

The Significant Cardiomyogenic Potential of Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells In Vitro

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ABSTRACT

We tested the cardiomyogenic potential of the human umbilical cord blood-derived mesenchymal stem cells (UCBMSCs). Both the number and function of stem cells may be depressed in senile patients with severe coronary risk factors. Therefore, stem cells obtained from such patients may not function well. For this reason, UCBMSCs are potentially a new cell source for stem cell-based therapy, since such cells can be obtained from younger populations and are being routinely utilized for clinical patients. The human UCBMSCs (5×10^3 per cm^2) were cocultured with fetal murine cardiomyocytes ([CM] 1×10^5 per cm^2). On day 5 of cocultivation, approximately half of the green fluorescent protein (GFP)-labeled UCBMSCs contracted rhythmically and synchronously, suggesting the presence of electrical communication between the UCBMSCs. The fractional shortening of the contracted UCBMSCs was $6.5\% \pm 0.7\%$ ($n = 20$). The

UCBMSC-derived cardiomyocytes stained positive for cardiac troponin-I (clear striation +) and connexin 43 (diffuse dot-like staining at the margin of the cell) by the immunocytochemical method. Cardiac troponin-I positive cardiomyocytes accounted for $45\% \pm 3\%$ of GFP-labeled UCBMSCs. The cardiomyocyte-specific long action potential duration (186 ± 12 milliseconds) was recorded with a glass microelectrode from the GFP-labeled UCBMSCs. CM were observed in UCBMSCs, which were cocultivated in the same dish with mouse cardiomyocytes separated by a collagen membrane. Cell fusion, therefore, was not a major cause of CM in the UCBMSCs. Approximately half of the human UCBMSCs were successfully transdifferentiated into cardiomyocytes in vitro. UCBMSCs can be a promising cellular source for cardiac stem cell-based therapy. STEM CELLS 2007;25:2017–2024

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Autologous stem cells are believed to be a potential cellular source for stem cell-based therapy, since they have the ability to proliferate and differentiate into cardiomyocytes [1–4]. Many types of cells, such as embryonic stem cells [5, 6], myoblasts [7, 8], bone marrow hematopoietic cells [9, 10], and mesenchymal stem cells (MSCs) [11–13], have been transplanted to restore damaged heart function in animal models. Autologous mononuclear cells [14–17] and myoblasts [18] have been injected into ischemic hearts clinically to improve impaired cardiac function. Despite the dramatic improvement of cardiac function by the stem-cell-based therapy in the animal model [10, 19], only modest effects were observed in the clinical study [14–17, 20]. One of the reasons for this may have been the extremely low rate of cardiomyogenesis of the stem cells in vitro and in vivo [2, 13, 21]. Therefore, the improvement of cardiac function may have been due to grafted stem cell-induced neovascularization [13, 22] and/or the paracrine effect [23]. Another reason

may have been the ages and disease histories of the patients. Recent papers have shown that the number and function of the circulating stem cells were depressed in older patients and in patients with diabetes mellitus [24, 25], suggesting that stem cells obtained from patients with coronary risk factors may not function well. This suggests limits to the utilization of autologous stem cells for the ischemic cardiomyopathy patient. On the other hand, in order to do allogenic stem cell transplantation, human leukocyte antigen (HLA)-type matching is very important for the stable survival of grafts. Therefore, the sample, which can be noninvasively collected from many volunteers, is a desirable source of stem cells due to the ease of establishing cell banks that can store all HLA-types.

Recently, umbilical cord blood (UCB) banking for transplantation of hematopoietic stem cells has become popular [26]. If we can utilize UCB for heart failure patients, we can utilize this UCB stem cell bank network system immediately. UCB-derived stem cells may be superior to marrow-derived stem cells because they are obtained from infants. UCB contains circulating stem/progenitor cells, and the cells contained in UCB are

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known to be quite distinct from those contained in bone marrow and adult peripheral blood [27]. UCB transplantation has been reported to improve cardiac function [28–30]. That study, however, used a fraction of hematopoietic lineage and failed to show any clear evidence for cardiomyogenesis *in vivo*. In the present study, we focus on the mesenchymal lineage of UCB.

Isolation, characterization, and differentiation of clonally expanded umbilical cord blood-derived mesenchymal stem cells (UCBMSCs) have been reported [31, 32]. UCBMSCs have been found to have multipotency, and the immunophenotype of the clonally expanded cells is consistent with that reported for bone marrow mesenchymal stem cells [33, 34]. Kim et al. [35] showed modest but significant functional recovery of impaired cardiac function by transplantation of human unrestricted somatic stem cells obtained from umbilical cord blood that expressed mesenchymal cell surface markers [34]; therefore, mesenchymal lineage of the cells obtained from UCB may have potential therapeutic advantage in cardiac stem cell therapy. However, *in vitro* [33] and *in vivo* [34, 35], cardiomyogenic transdifferentiation ability have not yet been extensively studied. In the present study, we find that UCBMSCs have a strong potential for cardiomyogenic transdifferentiation.

MATERIALS AND METHODS

Isolation and Cell Culture of UCBMSCs

The detailed isolation method has been described previously [33]. A few colonies were found in the culture dish bottom 1 month after the collected cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). One colony was trypsinized using a colony cylinder and then used for the experiment. We designated the monoclonal cell line as UCBMSC. The cells were prepared for infection with recombinant retroviruses expressing the human telomerase reverse transcriptase (TERT), as described previously [2, 33]. Stably transduced cells with an expanded life span were designated UCBMSC-TERT. The cells were cultured for further experiments under the approval of the Ethics Committee of our institute.

Preparation of Murine Fetal Cardiomyocytes

Fetal cardiomyocytes were obtained from the hearts of day 17 mouse fetuses [2]. Hearts were minced with scissors and washed with phosphate-buffered saline (PBS), and the minced hearts were incubated in PBS with 0.05% trypsin and 0.25 mM EDTA (ethylenediamine-*N,N,N',N'*-tetraacetic acid) (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) for 10 minutes at 37°C. After DMEM supplemented with 10% FBS was added, the cardiomyocytes were centrifuged at 1,000 rpm for 5 minutes. The pellet was then resuspended in 10 ml of DMEM with 10% FBS and incubated on glass dishes for 1 hour to separate the cardiomyocytes from fibroblasts. The floating cardiomyocytes were collected and replated at 1×10^5 per cm^2 .

