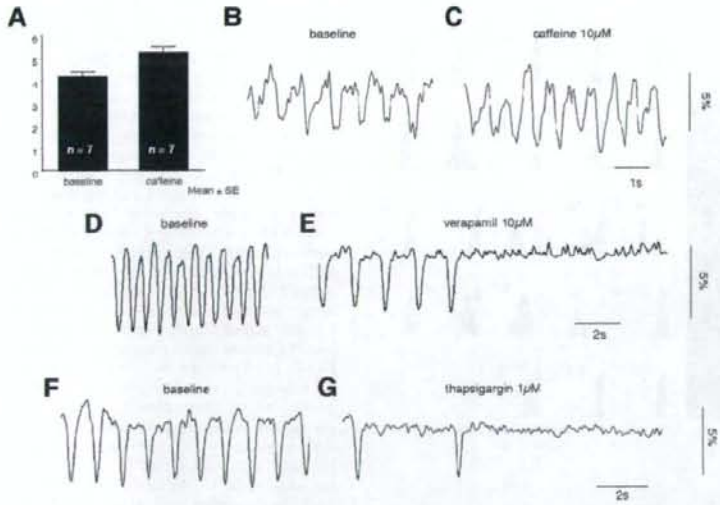

Figure 7. The effect of the drug administration on contraction of differentiated umbilical cord blood mesenchymal stem cells (UCBMSCs). The detected fractional shortening (% FS) of the differentiated UCBMSCs before and after the administration of caffeine (**A–C**), verapamil (**D, E**), and thapsigargin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

1 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.
2 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.
3 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.
4 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.
5 Klug MG, Soonpaa MH, Koh GY et al. Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. J Clin Invest 1996;98:216–224.
6 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.
7 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):I131–I136.
8 Taylor DA, Atkins BZ, Hungspreugs P et al. Regenerating functional myocardium: Improved performance after skeletal myoblast transplantation. Nat Med 1998;4:929–933.
9 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.
10 Orlic D, Kajstura J, Chimenti S et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701–705.
11 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.
12 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.
13 Gojo S, Gojo N, Takeda Y et al. In vivo cardiovasculogenesis by direct injection of isolated adult mesenchymal stem cells. Exp Cell Res 2003;288:51–59.
14 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.
15 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.
16 Assmus B, Schachinger V, Teupe C et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). Circulation 2002;106:3009–3017.
17 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.
18 Menasche P, Hagege AA, Scorsin M et al. Myoblast transplantation for heart failure. Lancet 2001;357:279–280.
19 Tomita S, Li RK, Weisel RD et al. Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 1999;100:II247–II256.
20 Stamm C, Westphal B, Kleine HD et al. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet 2003;361:45–46.
21 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.
22 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.
23 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.
24 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.
25 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.
26 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.
27 Mayani H, Lansdorp PM. Biology of human umbilical cord blood-derived hematopoietic stem/progenitor cells. STEM CELLS 1998;16:153–165.
28 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.
29 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.
30 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.
31 Lee OK, Kuo TK, Chen WM et al. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 2004;103:1669–1675.
32 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 Tsafrir 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.

See www.StemCells.com for supplemental material available online.

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

Stem Cells 2007;25;2017-2024; originally published online May 10, 2007;
DOI: 10.1634/stemcells.2006-0662

STEM CELLS
TISSUE-SPECIFIC STEM CELLS

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

NOBUHIRO NISHIYAMA,[a] SHUNICHIRO MIYOSHI,[a,b] NAOKO HIDA,[a,c] TARO UYAMA,[c] KAZUMA OKAMOTO,[d] YUKINORI IKEGAMI,[a] KENJI MIYADO,[c] KAORU SEGAWA,[f] MASANORI TERAI,[c] MICHIIE SAKAMOTO,[e] SATOSHI OGAWA,[a] AKIHIRO UMEZAWA[c]

[a]Cardiopulmonary Division, Keio University School of Medicine, Tokyo, Japan; [b]Keio University School of Medicine, Institute for Advanced Cardiac Therapeutics, Tokyo, Japan; [c]Department of Reproductive Biology and Pathology, National Research Institute for Child Health and Development, Tokyo, Japan; [d]Department of Surgery, [e]Department of Pathology, and [f]Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan

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 ([CM] 1×10^5 per cm^2). On day 5 of coculitivation, 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% ± 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% ± 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

Correspondence: Shunichiro Miyoshi, M.D., Ph.D., Cardiopulmonary Division of Keio University School of Medicine, 35-Shinanomachi Shinjuku-ku, Tokyo 160-8582, Japan. Telephone: +81-3-3353-1211 (ext. 62310); Fax: +81-3-3353-2502; e-mail: smiyoshi@cpnet.med.keio.ac.jp Received October 23, 2006; accepted for publication May 6, 2007; first published online in STEM CELLS *EXPRESS* May 10, 2007. 1066-5099/2007/$30.00/0 doi: 10.1634/stemcells.2006-0662

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 Sciences). 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 electrophoresis (−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 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% ± 3% of UCBMSCs became positive for human cardiac troponin-I antibody as a result of the cocultivation (Fig.

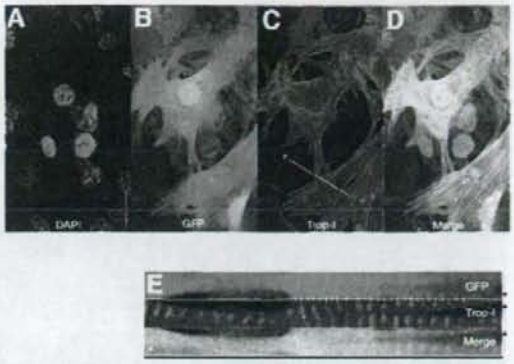

Figure 1. Cardiomyogenic transdifferentiation of umbilical cord blood mesenchymal stem cells. Laser confocal microscopic view of immunocytochemistry of differentiated umbilical cord blood mesenchymal stem cells with anti-cardiac troponin-I antibody. Superimposed images (Merge) of **(A–C)** are shown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

