

actin and myosin light chain-2 α (MLC-2 α) in the default state, implying that chorionic plate cells can differentiate into cardiomyocytes, like CMG cells in which *Csx/Nkx-2.5* and *GATA4* are constitutively expressed before induction [12].

Cardiomyogenic differentiation of chorionic plate cells

employed a co-culture system with murine fetal cardiomyocytes to induce cardiac differentiation, since *in vitro* simulation of the heart by the environment has been shown to be an efficient means of inducing the differentiation of human endothelial progenitor cells [15] and human marrow stromal cells [13]. EGFP-labeled chorionic plate cells were co-cultured with murine fetal cardiomyocytes without any chemical treatment. A few EGFP-positive chorionic plate cells started to contract on day 3 after the start of co-cultivation, and beat strongly and rigorously in a synchronized manner on day 5 (Supplementary movie 1). The cells continued to beat at least until day 21 during the period of observation. The frequency of cardiomyogenic differentiation from chorionic plate cells was calculated based on the number of cTnI-positive cells. In three independent experiments, the percentage of cells that underwent cardiomyogenic differentiation was similar ($15.1 \pm 5.1\%$) (Supplementary Fig. 1S). Our investigation of the cardiomyogenic-specific gene expression for differentiated chorionic plate cells (Fig. 2) found that cardiac actin was fully expressed, whereas *Csx/Nkx-2.5* and cardiac troponin T were only slightly expressed. *GATA4*, cTnI, MLC-2 α , and BNP were not expressed. Technical difficulties may have adversely affected these results, as some of the differentiated cells divided from murine fetal cardiomyocytes were physically damaged and so the ratio of differentiated to undifferentiated cells may well have been diminished in each case. In fact, in every experiment, cTnI was detected by immunocytochemical analysis. Immunocytochemical staining revealed that EGFP-labeled cells stained positive for cTnI (Figs. 3A–F). Immunostaining of longitudinal sagittal and axial transverse sections confirmed that cTnI was expressed in the EGFP-positive cells (Figs. 3G–I, Supplementary movie 2). These results imply that co-culture system of chorionic plate cells and murine embryonic cardiomyocytes induces differentiation of chorionic plate cells into cTnI-positive cells *in vitro*. cTnI-positive cells were evenly detected throughout the dish, suggesting that the cardiomyogenic induction rate was quite high in the present model. The EGFP-positive cells also expressed α -actinin, and connexin 43 (Figs. 4A–E). Clear striations were observed for the red fluorescence of cTnI (Fig. 3A) and α -actinin (Fig. 4A) in the differentiated chorionic plate cells. Connexin 43 staining (Figs. 4C–G) showed a clear and diffuse pattern around the margin of the cytoplasm, suggesting that these human transdifferentiated cardiomyocytes have tight electrical coupling with each other. We also performed *in vivo* implantation of EGFP-labeled chorionic plate donor cells into the ischemic heart model of nude rats (data not shown). EGFP-labeled chorionic plate donor cells exhibit positive cTnI reactivities at the implanted site. However, the frequency of cTnI-positive cells *in vivo* is not comparative with that *in vitro*.

To investigate if chorionic plate cells are capable of differentiating into osteoblasts and adipocytes [19,20], we induced chorionic to differentiate into osteocytes and adipocytes under specific culture conditions. Chorionic plate cells

did not show clear adipogenic and osteogenic differentiation: cells did not accumulate Oil Red O-positive fat droplets and calcium, and did not increase alkaline phosphatase osteogenic activity (Supplementary Fig. 2S), suggesting that chorionic plate cells have a cardiomyocyte potential, but not adipocyte or osteoblast potential.

The action potential of differentiated chorionic plate cells

To detect the electrophysiological coupling due to gap-junctional communication between beating cardiomyocytes, action potentials (APs) were recorded from spontaneously beating EGFP-positive cells. Alexa568 was injected into cells via a recording microelectrode to stain the cells and to confirm that the APs were generated by EGFP-positive cells (Figs. 5A–C). The dye did not diffuse into the murine cardiomyocytes, indicating that there were no tight cell-to-cell heterologous connections, i.e., gap junctions. Alexa568 dye did not diffuse into adjacent human and murine cells of the injected cells that exhibited action potentials. The action potentials obtained originate from human chorionic plate cells or may result from electrical coupling with adjacent cardiomyocytes [24]. The APs obtained from chorionic plate cells showed clear cardiomyocyte-specific sustained plateaux (Figs. 5D, E) and were therefore concluded to be APs of cardiomyocytes, not of smooth muscle cells, nerve cells, or skeletal muscle cells. The measured parameters of the recorded AP were averaged (Fig. 5F). Chorionic plate cells had the character of 'working' cardiomyocytes or ordinary cardiomyocytes. The rhythm of almost all the beating cells had become regular at 1 week. The fractional shortening (%FS) of the cells was analyzed (Supplementary Fig. 3S), using a cell-edge detection program developed by S.M. The EGFP-positive cells contracted simultaneously within the whole visual field, suggesting tight electrical communication among them. The average %FS was $5.46 \pm 0.40\%$ ($n=10$). In summary, human cardiomyocytes obtained from the chorionic plate cells were electrophysiologically and physiologically functional.

Discussion

Transdifferentiation of human extraembryonic mesodermal cells into embryonic mesodermal cells: functional working cardiomyocytes

This study was conducted to determine whether extensive *in vivo* propagation by cell culture would prevent the cardiomyogenic differentiation of placenta-derived cells. According to our previous study on the induction of cardiomyogenic differentiation of immortalized murine marrow stromal cells [12,15] and human marrow stromal cells [13] by demethylating agents, the transdifferentiation of human stromal cells was limited to working cardiomyocytes and did not include pacemaker cells. This was probably due to the origin of cells, that is, the default state of the chorionic plate-derived human fetal cells used in the experiment. The idea for the cardiomyogenic differentiation protocol using murine fetal cardiomyocytes without 5-azacytidine arose from reports that endothelial cells differentiate into cardiomyocytes as a result of co-cultivation with murine fetal cardiomyocytes [25].