Coculture System of UCBMSCs/UCBMSCs-TERT and Murine Fetal Cardiomyocytes

We employed a coculture system with fetal cardiomyocytes to induce cardiac transdifferentiation, since *in vitro* simulation of the heart by the environment has been shown to be an efficient means of inducing the transdifferentiation of human marrow-derived MSC [2]. Cryopreserved UCBMSCs and UCBMSCs-TERT were used for the experiment. After thawing, the cells were cultured for at least two passages to stabilize the condition of the cell before the cardiomyogenic induction. UCBMSCs and UCBMSCs-TERT were labeled with enhanced green fluorescent protein (GFP) by recombinant adenovirus transfection as described previously [2]. These cells were then exposed to 3 μM 5-azacytidine (5-azaC; Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) for 24 hours to induce cell transdifferentiation or were left untreated. Then, 5×10^3

per cm^2 of the cells were plated on the murine cardiomyocyte. The images were stored using a digital video system. The cell contraction was analyzed using a homemade image edge detection program made using Igor Pro 4 (WaveMetrics Inc., Portland, OR, <http://www.wavemetrics.com>). We administered 10 μM caffeine, 10 μM verapamil, or 1 μM thapsigargin to observe contraction of differentiated UCBMSCs.

Immunocytochemistry

A laser confocal microscope (FV1000; Olympus, Tokyo, <http://www.olympus-global.com>) was used for immunocytochemical analysis. The UCBMSCs and UCBMSCs-TERT were stained with mouse monoclonal anti-human cardiac troponin-I antibody (number 4T21 Lot 98/10-T21-C2; HyTest, Turku, Finland, <http://www.hytest.fi>) diluted 1:300, monoclonal anti- α actinin antibody (Sigma) diluted 1:300, or anti-connexin 43 antibody (Sigma) diluted 1:300. Nuclei were stained with 4'-6-diamidino-2-phenylindole (Wako Chemical, Osaka, Japan, <http://www.wako-chem.co.jp/english>) at 1:300. tetramethylrhodamine iso-thiocyanate (TRITC)-conjugated goat anti-mouse IgG (Sigma), TRITC-conjugated goat anti-rabbit IgG (Sigma), and Cy5-conjugated goat anti-mouse IgG (Chemicon, Temecula, CA, <http://www.chemicon.com>) were used as secondary antibodies.

Calculation of Induction Rate

After 1 week, UCBMSCs and UCBMSCs-TERT cultivated with or without murine fetal cardiomyocytes were detached from the dish by 0.1% trypsin and 0.25 mM EDTA for 5 minutes. The mass of cells obtained was then dissociated by 0.5% collagenase type-II (Worthington Biochemical, Lakewood, NJ, <http://www.worthington-biochem.com>) and 10 mM 2,3-butanedione monoxime (Sigma)-containing culture medium for 20–60 minutes. The isolated cells were seeded on poly-L-lysine coated dishes and stained. A confocal laser microscope was used to examine the cells. The cardiomyogenic induction rate was calculated as the fraction of human cardiac troponin-I-positive cells in the GFP-positive cells. The rate was calculated as the average from more than 10 separate experiments.

Examination of Chromosomes of UCBMSCs and Murine Cell Chimeras

Chromosomes from UCBMSCs cocultivated with murine cardiomyocyte for 1 week were stained by using a human chromosome-specific probe and a mouse chromosome-specific probe (Chromosome Science Labo, Hokkaido, Japan) and spectral karyotyping with fluorescence in situ hybridization (FISH) chromosome painting technique (Spectral Imaging, Vista, CA, <http://www.spectral-imaging.com>), according to the manufacturer's protocol.

Coculture of UCBMSCs-TERT and Murine Fetal Cardiomyocytes Separated by a Collagen Membrane

UCBMSCs-TERT and murine fetal cardiomyocytes were cocultured separately within the same dish. The murine fetal cardiomyocytes were seeded on top of a floating collagen film (CM-6; Koken, Tokyo, <http://www.kokenmpc.co.jp/english>), and the UCBMSCs-TERT were seeded on the bottom of the film. These two types of cells were, therefore, separated by a high-density atelocollagen film with a thickness of 30–40 μm , as shown in Figure 5E, that is permeable only for small molecules, less than 5,000 molecular weight (MW). After 1 week of cocultivation, the cells were analyzed immunocytochemically.

RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from the UCBMSCs and UCBMSCs-TERT with RNeasy (Qiagen, Hilden, Germany, <http://www1.qiagen.com>). Human cardiac RNA was purchased (Clontech, Palo Alto, CA, <http://www.clontech.com>). RNA for reverse transcription-polymerase chain reaction (RT-PCR) was converted to cDNA with a first-strand cDNA synthesis kit (GE Healthcare, Bucking-

hamshire, U.K., <http://www.gehealthcare.com>) according to the manufacturer's recommendations. RT-PCR was performed by using primers for the following genes: *Csx/Nkx-2.5*, *GATA4*; cardiac hormones: human atrial natriuretic peptide (hANP), human brain natriuretic peptide (hBNP); cardiac structural proteins: cardiac troponin-I, cardiac troponin T, myosin heavy chain (MHC), myosin light chain-2a (MLC2a), cardiac-actin; ion channel: hyperpolarization-activated cyclic nucleotide-gated potassium channel 2 (*HCN2*); and 18s rRNA (18s rRNA was used as an internal control). PCR primers were prepared such that they would amplify the human but not the mouse genes [2] (supplemental online Table 1).

Flow Cytometric Analysis

Cells were detached and stained for 30 minutes at 4°C with primary antibodies and immunofluorescent secondary antibodies. After washing, the cells were analyzed using a FACScan (BD Biosciences, San Diego, <http://www.bdbiosciences.com>), and the data were analyzed with the CellQuest software (BD Biosciences). Antibodies (anti-human CD13, CD14, CD24, CD29, CD31, CD34, CD44, CD45, CD54, CD55, CD59, CDw90, CD105, CD117, CD133, CD140a, CD157, CD164, CD166, Flk-1, SSEA-1, SSEA-3, and SSEA-4) were purchased from Beckman Coulter (Fullerton, CA, <http://www.beckmancoulter.com>), Immunotech (Luminy, France, http://www.beckmancoulter.com/products/pr_immunology.asp), Cytotech (Hellebaek, Denmark, <http://www.cytotech.dk>), Santa Cruz Biotechnology Inc. (Santa Cruz, CA, <http://www.scbt.com>), RDI (Concord, MA <http://www.researchd.com>), and Pharmingen Pharmaceutical Co. (San Diego).