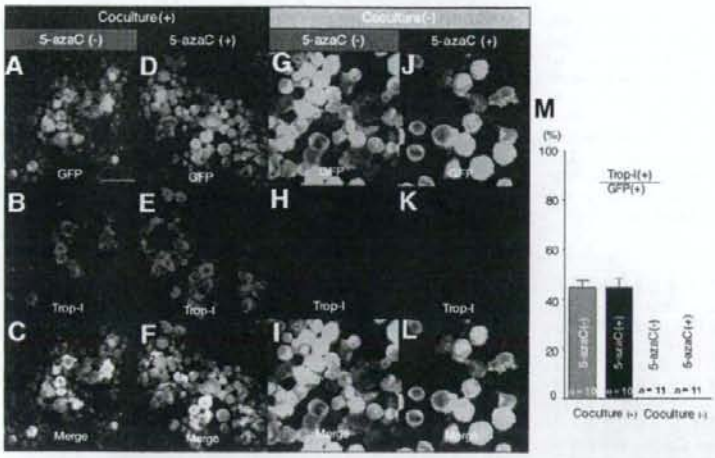

Figure 3. Calculation of cardiomyogenic transdifferentiation ratio of umbilical cord blood mesenchymal stem cells (UCBMSCs). **(A–L):** Representative laser confocal image of cardiac troponin-I ([**B, E, H, K**]; Trop-I, red) staining to calculate cardiomyogenic transdifferentiation rate of UCBMSCs. Upper bar denotes the culture conditions for each panel presented bellow. (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 μm. 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

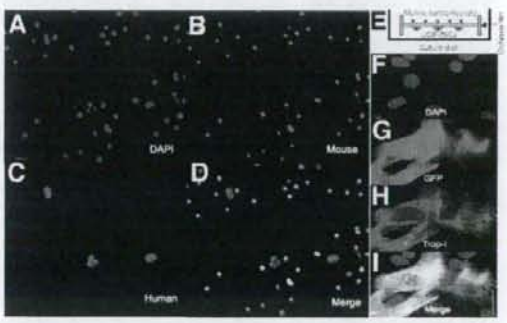

Figure 4. Cell fusion-independent cardiomyogenesis in UCBMSCs. **(A–D):** Representative images of fluorescent in situ hybridization for human nucleus and mouse nucleus are shown. Nuclei are stained with DAPI ([**A**]; blue). Mouse nuclei were detected as green (**B**) and human nuclei were detected 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-μm-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 μm. 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 β1), CD44 (Pgp-1/Iy-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.

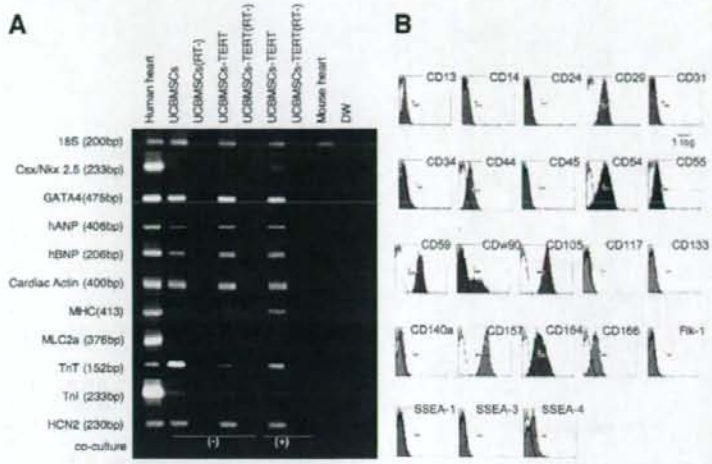

Figure 5. Expression of cardiomyocyte-specific genes in UCBMSCs and cell surface markers of UCBMSCs. **(A):** Expression of cardiomyocyte-specific genes in UCBMSCs and UCBMSCs-TERT. Reverse transcription-polymerase chain reaction (PCR) was performed with PCR primers with specificity for human genes encoding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

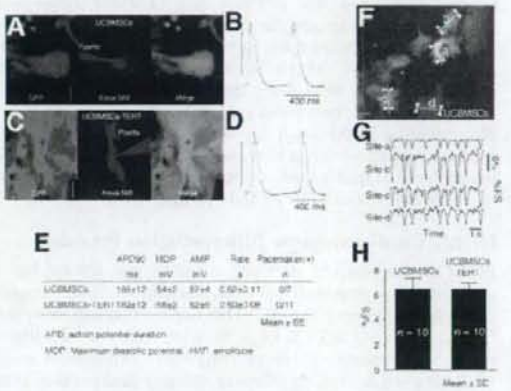

Figure 6. Functional analysis of UCBMSCs and UCBMSCs-TERT. Representative fluorescent microscopic images during action potential (AP) recording are shown **(A, C)**. Immediately after the AP recordings, alexa568 dye (red) was injected into the cell via the same recording electrode to confirm that the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

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

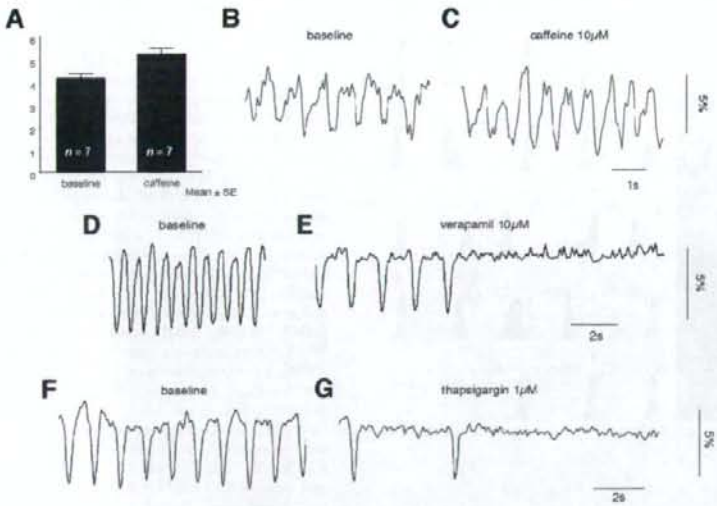

Figure 7. The effect of the drug administration on contraction of differentiated umbilical cord blood mesenchymal stem cells (UCBMSCs). The detected fractional shortening (% FS) of the differentiated UCBMSCs before and after the administration of caffeine (**A–C**), verapamil (**D, E**), and thapsigargin (**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 allogenic 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% ± 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% ± 3.6% of UCBMSCs; and (c) coculture 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