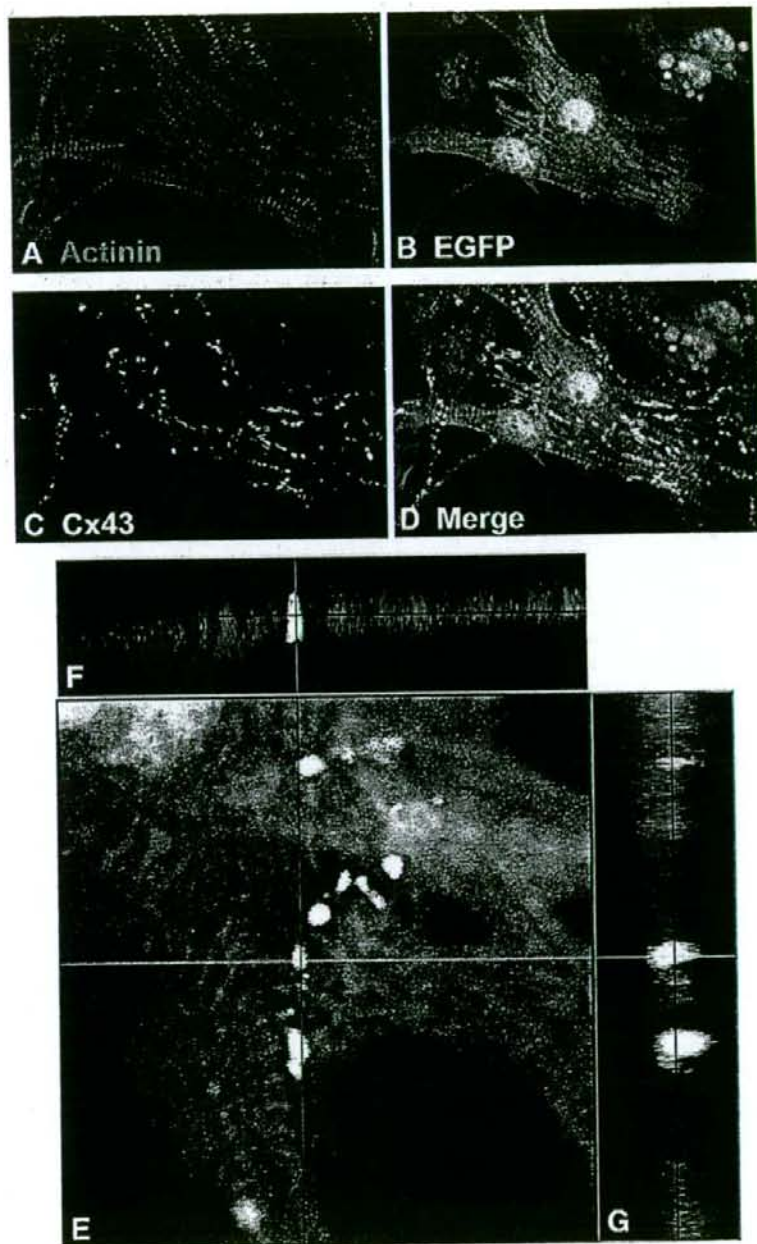


Fig. 4 - Immunocytochemistry of chorionic plate cells for α -actinin and connexin 43. (A-D) Immunocytochemistry of differentiated chorionic plate cells for α -actinin (Actinin) and connexin 43 (Cx43) antibody. Clear striations were observed for the red fluorescence of α -actinin (A) in the EGFP-positive cells (B). Connexin 43 (C) was stained along the attachment site of EGFP-positive cells. Merged images (α -actinin, connexin 43, EGFP) are shown in panel D. (E-G) A merged image for α -actinin, connexin 43, and EGFP is shown in panel E. (F) A longitudinal section at the green line in merged image E. (G) An axial section at the red line in merged image E. Panels F and G show that connexin 43 is located between EGFP-positive cells.

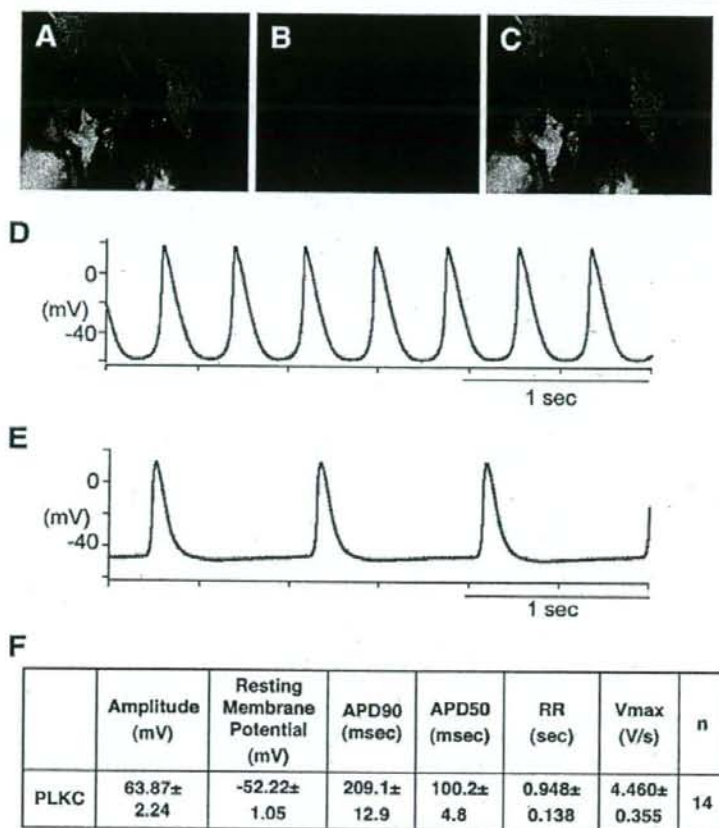


Fig. 5 – Electrophysiological and physiological analysis of chorionic plate cells. (A–C) EGFP-labeled chorionic plate cells were injected with Alexa568 solution by ionophoresis through a microelectrode. (D, E) Two different types of action potential, i.e., tachycardia type and bradycardia type, were recorded. Both types of cells had the features of working cardiomyocytes. The rhythm of their beating was regular. (F) The measured action potential parameters of EGFP-labeled chorionic plate cells are averaged.

The high frequency of cardiomyogenic differentiation makes it inconceivable that the transdifferentiation is due to fusion; in addition, bone marrow stromal cells [13] do not fuse with feeder cells in the co-cultivation system and the frequency of fusion in the co-culture system is not high [26] in contrast to myogenic differentiation [27]. The global gene expression pattern showed that the change in gene expression during differentiation was consistent with phenotypic alteration. The cells established from the placenta can be extensively and clonally expanded in vitro while retaining their potential to differentiate into cardiomyocytes that exhibit spontaneous beating and cardiomyocyte-specific action potential under in vitro conditions. This differentiation potential shown by the placenta-derived cells is the same as that reported for bone marrow MSCs [12,13,15]. It is also noteworthy that the transdifferentiation of chorionic plate cells represents the transition from extraembryonic cells to embryonic cells, while the transdifferentiation from bone-marrow-derived MSCs to neurogenic cells, which we previously reported [28,29], is transition between germ layers in embryonic tissues.

Most of the surface markers of the placenta-derived cells examined in this study are the same as those detected in their bone marrow counterparts [13,30], with both cord blood- and bone-marrow-derived mesodermal cells being positive for CD29, CD44, and CD59, and negative for CD34. Our finding of in vitro differentiation from extraembryonic mesodermal cells to embryonic mesodermal cells in this study, a key future goal for any cell-based therapy, could thus be achieved by exposing placenta-derived cells to murine fetal cardiomyocytes, at least in vitro. This technique allows the applications of the placenta to be further extended and permits it to be used as an alternative to bone marrow as a source of cells with cardiomyogenic potential.

Is the high rate of cardiomyogenic differentiation of placenta-derived cells due to the default cell state?

