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.


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Research Article

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

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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 7 , atrial myosin light chain, cardiac -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.

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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

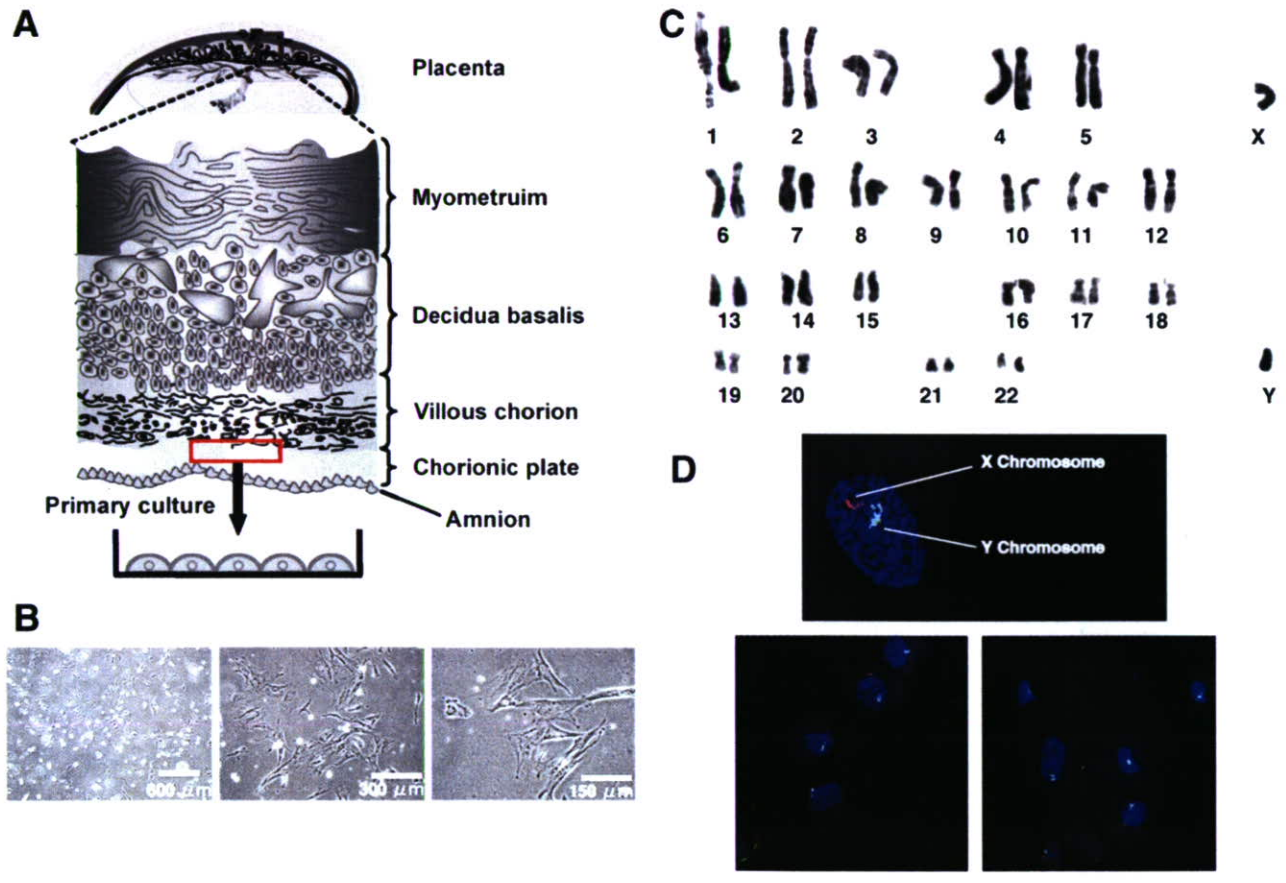


Fig. 1 – Establishment of chorionic plate cells. (A) Chorionic plate cells were established by primary culture of chorionic plate (red square in the chorionic mesoderm) in the human placenta. (B) Chorionic plate cells at PD 4 consisted of heterogeneous cell population. Three images show chorionic plate 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.

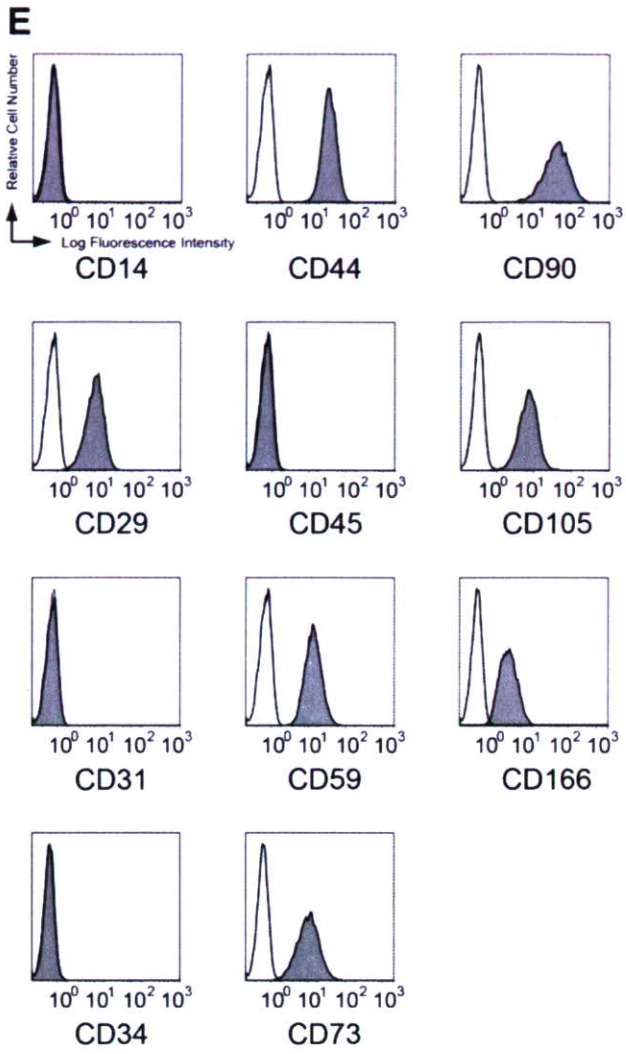


Fig. 1 (*continued*).

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) co-cultivation 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 g/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-2 (MLC-2), 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 l 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.

