Therapeutic Potential of Unrestricted Somatic Stem Cells Isolated from Placental Cord Blood for Cardiac Repair Post Myocardial Infarction

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Objective—Unrestricted somatic stem cells (USSCs) were successfully identified from human cord blood. However, the efficacy of USSC transplantation for improving left ventricular (LV) function post myocardial infarction (MI) is still controversial.

Methods and Results—PBS, 1×10^6 human fibroblasts (Fbr), 1×10^5 USSCs (LD), or 1×10^6 USSCs (HD) were transplanted intramyocardially 20 minutes after ligating the LAD of nude rats. Echocardiography and a microtip conductance catheter at day 28 revealed a dose-dependent improvement of LV function after USSC transplantation. Necropsy examination revealed dose-dependent augmentation of capillary density and inhibition of LV fibrosis. Dual-label immunohistochemistry for cardiac troponin-I and human nuclear antigen (HNA) demonstrated that human cardiomyocytes (CMCs) were dose-dependently generated in ischemic myocardium 28 days after USSC transplantation. Similarly, dual-label immunostaining for smooth muscle actin and class I human leukocyte antigen or that for von Willebrand factor and HNA also revealed a dose-dependent vasculogenesis after USSC transplantation. RT-PCR indicated that expression of human-specific genes of CMCs, smooth muscle cells, and endothelial cell markers in infarcted myocardium were significantly augmented in USSC-treated animals compared with control groups.

Conclusions—USSC transplantation leads to functional improvement and recovery from MI and exhibits a significant and dose-dependent potential for concurrent cardiomyogenesis and vasculogenesis. (***Arterioscler Thromb Vasc Biol.* 2009; 29:1830-1835.**)

Key Words: USSC ■ cardiomyogenesis ■ vasculogenesis ■ cell therapy ■ myocardial infarction

Irreversible myocardial damage post myocardial infarction (MI) results in congestive heart failure (CHF), which is a growing worldwide clinical issue.[1] The long-standing axiom explaining the pathophysiology of the cardiac pump failure was the limited capacity of the damaged myocardium for self-repair and tissue regeneration.[2] Currently, no medication or therapeutic procedure applied clinically, except for cardiac transplantation, has significant efficacy for replacing the myocardial scar with functioning contractile tissue. Therefore, given the major morbidity and mortality associated with MI and CHF, new approaches have been sought to address the principal pathophysiological deficits responsible for these conditions, namely loss of blood vessels and cardiomyocytes (CMCs). Recently, the identification of stem cells capable of contributing to tissue regeneration has ignited significant interest in the possibility that cell therapy could have the potency of repairing damaged myocardium.

A multipotent stem cell population with high proliferative potential was isolated from human umbilical cord blood and termed unrestricted somatic stem cells (USSCs) by Kogler and colleagues.[3] USSCs have been suggested as a more immature cell type than bone marrow (BM) mesenchymal stem cells (MSCs), by the potential to differentiate into osteoblasts, chondrocytes, adipocytes, neurons, and CMCs. The cells exhibit an extended life span and longer telomeres when compared with the MSCs. In addition, these cells grow adherently and can be expanded up to 10^{15} cells without losing pluripotency in culture.[3,4] The application of USSCs did not induce macroscopic or microscopic tumors 6 months after transplantation into a fetal sheep model, suggesting long-term safety of the USSC therapy in normal heart tissue.[3] These data suggest that USSC transplantation could be a promising strategy for the regeneration of damaged mesenchymal tissue such as infarcted myocardium. Kim et al[5]

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performed intramyocardial injection of USSCs 4 weeks after MI in swine. Engrafted USSCs were immunohistochemically identified in the infarct region 4 weeks after cell transplantation, and regional and global LV function significantly improved in pigs receiving USSCs compared with those receiving media. However, the differentiation fate of the transplanted USSCs especially into CMCs, smooth muscle cells (SMCs), and endothelial cells (ECs), and the precise mechanism is still unclear. Moelker et al[6] evaluated the outcome of intracoronary delivery of USSCs 1 week after MI in swine. Intracoronary infusion of USSCs caused micro infarctions, resulting in increase in infarct size. Global and regional left ventricular (LV) function was similar in swine receiving USSCs and those receiving medium. Immunohistochemical examination revealed that CMC and EC markers were not expressed in USSCs surviving in the border zone myocardium. Although mechanisms underlying the discrepant results between Kim's and Moelker's studies are unclear, cell administration route or timing of cell delivery after MI may relate to the different outcomes. Another issue is that such large animal studies are not ideal for evaluating the autocrine and paracrine effects of engrafted USSCs because of the limiting availability of antibodies and primers, which specifically distinguish human proteins/genes from those of swine. Therefore, in this study, we performed a series of experiments using intramyocardial transplantation of USSCs into immunodeficient rats with acute MI to precisely elucidate the vasculogenic and cardiomyogenic potential of USSCs by physiological, histological, and molecular approaches.

Materials and Methods

Detailed procedures in histological, physiological, and molecular analyses are described in the supplemental materials (available online at http://atvb.ahajournals.org).

Experimental Animals

Female athymic nude rats (F344/N Jcl rnu/rnu, CLEA Japan Inc, Tokyo, Japan) aged 7 to 8 weeks and weighting ≈130 to 145 g were used in this study. The institutional animal care and use committees of RIKEN Center for Developmental Biology approved all animal procedures, including human cell transplantation.