The cardiomyogenic differentiation rate of chorionic plate cells (15.1%) was relatively high compared to that of marrow-derived MSCs (less than 0.3%) [13]. The gene expression

pattern of chorionic plate cells before cardiomyogenic differentiation was different from that of marrow-derived MSCs. The expression of cardiomyocyte-associated genes in the chorionic plate cells, which we unexpectedly found by GeneChip analysis and confirmed by RT-PCR, is surprising. Constitutive expression of the *Csx/Nkx2.5* cardiogenic 'master' gene [31,32] in the chorionic plate cells with the ability of self-renewal suggests that the chorionic plate cells have cardiogenic potential [33] and may be termed "cardiac precursor cells" in the light of their biological characteristics like endometrium-derived myogenic precursor cells [27]. The mechanism of the drastic improvement in the differentiation rate of chorionic plate cells may be attributable to default characteristics as cardiac precursor cells of the placenta-derived cells in culture. Because of this improvement in the differentiation rate, it is possible to obtain a large number of cardiomyocytes without prolongation of their life span, i.e., transfer of oncogenic molecules into cells, to restore cardiac function. It is quite interesting that working cardiomyocytes can be generated from placenta-derived cells, since one of the types of target cells for regenerative medicine is heart cells.

Are human placenta-derived cells that are propagated in vitro useful for cell-based therapy?

Can primary placenta-derived cell 'culture' contribute to cell-based therapy or regenerative medicine? Primary placenta-derived cell culture obtained from the chorionic mesodermal layer succeeded in almost 100% of the attempts, and the cells were passaged only 3 or 4 times (6 to 7 PDs) before reaching premature senescence. The problems involved in cell-based therapy with human placenta-derived cells are the finite life span of the cells and the difficulty of obtaining a large enough number of cells. Based on the results of our previous study using cord blood-derived cells, the establishment of cells can be explained by: (1), lack of p16^{INK4a} in primary-cultured cells, or (2), selection of cells that do not express p16^{INK4a} from a heterogeneous population [22]. We cannot exclude either possibility, and we did observe two different types of cells, i.e., rapidly growing spindle cells and quiescent flat and elongated cells in the primary culture of placenta cells. Experimental settings that allow human placenta cells to double more than 100 times may be used to obtain a large number of cells at least from the placenta.

We believe that these placenta-derived extraembryonic mesodermal cells may be used to supply cardiomyocytes to patients with ischemic heart disease, dilated cardiomyopathy, and Kawasaki disease, which all have a poor prognosis and are sometimes lethal. The 'risk versus benefit' balance is essential when applying these multiplied cells clinically and the 'risk' or 'drawback' in this case is the transformation of implanted cells. In vivo experiments revealed that no tumor was observed for up to 4 weeks when chorionic plate cells at P5 (1×10^7) were subcutaneously inoculated into immunodeficient, non-obese diabetic (NOD)/severe combined immunodeficiency (SCID)/interleukin 2 receptor^{-/-} (NOG) mice (data not shown). Human placenta-derived cells spontaneously avoid premature senescence without gene induction and enter replicative senescence. Replicative senescence may be due to a tumor suppressor mechanism that avoids the risk of cell

transformation after implantation of cells as a source for cell-based therapy [34].

Our present study suggested the presence of a precursor cell type in the placenta which is destined to generate 'working cardiomyocytes'. Since the placenta is usually discarded, it can be collected at usual delivery or cesarean section and can be banked or stored. Cells with almost all the HLA types can be collected after several generations. A placenta-derived cell bank system covering all HLA types may be necessary for patients who cannot supply bone marrow cells but want to receive stem cell-based cardiac therapy.

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Data set from the gene chip analysis are available at the GEO database with accession number GSE7021: GSM162104 and GSM162105.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2007.04.028.

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

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Key Words. Physiology • Transplantation • Action potentials • Cells • Heart failure

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 (ICM) (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 allogeneic 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 1×10^2 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–E, 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. 1E). 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 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.

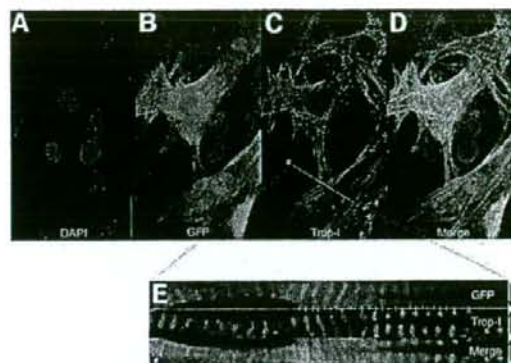


Figure 1. Cardiomyogenic transdifferentiation of umbilical cord blood mesenchymal stem cells. Laser confocal microscopic view of immunocytochemistry of differentiated umbilical cord blood mesenchymal stem cells with anti-cardiac troponin-I antibody. Superimposed images (Merge) of (A–C) are shown in (D). 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 Troponin-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; Troponin-I, troponin-I.

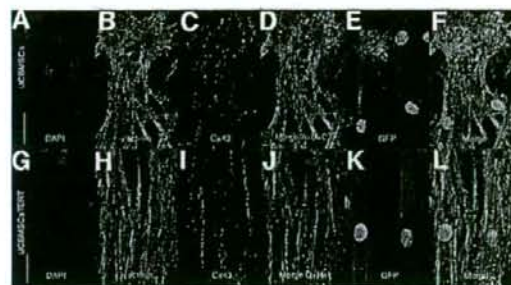


Figure 2. Immunocytochemical analysis of umbilical cord blood mesenchymal stem cell stained with anti-sarcomeric α -actinin and connexin 43. Laser confocal microscopic view of immunocytochemistry of differentiated umbilical cord blood mesenchymal stem cells and cells transduced with human TERT gene to 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.

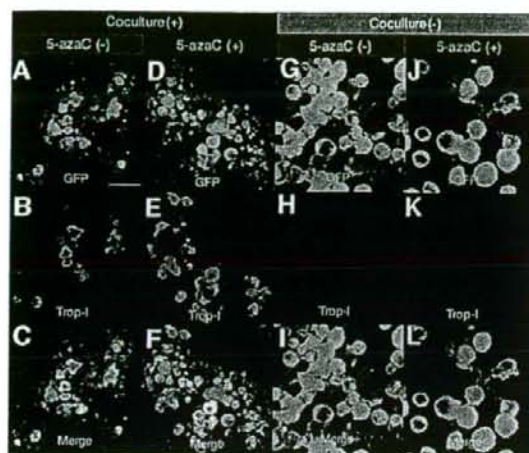


Figure 3. Calculation of cardiomyogenic transdifferentiation ratio of umbilical cord blood mesenchymal stem cells (UCBMSCs). (A–L): Representative laser confocal image of cardiac troponin-I (B, E, H, K); Troponin-I, red) staining to calculate cardiomyogenic transdifferentiation rate of UCBMSCs. Upper 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 Troponin-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 Troponin-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 Troponin-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; Troponin-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 cultured 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

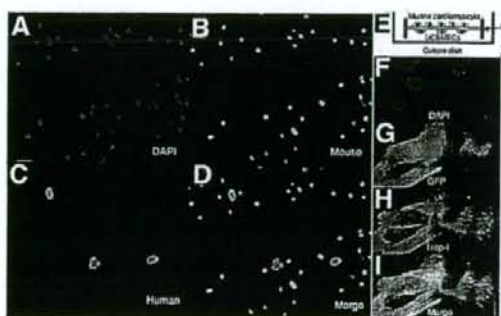


Figure 4. Cell fusion-independent cardiomyogenesis in UCBMSCs. (A–D): Representative images of fluorescent in situ hybridization for human nucleus and mouse nucleus are shown. Nuclei are stained with DAPI (A); blue). Mouse nuclei were detected as green (B) and human nuclei were detected as red (C). Superimposed 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, Troponin-I) are shown (I). Scale bars in the figure denote 20 μ m. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; Troponin-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.