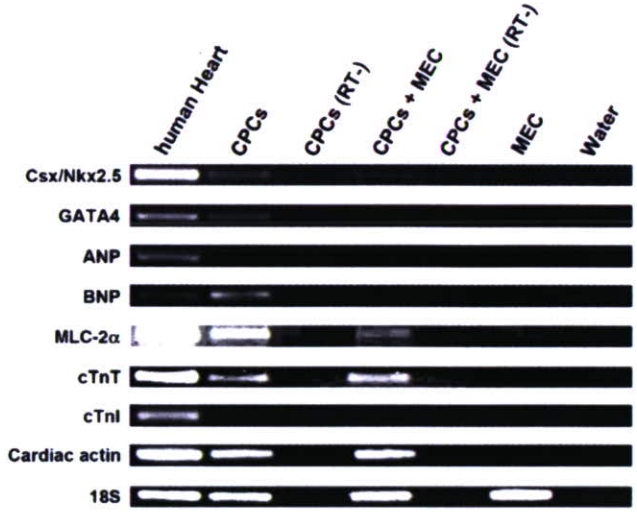


Fig. 2 – Gene expression of cardiomyocyte-specific/associated genes in chorionic plate cells. RT-PCR analysis revealed expression patterns of cardiomyocyte-specific or associated genes; *Csx/Nkx2.5, GATA4, ANP, BNP, cTnI, cTnT, cardiac actin* and *MLC-2* (from left to right) in human heart, chorionic 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- 1) (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-2	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

n cardiomyocyte-specific or -associated gene expression profiling of undifferentiated and differentiated chorionic plate cells (CPCs)

Common	Undifferentiated CPCs		Differentiated CPCs		Human heart	Description
CASQ2	34	A	12	A	P	Calsequestrin 2 (cardiac muscle)
CSRP3	8	A	5	A	P	Cysteine and glycine-rich protein 3 (cardiac LIM protein)
MYBPC3	56	A	117	A	P	Myosin binding protein C, cardiac
MYH6	11	A	50	A	P	Myosin, heavy polypeptide 6, cardiac n (cardiomyopathy, hypertrophic 1)
MYH7	5	A	161	P	P	Myosin, heavy polypeptide 7, cardiac n
MYH7	36	A	232	A	P	Myosin, heavy polypeptide 7, cardiac n
MYH7B	89	A	11	A	P	Myosin, heavy polypeptide 7B, cardiac
MYL2	267	A	153	A	P	Myosin, light polypeptide 2, regulatory,
MYL4	338	P	404	A	P	Myosin, light polypeptide 4, alkali; atrial
MYL4	220	P	412	P	P	Myosin, light polypeptide 4, alkali; atrial
MYL7	9	A	16	A	P	Myosin, light polypeptide 7, regulatory
RYR2	11	A	13	A	P	Ryanodine receptor 2 (cardiac)
RYR2	17	A	23	A	P	Ryanodine receptor 2 (cardiac)
TNNI3	96	A	195	A	P	Troponin I, cardiac
TNNT2	83	A	165	A	P	Troponin T2, cardiac
ACTC	289	P	671	P	P	Actin, alpha, cardiac muscle
ANKRD1	214	P	13	A	P	Ankyrin repeat domain 1 (cardiac mus
ATP5A1	5905	P	2657	P	P	ATP synthase, H^+ transporting, mitoch complex, alpha subunit, isoform 1, card
ATP2A1	107	A	53	A	A	ATPase, Ca^{++} transporting, cardiac mus fast twitch 1
ATP2A2(SERCA2A)	4465	P	2577	P	P	ATPase, Ca^{++} transporting, cardiac mus slow twitch 2
ATP2A2(SERCA2A)	814	P	338	A	P	ATPase, Ca^{++} transporting, cardiac mus slow twitch 2
ATP2A2(SERCA2A)	178	P	60	A	A	ATPase, Ca^{++} transporting, cardiac mus slow twitch 2
CASQ2	34	A	12	A	P	Calsequestrin 2 (cardiac muscle)
	6	A	17	A	A	qb80a04.x1 Soares_fetal_heart_NbHH1 *Homo Sapiens* cDNA clone IMAGE:17063 to TR:O21123 O21123 CYTOCHROME O mRNA sequence
BTN2A2	482	P	183	A	A	zd240d07.s1 Soares_fetal_heart_NbHH1 *Homo sapiens* cDNA clone IMAGE:34158 mRNA sequence
SNRP70	66	A	6	A	A	zd39c08.s1 Soares_fetal_heart_NbHH19 *Homo sapiens* cDNA clone IMAGE:34302 mRNA sequence
LOC51619	658	A	266	A	P	zd56g04.r1 Soares_fetal_heart_NbHH19 *Homo sapiens* cDNA clone IMAGE:34469 mRNA sequence

CDC42EP2	38	A	14	A	A	zd85d03.s1 Soares_fetal_heart_NbHH19 *Homo sapiens* cDNA clone IMAGE:34742 mRNA sequence
RRBP1	2174	P	1257	P	P	zf44f12.s1 Soares_fetal_heart_NbHH19 *Homo sapiens* cDNA clone IMAGE:37982 mRNA sequence
MAX	561	P	294	P	P	zg72g05.s1 Soares_fetal_heart_NbHH19 *Homo sapiens* cDNA clone IMAGE:39893 mRNA sequence
XYLB	24	A	13	A	A	zi99g02.s1 Soares_fetal_liver_spleen_1N *Homo sapiens* cDNA clone IMAGE:44894 mRNA sequence
BDH	59	A	8	A	P	3-hydroxybutyrate dehydrogenase (heart, mitochondrial)
PCDH7	8	A	205	P	A	BH-protocadherin (brain–heart)
PCDH7	7	A	75	P	P	BH-protocadherin (brain–heart)
PCDH7	157	A	168	A	P	BH-protocadherin (brain–heart)
PCDH7	14	A	3	A	A	BH-protocadherin (brain–heart)
CDH13	172	P	195	P	P	Cadherin 13, H-cadherin (heart)
HHL	210	P	117	P	P	Expressed in hematopoietic cells, heart
HHL	335	P	74	P	P	Expressed in hematopoietic cells, heart
FABP3	79	A	92	P	P	Fatty acid binding protein 3, muscle an (mammary-derived growth inhibitor)
FABP3	22	A	49	A	P	Fatty acid binding protein 3, muscle an (mammary-derived growth inhibitor)
HAND1	246	A	117	A	P	Heart and neural crest derivatives expr
HAND2	19	A	29	A	A	Heart and neural crest derivatives expr
ATP2A3	23	A	25	A	A	*Homo sapiens* SERCA3 gene, exons 1–7 (and joined CDS)
PLN	22	A	60	A	P	Phospholamban
PLN	71	A	99	A	P	Phospholamban
PLN	46	A	28	A	P	Phospholamban
NKX2-5	40	A	14	A	P	NK2 transcription factor related, locus 5
GATA4	16	A	46	A	P	GATA binding protein 4
GJA1	4792	P	2016	P	P	Gap junction protein, alpha 1, 43 kDa (c
ACTN1	7896	P	3182	P	P	Actinin, alpha 1
ACTN1	5400	P	2359	P	P	Actinin, alpha 1
ACTN1	5727	P	1529	P	A	Actinin, alpha 1
ACTN2	38	A	17	A	P	Actinin, alpha 2
ACTN2	53	A	16	A	P	Actinin, alpha 2
ACTN2	35	A	20	A	P	Actinin, alpha 2
ACTN2	189	A	123	A	P	Actinin, alpha 2
ACTN3	134	M	133	A	M	Actinin, alpha 3
ACTN4	1134	P	282	P	P	Human non-muscle alpha-actinin mRN complete cds
SLCBA1(NCX1)	53	A	228	A	P	Solute carrier family 8 (sodium/calcium member 1
SCN5A	32	A	61	A	P	Sodium channel, voltage-gated, type V, (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 g/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- -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