1 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.
2 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.
3 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.
4 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.
5 Klug MG, Soonpaa MH, Koh GY et al. Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. J Clin Invest 1996;98:216–224.
6 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.
7 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):I131–I136.
8 Taylor DA, Atkins BZ, Hungspreugs P et al. Regenerating functional myocardium: Improved performance after skeletal myoblast transplantation. Nat Med 1998;4:929–933.
9 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.
10 Orlic D, Kajstura J, Chimenti S et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701–705.
11 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.
12 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.
13 Gojo S, Gojo N, Takeda Y et al. In vivo cardiovasculogenesis by direct injection of isolated adult mesenchymal stem cells. Exp Cell Res 2003;288:51–59.
14 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.
15 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.
16 Assmus B, Schachinger V, Teupe C et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). Circulation 2002;106:3009–3017.
17 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.
18 Menasche P, Hagege AA, Scorsin M et al. Myoblast transplantation for heart failure. Lancet 2001;357:279–280.
19 Tomita S, Li RK, Weisel RD et al. Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 1999;100:II247–II256.
20 Stamm C, Westphal B, Kleine HD et al. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet 2003;361:45–46.
21 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.
22 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.
23 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.
24 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.
25 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.
26 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.
27 Mayani H, Lansdorp PM. Biology of human umbilical cord blood-derived hematopoietic stem/progenitor cells. STEM CELLS 1998;16:153–165.
28 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.
29 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.
30 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.
31 Lee OK, Kuo TK, Chen WM et al. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 2004;103:1669–1675.
32 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 Tsafrir 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.

See www.StemCells.com for supplemental material available online.

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

Stem Cells 2007;25;2017-2024; originally published online May 10, 2007;
DOI: 10.1634/stemcells.2006-0662

EXPERIMENTAL CELL RESEARCH 313 (2007) 2550–2562

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr

Research Article

'Working' cardiomyocytes exhibiting plateau action potentials from human placenta-derived extraembryonic mesodermal cells

Kazuma Okamoto[a,b], *Shunichiro Miyoshi*[c,d], *Masashi Toyoda*[a], *Naoko Hida*[a,c], *Yukinori Ikegami*[c], *Hatsune Makino*[a], *Nobuhiro Nishiyama*[c], *Hiroko Tsuji*[a,f], *Chang-Hao Cui*[a], *Kaoru Segawa*[e], *Taro Uyama*[a], *Daisuke Kami*[a], *Kenji Miyado*[a], *Hironori Asada*[f], *Kenji Matsumoto*[g], *Hirohisa Saito*[g], *Yasunori Yoshimura*[f], *Satoshi Ogawa*[c], *Ryo Aeba*[b], *Ryohei Yozu*[b], *Akihiro Umezawa*[a,*]

[a]*Department of Reproductive Biology and Pathology, National Research Institute for Child Health and Development, Tokyo, Japan*
[b]*Department of Surgery, Keio University School of Medicine, Tokyo, Japan*
[c]*Cardio-pulmonary Division of Keio University School of Medicine, Tokyo, Japan*
[d]*Institute for Advanced Cardiac Therapeutics, Keio University School of Medicine, Tokyo, Japan*
[e]*Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan*
[f]*Department of Obstetrics and Gynecology, Keio University School of Medicine, Tokyo, Japan*
[g]*Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo, Japan*

ARTICLE INFORMATION

Article Chronology:
Received 24 August 2006
Revised version received
19 April 2007
Accepted 24 April 2007
Available online 5 May 2007

Keywords:
Placenta
Co-culture
Cardiac differentiation

ABSTRACT

The clinical application of cell transplantation for severe heart failure is a promising strategy to improve impaired cardiac function. Recently, an array of cell types, including bone marrow cells, endothelial progenitors, mesenchymal stem cells, resident cardiac stem cells, and embryonic stem cells, have become important candidates for cell sources for cardiac repair. In the present study, we focused on the placenta as a cell source. Cells from the chorionic plate in the fetal portion of the human placenta were obtained after delivery by the primary culture method, and the cells generated in this study had the Y sex chromosome, indicating that the cells were derived from the fetus. The cells potentially expressed 'working' cardiomyocyte-specific genes such as cardiac myosin heavy chain 7β, atrial myosin light chain, cardiac α-actin by gene chip analysis, and Csx/Nkx2.5, GATA4 by RT-PCR, cardiac troponin-I and connexin 43 by immunohistochemistry. These cells were able to differentiate into cardiomyocytes. Cardiac troponin-I and connexin 43 displayed a discontinuous pattern of localization at intercellular contact sites after cardiomyogenic differentiation, suggesting that the chorionic mesoderm contained a large number of cells with cardiomyogenic potential. The cells began spontaneously beating 3 days after co-cultivation with murine fetal cardiomyocytes and the frequency of beating cells reached a maximum on day 10. The contraction of the cardiomyocytes was rhythmical and synchronous, suggesting the presence of electrical communication between the cells. Placenta-derived human fetal cells may be useful for patients who cannot supply bone marrow cells but want to receive stem cell-based cardiac therapy.

* *Corresponding author.* Fax: +81 3 5494 7048.
E-mail address: umezawa@1985.jukuin.keio.ac.jp (A. Umezawa).

0014-4827/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2007.04.028

Introduction

Major advances have been made in the prevention, diagnosis, and treatment of ischemic heart disease and cardiomyopathy, including the use of heart transplantation and artificial hearts. However, the number of patients suffering from heart disease is still increasing [1]. Morbidity and mortality from cardiovascular diseases continue to be an enormous burden experienced by many individuals, with substantial economic cost. Enthusiasm for cell therapy for the injured heart has already reached the clinical setting, with physicians in several countries involved in clinical trials using several types of cell populations [2,3]. Bone-marrow-derived mononuclear cells [4,5], unfractioned bone marrow cells [6], bone-marrow-derived $CD133^+$ cells [7], and myoblasts [8] have been injected into the ischemic heart clinically.