Human Unrestricted Somatic Stem Cell Preparation

USSCs and human fibroblasts were isolated, cultured, and prepared as previously described.[3]

Induction of Myocardial Infarction and Cell Transplantation

Rats were anesthetized with ketamine and xylazine (60 mg/kg and 10 mg/kg, IP, respectively). MI was induced by ligating the left anterior descending coronary artery (LAD) as described previously.[7] Twenty minutes after the LAD ligation, the cells were then transplanted into the periinfarct zone by injection with a 27G needle in a series of 6×20 μL injections of 1×10^5 (LD) USSCs, 1×10^6 (HD) USSCs, or 1×10^6 human fibroblasts (Fbr) resuspended in 120 μL of PBS or the same volume of PBS without cells (n=16 in each group).

Physiological Assessment of LV Function

Transthoracic echocardiography was performed to evaluate LV function immediately before and 5 and 28 days after MI as described previously.[7] Immediately after the final echocardiography on day 28, the rats underwent cardiac catheterization for more invasive and precise assessment of global LV function as described previously.[7,8]

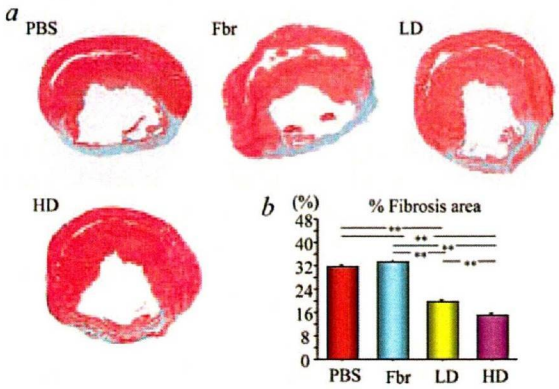

Figure 1. Histological evaluation of left ventricular (LV) remodeling after myocardial infarction (MI). a, Representative Masson-trichrome staining at day 28 in each group. Fbr indicates human fibroblasts; LD, 1×10^5 unrestricted somatic stem cells (USSCs); HD, 1×10^6 USSCs. b, Ratio of fibrosis area/ entire LV area (% fibrosis area) at day 28 in each group. $^{**}P<0.01$ (n=10 in each group).

Statistical Analysis

The results were statistically analyzed with the use of a software package (Statview 5.0, Abacus Concepts Inc). All values were expressed as mean±SE. Paired *t* tests were performed for comparison of data before and after treatment. The comparisons among 4 groups were made with 1-way ANOVAs. Post hoc analysis was performed by Scheffe test. Differences of $P<0.05$ were considered statistically significant.

Results

Morphometric Evaluation of Capillary Density and Infarct Size

LV remodeling as evaluated by % fibrosis area showed a dose-dependent inhibition in rats receiving USSCs ($P<0.01$ for HD versus LD, Fbr or PBS and LD versus Fbr or PBS). Percent fibrosis area was similar in Fbr and PBS groups (Figure 1).

Myocardial neovascularization assessed by capillary density on day 28 was enhanced in rats receiving USSC transplantation in a dose-dependent manner ($P<0.05$ for HD versus LD, $P<0.01$ for HD versus Fbr or PBS, and LD versus Fbr or PBS). Capillary density in Fbr group was similar as that in PBS group (supplemental Figure I).

Thus, transplantation of USSCs, not Fbr, significantly preserved LV structural integrity post MI. The histological efficacy of USSCs was dose-dependently observed.

Transplanted USSCs Dose-Dependently Preserve LV Function After MI

There were no significant differences in preoperative echocardiographic parameters, LVEDD, LVESD, FS, and RMWS among HD, LD, Fbr, and PBS groups (data not shown). Echocardiography on day 5 revealed that the functional parameters were also similar in all groups (data not shown). Left ventricular lateral wall motion on day 28 was better preserved in the USSC-treated groups compared with other groups (supplemental Figure IIa). Change in FS during 23

days (between day 5 and day 28 after cell transplantation) was significantly greater in the HD group than either Fbr or PBS group. Although a change in FS had a tendency to be greater in HD group than LD group, the difference was not statistically significant. The change in FS in Fbr group was also not significantly different from the PBS group ($P<0.01$ for HD versus LD, Fbr, or PBS and low versus Fbr or PBS). Similarly, the change in RWMS after transplantation was significantly lower (better preserved) in LD and HD groups compared with the Fbr or PBS group. The change in RWMS in the Fbr group was not significantly different from the PBS group ($P<0.05$ for HD versus LD and LD versus Fbr, $P<0.01$ for HD versus Fbr or PBS and LD versus PBS; supplemental Figure IIb).

Invasive hemodynamic study performed 4 weeks after transplantation revealed that heart rates were similar in each group (data not shown). The +dP/dt, absolute value of −dP/dt and EF were significantly greater in the HD group as compared to the LD, Fbr, or PBS groups. In addition, the LD group was significantly better than the Fbr or PBS group (+dP/dt: $P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS; −dP/dt: $P<0.01$ for HD versus Fbr or PBS and $P<0.05$ for HD versus LD and LD versus Fbr or PBS; EF: $P<0.01$ for HD versus Fbr or PBS and $P<0.05$ for HD versus LD and LD versus Fbr or PBS). The LVEDP 4 weeks after MI was significantly lower in HD and LD groups compared to the Fbr and PBS groups ($P<0.01$ for HD versus Fbr or PBS and LD versus Fbr or PBS). However, LVEDP 4 weeks after MI in HD group was similar as that in LD group. The +dP/dt, −dP/dt, EF and LVEDP 4 weeks after transplantation in the Fbr group were not significantly different from those in PBS group (supplemental Figure IIc).

Based on these data, transplantation of USSCs, not Fbr, significantly preserved global and regional LV function post MI and the functional effect of USSC transplantation was generally dose-dependent, where the HD USSC group exhibited the greatest effect on cardiac functional improvements.