STEM CELLS

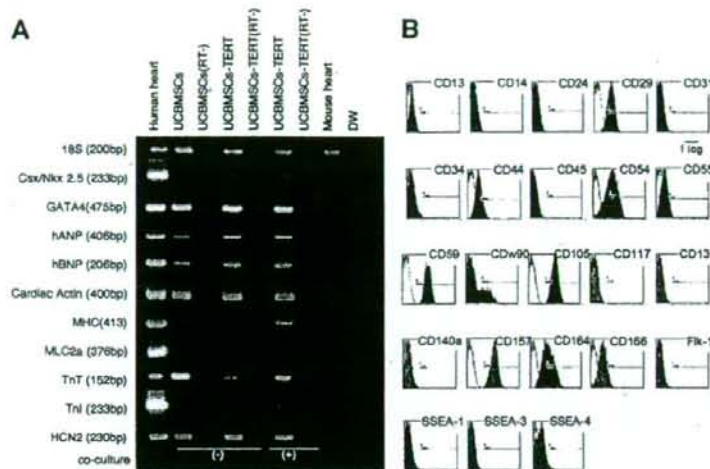


Figure 5. Expression of cardiomyocyte-specific genes in UCBMSCs and cell surface markers of UCBMSCs. (A): Expression of cardiomyocyte-specific genes in UCBMSCs and UCBMSCs-TERT. Reverse transcription-polymerase chain reaction (PCR) was performed with PCR primers with specificity for human genes encoding 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

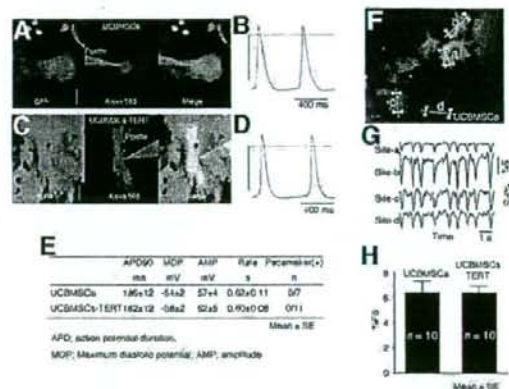


Figure 6. Functional analysis of UCBMSCs and UCBMSCs-TERT. Representative fluorescent microscopic images during action potential (AP) recording are shown (A, C). Immediately after the AP recordings, alexa568 dye (red) was injected into the cell via the same recording electrode to confirm that the recorded 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

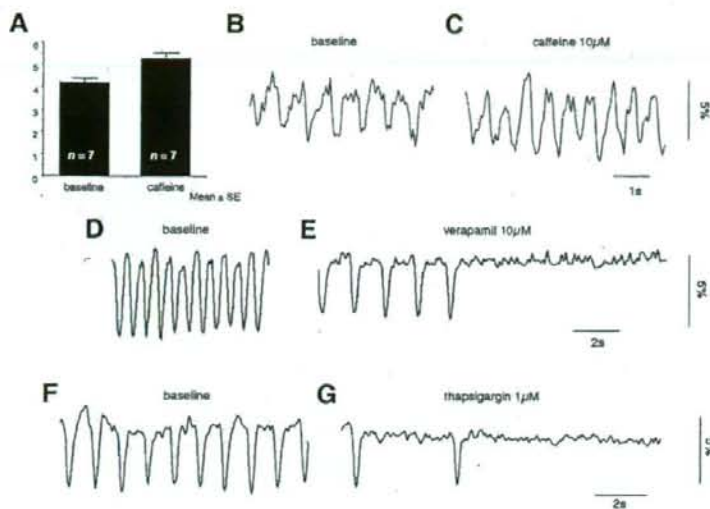


Figure 7. The effect of the drug administration on contraction of differentiated umbilical cord blood mesenchymal stem cells (UCBMSCs). The detected fractional shortening (% FS) of the differentiated UCBMSCs before and after the administration of caffeine (A–C), verapamil (D, E), and thapsigargin (F, 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 pluripotent 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.

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

Nobuhiro Nishiyama, Shunichiro Miyoshi, Naoko Hida, Taro Uyama, Kazuma Okamoto, Yukinori Ikegami, Kenji Miyado, Kaoru Segawa, Masanori Terai, Michiie Sakamoto, Satoshi Ogawa and Akihiro Umezawa
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 **AlphaMed Press**

Menstrual Blood-derived Cells Confer Human Dystrophin Expression in the Murine Model of Duchenne Muscular Dystrophy via Cell Fusion and Myogenic Transdifferentiation[□]

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Duchenne muscular dystrophy (DMD), the most common lethal genetic disorder in children, is an X-linked recessive muscle disease characterized by the absence of dystrophin at the sarcolemma of muscle fibers. We examined a putative endometrial progenitor obtained from endometrial tissue samples to determine whether these cells repair muscular degeneration in a murine mdx model of DMD. Implanted cells conferred human dystrophin in degenerated muscle of immunodeficient mdx mice. We then examined menstrual blood-derived cells to determine whether primarily cultured nontransformed cells also repair dystrophied muscle. In vivo transfer of menstrual blood-derived cells into dystrophic muscles of immunodeficient mdx mice restored sarcolemmal expression of dystrophin. Labeling of implanted cells with enhanced green fluorescent protein and differential staining of human and murine nuclei suggest that human dystrophin expression is due to cell fusion between host myocytes and implanted cells. In vitro analysis revealed that endometrial progenitor cells and menstrual blood-derived cells can efficiently transdifferentiate into myoblasts/myocytes, fuse to C2C12 murine myoblasts by in vitro coculturing, and start to express dystrophin after fusion. These results demonstrate that the endometrial progenitor cells and menstrual blood-derived cells can transfer dystrophin into dystrophied myocytes through cell fusion and transdifferentiation in vitro and in vivo.

INTRODUCTION

Skeletal muscle consists predominantly of syncytial fibers with peripheral, postmitotic myonuclei, and its intrinsic repair potential in adulthood relies on the persistence of a resident reserve population of undifferentiated mononuclear cells, termed "satellite cells." In mature skeletal muscle, most satellite cells are quiescent and are activated in response to environmental cues, such as injury, to mediate postnatal muscle regeneration. After division, satellite cell progeny, termed myoblasts, undergo terminal differentiation and become incorporated into muscle fibers (Bischoff, 1994). Myogenesis is regulated by a family of myogenic transcription factors including MyoD, Myf5, myogenin, and MRF4 (Sabourin and Rudnicki, 2000). During embryonic development, MyoD and Myf5 are involved in the establishment of the skeletal muscle lineage (Rudnicki *et al.*, 1993), whereas myogenin is required for terminal differentiation (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). During muscle

repair, satellite cells recapitulate the expression program of the myogenic genes manifested during embryonic development.