Mesenchymal stem cells (MSCs) are a potential cellular source for stem cell-based therapy, since they have the ability to proliferate and differentiate into mesodermal tissues, including the heart tissue, and entail no ethical problems [9]. Human MSCs have been used clinically to treat patients with graft versus host disease and osteogenesis imperfecta [10,11]. We previously showed that murine and human marrow-derived MSCs can differentiate into cardiomyocytes and start to beat synchronously in vitro [12,13]. In addition, we and other groups proposed that direct injection of murine MSCs into the heart is a feasible approach in murine models of ischemic heart disease and in the normal mouse heart [14,15]. Although MSC transplantation slightly improved impaired cardiac function, this effect was limited. One of the reasons for this may be due to an extremely low rate of cardiomyogenesis from marrow-derived MSCs in vitro [13] and in vivo [14–17]. In order to further improve cardiac function, we have been searching for another source of MSCs having highly cardiomyogenic potential.

The placenta is composed of the amniotic membrane, chorionic mesoderm, and decidua; the amniotic membrane and chorionic mesoderm are the fetal portion and the decidua is the maternal portion (Fig. 1A) [18]. Recently it was reported that the chorionic villi of the placenta differentiated into osteocytes, chondrocytes and adipocytes under specific culture conditions [19,20]. In this study, we generated cells with the mesenchymal phenotype from the chorionic mesoderm, and

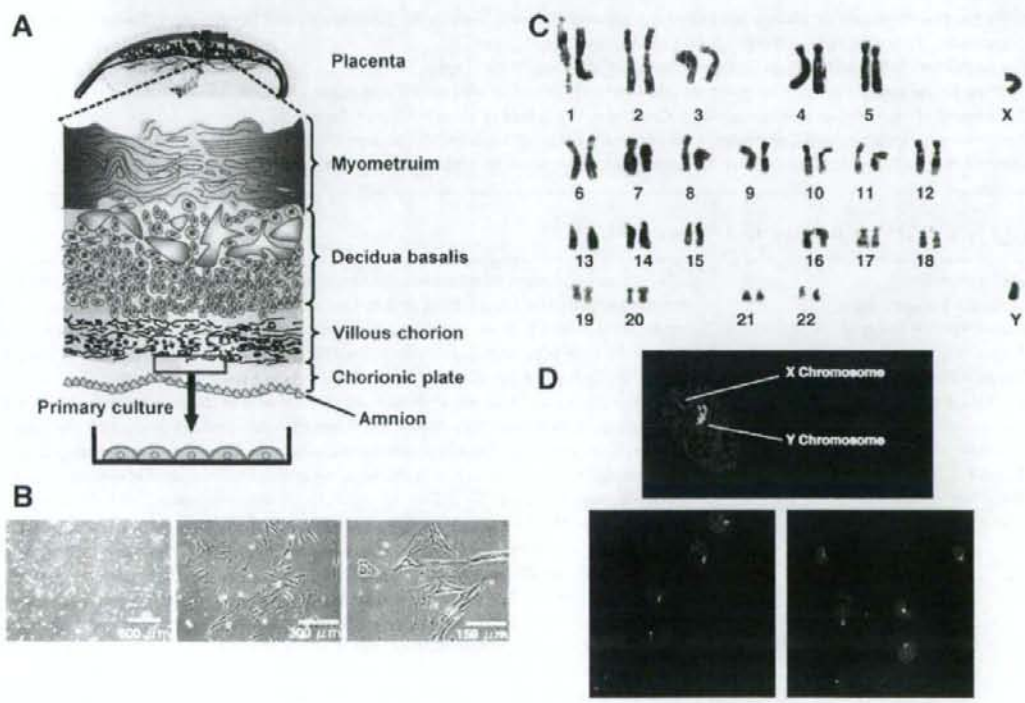

Fig. 1 – Establishment of chorionic plate cells. (A) Chorionic plate cells were established by primary culture of chorionic plate (red square in the chorionic mesoderm) in the human placenta. (B) Chorionic plate cells at PD 4 consisted of heterogeneous cell population. Three images show chorionic plate cells in the same culture dish. Their shape is different from that of fibroblasts. (C) Karyotyping by G-banding stain of chorionic plate cells. No chromosomal aberration was detected. (D) Chorionic plate cells have one X chromosome (red) and one Y chromosome (light blue). Nuclei were stained with DAPI (blue). (E) Flowcytometric analysis of chorionic plate cells using antibodies for CD14, CD29, CD31, CD34, CD44, CD45, CD59, CD73, CD90, CD105 and CD166. Black lines and shaded areas indicate reactivity of antibodies for isotype controls and that of antibodies for cell surface markers, respectively.

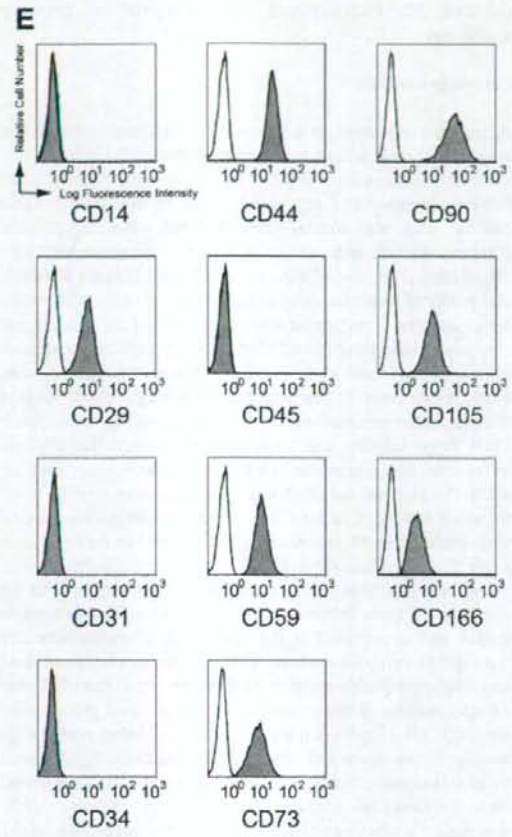

Fig. 1 (*continued*).