Transplanted USSCs Dose-Dependently Differentiate into CMCs

Double staining of GATA4 or Nkx2.5, an early cardiomyogenic marker, and HMA at day 5 revealed that GATA4 or Nkx2.5-positive immature cardiac stem/progenitor cells were negative for HMA in the PBS and Fbr groups. In contrast, in USSC-treated groups, double-positive cells for GATA4 or Nkx2.5 and HMA were observed as immature cardiac stem/progenitor cells derived from human USSCs (Figure 2a and 2b and supplemental Figure IIIa through IIIh). Double staining for cTn-I, a mature CMC marker, and HMA was performed to detect cardiomyogenic plasticity of transplanted USSCs at day 10. Human mitochondria-positive cells were identified in both Fbr and USSC groups, but human USSC-derived cardiomyogenic cells, which were double positive for HMA and cTn-I, were observed only in the USSC groups (Figure 2c). Differentiated human CMCs derived from the transplanted USSCs were mainly identified in the rat peri-infarct myocardium by double staining for cTn-I and HNA at day 28 (supplemental Figure IVa through IVe). The observation at day 28 was also confirmed by dual labeling for cTn-I and HMA (supplemental Figure IIIi through IIIl). These findings suggest that USSCs have the potential to differentiate into mature CMCs after transplantation into infarcted myocardium. A dose-dependent distribution of human CMCs in rat myocardium was observed in samples stained with cTn-I and HNA (Figure 3a through 3d). In fact, the density of human CMCs in ischemic myocardium detected as double-positive cells for HNA and cTn-I were dose-dependently increased in ischemic myocardium at day 28 ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS). Total (both human and rat) CMC density was also dose-dependently augmented in ischemic myocardium at day 28 ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS), suggesting that the USSCs also support myocardial regeneration through a paracrine mechanism (Figure 3e). Frequency of the human CMCs to total (rat and human) CMCs was dose-dependently increased after USSC trans-

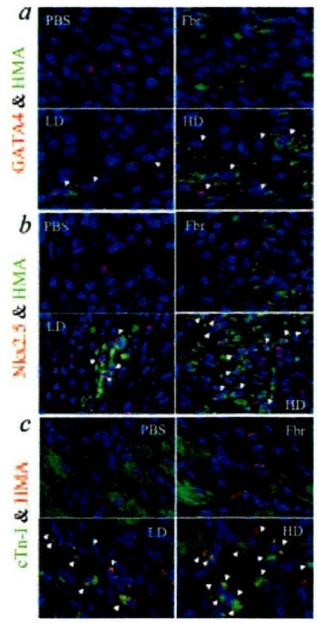

Figure 2. Representative immunofluorescent staining for immature cardiac markers and human mitochondria antigen (HMA) at days 5 and 10. a, Double immunofluorescent staining for GATA4 and HMA in each group at day 5. Few GATA4-positive immature CMCs (red) were identified, however no HMA-positive human cells (green) were detected in PBS group. HMA-positive and GATA4-negative cells or HMA-negative and GATA4-positive cells were observed in Fbr group. Few cells expressing both GATA4 and HMA were observed in LD group and relatively more double-positive cells were identified in HD group (×600). White arrows show nuclei of immature human CMCs. b, Double immunofluorescent staining for Nkx2.5 and HMA in each group at day 5. Few Nkx2.5-positive immature CMCs (red) were identified, however no HMA-positive human cells (green) were detected in PBS group. HMA-positive and Nkx2.5-negative cells or HMA-negative and Nkx2.5-positive cells were observed in Fbr group. Few cells expressing both Nkx2.5 and HMA were observed in LD group and relatively more double-positive cells were identified in HD group (×600). White arrows show nuclei of immature human CMCs. c, Representative double immunofluorescent staining for cardiac troponin-I (cTn-I; green) and HMA (red) in each group at day 10. Human CMCs expressing both cTn-I and HMA, were dose-dependently observed after USSC transplantation (×600). White arrows show human CMCs.

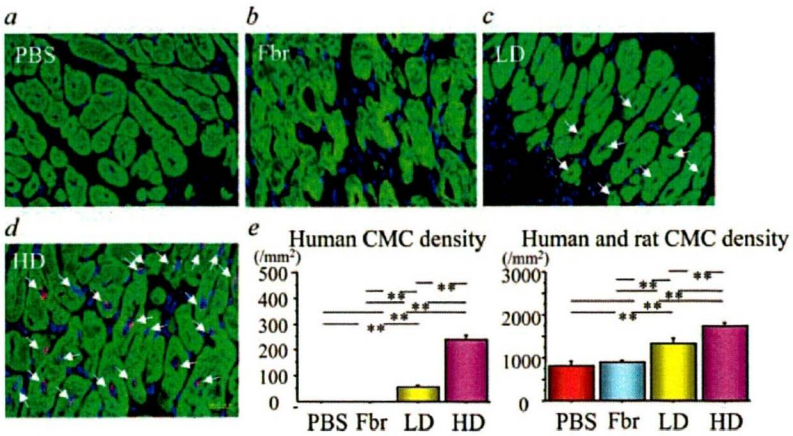

Figure 3. Histological evaluation of human CMC development in rat ischemic myocardium at day 28. a through d, Representative double immunofluorescent staining for cTn-I and HNA at day 28 in each group (×400). White arrows show nuclei of human CMCs. The double-positive cells for cTn-I and HNA derived from the transplanted USSCs were dose-dependently observed in ischemic myocardium (×400). e and f, Densities of human CMCs (the double-positive CMCs) and total (both human and rat) CMCs on day 28. *$P<0.05$; **$P<0.01$ (n=10 in all groups).

plantation ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS; supplemental Figure Ve).