Electrophysiological Experiment

Action potentials (APs) from the spontaneously beating GFP-positive UCBMSCs and UCBMSCs-TERT were recorded by use of standard microelectrodes, as described previously [2]. After the APs of the targeted cells were recorded, the dye (Alexa 568) was injected by electroporation (−5 nA for 10–20 seconds) to confirm the recorded APs obtained from GFP-positive cells. The extent of dye transfer was monitored under a fluorescent microscope.

RESULTS

Cardiomyogenic Transdifferentiation of UCBMSCs and UCBMSCs-TERT

On day 3 after starting the cocultivation, a few GFP-positive UCBMSCs and UCBMSCs-TERT started to contract ($n = 68$). On day 7, the beating of the murine cardiomyocytes stopped, whereas approximately half of the GFP-positive UCBMSCs and UCBMSCs-TERT beat strongly in a synchronized manner.

Immunocytochemistry revealed that a significant number of UCBMSCs and UCBMSCs-TERT expressing GFP were stained positive by the anti-human cardiac troponin-I antibody (Fig. 1A–1E, supplemental online Fig. 1A–1H). A clear striation pattern of cardiac troponin-I staining of UCBMSCs can be observed in higher magnification view (Fig. 1). Interestingly, troponin-I staining and GFP were observed alternately in a striated manner, suggesting that the troponin-I was expressed in the GFP-positive cells (Fig. 1 E). Clear striations were observed with red fluorescence of α -actinin in the differentiated UCBMSCs and UCBMSCs-TERT (Fig. 2B, 2H). Arrays of cardiomyocytes can be frequently observed (Fig. 2H). Connexin 43 staining (Fig. 2C, 2I) showed a clear and diffuse pattern around the margin of each GFP-positive cardiomyocyte, suggesting that these human transdifferentiated cardiomyocytes have tight electrical coupling with each other.

We also calculated the percentage of the human cardiac troponin-I-positive cells to determine the cardiomyogenic transdifferentiation rate of UCBMSCs and UCBMSCs-TERT. Since there was no essential difference between the UCBMSCs and

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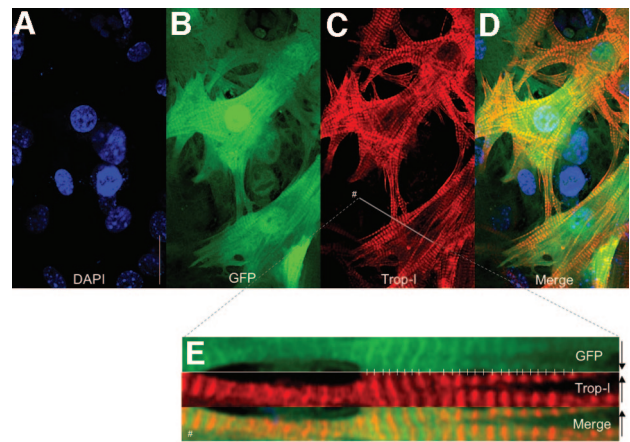


Figure 1. Cardiomyogenic transdifferentiation of umbilical cord blood mesenchymal stem cells. Laser confocal microscopic view of immunocytochemistry of differentiated umbilical cord blood mesenchymal stem cells with anti-cardiac troponin-I antibody. Superimposed images (Merge) of (A–C) are shown in (D). Significant numbers of differentiated GFP-positive umbilical cord blood-derived mesenchymal stem cells (green) had troponin-I (red) in their cytoplasm (yellow as a result of “merging” [D]). Nuclei are stained with DAPI (A), blue). Clear troponin-I (red) staining with striation pattern can be observed. GFP (B), green) and Trop-I (C), red) along the white line in (C) are magnified in panel (E). Interestingly, troponin-I staining and GFP were observed alternately in a striated manner, suggesting the troponin-I expressed in the GFP-positive cells. Scale bars in the figure denote 20 μ m. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; Trop-I, troponin-I.

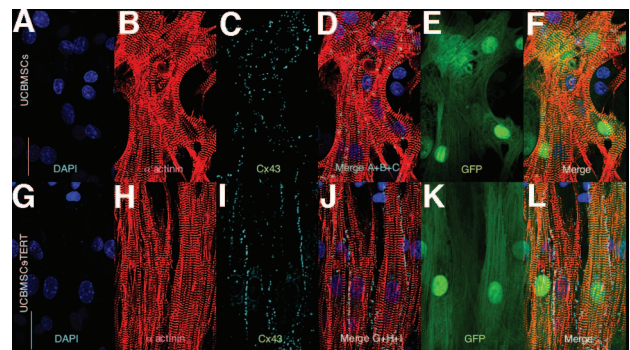


Figure 2. Immunocytochemical analysis of umbilical cord blood mesenchymal stem cell stained with anti-sarcomeric α -actinin and connexin 43. Laser confocal microscopic view of immunocytochemistry of differentiated umbilical cord blood mesenchymal stem cells and cells transduced with human TERT gene to prolong their life span (UCBMSCs-TERT) with anti-sarcomeric α -actinin (B, H); α -actinin, red) and connexin 43 (C, I); Cx43, cyan) antibody. Superimposed images (Merge) of (A–C) and (G–I) are shown in (D) and (J), respectively. Clear striation pattern of α -actinin and diffuse Cx43 dot-like staining around the margin of the UCBMSCs were observed. These cells are GFP-positive UCBMSCs (E, K); GFP, green. Merged images of (D, E) and (J, K) are (F) and (L), respectively). Nuclei are stained with DAPI (A, G), blue). It is noted that arrays of the UCBMSC-derived cardiomyocytes are sometimes observed (J). Scale bars in the figure denote 50 μ m. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; UCBMSCs, umbilical cord blood mesenchymal stem cells; UCBMSCs-TERT, umbilical cord blood mesenchymal stem cells-telomerase reverse transcriptase.