showed that: (a) physiologically functioning cardiomyocytes were transdifferentiated from human placenta-derived chorionic plate cells, but clear osteogenic and adipogenic phenotypes were not induced; (b) the cardiomyogenic induction rate obtained using our system was relatively high compared to that obtained using the previously described method [13]; (c) cocultivation with fetal murine cardiomyocytes alone without transdifferentiation factors such as 5-azaC or oxytocin is sufficient for cardiomyogenesis in our system; (d) chorionic plate cells have the electrophysiological properties of 'working' cardiomyocytes. The chorionic mesoderm contained a large number of cells with a cardiomyogenic potential.

Materials and methods

Chorionic plate cell culture

A human placenta was collected after delivery of a male neonate with informed consent. The study was approved by the ethics committee of Keio University, Tokyo, Japan (Number 17-44-1). To isolate chorionic plate cells, we used the explant culture method, in which the cells were outgrown from pieces of chorionic plate attached to dishes (Fig. 1A). Briefly, the decidua of the maternal part was separated and discarded. The chorionic plate from the fetal part were cut into pieces approximately 5 mm^3 in size. The pieces were washed in DMEM (high glucose; Kohjin Bio) supplemented with 100 U/ml penicillin-streptomycin (Gibco), 1 μg/ml Amphotericin B (Gibco) and 4 U/ml Novo-Heparin Injection 1000 (Mochidaseiyaku Co., Ltd.), until the supernatant was free of erythrocytes. Some pieces of chorionic plate were attached to the substratum in a 10-cm-diameter dish (Falcon, Becton, Dickinson and Company (BD), San Jose, CA, USA). Culture medium consisting of DMEM (high glucose; Kohjin Bio) supplemented with 10% FBS (CCT, Cansera, Canada) was added. The cells migrated out from the cut ends after approximately 20 days of incubation at 37 °C in 5% CO_2. The migrated cells were harvested with phosphate-buffered saline (PBS) with 0.1% trypsin and 0.25 mM EDTA (ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid) (Immuno-Biological Laboratories) for 5 min at 37 °C and counted. The harvested cells were re-seeded at a density of 3×10^5 cells in a 10-cm-diameter dish. Confluent monolayers of cells were subcultured at a 1:8 split ratio onto new 10-cm-diameter dishes and designated "chorionic plate cells". The culture medium was replaced with fresh culture medium every 3 or 4 days. The chorionic plate cells used in this study were within five to nine population doublings (approximately two to five passages).

Reverse transcriptase (RT)-PCR

Chorionic plate cells at PD 6 were dissociated with 0.1% trypsin and 0.25 mM EDTA for 5 min at 37 °C. Total RNA was extracted with RNeasy (Qiagen). Human cardiac RNA was purchased (Clontech). RNA for RT-PCR was converted to cDNA with Superscript (Invitrogen) according to the manufacturer's recommendations. RT-PCR was performed by using primers for the genes of cardiac transcription factors: Csx/Nkx-2.5, GATA4; a cardiac hormone: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP); cardiac structural proteins: cardiac troponin-I (cTnI), cardiac troponin T (cTnT), myosin light chain-2α (MLC-2α), cardiac actin; and 18s rRNA. 18s rRNA (18S) was used as an internal control. PCR was performed with recombinant Taq (Toyobo Co., Ltd.) or TaKaRa LA Taq with GC Buffer (Takara Shuzo Co., Ltd.) for 30 or 35 cycles, with each cycle consisting of 95 °C for 30 s, 55 °C, 61 °C or 65 °C for 45 s, and 2 °C for 45 s, with an additional 5-min incubation at 72 °C after completion of the final cycle. The PCR was performed in 50 μl of buffer (10 mmol/l Tris-HCl (pH 8.3), 2.5 mmol/l $MgCl_2$, and 50 mmol/l KCl) containing 1 mmol/l each of dATP, dCTP, dGTP, and dTTP, 2.5 U of Gene Taq (Nippon Gene), and 0.2 mol/l primers. The PCR products were size fractionated by 2% agarose gel electrophoresis.

Karyotyping of chorionic plate cells

Metaphase spreads were prepared from chorionic plate cells treated with 100 ng/ml colcemid (Karyo Max, Gibco Co. BRL) for 6 h. We performed karyotyping by G-banding stain on at least 30 metaphase spreads for each population. The CEP X/Y DNA Probe Kit (Vysis) was used to determine the proportion of XX and XY cells in accordance with the manufacturer's suggestions.

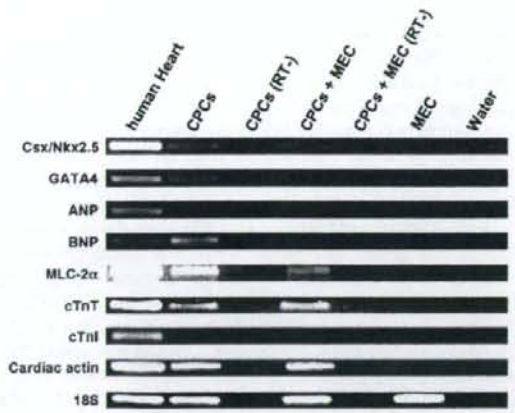

Fig. 2 – Gene expression of cardiomyocyte-specific/associated genes in chorionic plate cells. RT-PCR analysis revealed expression patterns of cardiomyocyte-specific or associated genes; *Csx/Nkx2.5, GATA4, ANP, BNP, cTnI, cTnT, cardiac actin* and *MLC-2α* (from left to right) in human heart, chorionic plate cells (CPCs), chorionic plate cells after co-culturing with murine embryonic cardiomyocytes (CPCs + MEC), murine embryonic cardiomyocytes (MEC) and water. "RT-" represented an omission of a reverse transcriptase treatment to RNA as negative control. Human heart RNA and water (without RNA) served as positive and negative control, respectively. 18s rRNA (18S) was amplified in parallel reactions as a housekeeping gene serving as an internal control.