We explored whether USSC transplantation may contribute to cardiac repair post MI partially by stimulating proliferation of resident CMCs. Double staining for Ki67, a marker of proliferating cells, and cTn-I revealed dose-dependent distribution of Ki67-positive CMCs in the ischemic myocardium 7 days after USSC transplantation, but not PBS or Fbr administration (supplemental Figure VIa through VId). In fact, density of the proliferative CMCs in the ischemic myocardium at day 7 was significantly greater in HD group than LD, Fbr, and PBS groups and in LD group than Fbr and PBS groups ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS; supplemental Figure VIe). These data indicate that transplanted USSCs may have the potential to stimulate proliferation of resident CMCs, thereby contribute to cardiac regeneration after MI.

The present results suggest that transplanted USSCs may have the potential to differentiate into mature CMCs and preserve the recipient's CMCs in the infarcted region. The data also demonstrate that a dose-dependent increase of the cardiac regenerative effect was observed between the two USSC transplant groups, whereas in the Fbr and PBS groups, no mature human CMCs were observed.

Transplanted USSCs Dose-Dependently Differentiate Into ECs

Differentiated human ECs derived from the transplanted USSCs were observed in the vasculatures within peri-infarct area by double staining for vWF and HNA (Figure 4a through 4d, supplemental Figure IVf through IVj). Density of the double-positive cells was greater in HD group than LD, Fbr, or PBS group ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS, $P<0.05$ for Fbr versus PBS). Density of total (both human and rat) ECs was also greater in HD group than LD, Fbr, or PBS group ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS; Figure 4e).

Thus, locally transplanted USSCs were incorporated into sites of neovascularization, resulting in contribution to both vasculogenesis by USSCs and angiogenesis by rat ECs in ischemic myocardium.

Transplanted USSCs Dose-Dependently Differentiate Into SMCs

Human SMCs derived from the transplanted USSCs were mainly identified in vascular structures within peri-infarct area by double staining for SMA and HLA-ABC (Figure 5a through 5d, supplemental Figure IVk through IVo). Human SMCs were observed after USSC transplantation and similar to the CMC and EC analyses, a dose-dependent increase in

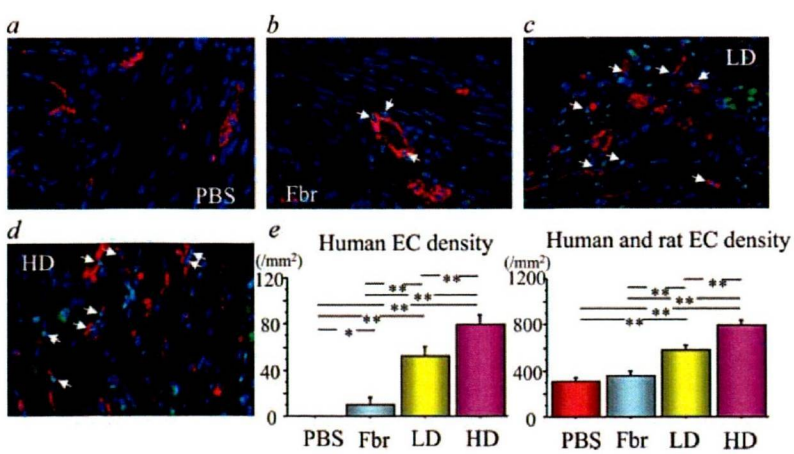

Figure 4. Histological evaluation of human endothelial cell (EC) development. a through d, Representative double immunofluorescent staining for von Willebrand factor (vWF; red) and human nuclear antigen (HNA; green) at day 28 in each group (×400). In PBS group (a), differentiated human ECs were not identified. In Fbr group (b), differentiated human ECs were rarely detected. In LD group (c), human ECs were more frequently demonstrated than Fbr and PBS groups. In HD group (d), human ECs were further more frequently identified than LD group. Arrows indicate human ECs. e, Densities of human ECs (the double-positive ECs) and total (both human and rat) ECs on day 28 in the ischemic myocardium. *$P<0.05$; **$P<0.01$ (n=10 in all groups).

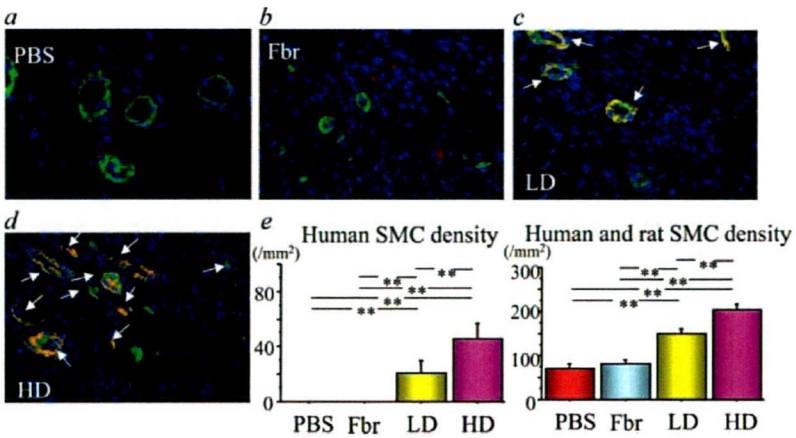

Figure 5. Histological evaluation of human smooth muscle cell (SMC) development. a through d, Representative double immunofluorescent staining for smooth muscle actin (SMA; green) and human leukocyte antigen (HLA)-ABC (red) at day 28 in each group (×400). Human SMCs were identified as double-positive cells (arrow). In PBS (a) and Fbr groups (b), differentiated human SMCs were not identified. In LD group (c), differentiated human SMCs were rarely identified. In HD group (d), human SMCs were more frequently identified than LD groups. e, Densities of human SMCs (the double-positive SMCs) and total (both human and rat) SMCs on day 28 in the ischemic myocardium. $^{**}P<0.01$ (n=10 in all groups).

SMCs was confirmed between the USSC transplant groups. In contrast, differentiated human SMCs were not identified in PBS and Fbr groups ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS). Total SMC density was also greater in HD group than LD, Fbr, or PBS group ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS; Figure 5e).