UCBMSCs-TERT, calculated data from both cell types are averaged and shown in Figure 3. Although UCBMSCs without cocultivation did not show any troponin-I expression (Fig. 3H, 3K), 45% \pm 3% of UCBMSCs became positive for human cardiac troponin-I antibody as a result of the cocultivation (Fig.

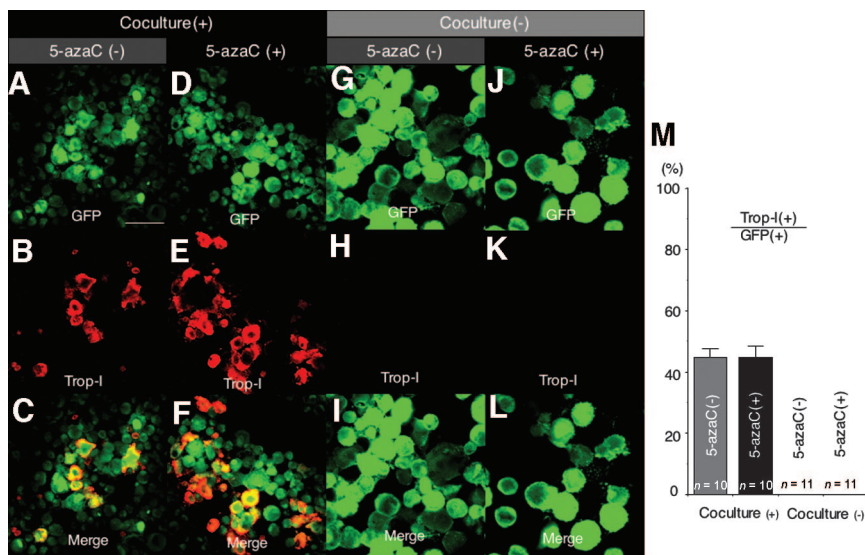


Figure 3. Calculation of cardiomyogenic transdifferentiation ratio of umbilical cord blood mesenchymal stem cells (UCBMSCs). (A–L): Representative laser confocal image of cardiac troponin-I ([B, E, H, K]; Trop-I, red) staining to calculate cardiomyogenic transdifferentiation rate of UCBMSCs. Upper bar denotes the culture conditions for each panel presented below. (Coculture; cocultivation with fetal murine cardiomyocyte, 5-azaC; pretreatment with 5-azacytidine.) Approximately half of the isolated GFP-positive UCBMSCs ([A, D]; GFP, green) stained positive for Trop-I ([B, E]) as a result of coculture. A superimposed image of (A) + (B) and (D) + (E) are shown in (C) and (F), respectively. On the other hand, UCBMSCs (G, J) do not show any Trop-I staining (H, K). Scale bars in the figure denote 50 μ m. The cardiomyogenic transdifferentiation rate of UCBMSCs was defined as the percentage of Trop-I-positive cells in the GFP-positive cells. Measured data were averaged and are shown (M). Error bars denote SEM ($n = 20$). Abbreviations: 5-azaC, 5-azacytidine; GFP, green fluorescent protein; Trop-I, troponin-I.

3B, 3E). It is noted that cardiomyogenic transdifferentiation could be observed in the cocultivated UCBMSCs and UCBMSCs-TERT without any 5-azaC pretreatment.

Cell Fusion-Independent Cardiomyogenic Transdifferentiation

Cell fusion has been shown to be quite a rare phenomenon [4, 36]; however, it may contribute to the generation of cardiomyocytes in our system. Nuclear fusion between the cocultivated UCBMSCs-TERT and fetal murine cardiomyocytes was observed in only approximately 0.09% (2/2165) of the cocultivated cells by FISH analysis (Fig. 4A–4D). In the differentiated cardiomyocyte, there is no cell having double nuclei in the isolated GFP-positive UCBMSCs. Furthermore, in cocultures of UCBMSCs-TERT with fetal murine cardiomyocytes separated by a collagen membrane (Fig. 4E), we observed beating GFP-positive cells and human cardiac troponin-I expression (Fig. 4F–4L) ($n = 8$). Because these two cell types were not attached directly to each other, it was concluded that the cardiomyogenesis in the present study was mainly caused by the transdifferentiation of the UCBMSCs.

Expression of Cardiomyocyte-Specific Genes and Surface Markers of UCBMSCs and UCBMSCs-TERT

We analyzed the cocultured UCBMSCs and UCBMSCs-TERT in terms of gene expression and by immunocytochemistry and electrical recording. RT-PCR was performed with primers that hybridized with human cardiomyocyte-specific genes but not with the murine orthologues (second column from the right, Fig. 5A). Differentiated UCBMSCs-TERT expressed *Csx/Nkx-2.5*, *GATA4*, *hANP*, *hBNP*, *cardiac-actin*, *MHC*, *MLC2a*, *cardiac troponin T*, *cardiac troponin-I*, and *HCN2*. Interestingly, all of the analyzed genes except for the *MHC* and *MLC2a* were expressed in UCBMSCs and UCBMSCs-TERT before the induction, implying that UCBMSCs may have cardiomyogenic potential as a default state, like CMG cells, in which *Csx/Nkx-2.5* and *GATA4* are constitutively expressed before induction [3]. Sequence analysis revealed that the sequences of the cDNAs matched those of the human genes.

Surface markers of the UCBMSCs-TERT were evaluated by flow cytometric analysis. The results showed that all of the

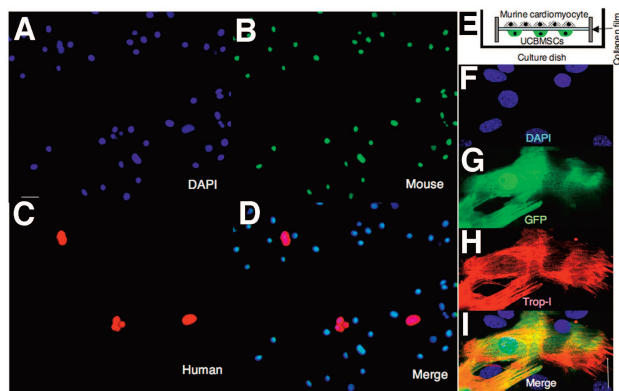


Figure 4. Cell fusion-independent cardiomyogenesis in UCBMSCs. (A–D): Representative images of fluorescent in situ hybridization for human nucleus and mouse nucleus are shown. Nuclei are stained with DAPI ([A]; blue). Mouse nuclei were detected as green (B) and human nuclei were detected as red (C). Superimposed image of (A–C) is shown in (D) (Merge). See text for details. (E): The experimental scheme is shown. The murine cardiomyocytes and UCBMSCs were cocultured on the top and the bottom of a collagen membrane, respectively. The cocultivated UCBMSCs and murine cardiomyocytes were separated by the 50- μ m-thick collagen membrane. Nuclei were stained with DAPI ([F], blue) and UCBMSCs were labeled with GFP ([G], green). UCBMSCs were stained with anti-human cardiac troponin-I antibody ([H], red), and the merged images (DAPI, GFP, Trop-I) are shown (I). Scale bars in the figure denote 20 μ m. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; Trop-I, troponin-I; UCBMSCs, umbilical cord blood mesenchymal stem cells.