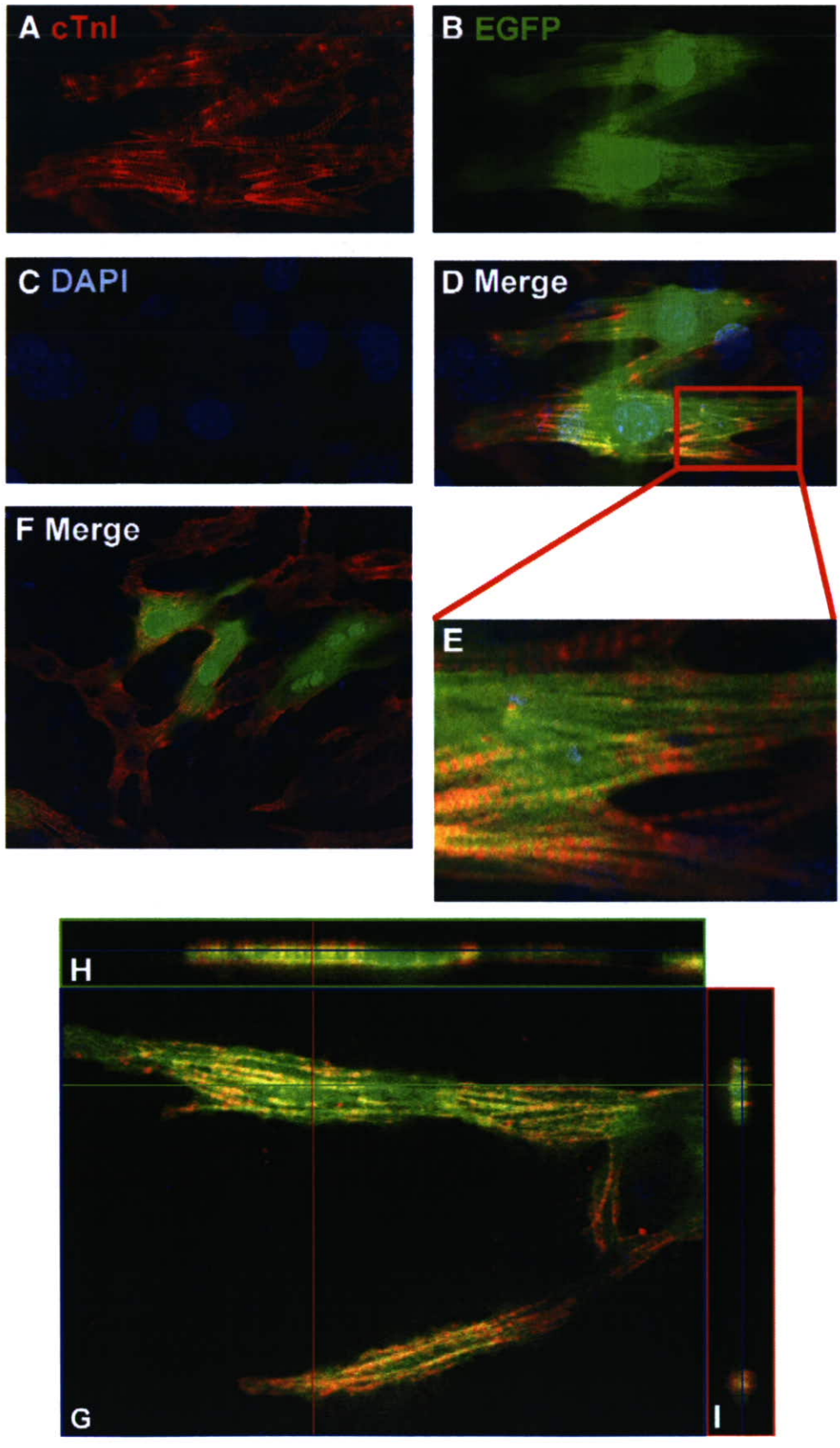
Almost all human tissues or organs can be a source of MSCs, which have been extracted from fat, muscle, menstrual blood, endometrium, placenta, umbilical cord, cord blood, skin, and eye. In this study, we focused on cells derived from fetuses, since fetus-derived cells tend to both differentiate and 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.

CD59, CD73, CD 90, CD105 and CD166, and negative for CD14, CD31, CD34 and CD45.

Next we investigated whether chorionic plate cells have cardiomyogenic potential by human cardiomyocyte-specific gene expression using RT-PCR method (Fig. 2, Table 1) and gene chip analysis (Table 2; GEO accession number, GSE7021: GSM162104 and GSM162105). Chorionic plate cells expressed Csx/Nkx-2.5, GATA4, BNP, cardiac troponin T (cTnT), cardiac



actin and myosin light chain-2 (MLC-2) 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±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 , 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 -actinin, and connexin 43 (Figs. 4A–E). Clear striations were observed for the red fluorescence of cTnI (Fig. 3A) and -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±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 ex 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].

