC (uMSCs), mononuclear cells (BMMNCs), or peripheral blood mononuclear cells (PBMNCs) on myocardial repair after induction of MI in rats. Ninety days later, we observed a better cardiac function assessed by ejection fraction, fraction of shortening and lung wet/dry weight ratios, less remodeling of left ventricle (LV), lower collagen density in the LV, and more vessels in the ischemic wall in the snMSCs transplantation group than in other cell-transplanted groups. Furthermore, the transplanted cells expressing cardiomyocyte specific proteins or vascular endothelial cell marker proteins were more in the snMSCs group than in other ones. We conclude that transplantation with single clonally purified MSCs seems to be more beneficial to the cardiac repair than with other stem cells after MI. J. Cell. Biochem. 99: 1132–1147, 2006.   © 2006 Wiley-Liss, Inc.

Key words: cardiac repair; myocardial infarction; single clonally purified MSCs; stem cell; transplantation

A growing body of evidence has demonstrated that bone marrow stem cell transplantation (BMT) could effectively repair an ischemically damaged heart [Fuchs et al., 2001; Orlic et al., 2001a; Shake et al., 2002], thereby setting the stage for early clinical trials [Strauer et al., 2002; Perin et al., 2003; Chen et al., 2004; Wollert et al., 2004]. The cell types mostly used to transplant include bone marrow or peripheral blood-derived mononuclear cells (BMMNCs or PBMNCs), and mesenchymal stem cells (MSCs). Most studies have been conducted using BMMNCs or PBMNCs, which were collected by gradient centrifugation, or unpurified MSCs that were isolated primarily by their tight adherence to culture plastic dishes. The cells isolated in these ways were initially heterogeneous, which caused different effects on the repair of the infarcted heart [Perin et al., 2003; Chen et al., 2004; Wollert et al., 2004]. The ability to purify, culture, and manipulate homogeneous stem cells from bone marrow would provide investigators with an invaluable cell source to study cell and organ development. Several methods have been developed to

prepare more homogeneous populations by the use of fluorescence activated cell sorting, which is only based on some specific surface markers [Zohar et al., 1997] or on their differences in size [Hung et al., 2002]. Krause et al. [2001] obtained a single bone marrow-derived stem cell from hematopoietic stem cells (HSCs). Recently it is demonstrated that HSCs could not transdifferentiate into cardiac myocytes in myocardial infarcts [Balsam et al., 2004; Murry et al., 2004; Nygren et al., 2004]. Kawada et al. [2004] found that clonally purified non-hematopoietic MSCs could differentiate into cardiomyocytes after myocardial infarction (MI). To date, however, there is no study evaluating the effects of transplantation with single clonally purified non-hematopoietic MSCs (snMSCs) in comparison with other cells on myocardium regeneration post-MI. Therefore, the present study was designed to compare the effects of snMSCs with BMMNCs, PBMNCs, and unpurified MSCs (uMSCs) transplantation on myocardial regeneration in a rat model of acute myocardial infarction (AMI).

METHODS

Preparation of Human Bone Marrow Cells and Peripheral Blood Cells

Bone marrow and peripheral blood were collected, respectively, from the iliac and the peripheral veins of 20–50 years old healthy adult donors following informed consent according to guidelines from the Fudan University Committee on the Use of Human Subjects in Research. BMMNCs and PBMNCs were isolated by Ficoll-Hypaque gradient centrifugation (Lymphoprep, Nycomed). uMSCs were obtained by adherent culture method from BMMNCs. BMMNCs were re-suspended in complete medium (Iscove's modified Dulbecco medium: IMDM with 20% fetal bovine serum, 2 mM L-glutamine, penicillin [100 U/ml] and streptomycin [100 ug/ml]). Cells were incubated with 95% air and 5% CO_2 at 37°C. Non-adherent cells were discarded after 24 or 48 h of culture, and adherent cells were washed twice with phosphate-buffered saline (PBS). The cultures were depleted of erythroid progenitor cells by removing cells that were not adherent to the culture dish at each change of medium every 3–4 days. At 80% confluence, cells were harvested with 0.25% trypsin for 5 min at 37°C and were passaged in a ratio of one to three. Fibroblast

was obtained from dermal tissues from healthy adult donors underwent prepucectomy, tissues were dissected, thoroughly minced and filtered through a 70 μm filter. Cell suspension was collected and centrifuged for 10 min at 300g.

Isolation, Expansion, and Purification of Human snMSCs

Human snMSCs were isolated and purified from bone marrow as described by Reyes et al. [2001]. Briefly, BMMNCs were depleted of $CD45^+/GlyA^+$ cells by means of micromagnetic beads (Miltenyi Biotec) and cultured in expansion medium. Once cell clones appeared, single cloned cells were expanded at a dilution of 1:4. At 80% confluence, MSCs were collected and incubated with MACS colloidal super-paramagnetic microbeads conjugated with anti-CD34 antibodies (Miltenyl Biotec), and then passed through a depletion column held within a magnetic apparatus. The $CD34^-$ cells were then collected and the purity of the cells was determined by using a fluorescence-activated cell sorter analyzer (FACS, Becton Dickinson).

Characterization of snMSCs

In order to analyze the cell surface markers of snMSCs, cells (2×10^5/ml) were incubated in PBS with 4 μl antibodies, including mouse anti-human monoclonal antibodies (Becton Dickinson): CD29 (555005), CD34 (553733), CD44 (553133), CD45 (555482), CD71 (554890), CD117 (553354), CD147 (555962), or SH2 (611527); mouse anti-human AC133 monoclonal (FAB1133F, R&D systems) or rabbit anti-human SH3 polyclonal antibody (#06-466, Upstate), for 1 h at room temperature. Mouse IgG1, IgG2a, and IgG2b (Becton Dickinson) were used as isotype controls. The samples were then analyzed by FACS equipped with CellQuest software (Becton Dickinson). Cell viability was assessed by flow cytometry using propidium iodide exclusion assays. Approximately 10,000 cells were analyzed and the data were expressed as a percentage of positive cells relative to the whole cell population. The data represent a mean value from five analyses. In order to observe the functional evidence of self-replication and pluripotency from snMSCs and fibroblast, octamer binding gene (Oct4), proto-oncogene Bmi1 and the ATP-binding cassette transporter G2 (ABCG2) were evaluated by RT-PCR and Western blot. Total RNA was isolated from snMSCs and fibroblasts using Trizol reagent (Gibco BRL).

The primers and the sizes of the expected products were as follows (forward and reverse, respectively): Oct4, CCC CCT GTC CCC CAT TCC TA/CGA CCA TCT GCC GCT TTG AG (577 bp); Bmi1, GAG GAG GTG AAT GAT AAA/CTA ATA ACC CAT TTG CTG (242 bp); ABCG2, GGC CTC AGG AAG ACT TAT GT/AAG GAG GTG GTG TAG CTG A (342 bp). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was served as an internal control: GGA AAG CTG TGG CGT GAT GG/GTA GGC CAT GAG GTC CAC CA (393 bp). Amplification conditions for these genes were: 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The PCR products were subjected to electrophoresis on 1.5% agarose gels and scanned using Image-Quant software (Kodak). For Western analysis, cell lysates were prepared as described previously [Zamber et al., 2003], and 100 μg of total protein per lane was loaded on the gel. Blots were probed with monoclonal anti-Oct4 (1:1,000, sc-5279, Santa Cruz), ABCG2 (1:500, MAB4155, Chemicon International), and Bmi-1 (1:500, 05-637, Upstate). GAPDH (1:300, MAB374, Chemicon International) was also detected as a protein control. A chemiluminescence detection kit (Amersham Biosciences) was used to detect the secondary antibody.

Differentiation In Vitro

uMSCs and snMSCs from the 10th passage were plated at a density of 1×10^4 cells/well in expansion medium containing 2% FCS, 10 ng/ml EGF, and 10 ng/ml PDGF-BB, with 3 μM 5-azacytidine, 1 nmol/L insulin, and 10 ng/ml transforming growth factor (TGFβ1) for 24 h. The medium was changed at 2 days interval for a week, and the cells were incubated with 5-azacytidine for another 24 h. At the 10th induction day, cells were evaluated for cardiac myocyte-specific protein expressions by Western blot and immunofluroscence.