UCBMSCs-TERT were positive for CD29 (integrin β 1), CD44 (Pgp-1/ly-24), CD54, CD55, CD59, CDw90, CD105, CD157, CD164, CD166, and SSEA-4 and negative for CD14 (a marker for macrophage and dendritic cells), CD31 (platelet endothelial cell adhesion molecule-1), CD34, CD45 (leukocyte common antigen), CD117 (c-kit), CD133, CD140a, Flk-1, SSEA-1, and SSEA-3 (Fig. 5B). Our UCBMSCs are negative for CD34, CD45, Flk-1, and CD133, thus differing from hematopoietic stem cell and from circulating endothelial progenitor cells. It is noted that our UCBMSCs are weakly positive for SSEA4 [37], an embryonic stem cell marker. Thus, UCBMSCs may be more plastic for transdifferentiation than other somatic stem cells.

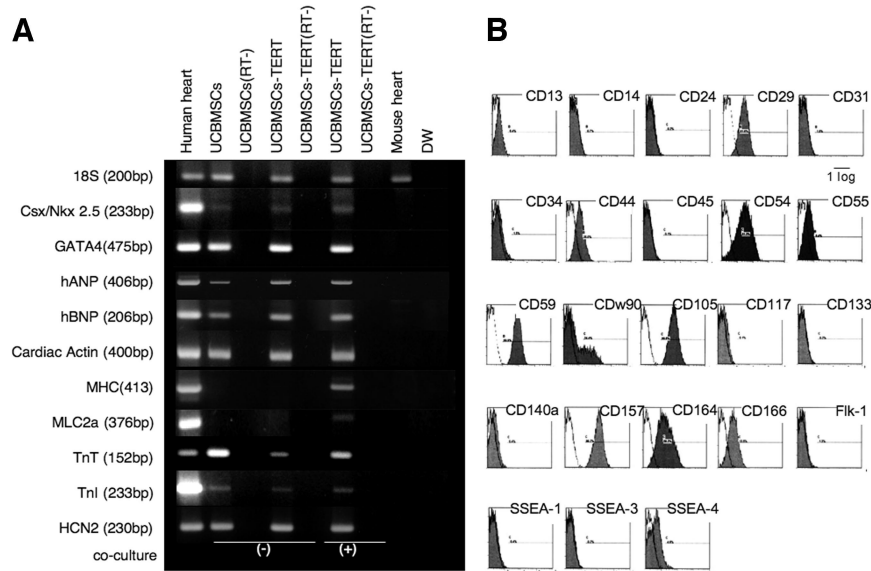


Figure 5. Expression of cardiomyocyte-specific genes in UCBMSCs and cell surface markers of UCBMSCs. **(A):** Expression of cardiomyocyte-specific genes in UCBMSCs and UCBMSCs-TERT. Reverse transcription-polymerase chain reaction (PCR) was performed with PCR primers with specificity for human genes encoding cardiac proteins but not for the corresponding murine genes. Only the 18S PCR primer used as a positive control reacted with both the human and murine genes. Human heart and mouse heart were used as a positive control and negative control, respectively. Almost all human cardiac genes were constitutively expressed in the default state. **(B):** Flow cytometric analysis of UCBMSCs with fluorescein isothiocyanate-coupled antibodies against the human surface antigens. Abbreviations: DW, distilled water; hANP, human atrial natriuretic peptide; hBNP, human brain natriuretic peptide; HCN2, hyperpolarization-activated cyclic nucleotide-gated potassium channel 2; MHC, myosin heavy chain; MLC2a, myosin light chain-2a; RT, reverse transcriptase; TnI, cardiac troponin I; TnT, cardiac troponin T; UCBMSCs, umbilical cord blood mesenchymal stem cells; UCBMSCs-TERT, umbilical cord blood mesenchymal stem cells-telomerase reverse transcriptase.

Functional Analysis of Differentiated UCBMSCs and UCBMSCs-TERT In Vitro

APs were recorded from spontaneously beating GFP-positive UCBMSCs and UCBMSCs-TERT. Alexa 568 was injected into cells via a recording microelectrode to stain the cells and confirm that the APs were generated by GFP-positive UCBMSCs (Fig. 6A, 6C). Since the dye did not diffuse into the murine cardiomyocytes, there were no tight cell-to-cell heterologous connections, that is, gap junctions. In most experiments, Alexa 568 diffused into the GFP-positive adjacent UCBMSCs and UCBMSCs-TERT, suggesting that homologous cell-to-cell connections had been established within 1 week after the start of cocultivation. The APs obtained from UCBMSCs and UCBMSCs-TERT showed clear cardiomyocyte-specific sustained plateaus. It was, therefore, concluded that they were the APs of cardiomyocytes, not of smooth muscle, nerve cells, or skeletal muscle (Fig. 6B, 6D). The measured parameters of the recorded APs were averaged (Fig. 6E). UCBMSCs and UCBMSCs-TERT did not have a marked pacemaker potential and had the character of working cardiomyocytes or ordinary cardiomyocytes. The rhythm of almost all of the UCBMSCs and UCBMSCs-TERT had become regular at 1 week. The fractional shortening (% FS) of the UCBMSCs and UCBMSCs-TERT was analyzed (Fig. 6F–6I) using a cell edge detection program. The GFP-positive cells contracted simultaneously within the whole visual field, suggesting tight electrical communication. There was no difference of % FS between the UCBMSCs and UCBMSCs-TERT. The % FS was augmented significantly by the administration of caffeine and inhibited by the administration of verapamil or thapsigargin (Fig. 7).