These findings suggest that transplanted USSCs exhibit a dose-dependent potency for differentiating into SMCs as well as preserving recipient SMCs in the infarcted myocardium.

Discussion

Cell transplantation is currently gaining a growing interest as a potent and novel means of improving prognosis of patients with cardiac failure. The basic assumption is that left ventricular dysfunction is largely attributable to the loss of a critical number of CMCs and that it can be partly reversed by implantation of new contractile cells into the postinfarction scars. The therapeutic strategy for cardiac failure with coronary artery disease should be focused on regenerating not only blood vessels but also cardiac muscle to improve the poor prognosis of the disease.

Many reports using various stem cells such as fat tissue–derived multipotent stem cells,[9] multipotent stem cells from BM or skeletal muscle,[10,11] and cardiac-resident progenitor cells,[12–15] which are capable of adopting the cardiomyogenic and vasculogenic fate, are also generating a great deal of interest. However, these novel cell therapies still have several problems for future clinical application. For example, techniques to efficiently and less invasively isolate, purify, and expand the numerically minor population of the potent stem cells will need to be optimized for clinical use, and experimental data from mammals larger than mice will surely be warranted. Moreover, other key questions such as (1) precise mechanism of tissue repair/regeneration and efficacy against LV dysfunction,[7,16–19] (2) optimization of cell dose, and (3) development of optimal delivery techniques also remains to be clarified.

Generally, umbilical cord blood is abundantly available, can be routinely harvested without any risk to the donors, and is seldom infected with agents, which give it a definite advantage for the development of cell therapeutics in regenerative medicine. In the case of autologous cell therapy, patients need to wait for the time of cell harvest, isolation, or expansion in a cell culture facility before undergoing transplantation. However, umbilical cord stem cells are routinely kept frozen after the whole procedure of the cell preparation is completed and therefore can be readily available for transplantation. The USSCs, which Kogler et al first identified from human cord blood in 2004, grow adherently, can be expanded up to 10^{15} cells without losing pluripotency in culture, and differentiates along mesodermal and endodermal lineages in animal models, suggesting significant potency of the USSC therapy in various clinical settings. In the present study, we have tried to confirm the multi-lineage developmental potency and the tissue plasticity of human USSCs after transplanting into immunodeficient (athymic nude) rats with acute MI. To detect the multi-lineage differentiation of the USSCs, we have performed not only immunohistochemistry but also RT-PCR for human-specific markers of CMCs, SMCs, and ECs. These sensitive assessments revealed dose-dependent augmentation of cardiomyogenesis and vasculogenesis of human USSCs in rat-infarcted myocardium. FISH analysis using human and rat genome probes indicated that cell fusion was not mainly involved in the process of the multi-lineage regeneration after transplantation of USSCs. The FISH analysis provided mechanistic information, indicating engraftment and differentiation versus cell fusion during cardiac regeneration by human USSCs. These results were consistent with the previous single cell PCR analysis in the case of sheep liver regeneration by human USSCs.[3] Immunohistochemical quantification of total (human and rat) CMCs, SMCs, or ECs in rat-infarcted myocardium also revealed dose-dependent preservation of the recipient cardiac cells probably because of a paracrine effect of the USSCs on recipient cell development. The multi-lineage potential was accompanied with dose-dependent enhancement of capillary density, inhibition of LV fibrosis, and preservation of LV function. These findings strongly suggest that USSCs may be useful for cardiomyogenic and vasculogenic regeneration in the infarcted myocardium by both autocrine and paracrine mechanisms. Considering future clinical application of the USSCs, a major limitation of the present study was a lack of assessing immune rejection, because the current study was performed using immunodeficient rats. Although a therapeutic effect of human USSCs using cyclosporine A immunosu-

pression was clearly demonstrated in a previous preclinical study using the swine model of chronic MI,[5] further investigation of long-term safety and efficacy of the cell therapy will be necessary, especially in the case of allogenic transplantation.

In conclusion, the multi-lineage differentiation potential of human USSCs for cardiomyogenic and vasculogenic regeneration of the infarcted myocardium was demonstrated by immunohistochemical and molecular assessments. The USSC therapy resulted in dose-dependent increase in capillary density, inhibition of LV remodeling, and improvement of LV function. Taken together with feasibility of the cell isolation and efficiency of the culture expansion, USSCs could be used as a highly valuable resource for cellular cardiomyoplasty in the future and could be the novel strategy to be translated from bench to bedside.

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Disclosures

Christina Willwerth, Stephan Wnendt, and William L. Fodor are employees of ViaCell Inc.

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Supplemental Materials

Supplementary Methods

Human Unrestricted Somatic Stem Cell Preparation

USSCs and human fibroblasts were isolated, cultured, and prepared as previously described [1]. The USSCs were characterized by cell surface markers using flow cytometry with antibodies against CD13, CD14, CD29, CD31, CD33, CD 34, CD44, CD45, CD49e, CD56, human leukocyte antigen class II (Beckmann Coulter) and CD133 (Miltenyi Biotech) according to the manufacturer's protocol. These cells were preserved at –80 °C until the day of cell transplantation.

Theses cells were washed with serum free Iscove's Modified Dulbecco's Medium (IMDM) twice by centrifugation, the supernatant was discarded, and the cells were resuspended in PBS. The number of surviving cells was enumerated using a hemacytometer and $1x10^6$ human fibroblasts, $1x10^5$ (Low dose, LD) or $1x10^6$ (High dose, HD) USSCs were prepared in 120 μl of PBS for transplantation.