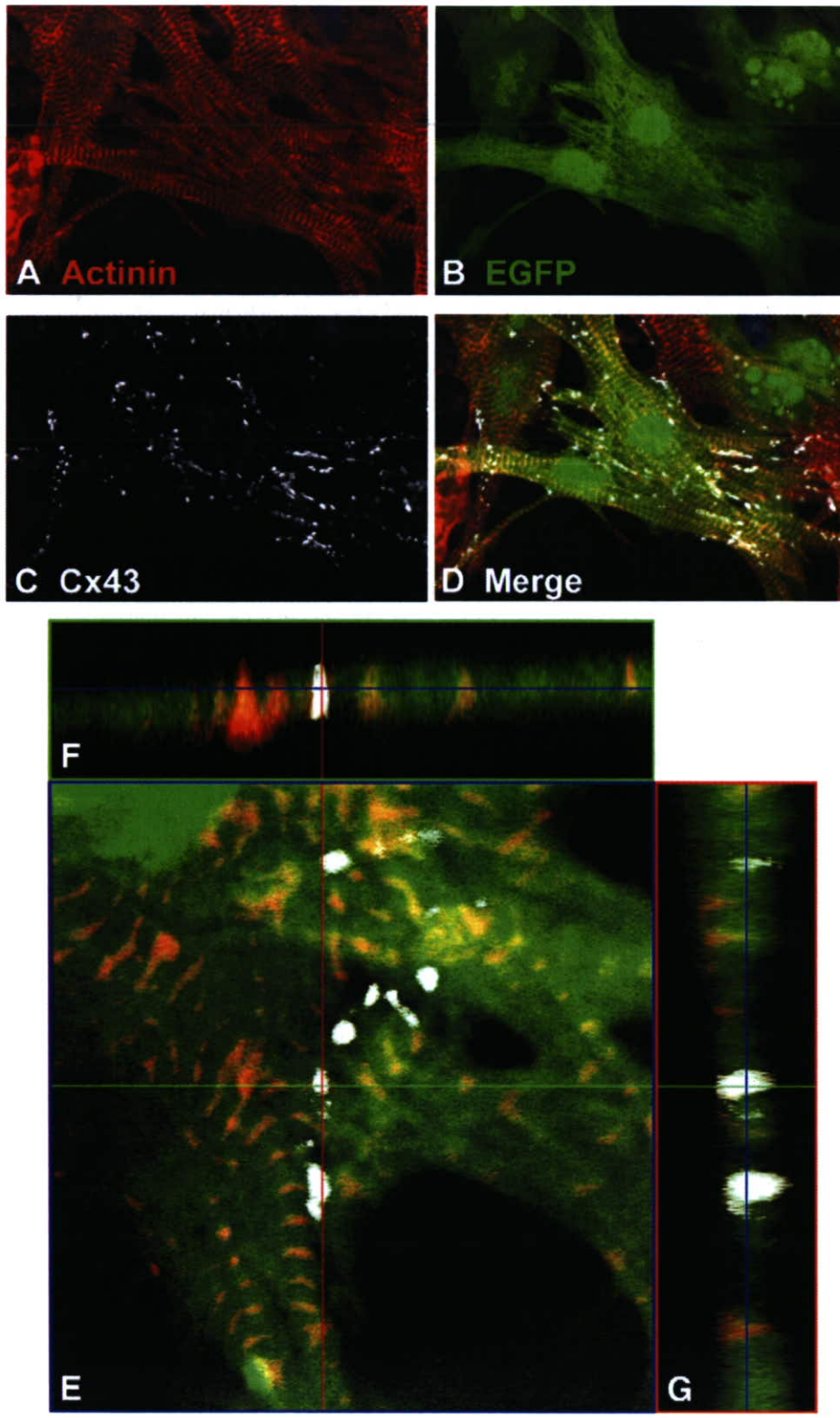
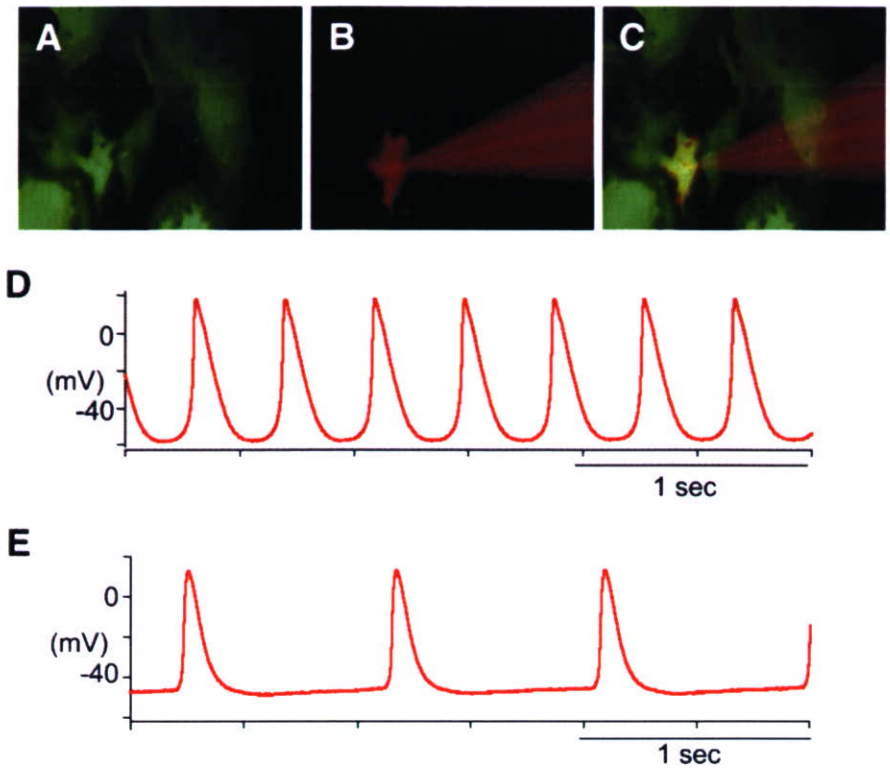


Fig. 4 – Immunocytochemistry of chorionic plate cells for -actinin and connexin 43. (A–D) Immunocytochemistry of differentiated chorionic plate cells for -actinin (Actinin) and connexin 43 (Cx43) antibody. Clear striations were observed for the red fluorescence of -actinin (A) in the EGFP-positive cells (B). Connexin 43 (C) was stained along the attachment site of EGFP-positive cells. Merged images (-actinin, connexin 43, EGFP) are shown in panel D. (E–G) A merged image for -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.