Dystrophin is associated with a large oligomeric complex of glycoproteins that provide linkage to the extracellular membrane (Ervasti and Campbell, 1991). In Duchenne muscular dystrophy (DMD), the absence of dystrophin results in destabilization of the extracellular membrane-sarcolemma-cytoskeleton architecture, making muscle fibers susceptible to contraction-associated mechanical stress and degeneration. In the first phase of the disease, new muscle fibers are formed by satellite cells. After depletion of the satellite cell pool in childhood, skeletal muscles degenerate progressively and irreversibly and are replaced by fibrotic tissue (Cossu and Mavilio, 2000). Like DMD patients, the mdx mouse lacks dystrophin in skeletal muscle fibers (Hoffman *et al.*, 1987; Sicinski *et al.*, 1989). However, the mdx mouse develops only a mild dystrophic phenotype, probably because muscle regeneration by satellite cells is efficient for most of the animal's life span (Cossu and Mavilio, 2000).

Myoblasts represent the natural first choice in cellular therapeutics for skeletal muscle because of their intrinsic myogenic commitment (Grounds *et al.*, 2002). However, myoblasts recovered from muscular biopsies are poorly expandable in vitro and rapidly undergo senescence (Cossu and Mavilio, 2000). An alternative source of muscle progenitor cells is therefore desirable. Cells with a myogenic potential are present in many tissues, and these cells readily

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form skeletal muscle in culture (Gerhart *et al.*, 2001). We report here that human dystrophin expression in the mdx model of DMD is attributed to cell fusion of mdx myocytes with human menstrual blood-derived stromal cells.

MATERIALS AND METHODS

Isolation of Human Endometrial Cells from Menstrual Blood

Menstrual blood samples ($n = 21$) were collected in DMEM with antibiotics (final concentrations: 100 U/ml penicillin/streptomycin) and 2% fetal bovine serum (FBS), and processed within 24 h. Ethical approval for tissue collection was granted by the Institutional Review Board of the National Research Institute for Child Health and Development, Japan. The centrifuged pellets containing endometrium-derived cells were resuspended in high-glucose DMEM medium (10% FBS, penicillin/streptomycin), maintained at 37°C in a humidified atmosphere containing 5% CO₂, and allowed to attach for 48 h. Nonadherent cells were removed by changing the medium. When the culture reached subconfluence, the cells were harvested with 0.25% trypsin and 1 mM EDTA and plated to new dishes. After 2–3 passages, the attached endometrial stromal cells were devoid of blood cells. Human EM-E6/E7/hTERT-2 cells, endometrium-derived progenitors, were obtained from surgical endometrial tissue samples and were immortalized by E6, E7, and hTERT (Kyo *et al.*, 2003). C2C12 myoblast cells were supplied by RIKEN Cell Bank (The Institute of Physical and Chemical Research, Japan).

Flow Cytometric Analysis

Flow cytometric analysis was performed as previously described (Terai *et al.*, 2005). Cells were incubated with primary antibodies or isotype-matched control antibodies, followed by additional treatment with the immunofluorescent secondary antibodies. Cells were analyzed on an EPICS ALTRA analyzer (Beckman Coulter, Fullerton, CA). Antibodies against human CD13, CD14, CD29, CD31, CD34, CD44, CD45, CD50, CD54, CD55, CD59, CD73, CD90, CD105, CD117 (c-kit), CD133, HLA-ABC, and HLA-DR were purchased from Beckman Coulter, Immunotech (Marseille, France), Cytotech (Hellebaek, Denmark), and BD Biosciences Pharmingen (San Diego, CA).

In Vitro Lentivirus-mediated Gene (EGFP) Transfer into EM-E6/E7/hTERT-2 Cells

Infection of EM-E6/E7/hTERT-2 cells with lentivirus having a CMV promoter and enhanced green fluorescent protein (EGFP) reporter resulted in high levels of EGFP expression in all cells. Cells were analyzed for EGFP expression by flow cytometry (Miyoshi *et al.*, 1997, 1998).

In Vitro Myogenesis

Menstrual blood-derived cells or EM-E6/E7/hTERT-2 cells were seeded onto collagen I-coated cell culture dishes (Bioscoat, BD Biosciences, Bedford, MA) at a density of 1×10^4 /ml in growth medium (DMEM, supplemented with 20% FBS). Forty-eight hours after seeding onto collagen I-coated dishes, cells were treated with 5-azacytidine for 24 h. Cell cultures were then washed twice with PBS and maintained in differentiation medium (DMEM, supplemented with either 2% horse serum (HS) or 1% insulin-transferrin-selenium supplement [ITS]). The differentiation medium was changed twice a week until the experiment was terminated.

RT-PCR Analysis of EM-E6/E7/hTERT-2 Cells and Menstrual Blood-derived Cells

Total RNA was prepared using IsoGen (Nippon Gene, Tokyo, Japan). Human skeletal muscle RNA was purchased from TOYOBO (Osaka, Japan). RT-PCR of Myf5, MyoD, desmin, myogenin, myosin heavy chain-I α /d (MyHC-I α /d), and dystrophin was performed with 2 μ g of total RNA. RNA for RT-PCR was converted to cDNA with a first-stand cDNA synthesis kit (Amersham Pharmacia Biotechnology, Piscataway, NJ) according to the manufacturer's recommendations. The sequences of PCR primers that amplify human but not mouse genes are listed in Supplementary Table 1. PCR was performed with TaKaRa recombinant Taq (Takara Shuzo, Kyoto, Japan) for 30 cycles, with each cycle consisting of 94°C for 30 s, 62°C or 65°C for 30 s, and 72°C for 20 s, with an additional 10-min incubation at 72°C after completion of the last cycle.

Immunohistochemical and Immunocytochemical Analysis

Immunohistochemical analysis was performed as previously described (Mori *et al.*, 2005). Briefly, the sections were incubated for 1 h at room temperature with mouse mAb against vimentin (Cone V9, DakoCytomation, Fort Collins, CO). After washing in PBS, sections were incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin, diluted, and washed in cold PBS. Staining was developed by using a solution containing diaminobenzidine and 0.01% H₂O₂ in 0.05 M Tris-HCl buffer, pH 6.7. Slides were

counterstained with hematoxylin. In the cases of fluorescence, frozen sections fixed with 4% PFA were used. The antibodies against human dystrophin (NCL-DYS3; Novocastra, Newcastle upon Tyne, United Kingdom) or anti-human nuclei mouse mAb (clone 235-1, Chemicon, Temecula, CA) was used as a first antibody, and goat anti-mouse IgG conjugated with Alexa Fluor 488 or goat anti-mouse IgG antibody conjugated with Alexa Fluor 546 (Molecular Probes, Eugene, OR) was used as a second antibody.