(a) Physiological Assessment of LV Function Using Echocardiography and Micro-tip Conductance Catheter

Transthoracic echocardiography (SONOS 5500, Philips Medical Systems) was

performed to evaluate LV function immediately before and 5 and 28 days after MI as described previously [2]. Under general anesthesia with ketamine and xylazine, LV end-diastolic and end-systolic dimensions (LVEDD and LVESD, respectively) and fractional shortening (FS) were measured at the midpapillary muscle level. Regional wall motion score (RWMS) was evaluated per published criteria [3]. Immediately after the final echocardiography on day 28, the rats underwent cardiac catheterization for more invasive and precise assessment of global LV function as described previously [2 4]. A 2.0 Fr micromanometer-tipped conductance catheter (SPR 838, Millar Instruments Inc, Houston, Tx) was inserted via right carotid artery into the LV cavity. LV pressure and its derivative (LV dP/dt) were continuously monitored using a multiple recording system (Pressure-Volume Conductance System ARIA and Pressure-Volume Analysis Using P-V Analysis Software [Millar Instruments Inc, Houston, Tx] and Power Lab® DAQ System [ADInstrument, Australia]).

Heart rate (HR), LV end-diastolic pressure (LVEDP), LV ejection fraction (EF) and the maximum and minimum LV dP/dt (+dP/dt and -dP/dt, respectively) were continuously recorded for 20 minutes. All data were acquired under stable hemodynamic conditions.

All procedures and analyses were performed by an experienced researcher who was blinded to the treatments.

(b) Tissue harvesting

In each treatment group, ten rats were euthanized 28 days after transplantation with an over dose of ketamine and xylazine At necropsy, hearts were sliced in a broad-leaf fashion into 4 transverse sections from apex to base, embedded in OCT compound, snap frozen in liquid nitrogen, and stored at -80°C for Masson-trichrome staining, immunohistochemistry and Fluorescence In Situ Hybridization (FISH). Rat hearts in OCT blocks were sectioned, and 5 μm serial sections were collected on slides followed by fixation with 4.0% paraformaldehyde at 4°C for 5 minutes and stained immediately. Selective dissection of a portion of the myocardium surrounding the peri-infarct area was taken to isolate total RNA for reverse transcriptase-PCR (RT-PCR) experiments. In each group, three rats were euthanized at day 5 and additional three were also killed at day 10 for histological evaluation.

(c) Morphometric Evaluation of Capillary Density and Infarct Size

Histochemical staining with isolectin B4 (Vector Laboratories, Burlingame, CA) was performed, and capillary density was morphometrically evaluated by histological examination of 5 randomly selected fields of tissue sections recovered from segments of LV myocardium subserved by the occluded LAD. Capillaries were recognized as tubular structures positive for isolectin B4.

To elucidate the severity of myocardial fibrosis, Masson-trichrome staining was

performed on frozen sections from each tissue block, and the stained sections were used to measure the average ratio of fibrosis area to entire LV area (percent fibrosis area).

(d) Immunofluorescent staining

To detect transplanted human cells in rat ischemic myocardium, immunohistochemistry was performed with the following human-specific antibodies; human leukocyte antigen (HLA)-ABC (BD Pharmingen, San Diego, CA), human mitochondria antigen (HMA) (Chemicon International, Temecula, CA) and human nuclear antigen (HNA) (Chemicon International). Staining specificity of HLA-ABC, HMA and HNA against human cells and lack of cross reactivity to rat cells were confirmed by histochemical staining with the use of human and rat heart samples as described previously [25]. Double staining for GATA4 or Nkx2.5 (Santa Cruz, Santa Cruz, CA) and HMA was performed to detect human cells expressing early cardiac transcription factor in rat ischemic myocardium at day 5. Double staining for cardiac troponin-I (cTn-I) (Chemicon International) as mature cardiomyocyte marker and HMA or HNA was performed to detect cardiomyogenic plasticity of transplanted human cells at day 10 and 28. Dual-labeled immunohistochemistry with HLA-ABC and smooth muscle actin (SMA) was performed to detect double-positive cells as human SMCs in rat myocardium. Similarly, double immunohistochemistry with von Willebrand factor (vWF) (Chemicon International) and HNA was performed to detect double positive cells as human ECs in ischemic myocardium.

Double staining for Ki67 (Pharmingen, San Diego, CA) and cTn-I was performed to detect proliferative CMCs in the ischemic myocardium 5 days after USSC transplantation. The secondary antibodies for each immunostaining are as follows: Alexa Flour 594-conjugated goat anti-mouse IgG_1 (Molecular Probes, Carlsbad, CA) for HLA-ABC staining, Alexa Flour 488 and 594-conjugated goat anti-mouse IgG_1 (Molecular Probes) for HMA staining, Alexa Flour 594 -conjugated goat anti-rabbit IgG (H+L) (Molecular Probes) for GATA4 or Nkx2.5 staining, Alexa Flour 488-conjugated goat anti-mouse IgG_{2a} (Molecular Probes) for cTn-I staining, Alexa Flour 488-conjugated goat anti-mouse IgG_{2a} (Molecular Probes) for SMA, Alexa Flour 488 and 594-conjugated goat anti-mouse IgG1 for HNA, Alexa Flour 594-conjugated goat anti-rabbit IgG for vWF (Molecular Probes) and Alexa Flour 596-conjugated goat anti-mouse IgG_1 (Invitrogen, Carlsbad, CA) for Ki67. DAPI solution was applied for 5 minutes for nuclear staining.

Number of total (both human and rat) CMCs was evaluated by counting cTn-I-positive cells in 5 randomly selected fields within rat ischemic myocardium at day 28. Similarly, the number of human CMCs in ischemic myocardium was examined by counting double-positive cells for HNA and cTn-I. Number of human SMCs detected as double-positive cells for HLA and SMA, and number of human ECs detected as double-positive cells for HNA and vWF 28 days after MI were also morphometrically quantified. Number of the proliferative CMCs in ischemic myocardium was examined by counting double-positive cells for Ki67 and cTn-I in 5 randomly selected fields within rat

ischemic myocardium at day 5.