Flow cytometric analysis

Chorionic plate cells were stained for 1 h at 4 °C with primary antibodies and immunofluorescent secondary antibodies. The cells were then analyzed on a Cytomics FC 500 (Beckman Coulter, Inc.) and the data were analyzed with the FlowJo Ver.7 (Tree Star, Inc.). Antibodies against human CD14 (6603511, Beckman Coulter), CD29 (Integrin-β1) (6604105, Beckman Coulter), CD31 (PECAM-1) (IM1431, Beckman Coulter), CD34 (IM1250, Beckman Coulter), CD44 (IM1219, Beckman Coulter, IM1219), CD45 (556828, Beckman Coulter), CD59 (IM3457, Beckman Coulter), CD73 (550257, BD Pharmingen), CD90 (Thy-1) (555596, BD Pharmingen), CD105 (Endoglin) (A07414, Beckman Coulter) and CD166 (ALCAM) (559263, BD Pharmingen) were adopted as primary antibodies.

Gene chip analysis

Human genomewide gene expression was examined with the Human Genome U133A Probe array (Affymetrix), which contains the oligonucleotide probe set for approximately 23,000 full-length genes and expressed sequence tags (ESTs). Total cellular RNA was immediately isolated with the RNeasy (Qiagen), according to the manufacture's instructions. Contaminating DNA was eliminated by DNase I (Takara Bio Inc.). The purity of RNA was assessed on the basis of the A260/A280 ratio, and the integrity of RNA was verified by agarose gel electrophoresis. Double-stranded cDNA was synthesized from DNase-treated total RNA, and the cDNA was subjected to in vitro transcription in the presence of biotinylated nucleoside triphosphates, according to the manufacturer's protocol (One-Cycle Target Labeling and Control Reagent package [http://www.affymetrix.com/support/technical/manual/expression_manual.affx]). The biotinylated cRNA was hybridized with a probe array for 16 h at 45 °C, and the hybridized biotinylated cRNA was stained with streptavidin-PE and scanned with a Hewlett-Packard Gene Array Scanner (Palo Alto). The fluorescence intensity of each probe was quantified by using the GeneChip Analysis Suite 5.0 computer program (Affymetrix). The expression level of a single mRNA was determined as the average fluorescence intensity among the intensities obtained with 11 paired (perfectly matched and single-nucleotide-mismatched) probes consisting of 25-mer oligonucleotides. If the intensities of mismatched probes were very high, gene expression was judged to be absent, even if high average fluorescence was obtained with the GeneChip Analysis Suite 5.0 program. The level of gene expression was determined with the GeneChip software as the average difference (AD). Specific AD levels were then calculated as the percentage of the mean AD level of six probe sets for housekeeping genes (*actin* and *GAPDH* [glyceraldehyde-3-phosphate dehydrogenase] genes). Further data analysis was performed with Genespring software version 5 (Silicon Genetics). To normalize the staining intensity variations among chips, the AD values for all genes on a given chip were divided by the median of all measurements on that chip. To eliminate changes within the range of background noise and to select the most differentially expressed genes, data were used only if the raw data values were less than 100 AD and gene expression was judged to be present by the Affymetrix data analysis.

Table 1 – RT-PCR primers used in this study

	Primer (sense)	Primer (anti-sense)	Annealing temperature (°C)	Product size (bp)
Csx/Nkx-2.5	CTTCAAGCCAGAGGCCTACG	CCGCCTCTGTCTTCTCCAGC	61	233
GATA4	GACGGGTCACTATCTGTGCAAC	AGACATCGCACTGACTGAGAAC	61	475
ANP	GAACCAGAGGGGAGAGACAGAG	CCCTCAGCTTGCTTTTTAGGAG	55	406
BNP	CATTTGCAGGGCAAACTGTC	CATCTTCCTCCCAAAGCAGC	55	206
MLC-2α	GAAGGTGAGTGTCCCAGAGG	ACAGAGTTTATTGAGGTGCCCC	65	376
cTnT	GGCAGCGGAAGAGGATGCTGAA	GAGGCACCAAGTTGGGCATGAACGA	65	152
cTnI	CCCTGCACCAGCCCCAATCAGA	CGAAGCCCAGCCCGGTCAACT	65	233
Cardiac actin	CTTCCGCTGTCCTGAGACAC	CCTGACTGGAAGGTAGATGG	61	400
18S	GTGGAGCGATTTGTCTGGTT	CGCTGAGCCAGTCAGTGTAG	55	200

Table 2 – Human cardiomyocyte-specific or -associated gene expression profiling of undifferentiated and differentiated chorionic plate cells (CPCs)