F

	Amplitude (mV)	Resting Membrane Potential (mV)	APD90 (msec)	APD50 (msec)	RR (sec)	Vmax (V/s)	n
PLKC	63.87± 2.24	-52.22± 1.05	209.1± 12.9	100.2± 4.8	0.948± 0.138	4.460± 0.355	14

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 procursor 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.

REFERENCES

[1] T. Thom, N. Haase, W. Rosamond, V.J. Howard, J. Rumsfeld, T. Manolio, Z.J. Zheng, K. Flegal, C. O'Donnell, S. Kittner, D. Lloyd-Jones, D.C. Goff Jr., Y. Hong, R. Adams, G. Friday, K. Furie, P. Gorelick, B. Kissela, J. Marler, J. Meigs, V. Roger, S. Sidney, P. Sorlie, J. Steinberger, S. Wasserthiel-Smoller, M. Wilson, P. Wolf, Heart disease and stroke statistics—2006 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee, Circulation 113 (2006) e85–e151.

[2] A. Leri, J. Kajstura, P. Anversa, Cardiac stem cells and mechanisms of myocardial regeneration, Physiol. Rev. 85 (2005) 1373–1416.

[3] M.A. Laflamme, C.E. Murry, Regenerating the heart, Nat. Biotechnol. 23 (2005) 845–856.

[4] H.F. Tse, Y.L. Kwong, J.K. Chan, G. Lo, C.L. Ho, C.P. Lau, Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation, Lancet 361 (2003) 47–49.
[5] E. Perin, Transendocardial injection of autologous mononuclear bone marrow cells in end-stage ischemic heart failure patients: one-year follow-up, Int. J. Cardiol. 95 (Suppl 1) (2004) S45–S46.
[6] K.C. Wollert, G.P. Meyer, J. Lotz, S. Ringes-Lichtenberg, P. Lippolt, C. Breidenbach, S. Fichtner, T. Korte, B. Hornig, D. Messinger, L. Arseniev, B. Hertenstein, A. Ganser, H. Drexler, Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial, Lancet 364 (2004) 141–148.
[7] O. Agbulut, S. Vandervelde, N. Al Attar, J. Larghero, S. Ghostine, B. Leobon, E. Robidel, P. Borsani, M. Le Lorc'h, A. Bissery, C. Chomienne, P. Bruneval, J.P. Marolleau, J.T. Vilquin, A. Hagege, J.L. Samuel, P. Menasche, Comparison of human skeletal myoblasts and bone marrow-derived CD133+ progenitors for the repair of infarcted myocardium, J. Am. Coll. Cardiol. 44 (2004) 458–463.
[8] P. Menasche, A.A. Hagege, M. Scorsin, B. Pouzet, M. Desnos, D. Duboc, K. Schwartz, J.T. Vilquin, J.P. Marolleau, Myoblast transplantation for heart failure, Lancet 357 (2001) 279–280.
[9] D.J. Prockop, Stem cell research has only just begun, Science 293 (2001) 211–212.
[10] K. Le Blanc, C. Gotherstrom, O. Ringden, M. Hassan, R. McMahon, E. Horwitz, G. Anneren, O. Axelsson, J. Nunn, U. Ewald, S. Norden-Lindeberg, M. Jansson, A. Dalton, E. Astrom, M. Westgren, Fetal mesenchymal stem-cell engraftment in bone after in utero transplantation in a patient with severe osteogenesis imperfecta, Transplantation 79 (2005) 1607–1614.
[11] K. Le Blanc, I. Rasmusson, C. Gotherstrom, C. Seidel, B. Sundberg, M. Sundin, K. Rosendahl, C. Tammik, O. Ringden, Mesenchymal stem cells inhibit the expression of CD25 (interleukin-2 receptor) and CD38 on phytohaemagglutinin-activated lymphocytes, Scand. J. Immunol. 60 (2004) 307–315.
[12] S. Makino, K. Fukuda, S. Miyoshi, F. Konishi, H. Kodama, J. Pan, M. Sano, T. Takahashi, S. Hori, H. Abe, J. Hata, A. Umezawa, S. Ogawa, Cardiomyocytes can be generated from marrow stromal cells in vitro, J. Clin. Invest. 103 (1999) 697–705.
[13] Y. Takeda, T. Mori, H. Imabayashi, T. Kiyono, S. Gojo, S. Miyoshi, N. Hida, M. Ita, K. Segawa, S. Ogawa, M. Sakamoto, S. Nakamura, A. Umezawa, 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. 6 (2004) 833–845.
[14] D. Orlic, J. Kajstura, S. Chimenti, I. Jakoniuk, S.M. Anderson, B. Li, J. Pickel, R. McKay, B. Nadal-Ginard, D.M. Bodine, A. Leri, P. Anversa, Bone marrow cells regenerate infarcted myocardium, Nature 410 (2001) 701–705.
[15] S. Gojo, N. Gojo, Y. Takeda, T. Mori, H. Abe, S. Kyo, J. Hata, A. Umezawa, In vivo cardiovasculogenesis by direct injection of isolated adult mesenchymal stem cells, Exp. Cell Res. 288 (2003) 51–59.
[16] J.S. Wang, D. Shum-Tim, J. Galipeau, E. Chedrawy, N. Eliopoulos, R.C. Chiu, Marrow stromal cells for cellular cardiomyoplasty: feasibility and potential clinical advantages, J. Thorac. Cardiovasc. Surg. 120 (2000) 999–1005.
[17] J.G. Shake, P.J. Gruber, W.A. Baumgartner, G. Senechal, J. Meyers, J.M. Redmond, M.F. Pittenger, B.J. Martin, Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects, Ann. Thorac. Surg. 73 (2002) 1919–1925 (discussion 1926).
[18] K.L. Moore, T.V.N. Persaud, The Developing Human: Clinically Oriented Embryology, Saunders, Philadelphia, Pa., 2003.
[19] K. Igura, X. Zhang, K. Takahashi, A. Mitsuru, S. Yamaguchi, T.A. Takashi, Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta, Cytotherapy 6 (2004) 543–553.
[20] X. Zhang, A. Mitsuru, K. Igura, K. Takahashi, S. Ichinose, S. Yamaguchi, T.A. Takahashi, Mesenchymal progenitor cells derived from chorionic villi of human placenta for cartilage tissue engineering, Biochem. Biophys. Res. Commun. 340 (2006) 944–952.
[21] J. Paquin, B.A. Danalache, M. Jankowski, S.M. McCann, J. Gutkowska, Oxytocin induces differentiation of P19 embryonic stem cells to cardiomyocytes, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 9550–9555.
[22] M. Terai, T. Uyama, T. Sugiki, X.K. Li, A. Umezawa, T. Kiyono, 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 16 (2005) 1491–1499.
[23] D.J. Prockop, I. Sekiya, D.C. Colter, Isolation and characterization of rapidly self-renewing stem cells from cultures of human marrow stromal cells, Cytotherapy 3 (2001) 393–396.
[24] I. Potapova, A. Plotnikov, Z. Lu, P. Danilo Jr., V. Valiunas, J. Qu, S. Doronin, J. Zuckerman, I.N. Shlapakova, J. Gao, Z. Pan, A.J. Herron, R.B. Robinson, P.R. Brink, M.R. Rosen, I.S. Cohen, Human mesenchymal stem cells as a gene delivery system to create cardiac pacemakers, Circ. Res. 94 (2004) 952–959.
[25] C. Badorff, R.P. Brandes, R. Popp, S. Rupp, C. Urbich, A. Aicher, I. Fleming, R. Busse, A.M. Zeiher, S. Dimmeler, Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes, Circulation 107 (2003) 1024–1032.
[26] K. Matsuura, H. Wada, T. Nagai, Y. Iijima, T. Minamino, M. Sano, H. Akazawa, J.D. Molkentin, H. Kasanuki, I. Komuro, Cardiomyocytes fuse with surrounding noncardiomyocytes and reenter the cell cycle, J. Cell Biol. 167 (2004) 351–363.
[27] C.H. Cui, T. Uyama, K. Miyado, M. Terai, S. Kyo, T. Kiyono, A. Umezawa, Menstrual blood-derived cells confer human dystrophin expression in the murine model of duchenne muscular dystrophy via cell fusion and myogenic transdifferentiation, Mol. Biol. Cell 18 (2007) 1586–1594.
[28] T. Mori, T. Kiyono, H. Imabayashi, Y. Takeda, K. Tsuchiya, S. Miyoshi, H. Makino, K. Matsumoto, H. Saito, S. Ogawa, M. Sakamoto, J. Hata, A. Umezawa, Combination of hTERT and bmi-1, E6, or E7 induces prolongation of the life span of bone marrow stromal cells from an elderly donor without affecting their neurogenic potential, Mol. Cell. Biol. 25 (2005) 5183–5195.
[29] J. Kohyama, H. Abe, T. Shimazaki, A. Koizumi, K. Nakashima, S. Gojo, T. Taga, H. Okano, J. Hata, A. Umezawa, Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent, Differentiation 68 (2001) 235–244.
[30] K.D. Lee, T.K. Kuo, J. Whang-Peng, Y.F. Chung, C.T. Lin, S.H. Chou, J.R. Chen, Y.P. Chen, O.K. Lee, In vitro hepatic differentiation of human mesenchymal stem cells Hepatology 40 (2004) 1275–1284.
[31] I. Komuro, S. Izumo, Csx: a murine homeobox-containing gene specifically expressed in the developing heart, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 8145–8149.
[32] I. Shiojima, I. Komuro, T. Mizuno, R. Aikawa, H. Akazawa, T. Oka, T. Yamazaki, Y. Yazaki, Molecular cloning and characterization of human cardiac homeobox gene CSX1, Circ. Res. 79 (1996) 920–929.
[33] Y. Yamada, K. Sakurada, Y. Takeda, S. Gojo, A. Umezawa, Single-cell-derived mesenchymal stem cells overexpressing Csx/Nkx2.5 and GATA4 undergo the stochastic cardiomyogenic fate and behave like transient amplifying cells, Exp. Cell Res. 313 (2007) 698–706.
[34] F. Ishikawa, Cellular senescence, an unpopular yet trustworthy tumor suppressor mechanism, Cancer Sci. 94 (2003) 944–947.