Cell Harvest and Labeling Before Transplantation

snMSCs and uMSCs were harvested from the 10th passage. Collected PBMNCs, BMMNCs, snMSCs, and uMSCs were stained with 4′, 6-diamidino-2′-phenylindole (DAPI, Roche Diagnostic) as previously described [Wang et al., 2000]. Cells were suspended in PBS at a density of 5×10^7 cells/ml and kept on ice for up to 4 h before transplantation.

Western Blot

Protein expressions of myosin heavy chain (MHC, 1:1,000, #05-716, Upstate), troponin T (1:500, MAB 1695, Chemicon International), and desmin (1:500, #MS-990-P0, NeoMarkers) were determined by Western blot in snMSCs pre-5-azacytidine induction as well as uMSCs and snMSCs post-5-azacytidine induction using respective antibodies [Jiang et al., 2000]. Adult normal rat cardiomycytes served as positive control.

Animal Models and Cell Transplantation

Adult male Sprague–Dawley rats (200–250 g) were purchased from Shanghai animal administration center. MI was induced by ligation of the left anterior descending coronary artery [Litwin et al., 1994]. Within 4 h post-MI, infarcted animals with EF < 70% and FS < 35% assessed by echocardiography were randomized to receive 80 μl of PBS (PBS group), BMMNCs (BMMNC group), PBMNCs (PBMNC group), unpurified MSCs (uMSC group, 10th passage) or snMSCs (snMSC group, 10th passage) suspension transplantation, which was equally injected into the infarct and peri-infarct regions (5×10^6 cells, four sites, two sites per the infarct or peri-infarct area, 20 μl per site, 1–2 cm apart). Cyclosporin A (CsA, Novartis Pharma) was administered (5 mg/kg, i.h.) daily beginning on day 1 post-MI until the scheduled study end. To observe whether CsA alone had an effect on the cardiac function and healing process post-MI, a separate group of MI rats received PBS transplantation with CsA injection (PBS + CsA group). In each group, the animals were further divided into two subgroups according to two time points: 3-day and 90-day post-MI. All animal experiment protocols were approved by the Animal Care and Use Committee of Fudan University and were in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Academy Press (NIH Publication No. 85-23, revised 1996).

Electrocardiography, Echocardiography and Tissue Weight Determination

Under general anesthesia with a mixture of ketamine/xylazine (100/15 mg/kg, i.p.), electrocardiography (ECG) was performed to observe malignant arrhythmia, and transthoracic echocardiography was operated on all animals using a modified method [Litwin et al.,

1994] before infarct (i.e., the baseline level), day 1 after operation, and at 3-day or 90-day post-MI. Left ventricular end-diastolic volume, internal diameter, anterior and posterior wall thickness at diastolic phase (LVEDV, LVDd, LVAWd, and LVPWd, respectively) were measured using the bi-plane area-length method. LV percent fractional shortening was calculated according to the modified Simpson's method: FS (%) $=[(\text{LVIDd-LVIDs})/\text{LVIDd}] \times 100$, EF (%) $= (\text{EDV}-\text{ESV})/\text{EDV} \times 100\%$, where FS and EF stand for left ventricular shortening fraction and ejection fraction, respectively; LVID is LV internal dimension, s is systole and d is diastole; EDV and ESV stand for end-diastolic and end-systolic LVID, respectively. All measurements were averaged for three consecutive cardiac cycles and were made by two independent researchers who were unaware of treatment condition. The rats were killed with intraperitoneal injection of overdose barbiturate (150 mg/kg) post-echocardiography examinations. The hearts and lungs were removed and free from adhering tissues. The body weight, left ventricular weight (LV weight), and wet lung weight were measured from all animals. LV weight/body weight was used as a measure of left ventricular hypertrophy by dividing the LV weight by body weight. The lungs were chopped into smaller pieces and placed in the oven at $65°C$ until a constant weight was obtained, which was usually after ~ 24 h. Relative weights of the lungs were expressed as wet/dry weight lung, which were calculated as a measure of pulmonary congestion by dividing the wet lung weight by the dry lung weight.

Histological and Immunohistochemical Analysis

Hearts of rats survived to the study were removed and the left ventricles were equally divided into three parts from the apex to the base. H&E, masson trichrome, or immunofluoroscence staining was performed to evaluate infarct size, collagen content, or vessel density, respectively. Infarct size was determined by planimetric measurement with a digital image program (Scion ImageJ) and calculated by dividing the sum of the planimetered endocardial and epicardial circumferences occupied by the infarct by the sum of the total epicardial and endocardial circumferences of the LV on three transversal sections from the apex to the base. The collagen density was expressed as

the proportion of collagen deposition area to normal left ventricular myocardium on middle LV transversal section [Nwogu et al., 2001]. von Willebrand factor (1:200, Factor VIII, 10665638, Zymed Lab) staining using immunofluorescence method was performed in middle LV transversal section and the number of vessels was expressed as the number of factor VIII$^+$ endothelial cells per square millimeter. For observation of cardiac differentiation of uMSCs and snMSCs post-5-azacytidine induction in vitro, cells were pre-labeled with DAPI and then stained with anti-MHC, troponin T, or desmin antibodies. In order to assess the fate of engrafted cells in the peri-infarct and infarct areas, the number of engrafted cells was counted under fluorescence microscope on a series of cryostat left ventricular apical tissue sections. The number of DAPI$^+$ cells was evaluated by counting ten randomly selected high-power fields. Immunofluorescence was performed with the primary antibodies including anti-MHC or factor VIII antibody (above mentioned), followed by incubation with FITC- or TRITC-conjugated secondary antisera. The differentiation ratios of the cells post-in vitro induction or in vivo transplantation were expressed as the percent proportion of the cells double positively stained with DAPI and MHC, troponin T, desmin, or factor VIII to the total positive-DAPI staining cells. In order to exclude cell fusion, positive-DAPI snMSCs slides were double stained with MHC and anti-human nuclei mononuclear antibody (1:20–1:30, MAB1281, Chemicon International, Inc.). Immunofluoroscence controls were performed to assess specificity, including exclusion of primary antibody and use of mouse, goat, and rabbit sera isotype in place of the antibodies. A pathologist who was blinded to the group identity of the slides evaluated the parameters.

Detection of Myocardium MPO, Inflammatory, or Immune Reaction

The infarct and peri-infarct areas from the rat basal regions were homogenized in saline (per 100 mg tissue in 0.9 ml saline). After centrifugation for 15 min at 3,000g, the supernatant was collected and stored at $-70°C$ until use. Myeloperoxidase (MPO) was detected, respectively, according to manufacture's instruction (A044, A018, NJBI, China). The change in absorbance of MPO was measured spectrophotometrically at 460 nm. One unit of MPO is

defined as that quantity of enzyme hydrolyzing peroxide at a rate of 1 mmol/min at 25°C. H&E staining were performed on a series of cryostat left ventricular apical tissue sections. The degree of inflammatory or immune reaction was expressed by counting the number of infiltrating neutrophil or lymphocyte in the infarct and the peri-infarct areas on two randomly selected low-power (50×) light microscope fields.

Statistical Analysis

Statistical analyses were performed with unpaired Student's t-test, or one-way ANOVA with Bonfferoni post hoc test or Chi-Square test to test for differences. Data (mean $\pm$ SD) were considered statistically significant at a value of $P < 0.05$.

RESULTS

snMSCs Culture and Morphology

Following two depletion steps using Macs immunomagnetic beads, more than 99% mononuclear cells were $CD45^- GlyA^-$ and 0.1–1% of $CD45^- GlyA^-$ mononuclear cells are BMMNCs. When these cells were cultured for 10–21 days, fibroblast-like cell clones formed. Purified snMSCs were collected from $CD34^- CD45^-$ cells at 10th, 20th, and 30th passages. snMSCs showed homogeneous morphology with normal proliferation and an undifferentiated status during culture expansion at 10, 20, and 30 cell doublings (Fig. 1A–D), respectively, while BMMNCs (Fig. 1E) and uMNCs (Fig. 1F) showed heterogeneous fibroblast-like, rod-like, disk-like appearances. Oct-4, Bmi-1, and ABCG2 were expressed in snMSCs but not in fibroblasts, as shown by RT-PCR (Fig. 1G) and Western blot (Fig. 1H).

Characterization of PBMNCs, BMMNCs, uMSCs, and snMSCs

FACS analysis revealed that snMSCs from 10 to 30 cell doublings uniformly expressed over 99% for MSC markers SH2 and SH3, integrins CD29 and CD71, matrix receptors CD44, and endothelial cell marker CD147, whereas were negative for the leukocyte common antigen CD45, and the hematopoietic lineage markers CD34, CD117, and AC133. snMSCs phenotype remained unchanged for more than 30-cell doublings, whereas uMSCs from 10th passage heterogonously expressed these markers (Table I). BMMNCs and PBMNCs contained

similar quantity of $SH2^+$, $SH3^+$, $CD29^+$, $CD71^+$, or $CD44^+$ cells (4–7%).