Systematic	Common	Undifferentiated CPCs		Differentiated CPCs		Human heart	Description
207317_s_at	CASQ2	34	A	12	A	P	Calsequestrin 2 (cardiac muscle)
205553_s_at	CSRP3	8	A	5	A	P	Cysteine and glycine-rich protein 3 (cardiac LIM protein)
208040_s_at	MYBPC3	56	A	117	A	P	Myosin binding protein C, cardiac
214468_at	MYH6	11	A	50	A	P	Myosin, heavy polypeptide 6, cardiac muscle, alpha (cardiomyopathy, hypertrophic 1)
204737_s_at	MYH7	5	A	161	P	P	Myosin, heavy polypeptide 7, cardiac muscle, beta
216265_x_at	MYH7	36	A	232	A	P	Myosin, heavy polypeptide 7, cardiac muscle, beta
215795_at	MYH7B	89	A	11	A	P	Myosin, heavy polypeptide 7B, cardiac muscle, beta
209742_s_at	MYL2	267	A	153	A	P	Myosin, light polypeptide 2, regulatory, cardiac, slow
210088_x_at	MYL4	338	P	404	A	P	Myosin, light polypeptide 4, alkali; atrial, embryonic
210395_x_at	MYL4	220	P	412	P	P	Myosin, light polypeptide 4, alkali; atrial, embryonic
219942_at	MYL7	9	A	16	A	P	Myosin, light polypeptide 7, regulatory
207557_s_at	RYR2	11	A	13	A	P	Ryanodine receptor 2 (cardiac)
214044_at	RYR2	17	A	23	A	P	Ryanodine receptor 2 (cardiac)
205742_at	TNNI3	96	A	195	A	P	Troponin I, cardiac
215389_s_at	TNNT2	83	A	165	A	P	Troponin T2, cardiac
205132_at	ACTC	289	P	671	P	P	Actin, alpha, cardiac muscle
206029_at	ANKRD1	214	P	13	A	P	Ankyrin repeat domain 1 (cardiac muscle)
213738_s_at	ATP5A1	5905	P	2657	P	P	ATP synthase, H^+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1, cardiac muscle
205444_at	ATP2A1	107	A	53	A	A	ATPase, Ca^{++} transporting, cardiac muscle, fast twitch 1
209186_at	ATP2A2(SERCA2A)	4465	P	2577	P	P	ATPase, Ca^{++} transporting, cardiac muscle, slow twitch 2
212361_s_at	ATP2A2(SERCA2A)	814	P	338	A	P	ATPase, Ca^{++} transporting, cardiac muscle, slow twitch 2
212362_at	ATP2A2(SERCA2A)	178	P	60	A	A	ATPase, Ca^{++} transporting, cardiac muscle, slow twitch 2
207317_s_at	CASQ2	34	A	12	A	P	Calsequestrin 2 (cardiac muscle)
65472_at		6	A	17	A	A	qb80a04.x1 Soares_fetal_heart_NbHH19W *Homo Sapiens* cDNA clone IMAGE:1706382 3' similar to TR:O21123 O21123 CYTOCHROME OXIDASE I; mRNA sequence
205298_s_at	BTN2A2	482	P	183	A	A	zd240d07.s1 Soares_fetal_heart_NbHH19W *Homo sapiens* cDNA clone IMAGE:341581 3', mRNA sequence
213121_at	SNRP70	66	A	6	A	A	zd39c08.s1 Soares_fetal_heart_NbHH19W *Homo sapiens* cDNA clone IMAGE:343022 3', mRNA sequence
65521_at	LOC51619	658	A	266	A	P	zd56g04.r1 Soares_fetal_heart_NbHH19W *Homo sapiens* cDNA clone IMAGE:344694 5', mRNA sequence

214014_at	CDC42EP2	38	A	14	A	A	zd85d03.s1 Soares_fetal_heart_NbHH19W *Homo sapiens* cDNA clone IMAGE:347429 3', mRNA sequence
201204_s_at	RRBP1	2174	P	1257	P	P	zf44f12.s1 Soares_fetal_heart_NbHH19W *Homo sapiens* cDNA clone IMAGE:379823 3', mRNA sequence
209331_s_at	MAX	561	P	294	P	P	zg72g05.s1 Soares_fetal_heart_NbHH19W *Homo sapiens* cDNA clone IMAGE:398936 3', mRNA sequence
214776_x_at	XYLB	24	A	13	A	A	zi99g02.s1 Soares_fetal_liver_spleen_1NFLS_S1 *Homo sapiens* cDNA clone IMAGE:448946 3', mRNA sequence
211715_s_at	BDH	59	A	8	A	P	3-hydroxybutyrate dehydrogenase (heart, mitochondrial)
205534_at	PCDH7	8	A	205	P	A	BH-protocadherin (brain-heart)
205535_s_at	PCDH7	7	A	75	P	P	BH-protocadherin (brain-heart)
210273_at	PCDH7	157	A	168	A	P	BH-protocadherin (brain-heart)
210941_at	PCDH7	14	A	3	A	A	BH-protocadherin (brain-heart)
204726_at	CDH13	172	P	195	P	P	Cadherin 13, H-cadherin (heart)
203020_at	HHL	210	P	117	P	P	Expressed in hematopoietic cells, heart, liver
213982_s_at	HHL	335	P	74	P	P	Expressed in hematopoietic cells, heart, liver
205738_s_at	FABP3	79	A	92	P	P	Fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
214285_at	FABP3	22	A	49	A	P	Fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
220138_at	HAND1	246	A	117	A	P	Heart and neural crest derivatives expressed 1
220480_at	HAND2	19	A	29	A	A	Heart and neural crest derivatives expressed 2
213036_x_at	ATP2A3	23	A	25	A	A	*Homo sapiens* SERCA3 gene, exons 1–7 (and joined CDS)
204938_s_at	PLN	22	A	60	A	P	Phospholamban
204939_s_at	PLN	71	A	99	A	P	Phospholamban
204940_at	PLN	46	A	28	A	P	Phospholamban
206578_at	NKX2-5	40	A	14	A	P	NK2 transcription factor related, locus 5 (*Drosophila*)
205517_at	GATA4	16	A	46	A	P	GATA binding protein 4
201667_at	GJA1	4792	P	2016	P	P	Gap junction protein, alpha 1, 43 kDa (connexin 43)
208636_at	ACTN1	7896	P	3182	P	P	Actinin, alpha 1
208637_x_at	ACTN1	5400	P	2359	P	P	Actinin, alpha 1
211160_x_at	ACTN1	5727	P	1529	P	A	Actinin, alpha 1
203861_s_at	ACTN2	38	A	17	A	P	Actinin, alpha 2
203862_s_at	ACTN2	53	A	16	A	P	Actinin, alpha 2
203863_at	ACTN2	35	A	20	A	P	Actinin, alpha 2
203864_s_at	ACTN2	189	A	123	A	P	Actinin, alpha 2
206891_at	ACTN3	134	M	133	A	M	Actinin, alpha 3
200601_at	ACTN4	1134	P	282	P	P	Human non-muscle alpha-actinin mRNA, complete cds
211805_s_at	SLCBA1(NCX1)	53	A	228	A	P	Solute carrier family 8 (sodium/calcium exchanger), member 1
207413_s_at	SCN5A	32	A	61	A	P	Sodium channel, voltage-gated, type V, alpha (long QT syndrome 3)

Introduction of the EGFP gene

Recombinant adenovirus carrying the enhanced green fluorescent protein (EGFP) gene was prepared as described [13]. Chorionic plate cells were plated on dishes at $2\times10^5/cm^2$, and infected with EGFP-expressing adenovirus at 10 plaque-forming units/cell on the next day. Chorionic plate cells were examined in vitro by fluorescent confocal microscopy for expression of the EGFP gene. By 7 days post-infection, nearly all of the cells expressed EGFP. To eliminate the possibility of free adenovirus in the cell supernatant, we infected murine fetal cardiomyocytes with chorionic plate cell supernatants after infection. No murine fetal cardiomyocytes expressed EGFP, implying that the cells are not transfected with free adenovirus.