Immunocytochemical analysis was performed as previously described (Mori *et al.*, 2005), with antibodies to skeletal myosin (Sigma, St. Louis, MO; product no. M 4276), MF20 (which reacts with all sarcomere myosin in striated muscles, Developmental Studies Hybridoma Bank, University of Iowa, IA), α -sarcomeric actin (Sigma, product no. A 7811), and desmin (BioScience Products, Emmenbruecke, Switzerland; no. 010031, clone: D9) in PBS containing 1% bovine serum albumin. As a methodological control, the primary antibody was omitted. In the cases of fluorescence, slides were incubated with Alexa Fluor 546-conjugated goat anti-mouse IgG antibody.

Western Blotting

Western blot analysis was performed as previously described (Mori *et al.*, 2005). Blots were incubated with primary antibodies (desmin, myogenin [Clone F5D, Santa Cruz Biotechnology], and dystrophin [NCL-DYSA, Novocastra]) for 1–2 h at room temperature. After washing three times in the blocking buffer, blots were incubated for 30 min with a horseradish peroxidase-conjugated secondary antibody (0.04 μ g/ml) directed against the primary antibody. The blots were developed with enhanced chemiluminescence substrate according to the manufacturer's instructions.

Fusion Assay

EM-E6/E7/hTERT-2 cells (2500/cm²) or EGFP-labeled EM-E6/E7/hTERT-2 cells (2500/cm²) were cocultured with C2C12 myoblasts (2500/cm²) for 2 d in DMEM supplemented with 10% FBS and then cultured for 7 additional days in DMEM with 2% HS to promote myotube formation. The cultures were fixed in 4% paraformaldehyde and stained with a mouse anti-human nuclei IgG1 mAb and the mouse anti-human dystrophin IgG2a mAb (or anti-myosin heavy chain IgG2b mAb MF-20). The cells were visualized with appropriate Alexa-fluor-conjugated goat anti-mouse IgG1 and IgG2a (or IgG2b) secondary antibodies (Molecular Probes). Total cell nuclei were stained with DAPI (4',6'-diamidino-2-phenylindole).

In Vivo Cell Implantation

Six- to 8-wk-old NOD/Shi-scid/IL-2 receptor $-/-$ (NOG, CREA, Shizuoka, Japan) mice and 6- to 8-wk-old mdx-scid mice were implanted with EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells in seven independent experiments. The cells (2×10^7) were suspended in PBS in a total volume of 100 μ l and were directly injected into the right thigh muscle of NOG mice or mdx-scid mice. The mice were examined 3 wk after cell implantation, and the right thigh muscle was analyzed for human vimentin and dystrophin by immunohistochemistry. The antibodies to vimentin and dystrophin (NCL-DYS3) react with human vimentin and dystrophin-equivalent protein, but not murine protein.

RESULTS

Surface Marker Expression of Endometrium-derived Cells

We investigated myogenic differentiation of primary cells without gene introduction from menstrual blood, because menstrual blood on the first day of the period is considered to include endometrial tissue. We successfully cultured a large number of primary cells from menstrual blood. Menstrual blood-derived cells showed at least two morphologically different cell groups: small spindle-like cells and large stick-like cells, regarded as being passage day (PD) 1 or 2 (Figure 1, A and B, respectively). Surface markers of the menstrual blood-derived cells were evaluated by flow cytometric analysis. Surface markers of EM-E6/E7/hTERT-2 cells (Figure 1C) and menstrual blood-derived cells (Figure 1D) were evaluated by flow cytometric analysis (Figure 1E). In these experiments, the cells were cultured in the absence of any inductive stimuli. EM-E6/E7/hTERT-2 cells were positive for CD13, CD29 (integrin β 1), CD44 (Pgp-1/ly24), CD54, CD55, CD59, CD73, and CD90 (Thy-1), implying that EM-E6/E7/hTERT-2 cells expressed mesenchymal cell-related antigens in our experimental setting. Menstrual blood-derived cells were positive for CD13, CD29, CD44, CD54, CD55, CD59, CD73, CD90, and CD105, implying that proliferating

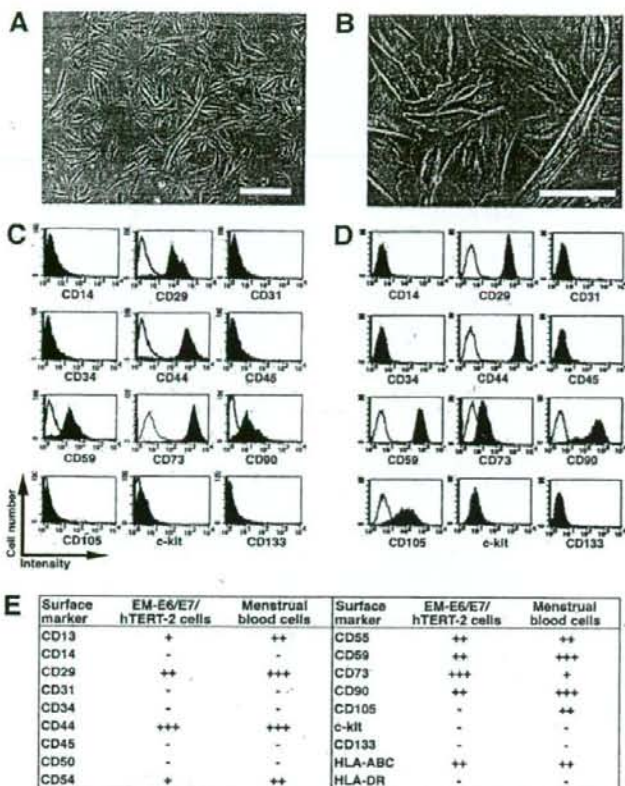


Figure 1. Surface marker expression of endometrium-derived cells. (A and B) Morphology of menstrual blood-derived cells, regarded as being PD 1 or 2. Scale bars, 200 μ m (A), 100 μ m (B). (C and D) Flow cytometric analysis of cell surface markers of EM-E6/E7/hTERT-2 cells (C) and menstrual blood-derived cells (D). (E) Further phenotypic analysis in EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells are summarized. Peak intensity was estimated in comparison with isotype controls. +++, strongly positive (>100 times the isotype control); ++, moderately positive (<100 times but more than 10 times the isotype control); +, weakly positive (<10 times but more than twice the isotype control); -, negative (less than twice the isotype control).

erated and propagated cells express mesenchymal cell-related cell surface markers. Unlike EM-E6/E7/hTERT-2 cells, the menstrual blood-derived adherent cells were positive for CD105. EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells expressed neither hematopoietic lineage markers, such as CD34, nor monocyte-macrophage antigens such as CD14 (a marker for macrophage and dendritic cells), or CD45 (leukocyte common antigen). The lack of expression of CD14, CD34, or CD45 suggests that EM-E6/E7/hTERT-2 cells and the menstrual blood-derived cell culture in the present study is depleted of hematopoietic cells. The cells were also negative for expression of CD31 (PECAM-1), CD50, c-kit, and CD133. The cell population was positive for HLA-ABC, but not for HLA-DR. These results demonstrate that almost all cells derived from endometrium are of mesenchymal origin or stromal origin.