In Vitro Cardiomyocyte Differentiation

After in vitro 5-azacytidine induction, immunofluoroscence showed that over 90% of snMSCs expressed the caridomyocyte-like marker proteins MHC, troponin T, and desmin, whereas unpurified MSCs expressed only 40% of these proteins (data not shown). MHC, troponin T, and desmin expressions were higher in snMSCs compared with uMSCs by Western blot (Fig. 2). There were no desmin, troponin T, and MHC expressions in snMSCs during culture expansion before 5-azacytidine induction.

Myocardial Infarction Model

MI was created in 150 animals. Thirty animals were excluded because of lethal ventricular fibrillation at the time of coronary ligation, which left a total group of 120 animals and were used as treatment. There was no death in the process of cell transplantation or PBS injection. Ninety one rats were available for serial functional studies in the end. There was no significant difference in mortality between six groups (Table II, $P > 0.5$) at day 3 or day 90 post-MI. No malignant arrhythmia was found on ECG recordings on scheduled study end and no tumor formation was observed at autopsy.

Cardiac Geometry, Function, and Tissue Weights

Echocardiography studies showed that baseline EF and FS were not significantly different between the PBS-injected, PBMNCs-grafted, BMMNCs-grafted, uMSCs-grafted, and snMSCs-grafted hearts. On day 1 post-infarct, animals in all six groups developed typical changes of acute heart failure and LV early remodeling, in comparison with data obtained at the baseline levels (data not shown). As shown in Table II, there were no significant differences in LV function and remodeling indexes among the six groups on 3-day post-MI. On 90-day post-MI, LV ejection fraction in the PBS groups showed no significant change, but exhibited significant improvement in all cell transplantation groups, the maximal improvement was seen in snMSCs treated rats ($P < 0.05$). This change was associated with ordinal reduction of lung wet/dry weight ratios in the cell transplantation groups compared
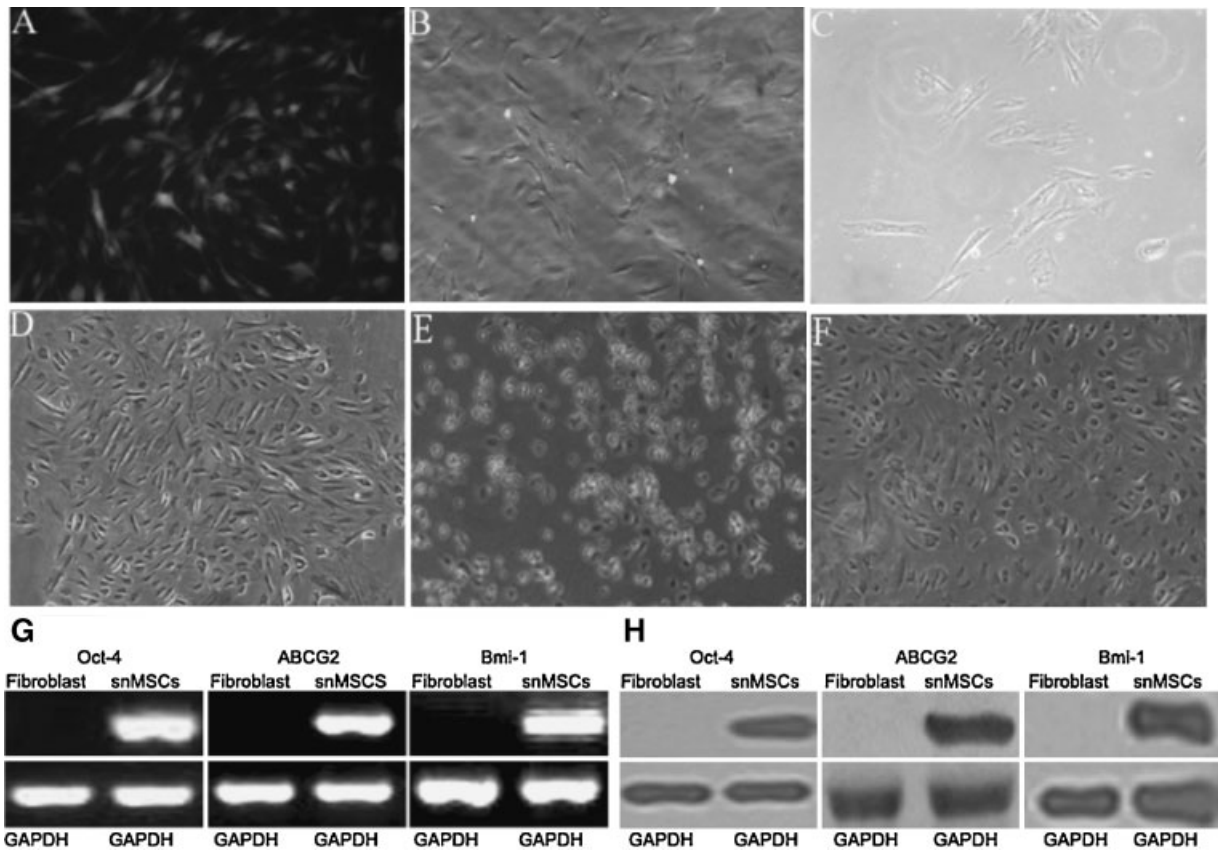
Fig. 1. snMSCs morphology and differentiation potential. Ten to Twenty days after the first seeding of BMMNCs into culture, cell clones grew and appeared as fibroblast-like (**A**), snMSCs maintained homogenous fibroblast-like appearance at 10 (**B**), 20 (**C**), and 30 (**D**) cell doublings. BMMNCs (**E**) and uMSCs (**F**) showed heterogeneous appearances: fibroblast-like, rod-like, discus-like (magnification = 400×). Oct4, Bmi1, and ABCG2 expressions by RT-PCR (**G**) and Western blot results (**H**) in human-derived fibroblasts and human-derived snMSCs.

TABLE I. Cell Characterization Assessed by FACS, Including the Mean Percentage of Cell Surface Marker and the Viability of Cells Pre-Transplantation

	PBMNCs	BMMNCs	UMSCs	snMSCs
MSC markers				
SH2	1.93 ± 0.16	2.04 ± 0.28	84.88 ± 3.72*,†	99.56 ± 0.23*,†,*
SH3	1.92 ± 0.21	2.13 ± 0.33	85.65 ± 3.21*,†	99.34 ± 0.31*,†,*
Integrin markers				
CD29	2.36 ± 0.26	2.10 ± 0.32	89.25 ± 2.60*,†	99.44 ± 0.31*,†,*
CD71	3.64 ± 1.43	7.03 ± 1.31	80.04 ± 5.09*,†	99.08 ± 0.33*,†,*
Matrix receptors				
CD44	4.33 ± 1.56	4.70 ± 2.06	77.84 ± 6.52*,†	99.36 ± 0.38*,†,*
Endothelial marker				
CD147	7.46 ± 2.18	13.47 ± 3.10*	86.390 ± 4.15*,†	99.55 ± 0.23*,†,*
Leukocyte common antigen				
CD45	41.49 ± 3.57	31.01 ± 3.66*	1.65 ± 0.17*,†	0.15 ± 0.06*,†
Hematopoietic line-age markers				
CD34	2.58 ± 0.36	3.08 ± 0.56*	0.67 ± 0.19*,†	0.08 ± 0.02*,†,*
CD117	1.11 ± 0.30	2.48 ± 0.58*	8.20 ± 1.30*,†	0.26 ± 0.07†,*
AC133	1.48 ± 0.33	2.63 ± 1.54*	0.35 ± 0.15*,†	0.15 ± 0.06*,†
Cell viability	93.75 ± 3.55	94.54 ± 2.96	94.75 ± 3.11	95.26 ± 3.47