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Ways for a Mesenchymal Stem Cell to Live on Its Own: Maintaining an Undifferentiated State Ex Vivo

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Abstract

Like all stem cells, mesenchymal stem cells (MSC) must balance self-renewal and differentiation. Complex regulatory mechanisms are required to keep stem cells in an undifferentiated, self-renewing state and to mediate their subsequent differentiation and proliferation. In this review, we discuss how adequate numbers of MSC can be maintained in culture. In particular, we focus on identification of the cell culture conditions needed to maintain general, nonspecific potential as a stem cell over time and through replication. It would be extremely advantageous to be able to maintain MSC populations in a completely undifferentiated state and to determine and switch on specific differentiation as and when required.
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Key words: Mesenchymal stem cells; Cell culture; Differentiation; Cell growth; Senescence

1. Introduction

Mesenchymal stem cells (MSC) are attracting a great deal of attention, because they represent a valuable source of cells for use in regenerative medicine [1]. Use of MSC entails no ethical or immunologic problems, and they provide an excellent model of cell differentiation in biology. Not surprisingly, mesenchymal cell biology is a complex and rapidly evolving field. Critical unanswered questions remain: What defines an MSC (as opposed to just a mesenchymal cell), and what provides MSC with their unique properties?

Stem cell biology is based on the principle that any tissue may contain cells that possess the potential for both self-renewal and differentiation into one or more cell types. Mesenchymal cells are derived from an organ's supporting tissue, as opposed to parenchyma or the supporting framework of an animal organ, which typically consists of connective tissue. Among these cells exist stem cells, which have two basic processes, ie, self-renewal and differentiation. MSC were first discovered in 1976 by Friedenstein, who described clonal, plastic-adherent cells from bone marrow that provided a physical scaffold for hematopoiesis and that could differentiate into osteoblasts, chondrocytes, and adipocytes in vitro [2]. To date, investigators have demonstrated that MSC per se can be recovered from a variety of adult tissues and have the capacity to differentiate into a variety of specific cell types [3-8]. The mesenchymal phenotype can be maintained under optimal culture conditions, and MSC in vitro are recognized as adherent fibroblastic cells with a generally spindle shape, although some candidate populations of cells are more spherical with few spindle-shaped cells [9]. This heterogeneous population is too "crude" to consist solely of MSC. Although MSC can be harvested from a variety of tissues and have multipotency (ie, the capability to differentiate into numerous tissue lineages, including myoblasts, cardiomyocytes, osteoblasts, adipocytes, chondrocytes, and possibly even neural cells), MSC populations cannot easily be induced to differentiate into one lineage at the same time. There are some cell populations that behave like progenitor cells with monopotency or bipotency. In this review, we describe how MSC derived from a variety of tissues have tissue-specific characteristics and discuss optimal culture conditions that can allow the cells both to keep proliferating in an undifferentiated, self-renewing state and to permit mediation of their future differentiation.