Implanted Endometrium-derived Cells Induce De Novo Myogenesis in Immunodeficient NOG Mice

EM-E6/E7/hTERT-2 cells originate from the endometrial gland and are considered as endometrial progenitor cells or bipotential cells capable of differentiating into both glandular epithelial cells and endometrial stromal cells (Kyo et al., 2003). To determine whether EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells generate complete endometrial structure in vivo, like endometriosis, the cells without any treatment or induction were injected into the right thigh

muscle of immunodeficient NOG mice. PBS without cells was injected into the left thigh muscles as a control. We failed to detect any endometrial structure in the cell-injected site. Immunohistochemical analysis using an antibody specific to human vimentin, an intermediate filament associated with a mesenchymal cell, revealed that the injected EM-E6/E7/hTERT-2 cells (Figure 2, A–F) or menstrual blood-derived cells (Figure 2, G–J) extensively migrated or infiltrated between muscular fibers (Figure 2, arrowheads). To investigate if the donor cells between muscular fibers occur as a result of cell migration, we performed a time-course analysis of implanted cells, as probed by human-specific antibody to vimentin (Supplementary Figure 1). Donor cells at 3 h after implantation are observed at the injection site, which is considered to be due to just injection of cells. Cells at 1–3 wk after implantation are detected between myocytes in the muscle bundle or muscular fascicle as well as in the interstitial tissue, implying that the donor cells between myotubes result from cell migration. Interestingly, some of the vimentin-positive implanted cells exhibited round-shaped structure (Figure 2, D, F, and J, arrows), suggesting that endometrium-derived cells are capable of differentiating into myoblasts/myotubes, and can contribute to skeletal muscle repair in patients suffering from genetic disorders such as DMD, similar to previous reports for marrow stromal cells (Dezawa et al., 2005) and synovial membrane cells (De Bari et al., 2003).

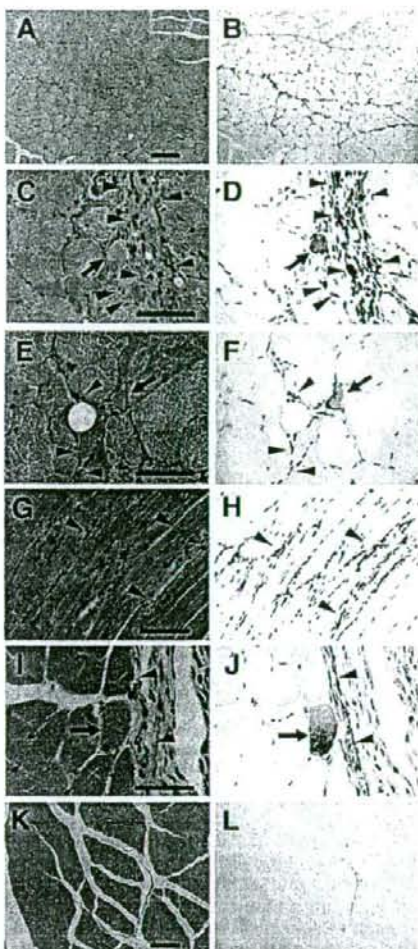


Figure 2. Implantation of endometrium-derived cells into the muscle of NOG mice. EM-E6/E7/hTERT-2 cells (A–F) or menstrual blood-derived cells (G–L) cultured in absence of any stimuli were directly injected into the right thigh muscle of NOG mice. Immunohistochemical analysis was performed using antibody that reacts to human vimentin but not to murine vimentin. (A, C, E, G, I, and K) hematoxylin and eosin stain. (B, D, F, H, J, and L) immunohistochemistry. Note that vimentin-positive EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells with a spindle morphology (C–J, arrowheads) extensively migrated into muscular bundles at 3 wk after injection, and some of the injected cells exhibited round structure (D, F, and J, arrows). Isotype mouse IgG1 served as a negative control (L). Scale bars, 100 μ m (A, B, K, and L), 50 μ m (C–F, I, and J), 90 μ m (G and H).

Induction of Myogenic Differentiation in Endometrial Progenitor Cells *In Vitro*

EM-E6/E7/hTERT-2 cells at 2 wk (cultured in the DMEM supplemented with 20% FBS) after exposure to different concentrations (5, 10, and 100 μ M) of 5-azacytidine were analyzed by immunostaining using anti-desmin antibody (Figure 3, A–E). The number of desmin-positive cells was

significantly higher in experimental groups with 5 or 10 μ M 5-azacytidine than in untreated control groups ($p < 0.05$) (Figure 3F). To investigate whether EM-E6/E7/hTERT-2 cells are capable of differentiating into skeletal muscle cells *in vitro*, the cells were exposed to 5 μ M 5-azacytidine for 24 h and then subsequently cultured in the DMEM supplemented with 2% HS or serum-free ITS for up to 21 d. Skeletal myoblastic differentiation of the cells was analyzed by evaluating expression of MyoD, Myf5, desmin, myogenin, MyHC-IIx/d, and dystrophin by RT-PCR. The MyoD, desmin, myogenin, and dystrophin genes were constitutively expressed, but MyHC-IIx/d and Myf5 genes were not. The decline of MyoD was observed in both the 2% HS (Figure 3, G and H) and the serum-free ITS (Figure 3K). The expression of MyHC-IIx/d, as determined by RT-PCR and immunocytochemistry, significantly increased with 2% HS (Figure 3G) and serum-free ITS (Figure 3K). Immunocytochemical analysis indicated that α -sarcomeric actin (Figure 3I) and MyHC (Figure 3J) were detected in the cells incubated with 2% HS for 21 d.

In Vitro Myogenic Differentiation of Menstrual Blood-Derived Cells

Menstrual blood-derived cells at 3 wk (cultured in DMEM supplemented with 20% FBS) after exposure to different concentrations (5, 10, and 100 μ M) of 5-azacytidine were analyzed by immunostaining using anti-desmin antibody (data not shown). The number of desmin-positive cells was significantly higher in experimental groups with 5 or 10 μ M 5-azacytidine than with 100 μ M 5-azacytidine; for further *in vitro* experiments, the menstrual blood-derived cells were exposed to 5 μ M 5-azacytidine for 24 h and then subsequently cultured in DMEM supplemented with low serum (2% HS) or serum-free ITS for up to 21 d (Figure 4). Myogenic potential of human menstrual blood-derived cells was analyzed by evaluating the expression of Myf5, MyoD, desmin, myogenin, MyHC-IIx/d, and dystrophin by RT-PCR. MyoD, desmin, and dystrophin genes were constitutively expressed in menstrual blood-derived cells, but MyHC-IIx/d and Myf5 were not (Figure 4A). For cells treated with 2% HS or serum-free ITS, the mRNA level of desmin and myogenin significantly increased after 3 d, and desmin steadily increased until day 21 (Figure 4, C and D). MyHC-IIx/d started to be expressed at a low level at day 21 of induction (Figure 4C). We then analyzed desmin expression by immunocytochemistry. Menstrual blood-derived cells were exposed to 5 μ M 5-azacytidine for 24 h and then subsequently cultured in DMEM supplemented with 20% FBS for up to 2 wk. Desmin was readily detected in colonies of the menstrual blood-derived cells (Figure 4B). Western blot analysis indicated that desmin, myogenin, and dystrophin were highly expressed in the cells incubated for 3 wk (Figure 4, E–G). These results suggest that menstrual blood-derived cells are, like the EM-E6/E7/hTERT-2 cells, able to differentiate into skeletal muscle.