Data are expressed as the mean ± SD (n = 5).
*$P < 0.05$ versus the PBMNCs.
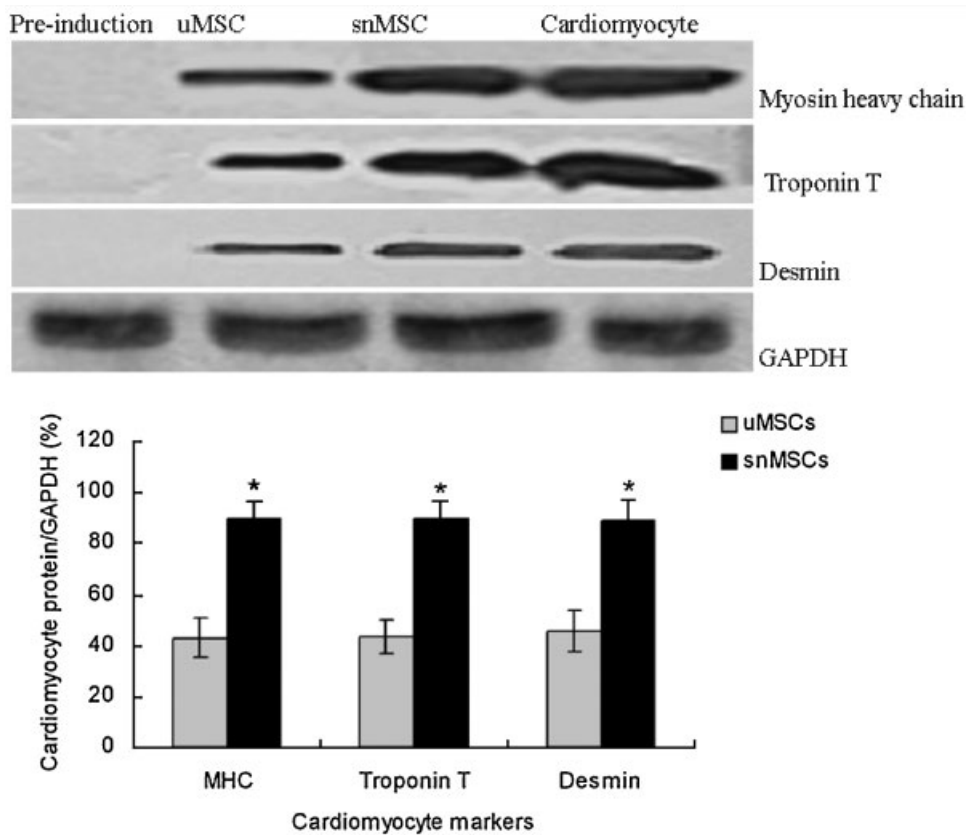†$P < 0.05$ versus the BMMNCs.
‡$P < 0.05$ versus the UMSCs.

Fig. 2. Cardiomyocyte markers post-in vitro 5-azacytidine induction in uMSCs and snMSCs. *$P < 0.05$ versus the uMSCs group (mean ± SD, n = 6). Each experiment was performed twice. The results were the average of six experiments on three times of cell analysis in each subgroup.

with the PBS groups. In comparison with 3-day post-MI, injection of PBS and PBMNCs on 90-day post-MI indicated similarly sustained exacerbation with thickening of LVPWd, thinning of LVAWd, and dilation of LVDd and LVEDV, whereas transplantation of BMMNCs, uMSCs, and snMSCs significantly ameliorated these indices on day 90 post-MI, and the effect was greatest in snMSCs treated rats ($P < 0.05$). There were no significant differences in heart rate between the six groups. Consistent with the changes in LV structure, LV/body weight ratios at day 90 post-MI were significantly lower in the BMMNCs, the uMSCs, and the snMSCs groups than in the PBS and the PBMNCs groups, and the maximal decrease was seen in the snMSCs group. However, the final body weight was significantly increased at 90-day post-MI in the snMSCs group than in the PBS, the PBS + CsA, the PBMNCs, the BMMNCs, and the uMSCs group. LV/body weight ratios and body weight showed no significant difference at 3-day post-MI between six groups (data not shown).

Assessment of Regional Angiogenesis

As shown in Figure 3A, there was no significant difference in the vessel density between six groups at 3-day post-MI. However, in comparison with 3-day post-MI, the vessel density at peri-infarct area 90 days after MI was 8.5-fold, 7.8-fold, 4.0-fold, 2.7-fold, and 1.2-fold greater in the snMSC group than in the PBS, the PBS + CsA, the PBMNC, the BMMNC, and the uMSC groups, respectively ($P < 0.05$), and was 4.7-fold, 4.3-fold, 1.1-fold, 0.6-fold, and 0.3-fold greater at the infarct area than in the PBS, the PBS + CsA, the PBMNCs, the BMMNCs, and the uMSCs groups, respectively ($P < 0.05$). Moreover, the vessel density at peri-infarct area in both two time points was greater than at infarct area in the six groups ($P < 0.05$), and was seen the most obvious in the snMSCs group at day 90 post-MI (2.9-fold, $P < 0.05$). The vessel density at both the peri-infarct and the infarct areas was similar between both PBS groups. The vessel density in the normal area was not

TABLE II. Morphological and Functional Consequences of Echocardiographic Study, the Survival (%), and Tissue Weights

	PBS	PBS + CsA	PBMNC	BMMNC	uMSC	snMSC
Day-3 post-MI, n	7	7	8	9	9	9
LV ejection fraction, %	54.4 ± 7.8	55.4 ± 7.2	53.6 ± 8.7	54.0 ± 5.2	56.9 ± 2.9	59.1 ± 4.9
LVAWd, mm	1.11 ± 0.18	1.13 ± 0.17	1.13 ± 0.09	1.24 ± 0.17	1.19 ± 0.15	1.26 ± 0.15
LVPWd, mm	1.43 ± 0.28	1.40 ± 0.32	1.28 ± 0.09	1.37 ± 0.30	1.47 ± 0.28	1.37 ± 0.10
LVDd, mm	5.44 ± 0.44	5.46 ± 0.41	5.54 ± 0.35	5.48 ± 0.59	5.69 ± 0.31	5.39 ± 0.50
LVEDV, ml	0.63 ± 0.02	0.62 ± 0.03	0.60 ± 0.04	0.62 ± 0.04	0.60 ± 0.42	0.62 ± 0.04
Day-90 post-MI, n	6	6	7	8	7	8
LV ejection fraction, %	48.0 ± 7.8	49.7 ± 8.3	60.3 ± 3.4*,†,‡	67.1 ± 3.5*,†,‡,§	74.8 ± 7.8*,†,‡,§,‡	82.5 ± 6.9*,†,‡,§,¶,‡
Lung wet/dry weight ratio, %	6.89 ± 0.49	6.78 ± 0.19	6.85 ± 0.19	6.59 ± 0.35	6.49 ± 0.32	4.14 ± 0.54*,†,‡,§,¶
LVAWd, mm	1.12 ± 0.16	1.08 ± 0.08	1.20 ± 0.08	1.55 ± 0.09*,†,‡,§	1.60 ± 0.10*,†,‡,‡	1.83 ± 0.28*,†,‡,§,¶,‡
LVPWd, mm	2.45 ± 0.19‡	2.40 ± 0.14‡	2.37 ± 0.14*,†	1.70 ± 0.3*,†,‡,§	1.74 ± 0.26*,†,‡,§	2.04 ± 0.22*,†,‡,§,¶,‡
LVDd, mm	7.37 ± 0.81	7.52 ± 0.83	5.47 ± 0.48	4.58 ± 0.28	4.54 ± 0.42*,†,‡,§	4.00 ± 0.27*,†,‡,§,¶,‡
LVEDV, ml	0.72 ± 0.04‡	0.73 ± 0.03‡	0.63 ± 0.03*,†	0.58 ± 0.03*,†,‡	0.55 ± 0.05*,†,‡	0.51 ± 0.03*,†,‡,§,¶,‡
LV/body weight ratios, g/kg	2.29 ± 0.11	2.27 ± 0.13	2.15 ± 0.27	1.80 ± 0.08*,†,‡	1.65 ± 0.07*,†,‡	1.42 ± 0.08*,†,‡
Body weight, g	291.5 ± 22.5	291.8 ± 29.6	263.7 ± 37.1	314.0 ± 67.9	297.0 ± 34.5	363.0 ± 26.9*,†,‡

LVDd, LVEDV, LVAWd, or LVPWd stand for LV end-diastolic internal diameter or volume, anterior or posterior wall thickness, respectively.

*$P < 0.05$, versus the PBS.

†$P < 0.05$, versus the PBS + CsA.

‡$P < 0.05$, versus the PBMNCs.

§$P < 0.05$, versus the BMMNCs.