DISCUSSION

Physiologically Functioning Cardiomyocytes Can Be Generated from UCBMSCs In Vitro

Compared with the cardiomyogenic differentiation efficiency of the marrow-derived MSC (0.3%) [2], a significant number of

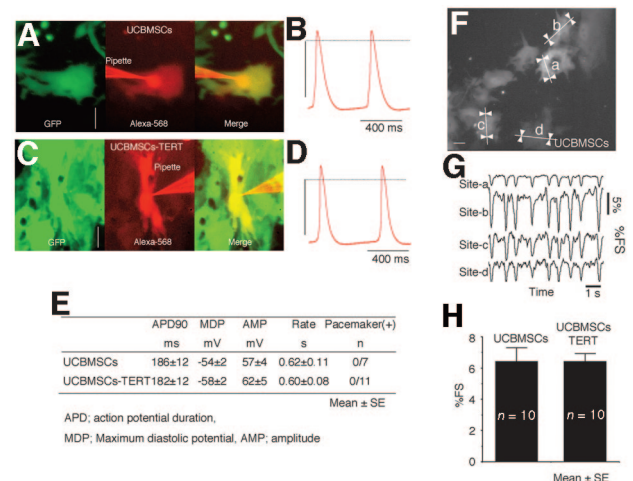


Figure 6. Functional analysis of UCBMSCs and UCBMSCs-TERT. Representative fluorescent microscopic images during action potential (AP) recording are shown **(A, C)**. Immediately after the AP recordings, alexa568 dye (red) was injected into the cell via the same recording electrode to confirm that the recorded AP was obtained from GFP-positive UCBMSCs. **(B, D):** Representative APs obtained from **(A)** and **(C)** respectively. The dotted line denotes the 0 mV level and the vertical line denotes 50 mV. **(E):** The measured AP parameters were averaged and are shown. **(F):** A representative still image from cell motion analysis is shown. The white arrowheads point to the automatically detected cell edge. The detected fractional shortening along the white line obtained from site-a, -b, -c, -d **(G)**. **(H):** The measured % FS was averaged and is shown. Abbreviations: AMP, amplitude; APD, action potential duration; % FS, fractional shortening; GFP, green fluorescent protein; MDP, maximum diastolic potential; ms, milliseconds; s, second; UCBMSCs, umbilical cord blood mesenchymal stem cells; UCBMSCs-TERT, umbilical cord blood mesenchymal stem cells-telomerase reverse transcriptase.

the UCBMSCs transdifferentiated into cardiomyocytes in vitro in the present study. Generated cardiomyocytes showed physiologically functioning ability, that is, cardiomyocyte-specific

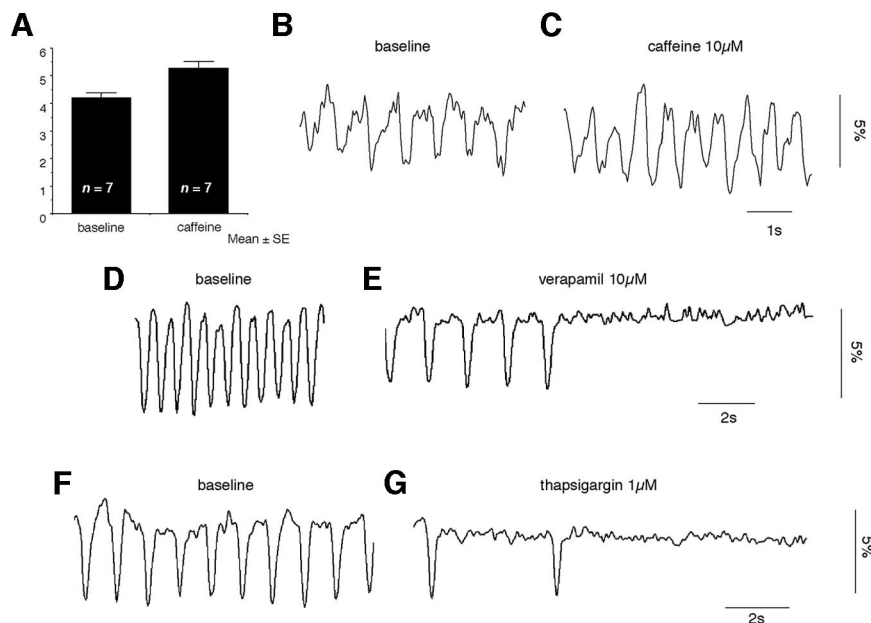


Figure 7. The effect of the drug administration on contraction of differentiated umbilical cord blood mesenchymal stem cells (UCBMSCs). The detected fractional shortening (% FS) of the differentiated UCBMSCs before and after the administration of caffeine (A–C), verapamil (D, E), and thapsigargin (F, G) are shown. The caffeine slightly increased the beating rate and increased the % FS significantly (A). On the other hand, immediately after the administration of verapamil (D) or thapsigargin (F), the beating rate decreased, then ceased (E, G). Abbreviation: s, second.

APs with long duration (more than 100 milliseconds) and spontaneous contraction. The fact that each UCBMSC beats in a synchronized manner and the fact of the diffuse connexin 43 staining together suggest the formation of tight electrical coupling among the UCBMSCs. In our previous paper, we used the cells immediately after being quickly thawed from cryopreserved UCBMSCs, then failed to observe cardiomyogenic transdifferentiation in the small number of observations [33]. Recently, however, we felt at least two passages should be required to stabilize and regain cardiomyogenic transdifferentiation ability in UCBMSCs and our several cell lines.