***Fluorescence In Situ Hybridization* (FISH)**

To identify whether cardiac repair occurred through cell fusion or not, FISH was performed with biotin-conjugated DNA probe for human genome and digoxigenin-conjugated DNA probe for rat genome (Chromosome Science Lab, Sapporo, Japan) in MI tissue. Tissue sections were fixed immediately with Carnoy's fixation (MEOH and acetic acid) at room temperature for 10 minutes, dehydrated, and denatured according to the manufacture's protocol as described previously [2]. Sections were hybridized with the DNA probes for human and rat genome overnight at 37°C. After post-hybridization wash, Alexa 488-conjugated streptavidin and Cy-3-conjugated anti-digoxigenin was applied and slides were counterstained with DAPI, then fluorescent microscopically examined. The directly differentiated cells or the fused cells were identified as the cells positive for human genome only or the double-positive cells for both genomes, respectively. The directly differentiated or fused cells were quantified in 5 randomly selected fields in the ischemic myocardium at day 28.

(e) RT-PCR analysis of USSCs and ischemic heart tissue

Total RNA was obtained from USSCs before transplantation and from tissues of rat ischemic LV at day 28 using Tri-zol (Life Technologies) according to the manufacture's

instructions [2]. In brief, the first-strand cDNA was synthesized using the RNA LA PCR Kit Ver1.1 (Takara, Otsu, Japan), amplified by Taq DNA polymerase (Advantage–GC cDNA PCR Kit (Clontech, Mountain View, CA) and AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA)). PCR was performed using a PCR thermalcycler (MJ Research PTC-225, Bio-Rad Laboratories, Waltham, MA). The human GAPDH (hGAPDH), total (human and rat) GAPDH, human CD34 (hCD34), human brain natriuretic peptide (hBNP), human cardiac troponin-I (hcTn-I), human myosin heavy chain-β (hMHC-β), human KDR (hKDR), human eNOS (heNOS) and human Nkx 2.5 (hNkx 2.5) were amplified by Taq DNA polymerase (AmpliTaq Gold DNA polymerase, Applied Biosystems) at the following conditions: 35 cycles of 30 seconds initial denaturation at 94°C, annealing at 56°C for 1 minute, and 30 seconds of extension at 72°C according to the manufacture's instructions. Human SMA (hSMA), human sm22α (hsm22α) and human CD31 (hCD31) were amplified by Taq DNA polymerase (Advantage–GC cDNA PCR Kit) under the following conditions: 37 cycles of 30 seconds initial denaturation at 94°C, annealing at 68°C for 3 minute, and 7 minutes of elongatation at 64°C according to the manufacture's instructions. Subsequently, PCR products were visualized in 1.5% ethidium bromide-stained agarose gels. Human heart RNA distributed from Clontech (premium RNA) was used as positive control.

Primers : To avoid interspecies cross-reactivity of the primer pairs between human and rat genes, we designed following human-specific primers using Oligo software

(Takara). All primer pairs did not show cross-reactivity to rat genes as described previously [2]. hBNP primer sequence (146 bp): sense GCA AAA TGG TCC TCT ACA CC; antisense CAG GAC TTC CTC TTA ATG CC; hcTn-I primer sequence (218 bp): sense AAT TGC AGC TGA AGA CTC TG; antisense GAC TTT TGC CTC TAT GTC GT; hMHC-β primer sequence (214 bp): sense GCT AAA GGT CAA GGC CTA CA; antisense GCA GAT CAA GAT CTG GCA AA; hNkx 2.5 primer sequence (205 bp): sense GAG AGT TTG TGG CGG CGA TT; antisense CGA CGG CGA GAT AGC AAA GG; hSMA primer sequence (485 bp): sense TCT GGA CGC ACA ACT GGC ATC GT; antisense TAC ATA GTG GTG CCC CCT GAT AG; hsm22-α primer sequence (468 bp): sense CGG CTG GTG GAG TGG ATC ATA; antisense CCC TCT GTT GCT GCC CAT CTG A; hCD31 primer sequence (469 bp): sense AGG TCA AGC AGC ATC GTG GTC AAC AT; antisense TTG TCT TTG AAT ACC GCA G; hCD34 primer sequence (380 bp): sense AAT GAG GCC ACA ACA AAC ATC ACA; antisense CTG TCC TTC TTA AAC TCC GCA CAG C; hKDR primer sequence (468 bp): sense CAA ATG TGA AGC GGT CAA CAA AGT T; antisense ATG CTT TCC CCA ATA CTT GTC GTC T; heNOS primer sequence (456 bp): sense AAC CAC ATC AAG TAT GCC ACC AAC C; antisense CGT GCC GAT CTC AGT GCT CA; hGAPDH primer sequence (596 bp): sense CTG ATG CCC CCA TGT TCG TC; antisense CAC CCT GTT GCT GTA GCC AAA TTC G; Total GAPDH primer sequence (320 bp): sense GTG CCA GCC TCA TGT TCG TC; antisense CGC CAG TGT ACT CCA CGA CAT TTC G

Real-time PCR for analyzing the human-specific gene expression of angiogenic growth factors and the cardiomyogenic and vasculogenic lineage markers in rat ischemic myocardium following USSC transplantation

To quantify the mRNA expression of human-specific genes of angiogenic factors and the cardiomyogenic and vasculogenic markers in rat ischemic myocardium following human cell transplantation, real-time PCR was performed on an ABI PRISM 7000 sequence detector (Applied Biosystems) as described previously [11]. Total RNA was prepared as described above and the expression of the related genes was quantified using the SYBR green reagent (2x SYBR Green Supermix; Bio-Rad, Hercules, CA) following the instructions of the manufacturer on a Bio-Rad Cycler. PCR of anigiogenic factors was performed in multiplicate in optimized conditions: 95°C denatured for 3 minutes, followed by 40 cycles of 45 seconds at 94°C, 45 seconds at 55°C, and 45 seconds at 72°C. PCR of human-specific cardiomyogenic and vasculogenic genes was also performed in multiplicate in optimized conditions: 95°C denatured for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Primer for human-specific angiogenic factors (VEGF, bFGF, HGF and SDF-1) and human-specific CMC, SMC and EC markers (hcTn-I, hSM22α, and hKDR) were confirmed not to cross-react with rat genes by using rat heart samples. (data not shown).