¶$P < 0.05$, versus the uMSCs groups, respectively.
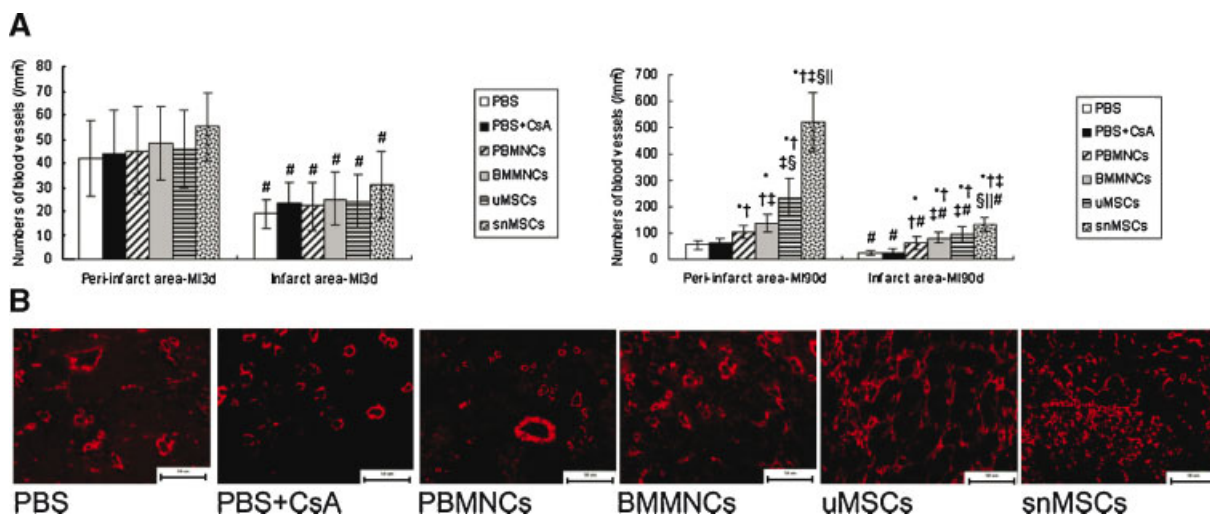
‡$P < 0.05$ versus at day 3 post-MI, respectively.

Fig. 3. Assessment of vessel density. **A:** Quantitative analysis of vessel density in individual groups. *$P < 0.05$, †$P < 0.05$, ‡$P < 0.05$, §$P < 0.05$, and ‖$P < 0.05$ versus the PBS, the PBS + CsA, the PBMNCs, the BMMNCs, and the uMSCs groups, respectively; #$P < 0.05$ versus at the peri-infarct area (3-day post-MI, n = 7, 7, 8, 9, 9, and 9 in the PBS, the FBT, the PBMNC, the BMMNC, the uMSC, and the snMSC groups; 90-day post-MI, n = 6, 6, 7, 8, 7, and 8 in the PBS, the PBS + CsA, the PBMNCs, the BMMNCs, the uMSCs, and the snMSCs groups, respectively. The number of vessels in each slide was calculated based on ten microscopic fields. The results were the average of five slides from each animal). **B:** Representative photographs of the infarct-related artery area from different groups are shown at the peri-infarct regions in 90-day post-MI. Heart sections were stained with anti-factor VIII antibody (red). Each bar stands for 50 µm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

significantly different among the six groups (data not shown). Figure 3B showed the representative immunofluorescence staining of factor VIII at the peri-infarct of the individual group at day 90 post-MI.

The Number and Differentiation of Transplanted Cells

DAPI⁺ cells were counted under high-power fluorescence microscope. DAPI⁺ cells were similar among six groups at 3-day post-MI (data now shown). On 90-day post-MI, however, the number of DAPI⁺ cells in snMSCs treated hearts was significantly higher than those in BMMNCs and PBMNCs treated hearts. Moreover, the number of engrafted snMSCs was higher than that of engrafted uMSCs in the heart (Fig. 4G). Transplanted snMSCs at the peri-infarct and infarct areas expressed MHC or factor VIII more than transplanted BMMNCs and uMSCs (arrowhead, Fig. 4A–D), whereas transplanted PBMNCs did not express these proteins. As shown in Figure 4H, I, quantitative analysis indicated that the percent proportions of the cells double positively stained with DAPI and MHC and factor VIII to the total positive-DAPI staining cells at the peri-infarct and infarct areas were the greatest in the snMSCs

group, the secondary in the uMSCs group, and the smallest in the BMMNCs group ($P < 0.05$). Three staining showed that positive-DAPI snMSCs (Fig. 4E(a)) were positively stained with human nuclei (Fig. 4E(b),(c)) and also expressed MHC (Fig. 4E(d)). All transplanted DAPI⁺ cells did not express MHC or factor VIII at 3-day post-MI.

Immune or Inflammatory Reaction

The degree of immune or inflammatory reaction was expressed by counting the number of infiltrating lymphocyte or neutrophil in the infarct and the peri-infarct areas. Histological H&E staining showed that there was no obvious lymphocyte or neutrophil infiltrating into the infarct and the peri-infarct areas in all groups (Fig. 4F). MPO levels in the infarcted and border myocardium showed no obvious difference among all the groups (Fig. 4J).

Assessment of Collagen Density and Infarct Size

As shown in Figure 5A, the collagen-rich myocardial scar in the infarcted wall stained blue, whereas viable myocardium stained red. On day 90 post-MI, under low magnification light microscopy, the collagen density was similar in the PBS, the CsA + PBS, and the
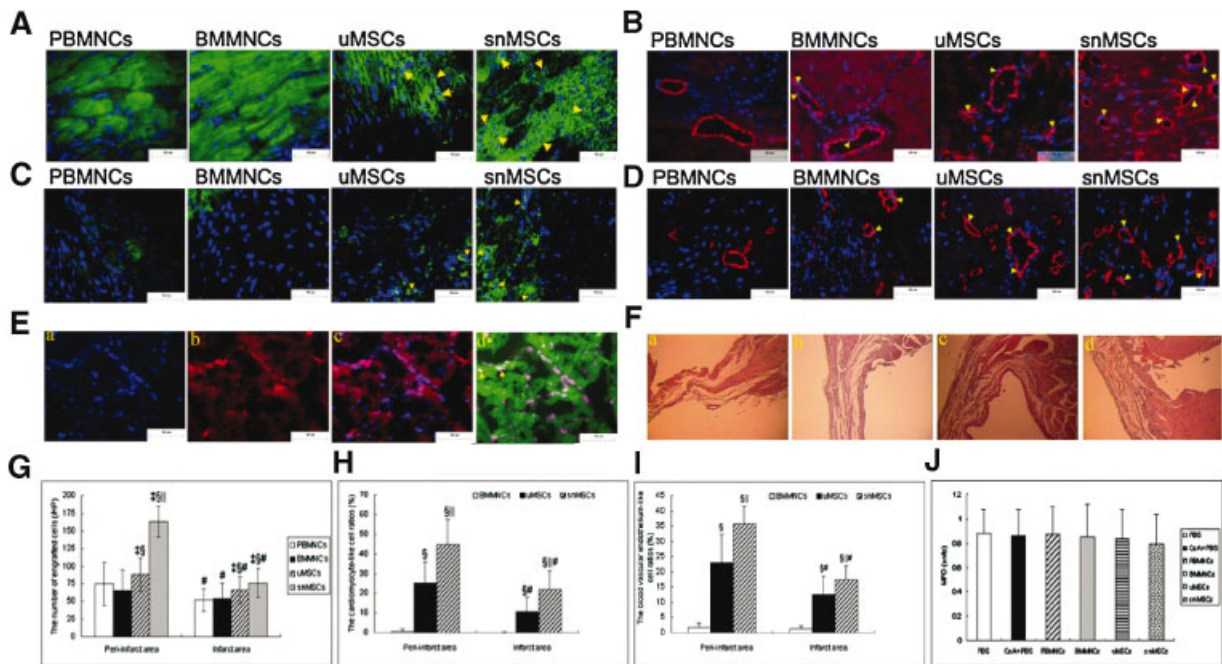
Fig. 4. Differentiation of transplanted cells at day 90 post-MI. **A** and **C**: showed that transplanted cells expressed MHC (arrowheads) at the peri-infarct and the infarct areas, respectively. **B** and **D**: showed that transplanted cells expressed factor VIII (arrowheads) at the peri-infarct and the infarct areas, respectively. The nuclei of transplanted cells were stained with DAPI and revealed as blue, and the cytoplasm of the myocardiocytes or blood endothelial cells was stained as green or red with anti-myosin heavy chain protein or factor VIII. **E**: Showed the condition of transplanted snMSCs tri-stained with DAPI, MHC, and human nuclei protein. The nuclei of transplanted snMSCs **(a)** were stained with human nuclei protein as red **(b)**. Some of transplanted snMSCs double stained by DAPI and human nuclei protein **(c)** were also stained with MHC **(d,** arrowheads). Each bar in (A–E) stands for 50 μm. **F**: Revealed H&E stains of rat infarcted hearts in the PBS, the PBS + CsA, the uMSCs, and the snMSC groups low magnification (50×) light microscope. **G**: Showed the