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2. MSC Optimally Require Their Original Milieu

Stem cells are indispensable entities in most multicellular organisms in that they are responsible for forming tissues during early development and for maintaining them in the adult stage. Because all somatic stem cells, including MSC, have the same genetic material, every cell type has the potential to express a stem cell phenotype under specific conditions; that is, tissue-specific flexibility is inherent. Mesenchymal cells, of which connective tissue is mainly composed, do not develop into a tissue or an organ. The cells synthesize the extracellular matrix by themselves and in vitro establish favorable environments for growth. Mesenchymal cell cultures can be made from suspensions of cells dissociated from tissues, and microscopical and biochemical analyses facilitate exploration of the effects of adding or removing specific molecules, such as hormones or growth factors.

Cells vary in their needs in a cell type–specific manner, and cells can therefore be categorized or defined by their requirements. Bone marrow–derived MSC, for example, can be compared with umbilical cord blood–derived MSC. Several methods are available for distinguishing between these two types of cells. One such method is flow cytometric analysis of markers that appear on the surfaces of the cells. Some surface marker characteristics of bone marrow–derived MSC are the same as those of cord blood–derived MSC (eg, $CD29^+$, $CD44^+$, $CD55^+$, $CD59^+$, $CD34^-$, and $CD117^-$ [10-12]). On the other hand, the CD90 and CD133 markers can be used to distinguish cord blood–derived cells from bone marrow–derived cells, because these markers are expressed in multipotent marrow-derived cells but not in cord blood–derived cells [12]. As yet, no surface markers have been identified that define MSC. Another method is complementary DNA microarray/chip technology. Because of the logical connection between gene expression and cell function, gene expression patterns can predict the variation in cell phenotypes and reveal novel phenotypic aspects of the cells and tissues studied. In mesenchymal cell culture, expression of genes encoding growth factor receptors is an important factor in microarray analysis–based investigations of the effects of growth factors, because cells dissociated from different tissues, such as bone marrow and cord blood, have different responses to growth factors (Table 1). This response affects differentiation potential. Bone marrow–derived cells can differentiate into osteoblasts, chondrocytes, cardiomyocytes, adipocytes, skeletal myocytes, and neural cells and do so according to the specific cell culture conditions, whereas cord blood–derived cells exhibit only osteogenic and adipogenic potential under the same conditions.

How can the microenvironment, culture conditions, or growth factors dictate the identity of MSC and their production of different progeny? Serum plays a critical role in the growth of cells in vitro by providing components such as amino acids, lipids, growth factors, vitamins, hormones, and attachment factors, by acting as a pH buffer, and by providing protease inhibitors. Most media for mesenchymal cell culture include a poorly defined mixture of macromolecules in the form of fetal calf serum; however, the use of serum for MSC culture makes it difficult to know which specific macromolecules a particular type of cell requires for survival and normal function. This difficulty led to the development of serum-free chemically defined media. In addition to the usual molecules, such specialized media will need to contain essential growth factors that the MSC require in culture. The primary requirements of MSC in culture reflect the origin of the cells; that is, investigators have to try to recreate the specific native tissue/organ milieu of the cells' origin. Identification of a means to create an "Elysium" or ideal stress-free native environments for cultivating and maintaining MSC will bestow significant benefits for future stem cell therapeutics.

3. The Paradox of Cell Growth versus Life Span

In cell culture it is important not only to remove animal serum from the culture medium (for reasons of medical safety with respect to infectious diseases [13-15]) but also to obtain large numbers of cells for use in therapy. Cells must be propagated in vitro to obtain the large numbers needed for biomedical procedures; however, Hayflick's problem is unavoidable in normal cells [16,17]. Most vertebrate cells stop dividing after a finite number of divisions in culture, a process called *senescence* [17,18]. Senescence is classified into two categories: "stress-induced premature senescence" (or "telomere-independent senescence") and "replicative senescence" (or "telomere-dependent senescence") [19-21]. Marrow-derived mesenchymal cells divide approximately 25 to 40 times [11,22] in culture before they cease dividing or reach senescence (M0, mortality stage 0, ie, premature senescence), whereas a few cells that overcome this step restart proliferation but stop dividing again in replicative senescence (M1) (Figure 1).

To resolve these problems, investigators can extend the life span of bone marrow–derived MSC via retroviral transduction of human telomerase reverse transcriptase (hTERT) and human papillomavirus type 16 E6 and/or E7 genes [11]. A significant observation is that an increase in telomerase

Table 1.
Reactivities of Mesenchymal Stem Cells to Growth Factors*

	MSC	UCB	UE6E7T-12	UE7T-13
PDGF	+++	+++	+++	+++
EGF	+++	++	+++	++
aFGF	+++	++	++	+
bFGF	+++	+++	+++	++
LIF	–	+	++	+
VEGF	+	+	+	–
HGF	–	–	+	–
IGF-1	+	+	–	–
IL-1	–	–	–	++
IL-6	–	–	+	–

*Plus and minus symbols show the strength of growth factor reactivity based on cell proliferation. MSC indicates human bone marrow–derived mesenchymal stem cells; UCB, human umbilical cord blood–derived mesenchymal stem cells; UE6E7T-12 and UE7T-13, MSC transduced with human papillomavirus type 16 E6 and/or E7, and human telomerase reverse transcriptase (hTERT); PDGF, platelet-derived growth factor; EGF, epidermal growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic FGF; LIF, leukemia inhibitory factor; VEGF, vascular endothelial growth factor; IGF-1, insulin-like growth factor 1; IL-1, interleukin 1.