Highly Cardiomyogenic Differentiation Potential

In the marrow-derived stem cell, mesenchymal lineage has a cardiomyogenic transdifferentiation potential [2, 3]; hematopoietic cell lineage does not [21]. This implies that mesenchymal lineage of the cell in UCB might have the ability to transdifferentiate into cardiomyocytes. Several *in vivo* experiments using UCB have shown feasible effects in restoring cardiac function in the myocardial infarction model [28–30]. However, these experiments used CD34+ or CD133+ hematopoietic lineage of the cell in UCB and failed to show any clear evidence of cardiomyogenesis. Surface marker analysis revealed UCBMSCs as differing from hematopoietic stem cells and from circulating endothelial progenitor cells. Kögler et al. [34] reported that stem cells obtained from UCB, so-called unrestricted somatic stem cells (USSCs), have a pluripotent differential potential with a similar surface marker pattern, that is, negative for CD34 and CD45 and positive for CD29 and CD44, that is typical for mesenchymal cells. Furthermore, Kim et al. [35] showed that USSCs improved impaired cardiac function *in vivo*. Although the two papers showed modest evidence for cardiomyogenic potential of USSCs *in vivo*, experiments had not been extensively done to show the evidence of cardiac transdifferentiation. Finally, these papers failed to show clear evidence for cell fusion-independent cardiomyogenesis and efficiency of cardiomyogenic differentiation. In the present study, we show significant potential of cell fusion-independent cardiomyogenesis of UCBMSCs.

Comparisons with Other Stem Cells for Cardiology

Cardiac precursor cells (CPCs) [38] should be a promising stem cell source for cardiac regeneration therapy. However, CPCs failed to differentiate to the physiologically functioning cardiomyocyte *in vitro*, and cardiomyogenic differentiation efficiency *in vivo* was 29%–40%. Thus, cardiomyogenic differentiation efficiency might not be so markedly high compared with the UCBMSCs. Moreover, it is very difficult to match the donor-recipient HLA-type, and there is still a longstanding ethical problem. An embryonic stem cell is a pluripotential stem cell that has a cardiomyogenic differentiation potential. But there are still critical underlying problems, that is, teratoma formation [39], genomic alteration in long-term culture [40], and the ethical problem. Differing from embryonic stem cells, our RT-PCR data suggest constitutive expression of Nkx2.5/Csx and GATA4 and other cardiac structure mRNA with the ability of self-renewal. This suggests that some population of the UCBMSCs has cardiomyogenic potential as the default state, and they may be termed cardiac precursor cells in light of their biological features. Recently, we reported that human endometrial gland-derived mesenchymal cells also have a high cardiomyogenic potential [41]. This suggests that they may be a stem cell source for heart disease. However, for male patients, there is no choice for autologous transplantation of this cell and no running stem cell bank for this cell. On the other hand, if UCBMSCs were isolated and frozen at the time of birth, they could later be thawed for use by the donor who required cardiac stem cell therapy at a later age. Furthermore, UCB banking has played a major role for hematopoietic stem cell transplantation for leukemia treatment. If we utilize a world-wide UCB bank system for cardiac stem cell therapy, we may be able to utilize UCBMSCs for cardiac stem cell therapy in the near future. Since several reports showed that mesenchymal cells cause immunological tolerance [42–44], we speculate that only a minimum administration of immunosuppressive agents may be sufficient to control rejection of the allogeneic UCBMSC transplantation, if we match the other MHC antigen by utilizing the stem cell bank system.

Study Limitations

From a single stem cell we can obtain approximately 2^{32} cells with extremely high cardiomyogenic potential; however, the number of MSCs in the UCB is quite low, as was described previously [33, 34]. Thus, further experiments should be done to establish a method to collect the UCBMSCs efficiently. The transfection of the TERT gene may alter the phenotype of UCBMSCs to some extent. However, TERT-gene transfection was not essential for causing cardiomyogenic differentiation of UCBMSCs, and there was no essential difference between the UCBMSCs and UCBMSCs-TERT in the present study.

Our *in vitro* cardiomyogenic induction system provided a substantial environmental factor to cause cardiomyogenic transdifferentiation of UCBMSCs *in vitro*; however, specific key factors (e.g., humoral factors) for cardiomyogenesis were still unclear. It is still undetermined whether such key factors for cardiomyogenesis are sufficiently provided by the surrounding host heart when UCBMSCs are engrafted *in vivo*. We believe that the definition of these specific factors *in vitro* should be extremely important to improve cardiomyogenesis *in situ*; therefore, in the present study, we focused on *in vitro* cardiomyogenesis of UCBMSCs.

Cell fusion is a rare phenomenon (0.6%–0.05%) [36], and the frequency of nuclear fusion was low (0.1%) in the present study. On the other hand, the cardiomyogenic differentiation

efficiency of UCBMSCs was extremely high ($44.9\% \pm 3.6\%$). Furthermore, a 40- μm -thick atelocollagen membrane is not permeable for molecules larger than 5,000 MW, and no cell migration from the top of the membrane to the bottom was observed in our culture condition. On this basis, we concluded that cell fusion did not play a major role in the UCBMSC-derived cardiomyogenesis in the present study.

Summary

Our major findings in the present study are: (a) for the first time, physiologically functioning cardiomyocytes were transdifferentiated from human UCBMSCs *in vitro*; (b) the observed cardiomyogenic transdifferentiation, independent of cell fusion, was approximately $44.9\% \pm 3.6\%$ of UCBMSCs; and (c) cocultivation with fetal murine cardiomyocytes alone without other transdifferentiation factors, that is, 5-azaC, is sufficient for cardiomyogenesis in our system. Therefore, UCBMSCs may be a promising cellular source for cardiac stem cell-based therapy, by which cardiomyogenesis can be expected.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