number of engrafted cells per high-power field. **H** and **I**: Showed the percent proportion of the cells double positively stained with DAPI and MHC, or factor VIII to the total positive-DAPI staining cells at the peri-infarct and the infarct areas, respectively. **J**: Showed that quantitative analysis of MPO in the individual groups (n = 6, 6, 7, 8, 7, and 8 in the PBS, the PBS + CsA, the PBMNC, the BMMNC, the uMSC, and the snMSC groups, respectively. The number of the double positively stained cells in each slide was calculated based on ten microscopic fields. The results were the average of five slides from each animal. The MPO result in each animal was the average of two times of tissue analysis). $^{*}P < 0.05$, $^{†}P < 0.05$, $^{‡}P < 0.05$, $^{§}P < 0.05$, and $^{||}P < 0.05$ versus the PBS, the PBS + CsA, the PBMNCs, the BMMNCs, and the uMSCs groups, respectively; $^{#}P < 0.05$ versus at the peri-infarct area. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

PBMNCs groups, whereas collagen density was reduced in rats receiving BMMNCs, uMSCs, and snMSCs (Fig. 5A). Statistical analysis showed that the collagen density in the BMMNCs, the uMSCs, and the snMSCs groups was in inverse proportion to these transplanted cell purity ($P < 0.05$, Fig. 5C). Consistent with the changes in the collagen density, the infarct size at 90 day post-MI were significantly smaller in the snMSCs group than other groups, whereas no significant difference was seen between the PBS and the PBMNCs groups (Fig. 5B,D).

DISCUSSION

This is the first study to demonstrate that snMSCs isolated and purified from BMMNCs

and uMSCs leads to the greatest differentiation potential and maintenance of homogenous appearance and cell surface markers not only in vitro but also in vivo. When grafted into the infarcted hearts, snMSCs caused the greatest effects on impeding the development of heart failure, preserving LV function and dimensions, and inhibiting infarct expansion. These structural and functional changes were associated with reduced myocardial collagen, enhanced angiogenesis, and increased vasculogenesis and myocardiogenesis. No malignant arrhythmia and no obvious immune responses were observed in the transplanted hearts, which suggested that transplantation of the novel population of snMSCs could be performed safely. Together, the findings of this study imply that single
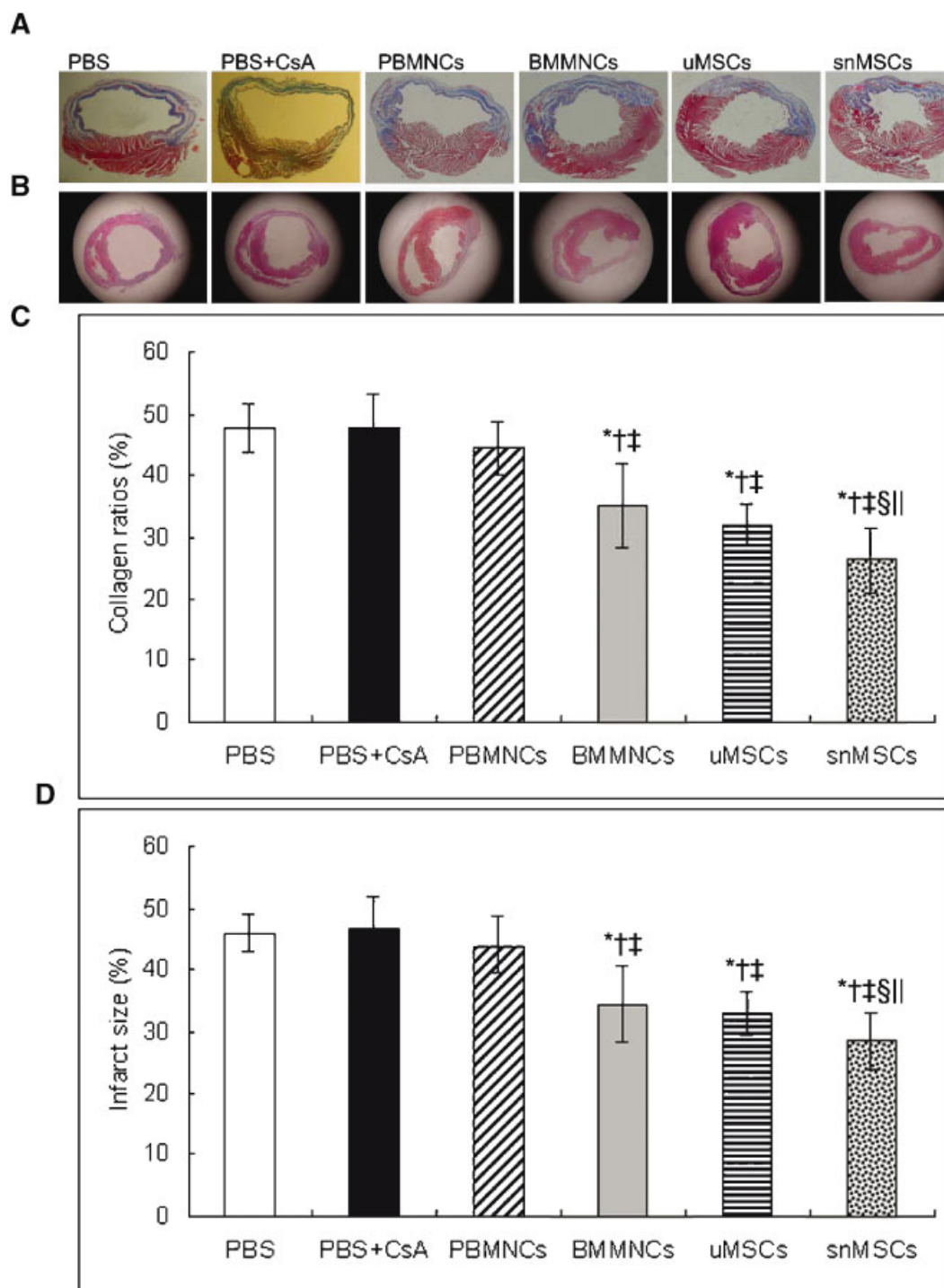
Fig. 5. Assessment of collagen density and infarct size on day 90 post-MI. **A:** Transversal LV sections were stained by Masson trichrome method under low magnification (2.5×). **B:** Typical H&E stains of infarct area in the six groups under low magnification (2.5×). **C** and **D:** Respectively, the quantitative analysis of the collagen ratios or infarct size in all the groups. $^*P < 0.05$, $^\dagger P < 0.05$, $^\ddagger P < 0.05$, $^\S P < 0.05$, and $^{||}P < 0.05$ versus the PBS, the PBS + CsA, the PBMNCs, the BMMNCs, and the uMSCs groups, respectively; $^\#P < 0.05$ versus at day 3 post-MI, respectively ($n = 6, 6, 7, 8, 7$, and 8 in the PBS, the PBS + CsA, the PBMNCs, the BMMNCs, the uMSCs, and the snMSCs groups, respectively. The number of collagen density and infarct size in each animal was calculated based on two low-magnification microscopic fields). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

clonally purified non-hematopoietic mesenchymal stem cells serve as a superior transplantation cell source for myocardial repair post-MI in rats.