Regeneration of Dystrophin by Cell Implantation in the DMD Model *mdx-scid* Mouse

To investigate whether human EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells can generate muscle tissue *in vivo*, cells without any treatment or induction were implanted directly into the right thigh muscles of *mdx-scid* mice (Supplementary Figure 2). The left thigh muscles were injected with PBS as an internal control. After 3 wk, myotubes in the muscle tissues injected with human EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells expressed human dystrophin as a cluster (Figure 5, A, C, and D, EM-

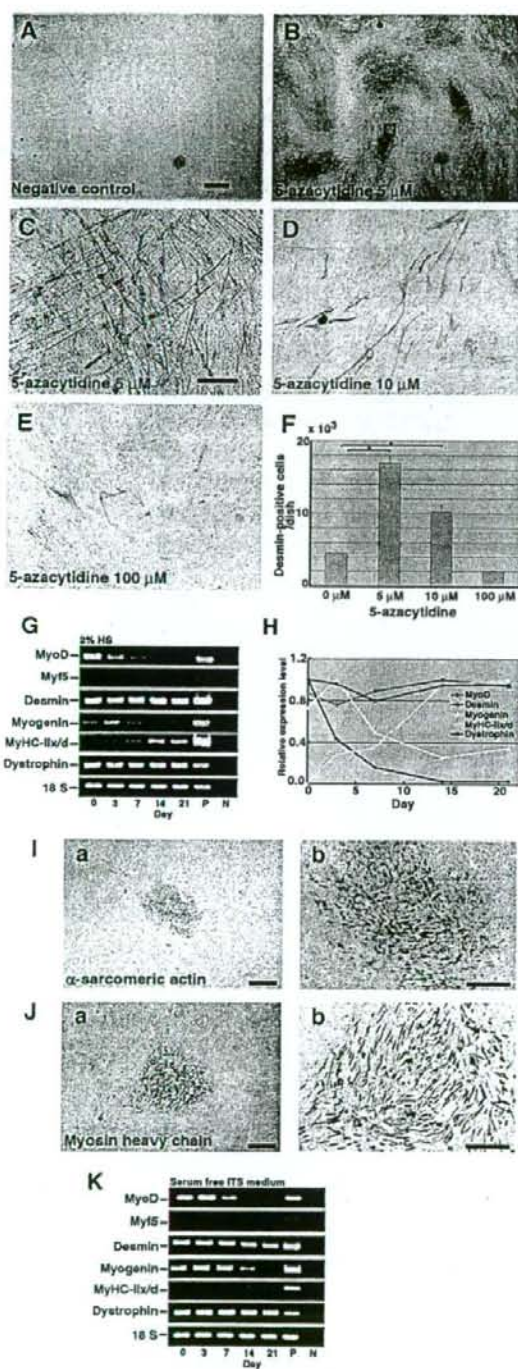


Figure 3. Expression of myogenic-specific genes during myogenic differentiation of EM-E6/E7/hTERT-2 cells. (A–E) Immunocytochemical

E6/E7/hTERT-2 cells, and 5B, menstrual blood-derived cells). Quantification analysis revealed that the percentage of dystrophin-positive myofibers after implantation of menstrual blood-derived cells was high, compared with that after implantation of EM-E6/E7/hTERT-2 cells (Figure 5E). Donor cells with EGFP fluorescence participated in myogenesis 3 wk after implantation (Supplementary Figure 3). EGFP-labeled EM-E6/E7/hTERT-2 cells became positive for human dystrophin (Figure 5C). Dystrophin was not detected in the muscle of mdx-scid mice and NOG mice without cell implantation because the antibody to dystrophin used in this study is human-specific, implying that dystrophin is transcribed from dystrophin genes of human donor cells but not from reversion of dystrophied myocytes in mdx-scid mice.

To determine if dystrophin expression in the donor cells is due to transdifferentiation or fusion, immunohistochemistry with an antibody against human nuclei (HuNucl) and DAPI stain was performed. If dystrophin expression is explained by fusion, dystrophin-positive myocytes must be demonstrated to have both human and murine nuclei. We examined almost all the 7- μ m-thick serial histological sections parallel to the muscular bundle (longitudinal section) of the muscular tissues by confocal microscopy and found that dystrophin-positive myocytes have nuclei derived from both human and murine cells in the longitudinal section of the myocytes (Figure 5D), implying that dystrophin expression is attributed to fusion between murine host myocytes and human donor cells, rather than myogenic differentiation of EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells per se.

Detection of Human Endometrial Cell Contribution to Myotubes in an In Vitro Myogenesis Model

To simulate in vivo phenomena, human endometrial cells were cocultured in vitro with murine C2C12 myoblasts for 2 d under proliferative conditions and then switched to differentiation conditions for an additional 7 d. Figure 6A provides an example of how human and mouse nuclei in the

analysis of EM-E6/E7/hTERT-2 cells using an antibody to desmin. (A) Omission of only the primary antibody to desmin serves as a negative control. (C) Higher magnification of inset in B. (F) Myogenic differentiation of EM-E6/E7/hTERT-2 cells with exposure to different concentrations (B, 5 μ M; C, 5 μ M; D, 10 μ M; E, 100 μ M) of 5-azacytidine. To estimate myogenic differentiation, the number of all the desmin-positive cells was counted for each dish ($n = 3$). Data were analyzed for statistical significance using ANOVA. EM-E6/E7/hTERT-2 cells were cultured in the DMEM supplemented with 2% HS, and serum-free ITS. (G and K) RT-PCR analysis with PCR primers allows amplification of the human MyoD, Myf5, desmin, myogenin, myosin heavy chain type IIx/d (MyHC-IIx/d), and dystrophin cDNA (from top to bottom). RNAs were isolated from EM-E6/E7/hTERT-2 cells at the indicated day after treatment with 5-azacytidine. RNAs from human muscle and H₂O served as positive (P) and negative (N) controls, respectively. Only the 18S PCR primer reacted with the human and murine cDNA. (H) Time course of MyoD, desmin, myogenin, MyHC-IIx/d, and dystrophin expression in the cells incubated with 2% HS for up to 21 d after 5-azacytidine treatment. Relative mRNA levels were determined using Multi Gauge Ver 2.0 (Fuji Film). The signal intensities of MyoD, desmin, and dystrophin mRNA at day 0, myogenin mRNA at day 3, and MyHC-II/d mRNA at day 21 were regarded as equal to 100%. (I and J) The cells were exposed to 5 μ M 5-azacytidine for 24 h and then subsequently cultured in DMEM supplemented with 2% HS for 21 d. α -Sarcomeric actin (I) and skeletal myosin heavy chain (J) was detected by immunocytochemical analysis. Scale bars, 2 mm (A and B), 300 μ m (C–E), 900 μ m (Ia and Ja), 425 μ m (Ib and Jb).