- Koyanagi M, Haendeler J, Badorff C et al. Non-canonical Wnt signaling enhances differentiation of human circulating progenitor cells to cardiomyogenic cells. *J Biol Chem* 2005;280:16838–16842.
- Takeda Y, Mori T, Imabayashi H et al. Can the life span of human marrow stromal cells be prolonged by bmi-1, E6, E7, and/or telomerase without affecting cardiomyogenic differentiation? *J Gene Med* 2004;6: 833–845.
- Makino S, Fukuda K, Miyoshi S et al. Cardiomyocytes can be generated from marrow stromal cells *in vitro*. *J Clin Invest* 1999;103: 697–705.
- Iijima Y, Nagai T, Mizukami M et al. Beating is necessary for transdifferentiation of skeletal muscle-derived cells into cardiomyocytes. *FASEB J* 2003;17:1361–1363.
- Klug MG, Soonpaa MH, Koh GY et al. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. *J Clin Invest* 1996;98:216–224.
- Min JY, Yang Y, Converso KL et al. Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats. *J Appl Physiol* 2002;92:288–296.
- Ghostine S, Carrion C, Souza LC et al. Long-term efficacy of myoblast transplantation on regional structure and function after myocardial infarction. *Circulation* 2002;106(12 suppl 1):1131–1136.
- Taylor DA, Atkins BZ, Hungspreugs P et al. Regenerating functional myocardium: Improved performance after skeletal myoblast transplantation. *Nat Med* 1998;4:929–933.
- Jackson KA, Majka SM, Wang H et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest* 2001;107:1395–1402.
- Orlic D, Kajstura J, Chimenti S et al. Bone marrow cells regenerate infarcted myocardium. *Nature* 2001;410:701–705.
- Wang JS, Shum-Tim D, Galipeau J et al. Marrow stromal cells for cellular cardiomyoplasty: Feasibility and potential clinical advantages. *J Thorac Cardiovasc Surg* 2000;120:999–1005.
- Shake JG, Gruber PJ, Baumgartner WA et al. Mesenchymal stem cell implantation in a swine myocardial infarct model: Engraftment and functional effects. *Ann Thorac Surg* 2002;73:1919–1925.
- Gojo S, Gojo N, Takeda Y et al. *In vivo* cardiovascularogenesis by direct injection of isolated adult mesenchymal stem cells. *Exp Cell Res* 2003; 288:51–59.
- Strauer BE, Brehm M, Zeus T et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation* 2002;106:1913–1918.
- Hamano K, Nishida M, Hirata K et al. Local implantation of autologous bone marrow cells for therapeutic angiogenesis in patients with ischemic

- heart disease: Clinical trial and preliminary results. *Jpn Circ J* 2001;65: 845–847.
- Assmus B, Schachinger V, Teupe C et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOP-CARE-AMI). *Circulation* 2002;106:3009–3017.
- Tse HF, Kwong YL, Chan JK et al. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. *Lancet* 2003;361:47–49.
- Menasche P, Hagege AA, Scorsin M et al. Myoblast transplantation for heart failure. *Lancet* 2001;357:279–280.
- Tomita S, Li RK, Weisel RD et al. Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation* 1999;100: II247–II256.
- Stamm C, Westphal B, Kleine HD et al. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. *Lancet* 2003;361:45–46.
- Murry C, Soonpaa M, Reinecke H et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 2004;428:664–668.
- Tang YL, Zhao Q, Zhang YC et al. Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium. *Regul Pept* 2004;117:3–10.
- Gnecchi M, He H, Liang O et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med* 2005;11:367–368.
- Fadini GP, Miorin M, Facco M et al. Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. *J Am Coll Cardiol* 2005;45:1449–1457.
- Heiss C, Keymel S, Niesler U et al. Impaired progenitor cell activity in age-related endothelial dysfunction. *J Am Coll Cardiol* 2005;45:1441–1448.
- Warkentin P and Foundation for the Accreditation of Cellular Therapy. Voluntary accreditation of cellular therapies: Foundation for the Accreditation of Cellular Therapy (FACT). *Cytotherapy* 2003;5:299–305.
- Mayani H, Lansdorp PM. Biology of human umbilical cord blood-derived hematopoietic stem/progenitor cells. *STEM CELLS* 1998;16: 153–165.
- Hirata Y, Sata M, Motomura N et al. Human umbilical cord blood cells improve cardiac function after myocardial infarction. *Biochem Biophys Res Commun* 2005;327:609–614.
- Leor J, Guetta E, Feinberg M et al. Human umbilical cord blood-derived CD133+ cells enhance function and repair of the infarcted myocardium. *STEM CELLS* 2006;24:772–780.
- Ma N, Stamm C, Kaminski A et al. Human cord blood cells induce angiogenesis following myocardial infarction in NOD/scid-mice. *Cardiovasc Res* 2005;66:45–54.
- Lee OK, Kuo TK, Chen WM et al. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* 2004;103:1669–1675.
- Goodwin HS, Bicknese AR, Chien SN et al. Multilineage differentiation

- activity by cells isolated from umbilical cord blood: Expression of bone, fat, and neural markers. *Biol Blood Marrow Transplant* 2001;7:581–588.
- 33 Terai M, Uyama T, Sugiki T et al. Immortalization of human fetal cells: The life span of umbilical cord blood-derived cells can be prolonged without manipulating p16INK4a/RB braking pathway. *Mol Biol Cell* 2005;16:1491–1499.
 - 34 Kögler G, Sensken S, Airey J et al. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J Exp Med* 2004;200:123–135.
 - 35 Kim BO, Tian H, Prasongsukarn K et al. Cell transplantation improves ventricular function after a myocardial infarction: A preclinical study of human unrestricted somatic stem cells in a porcine model. *Circulation* 2005;112(suppl 9):I96–I104.
 - 36 Matsuura K, Wada H, Nagai T et al. Cardiomyocytes fuse with surrounding noncardiomyocytes and reenter the cell cycle. *J Cell Biol* 2004;167:351–363.
 - 37 Kannagi R, Cochran N, Ishigami F et al. Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. *EMBO J* 1983;2:2355–2361.
 - 38 Beltrami A, Barlucchi L, Torella D et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 2003;114:763–776.
 - 39 Leor J, Gerecht-Nir S, Cohen S et al. Undifferentiated human embryonic stem cells are not guided to form new myocardium by transplantation into normal and infarcted heart. Paper presented at: American College of Cardiology Meeting; March 6–9, 2005; Orlando, FL.
 - 40 Maitra A, Arking D, Shivapurkar N et al. Genomic alterations in cultured human embryonic stem cells. *Nat Genet* 2005;37:1099–1103.
 - 41 Miyoshi S, Hida N, Nishiyama N et al. Human menstrual blood is a potential cell source for cardiac stem cell therapy. Paper presented at: American College of Cardiology Meeting; March 6–9, 2005; Orlando, FL.
 - 42 Makkar RR, Price MJ, Lill M et al. Intramyocardial injection of allogenic bone marrow-derived mesenchymal stem cells without immunosuppression preserves cardiac function in a porcine model of myocardial infarction. *J Cardiovasc Pharmacol Ther* 2005;10:225–233.
 - 43 Beyth S, Borovsky Z, Mevorach D et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* 2005;105:2214–2219.
 - 44 Tsafirir A, Brautbar C, Nagler A et al. Alloreactivity of umbilical cord blood mononuclear cells: Specific hyporesponse to noninherited maternal antigens. *Hum Immunol* 2000;61:548–554.



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