In previous reports, MSCs isolated by their adherence to tissue culture surfaces [Zohar et al., 1997], or on the basis of their differences in size [Hung et al., 2002], or immunoselected by specific surface markers [Cheng et al., 2000] are usually contaminated by hematopoietic precursors, and heterogeneous in morphology, surface marker profile, and phenotype characterization. In this report, MSCs obtained by adherent culture method from BMMNCs also showed heterogeneous morphology, such as fibroblast-like, rod-like, disk-like appearances, and contained a few hematopoietic cells. However, when purified by Macs immunomagnetic beads immunoselection in combination with limit dilution culture (e.g., single clone culture), MSCs turned into a relatively homogeneous population of human MSCs, which maintained homogeneous morphology and extensive proliferation during culture expansion. These cells expressed over 99% of SH2, SH3, CD44, CD29, CD71, and CD147, and were negative for CD34, CD45, CD117, and AC133 for more than 30 cell doublings suggesting that these single clonally MSCs contained none of hematopoietic lineage cells (snMSCs). Moreover, snMSCs expressed functional specific markers of self-replication and pluripotency, Oct4, Bmi1, and ABCG2, whereas fibroblast did not express these proteins. Oct4 is known a key regulator of stem cell pluripotency and differentiation [Pesce and Scholer, 2000, 2001]. ABCG2, the ATP-binding cassette (ABC) transporter G2, is a phenotypic marker, functional regulator of stem cells and a novel stem cell transporter [Bunting, 2002]. Bmi1 is required for maintenance of adult self-renewing stem cells and determines the proliferating capacity of stem cells [Lessard and Sauvageau, 2003; Park et al., 2003]. These findings suggest that snMSCs in the present study might be capable of self-renewal and could differentiate into more than one-cell types (pluripotential). After 5-azacytidine induction, snMSCs showed cardiomyocyte-like structure. Over 90% of snMSCs expressed the caridomyocyte-like marker proteins MHC, troponin T, and desmin, whereas unpurified MSCs expressed only 40% of these proteins, which suggested that snMSCs have high efficacy of differentiation in vitro. Their cultivation and selective differentiation in vitro should provide further understanding of this important progenitor of multiple tissue types and the potential advantages of new therapeutic approaches for the restoration of damaged hearts.

Transplantation of snMSCs for cardiac repair has not been reported. Bel et al. [2003] and Hamano et al. [2002] demonstrated that transplantation of fresh unfractionated bone marrow into post-infarction scars did not improve cardiac function, which correlated with a lack of new angiogenesis and differentiation of grafted cells into cardiomyocytes or endothelial cells. In the present study, the cardiac function at day 90 post-MI was improved in the order as snMSCs > uMSCs > BMMNCs > PBMNCs > P-PBS + CsA ≈ PBS, indicating that cardiac function improvement correlated with differences in purity of the transplanted cells. In parallel with changes in cardiac function, rats receiving snMSCs showed significantly prevented dilation of LVDd and LVEDV, thickening of LVPWd, and thinning of LVAWd, decreased LV/body weight ratios, reduced collagen density and infarct size, and increased mass of viable myodium within the infarcted area than those receiving BMMNCs, uMSCs, or PBMNCs, suggesting attenuation in left ventricular remodeling was also purity-related. We also found that the final body weight was significantly increased in the snMSCs group than in the PBS, the PBS + CsA, the PBMNCs, the BMMNCs, and the uMSCs group, which may be relative to snMSCs transplantation improving cardiac function, enhancing the appetite, and resulting in increased rat body weight. Therefore, efficacy of purified MSCs for cardiac repair and prognosis was greater than that of unpurified MSCs. Our data on the effect of direct injection of snMSCs into rat acute ischemic myocardium are consistent with those of Yoon et al. [2005] reported that transplantation of clonally expanded multipotent stem cells from human bone marrow improves cardiac function, augments proliferation and survival of host myocardium, increases capillary, and decreases myocardial fibrosis in a rat model of MI. However, Dai et al. [2005] demonstrated that transplanted allogenic MSCs survived in infarcted myocardium, and indeed expressed cardiospecific surface markers, but differentiation was incomplete as only immature myofibrillar organization was detectable. Moreover, MSCs improved global LV function at 4 weeks while these benefits of

MSC treatment were lost at 6 months, which suggests a possible transient and early paracrine effect. In this present study, a similar beneficial effect of snMSCs transplantation on the infarcted heart was observed even at 3 months (90 days) post-transplantation, suggesting that the snMSCs are better than MSCs used in Dai et al.'s study for cardiac repair. In comparison with day 90 post-MI, LV function, the vessel density and the number of survived DAPI^{+} cells were similar at day 3 post-MI among the various groups, which suggested that snMSCs transplantation showed no better effect within the early stage of transplantation. The effects beyond this observation period remain unknown and warrant further investigation.

In animals receiving snMSC transplantation, the vessel density in the peri-infarct and infarcted wall was higher than that of the other heterogeneous stem cell groups on 90-day post-MI. Our previous research found that increased VEGF expression, vascular density and regional blood flow in the infarct zone, decreased apoptosis of hypertrophied myocytes post-uMSCs transplantation [Tang et al., 2004]. In the present study, we further demonstrated that the extent of vessel density increase induced by these unpurified and heterogeneous MSCs, BMMNCs, or PBMNCs transplantation was less at both the peri-infarct and the infarct areas than that of snMSCs transplantation. Our study showed that rats receiving BMMNCs and uMSCs demonstrated markedly increased vessel density at both the peri-infarct and the infarct areas in comparison with rats receiving PBMNCs, this result is in agreement with former studies demonstrating that BMMNCs implantation increased capillary numbers more than PBMNCs implantation in an ameroid-induced chronic myocardial ischemia model in pig [Kamihata et al., 2002]. Moreover, the vessel density increase and collagen/infarct size reduction were positively correlated with cardiac function improvement and LV remodeling improvement suggesting that increased angiogenesis, decreased collagen, and infarct size contributed to the cardiac function recovery and LV remodeling reverse in rats receiving various cell therapy.

Apart from angiogenesis, vasculogenesis may also contribute to the enhancement of cardiac function [Kocher et al., 2001]. Neoangiogenesis, including vasculogenesis and angiogenesis, could enhance the blood supply to ischemic myocardium and improve post-ischemic myocardial salvage. Formation of new myocardium within the infarcted area could attenuate myocardial remodeling and lead to sustained improvement in cardiac function [Orlic et al., 2001b]. In this study, endothelial phenotypes were seen post-snMSCs transplantation, which might relate to new vessels formation. In addition, we observed transplanted snMSCs expressed myosin heavy chain proteins. These cells also expressed human nuclei protein, which indicated these cells were originated from human not from rat suggesting that these single colonal MSCs could differentiate in vivo. We also found that the number of expressed MHC or factor VIII in both peri-infarct and infarct areas were greater in rats receiving snMSCs than those receiving uMSCs and BMMNCs. None of transplanted PBMNCs expressed MHC or factor VIII. This result indicated that purified MSCs have the greater differentiation potential than those unpurified bone marrow cells or peripheral blood cells.

One interesting finding from this study is that uMSCs transplantation significantly increased cardiac function, but caused no markedly further improvement of LV remodeling compared with BMMNCs transplantation. This finding is compatible with similar changes in collagen density and infarct size at the whole LV, and the blood vessel density at the infarct region between the two groups. These results suggested that unpurified MSCs obtained by the tight adherence might bring no more beneficial effects on the prognosis of the remodeled hearts in comparison with freshly isolated BMMNCs. Thus, cell purity plays a key role for the efficacy in cardiac repair post-MI. Furthermore, no obvious difference was seen in the number of lymphocyte or neutrophil, and MPO levels in the infarct and the peri-infarct areas among all the groups, which indicated that transplanted snMSCs did not induce inflammation aggravation. Our study showed that there were no differences between PBS and PBS + CsA groups indicating the immunosuppression (CsA) per se could not be an important factor affecting cardiac function and myocardial healing. Previous study had shown that immunosuppressive therapy may greatly alter potential to secrete angiogenic molecules [Johnson et al., 1999] and the effects of transplanted stem cells on angiogenesis were mediated at least in part by secretion of a variety of paracrine mediators

[Urbich et al., 2005], therefore, the effects on various stem cell groups might differ with or without cyclosporin A. Cyclosporin A was added to all stem cell groups in this study and all effects were obtained "on top of cyclosporin A," the potential effects of cyclosporin A on different stem cell populations were not studied in the present study and future studies are warranted to address this issue.

In addition to our present snMSCs, there have been other "purified and clonal" mesenchymal stem cells isolated and reported, starting with Pittenger et al. [1999], and later by Kawada et al. [2004] and Yoon et al. [2005]. Thus, further study remained to investigate which one would be the "best source" among these pure bone marrow mesenchymal stem cells as donor cells for cell therapy in MI.