Preparation of murine fetal cardiomyocytes

Fetal cardiomyocytes were obtained from the hearts of day 17 mouse fetuses. The hearts were minced with scissors and washed with PBS, and then incubated in PBS with 0.1% trypsin and 0.25 mM EDTA for 10 min at 37 °C. After DMEM supplemented with 10% FBS was added, the cardiomyocytes were centrifuged at 1000 rpm for 5 min. The pellet was then re-suspended in 10 ml of DMEM with 10% FBS and incubated on glass dishes for 1 h to separate the cardiomyocytes from fibroblasts. The floating cardiomyocytes were collected and re-plated at $5\times10^4/cm^2$.

Co-culture system of chorionic plate cells and murine fetal cardiomyocytes

Neither 5-azaC [12] nor oxytocin [21] was used in this process as they are known to initiate cardiomyogenic differentiation. EGFP-labeled chorionic plate cells were harvested with 0.25% trypsin and 1 mM EDTA and overlaid onto the cultured fetal cardiomyocytes at $7\times10^3/cm^2$. Every 2 days the culture medium was replaced with fresh culture medium that was supplemented with 10% FBS and 1 μg/ml Amphotericin B (Gibco). The morphology of the beating EGFP-labeled chorionic plate cells was evaluated under a fluorescent microscope. The image was monitored using a CCD camera and stored as digital video. The cell contraction was analyzed using an image-edge detection program made by Igor Pro 4 (Wavemetrics Inc., Lake Oswego, Oregon).

Electrophysiological analysis

On day 10 of co-cultivation, action potentials (APs) were recorded as described previously [12,13] from spontaneously beating EGFP-labeled cells. Spontaneously beating EGFP-positive chorionic plate cells were selected as targets. The APs of the targeted cells had been recorded and Alexa568 dye was injected by iontophoresis to confirm that the APs were generated by EGFP-positive chorionic plate cells. The extent of dye transfer was monitored under a fluorescence microscope, and digital images were recorded with a digital photo camera (D100; Nikon, Tokyo, Japan) mounted on a microscope with a fluorescence filter (UMWIG2; Olympus).

Immunocytochemistry

A laser confocal microscope (LSM510, Zeiss) was used for immunocytochemical analysis. The chorionic plate cells co-cultured with fetal cardiomyocytes in vitro were fixed with 2% paraformaldehyde (PFA) in PBS for 20 min at 4 °C and treated with 0.1% Triton-X PBS for 20 min at room temperature. These cells were then stained with mouse monoclonal anti-human cardiac troponin-I antibody (#4T21/19-C7 HyTest, Euro, Finland) diluted 1:300, monoclonal anti-α-actinin antibody (Sigma) diluted 1:300, and anti-connexin 43 antibody (Sigma) diluted 1:300. To prevent fading and to stain nuclei, a Slow Fade Light Antifade kit with 4′-6-diamidino-2-phenylindole (DAPI) (Molecular Probes) was used.

Results

Establishment of chorionic plate cells

Almost all human tissues or organs can be a source of MSCs, which have been extracted from fat, muscle, menstrual blood, endometrium, placenta, umbilical cord, cord blood, skin, and eye. In this study, we focused on cells derived from fetuses, since fetus-derived cells tend to both differentiate and proliferate better than adult cells [22]. In that sense, human placenta is a good source of fetus-derived MSCs. We cultivated chorionic plate cells that were obtained from the chorionic mesoderm of the placenta (Fig. 1A). The chorionic plate cells regarded as being Population Doubling (PD) 0 or Day 0 were fibroblast-like in morphology, indistinguishable in appearance from the marrow-derived MSCs, and relatively larger in size than rapidly self-renewing stem cells [23] (Fig. 1B). The cells from PD 9 to PD 18 rapidly proliferated in culture and were propagated continuously. Chorionic plate cells did not undergo malignant transformation. They stopped dividing after reaching confluence and they did not form any foci after reaching confluence in vitro.

To clarify the character of the established chorionic plate cells, we first performed karyotypic analysis of 30 cells at PD 3. All cells had normal chromosomes without any chromosomal aberration (Fig. 1C). The sex chromosomes were found to be XY, implying that all cells were of fetal origin. Genomic FISH analysis also revealed that all cells had XY chromosomes (Fig. 1D). We examined the cell surface marker of the placenta-derived cells (chorionic plate cells) by FACS analysis (Fig. 1E). The surface markers of chorionic plate cells are exactly the same as those of previously reported bone-marrow- and cord blood-derived mesodermal cells, i.e., positive for CD29, CD44,

Fig. 3 – Immunocytochemistry of chorionic plate cells for human cardiac troponin-I. (A–F) Immunocytochemistry of differentiated chorionic plate cells with anti-human cardiac troponin-I (cTnI) antibody. The EGFP-positive cells (B) were stained with anti-human cTnI antibody (A) and the merged image (DAPI, EGFP, cTnI) is shown in panels D and F. An enlarged image (red square in D) is shown in panel E. Clear striations were observed with red fluorescence of cTnI in the differentiated cells. (G–I) A merged image for EGFP and cTnI is shown in panel G. A longitudinal section at the green line in the merged image G is shown in panel H. An axial section at the red line in merged image G is shown in panel I.