Primers: human VEGF (hVEGF), forward, 5-TCTCCCTGATCGGTGACAGT-3,

reverse, 5-GGCAGAGCTGAGTGTTAGCA-3; human bFGF (hbFGF), forward, 5-TTTCTAGCTTCCATCCTTTCTCC-3, reverse, 5–AGTTACCAGCTCCCCCAAAA-3; human HGF (hHGF), forward, 5-GCACCCACCAATACAACTGTC-3, reverse, 5-TGACTCTCCAGTAGTTGTCTTAGGATT-3; human SDF-1 (hSDF-1), forward, 5-CCTCCCCCAACCTTAGATGT-3, reverse, 5-CAGACGATCACACCATGGAA; human GAPDH (hGAPDH), forward, 5-CACTGAATCTCCCCTCCTCA-3, reverse, 5-TCCCCTCTTCAAGGGGTCTA-3; hcTn-I, forward: 5-CGGAGAGTGAGGATCTCTGC-3, reverse: 5-TCGGTGTCCTCCTTCTTCAC-3; hSM22α, forward: 5-AAGAAAGCGCAGGAGCATAA-3, reverse: 5-AAGGCCAATGACATGCTTTC-3; and hKDR, forward: 5-TTTTTGCCCTTGTTCTGTCC-3, reverse: 5-TCATTGTTCCCAGCATTTCA-3.

No other products were amplified because melting curves showed only one peak in each primer pair. Fluorescence signals were measured over 40 PCR cycles. The cycle number at which the signals crossed a threshold set within the logarithmic phase was recorded. For quantification, we evaluated the difference in cycle threshold between the AP-treated group and vehicle control of each gene. The efficiency of amplification of each pair of primers was determined by serial dilutions of templates and all were 0.9. Each sample was normalized with the loading references of hGAPDH. *Ct* values used were the means of the triplicate replicates. Experiments were repeated at least three times.

Supplementary Results

FISH Analysis to Assess the Mechanism of Cardiac Regeneration Following USSC Transplantation

To ensure whether cardiac repair occurred though cell fusion or not, we performed FISH with human genome and rat genome probes using tissue samples 28 days post MI and USSC transplantation. The specificity of the probes was tested in tissues of normal rat heart and rat heart immediately after human cell transplantation. We confirmed that these two probes did not cross-react with the other species cells (data not shown). The FISH analysis revealed that most of the USSC-derived cells expressing human genome were not paired with rat genome, indicating very few incidence of cell fusion between transplanted USSCs and recipient cells (cell fusion ratio, 1.5±0.8%) **(Supplementary Figure VII)**.

These findings indicate that multi-lineage differentiation without cell fusion may be a major contributing factor to the process of cardiac repair following transplantation of USSCs.

Human-Specific Gene expression of Cardiomyogenic, Smooth Muscle and Endothelial Lineage Markers in Rat Ischemic Myocardium Following USSC Transplantation

To further confirm the immunohistochemical results regarding cardiomyogenesis

and vasculogenesis using a molecular approach, we performed RT-PCR with rat ischemic myocardium from each of the different transplant groups using human-specific primers for BNP, cTn-I, MHC-β and Nkx 2.5 as human CMC lineage markers, human-specific primers for sm22α and SMA as human SMC markers and human-specific primers for CD31, eNOS and KDR as human EC markers. The RT-PCR analysis revealed the expression of human-specific cardiomyogenic, arteriogenic and vasculogenic genes in rat ischemic myocardium 28 days following USSC transplantation. Notably, the gene expression profile of all markers, except hSMA and sm22α, were not detected in USSCs before transplantation, but were significantly augmented in ischemic myocardium after USSC transplantation. In addition, to quantify the expression of cariomyogenic and vasculogenic genes, we performed real-time PCR with rat ischemic myocardium, using human-specific primer cTn-I, human-specific primer sm22α and human-specific primer KDR. The gene expression profile of all markers was significantly augmented in ischemic myocardium after USSC transplantation compared with Fbr or PBS treatment (**Supplementary Figure VIII**).

Transplanted USSCs highly express the angiogenic growth factors

We analyzed by real-time RT-PCR whether transplanted USSCs may express the angiogenic cytokines such as human VEGF (hVEGF), human bFGF (hbFGF), human HGF (hHGF) and human SDF-1 (hSDF-1) at day 5. Expression of hVEGF, hbFGF, hSDF-1 and

hHGF in the infarct and peri-infarct borders was confirmed at higher levels in the USSC-transplanted animals than the Fbr or PBS-treated groups ($P<0.05$) **(Supplementary Figure IX)**. Thus, transplanted USSCs may enhance myocardial angiogenesis by overexpressing hVEGF, hbFGF, hHGF and hSDF-1.

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Supplementary Figure Legends

Supplementary Figure I

Histological evaluation of myocardial neovascularization after myocardial infarction (MI).

a: Representative histochemical staining for isolectin B4 in each group at day 28 (x200).

b: Capillary density in rats receiving USSCs, Fbr or PBS at day 28. *, $P<0.05$; **, $P<0.01$