CONCLUSIONS

We conclude that $SH2^+SH3^+CD29^+CD71^+$ $CD44^+CD147^+CD34^-CD45^-CD117^-AC133^-$ cells represent a novel non-hematopoietic mesenchymal subpopulation of bone marrow derived stem cells capable of successful and substantial engraftment in areas of transmural myocardial scar, with in the de-novo formation of myocardium both in vitro and in vivo. The single clonally purified non-hematopoietic mesenchymal stem cells functioned more effectively than those unpurified and heterogeneous cells on long-term period post-MI, which may represent a novel and more effective cell type for MI patients.

Abbreviations

BMMNCs	bone marrow mononuclear cells
BMMNCs group	rats received BMMNCs transplantation
DAPI	4′, 6-diamidino-2′-phenylindole
EF	ejection fraction
FACS	fluorescence activated cell sorter
Factor VIII	von Willebrand factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
H&E	hematoxylin and eosin staining
HSCs	hematopoietic stem cells
MHC	myosin heavy chain
MI	myocardial infarction
MPO	myeloperoxidase
PBMNCs	peripheral blood mononuclear cells
PBMNCs group	rats received PBMNCs transplantation
PBS	phosphate-buffered saline
CsA	cyclosporin A
PBS + CsA or PBS groups	rat received PBS injection with or without CsA injection
snMSCs	single clonally purified non-hematopoietic bone marrow mesenchymal stem cells
uMSCs	unpurified bone marrow mesenchymal stem cells

REFERENCES

Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. 2004. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 428:668–673.

Bel A, Messas E, Agbulut O, Richard P, Samuel JL, Bruneval P, Hagege AA, Menasche P. 2003. Transplantation of autologous fresh bone marrow into infarcted myocardium: A word of caution. Circulation 108(Suppl I): II247–II252.

Bunting KD. 2002. ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells 20:11–20.

Chen SL, Fang WW, Ye F, Liu YH, Qian J, Shan SJ, Zhang JJ, Chunhua RZ, Liao LM, Lin S, Sun JP. 2004. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol 94:92–95.

Cheng L, Qasba P, Vanguri P, Thiede MA. 2000. Human mesenchymal stem cells support megakaryocyte and pro-platelet formation from CD34(+) hematopoietic progenitor cells. J Cell Physiol 184:58–69.

Dai W, Hale SL, Martin BJ, Kuang JQ, Dow JS, Wold LE, Kloner RA. 2005. Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium: Short- and long-term effects. Circulation 112:214–223.

Fuchs S, Baffour R, Zhou YF, Shou M, Pierre A, Tio FO, Weissman NJ, Leon MB, Epstein SE, Kornowski R. 2001. Transendocardial delivery of autologous bone marrow

enhances collateral perfusion and regional function in pigs with chronic experimental myocardial ischemia. J Am Coll Cardiol 37:1726–1732.

Hamano K, Li TS, Kobayashi T, Hirata K, Yano M, Kohno M, Matsuzaki M. 2002. Therapeutic angiogenesis induced by local autologous bone marrow cell implantation. Ann Thorac Surg 73:1210–1215.

Hung SC, Chen NJ, Hsieh SL, Li H, Ma HL, Lo WH. 2002. Isolation and characterization of size-sieved stem cells from human bone marrow. Stem Cells 20:249–258.

Jiang Y, Prosper F, Verfaillie CM. 2000. Opposing effects of engagement of integrins and stimulation of cytokine receptors on cell cycle progression of normal human hematopoietic progenitors. Blood 95:846–854.

Johnson DW, Saunders HJ, Johnson FJ, Huq SO, Field MJ, Pollock CA. 1999. Fibrogenic effects of cyclosporin A on the tubulointerstitium: Role of cytokines and growth factors. Exp Nephro l 7:470–478.

Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Amano K, Iba O, Imada T, Iwasaka T. 2002. Improvement of collateral perfusion and regional function by implantation of peripheral blood mononuclear cells into ischemic hibernating myocardium. Arterioscler Thromb Vasc Biol 22:1804–1810.

Kawada H, Fujita J, Kinjo K, Matsuzaki Y, Tsuma M, Miyatake H, Muguruma Y, Tsuboi K, Itabashi Y, Ikeda Y, Ogawa S, Okano H, Hotta T, Ando K, Fukuda K. 2004. Non-hematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. Blood 104:3561–3587.

Kocher A, Schuster M, Szabolcs M, Burkhoff D, Wang J, Homma S, Edwards N, Itescu S. 2001. Neovascularization of ischemic myocardium by human bone-morrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 7:430–436.

Krause D.S., Theise N.D., Collector M.I., Gardner R, Neutzel S, Sharkis SJ. 2001. Multi-organ, multi-lineage engrafment by a single bone marrow-derived stem cell. Cell 105:369–377.

Lessard J, Sauvageau G. 2003. Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. Nature 423:255–260.

Litwin SE, Katz SE, Morgan JP, Douglas PS. 1994. Serial echocardiographic assessment of left ventricular geometry and function after large myocardial infarction in the rat. Circulation 89:345–354.

Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. 2004. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 428:664–668.

Nwogu JI, Geenen D, Bean M, Brenner MC, Huang X, Buttrick PM. 2001. Inhibition of collagen synthesis with prolyl 4-hydroxylase inhibitor improves left ventricular function and alters the pattern of left ventricular dilatation after myocardial infarction. Circulation 104:2216–2221.

Nygren JM, Jovinge S, Breitbach M, Sawen P, Roll W, Hescheler J, Taneera J, Fleischmann BK, Jacobsen SE. 2004. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nat Med 10:494–501.

Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. 2001a. Bone marrow cells regenerate infracted myocardium. Nature 410:701–705.

Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. 2001. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA 98:10344–10349.

Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, Morrison SJ, Clarke MF. 2003. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature 423:302–305.

Perin EC, Dohmann HF, Borojevic R, Silva SA, Sousa AL, Mesquita CT, Rossi MI, Carvalho AC, Dutra HS, Dohmann HJ, Silva GV, Belem L, Vivacqua R, Rangel FO, Esporcatte R, Geng YJ, Vaughn WK, Assad JA, Mesquita ET, Willerson JT. 2003. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. Circulation 107:2294–2302.

Pesce M, Scholer H. 2000. Oct4: Control of totipotency and germline determination. Mol Rep Dev 55:452–457.

Pesce M, Scholer H. 2001. Oct4: Gatekeeper in the beginnings of mammalian development. Stem Cells 19:271–278.

Pittenger M, Mackay A, Beck S, Jaiswal R, Douglas R, Mosca J, Moorman M, Simonetti D, Craig S, Marshak D. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie C. 2001. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 98:2615–2625.

Shake JG, Gruber PJ, Baumgartner WA, Senechal G, Meyers J, Redmond JM, Pittenger MF, Martin BJ. 2002. Mesenchymal stem cell implantation in a swine myocardial infarct model: Engraftment and functional effects. Ann Thorac Surg 73:1919–1925.

Strauer BE, Brehm M, Zeus T, Kostering M, Hernandez A, Sorg RV, Kogler G, Wernet P. 2002. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation 106:1913–1918.

Tang YL, Zhao Q, Zhang YC, Cheng L, Liu M, Shi J, Yang YZ, Pan C, Ge J, Phillips MI. 2004. Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium. Regul Pept 117:3–10.

Urbich C, Aicher A, Heeschen C, Dernbach E, Hofmann WK, Zeiher AM, Dimmeler S. 2005. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 39:733–742.

Wang JS, Shum-Tim D, Galipeau J, Chedrawy E, Eliopoulos N, Chiu RC. 2000. Marrow stromal cells for cellular cardiomyoplasty: Feasibility and potential clinical advantages. J Thorac Cardiovasc Surg 120:999–1005.

Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, Fichtner S, Korte T, Hornig B, Messinger D, Arseniev L, Hertenstein B, Ganser A, Drexler H. 2004. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: The BOOST

randomised controlled clinical trial. Lancet 364:141–148.

Yoon YS, Wecker A, Heyd L, Park JS, Tkebuchava T, Kusano K, Hanley A, Scadova H, Qin G, Cha DH, Johnson KL, Aikawa R, Asahara T, Losordo DW. 2005. Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. J Clin Invest 115:326–338.

Zamber CP, Lamba JK, Yasuda K, Farnum J, Thummel K, Schuetz JD, Schuetz EG. 2003. Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine. Pharmacogenetics 13:19–28.

Zohar R, Sodek J, McCulloch CA. 1997. Characterization of stromal progenitor cells enriched by flow cytometry. Blood 90:3471–3481.