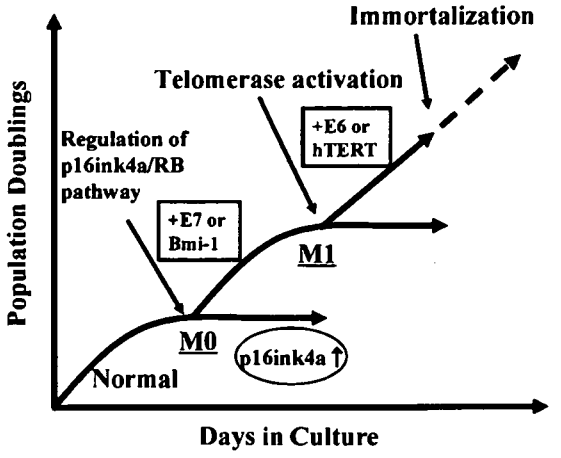

Figure 1. Life span of mesenchymal stem cells. Marrow-derived mesenchymal cells divide approximately 25 to 40 times in culture before they cease dividing or reach senescence (M0, mortality stage 0, ie, premature senescence). Some cells that overcome this step restart proliferation but stop dividing again in replicative senescence (M1). The system in which the p16ink4a/RB pathway is inhibited and telomerase is activated via transduction of *bmi-1*, human papillomavirus type 16 E6, E7, and hTERT is efficient in extending cellular life span.

activity produced via hTERT induction is insufficient to prolong the life span of bone marrow–derived MSC [23], because p16ink4a, a cyclin-dependent kinase inhibitor, is up-regulated [22]. This up-regulation of p16ink4a is directly linked to an increase in the number of cell doublings [24]. Inhibition of the p16/Rb pathway is sufficient to prolong the life span of cells in cultures of marrow-derived cells [11,22]. The p16ink4a/RB braking pathway leading to senescence can be inhibited by inducing the human papillomavirus type 16 E7 gene and/or *bmi-1*. *bmi-1*, one of the polycomb-group genes, has been used to inhibit p16ink4a transcription in order to prolong life span [25,26]. In addition, induction of the human papillomavirus type 16 E6 gene, which inhibits the p53 pathway, allows long-term cultivation of these cells. This system, in which the p16ink4a/RB pathway is inhibited and telomerase is activated, is highly efficient in extending the life span of bone marrow–derived MSC [23]. The life span of cord blood–derived MSC can be extended with hTERT alone [12].

Cell characteristics remain unaffected by *bmi-1*, E6, E7, and hTERT. The surface markers and the growth factor reactivities of transduced cells are unchanged (Table 1), and the transduced cells maintain their capabilities as stem cells [12,22]. So, can multipotent MSC with an extended life span be made available for cell-based therapy? It appears that transduced cells do not transform according to classic criteria: they do not generate a tumor in immunosuppressed interleukin 2 receptor knock-out NOD-SCID mice [22], they do not form foci in vitro, and they stop dividing after confluence. We cannot rule out the possibility, however, that gene-transduced bone marrow cells will become tumorigenic in patients several decades after commencement of cell therapy. What must be taken into account is that even when nononcogenic genes are introduced for cell-based therapy to increase cell growth and prolong life span, cases of leukemia have occurred in severe combined-immunodeficient patients treated with gene-modified lymphocytes [27]. Because of these failures, more time will be required before gene-modified cells can be used for regenerative medicine. Alleviation of culture stress is thus necessary to prolong the life span of mesenchymal cells.

Signaling from growth factor receptors caused by exogenously added growth factors induces p16ink4a protein through p38 and should selectively inhibit the prolongation of the cell life span without affecting growth factor–dependent cell proliferation through the classic Mek-Erk MAPK pathway (Figure 2A). Excessive stimulation by growth factors can be a cell senescence inducer, like oxidative stress and "culture shock" [28,29]. Growth factor–dependent acceleration of premature senescence or growth arrest is rather unexpected and unfavorable and is analogous to pressing down on the gas and brake pedals simultaneously [30,31]. Up-regulation of cell growth without affecting the cell life span, a key future goal of any cell-based therapy, would thus be a trade-off and create a fundamental quandary (Figures 2B and 2C).

4. Conclusion

Mammalian aging is associated with reduced regenerative capacity in tissues that contain stem cells. The aging process has been proposed to be at least partially caused by the senescence of progenitors with age; however, whether genes associated with senescence functionally contribute to physiological declines in progenitor activity has not yet been tested. Quantitative and qualitative changes do occur in stem cell populations with age [32-36]. The transition of multipotent stem cells to a more specific differentiated state is associated with simultaneous activation or inactivation of specific genes, and the promiscuous expression of many lineage-specific genes in primitive stem cells gradually decreases as cells reach a more mature state. Covalent modification

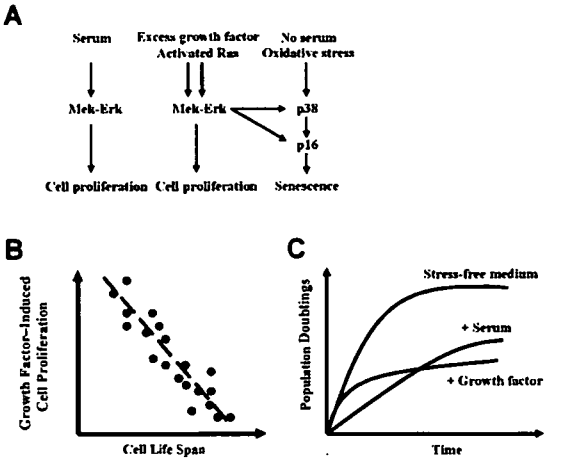

Figure 2. Signaling cascade and growth curves of mesenchymal stem cells for cell therapy. A, Scheme of the signaling cascade induced by stimuli leading to cell proliferation and senescence. B, Cell proliferation and the life span of mesenchymal cells are inversely correlated. C, Idealized cell growth profile in a stress-free medium. An ideal medium will help promote cell growth without affecting cellular aging.

includes acetylation, methylation, phosphorylation, and ubiquitination [37], and epigenetic regulation plays important roles in regulating gene expression. Consequently, the aging process influences stem cell–specific gene transcription. MicroRNA have recently been reported to be engaged in this regulation [38]. The goal of developing a clinically suitable medium for proliferating mesenchymal cells without affecting multipotency ex vivo appears feasible (Figure 2C), and its achievement should be beneficial to patients requiring an autologous or allogeneic transplant.

References

1. Alhadlaq A, Mao JJ. Mesenchymal stem cells: isolation and therapeutics. *Stem Cells Dev.* 2004;13:436-448.
2. Friedenstein AJ. Precursor cells of mechanocytes. *Int Rev Cytol.* 1976;47:327-359.
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. Umezawa A, Maruyama T, Segawa K, Shadduck RK, Waheed A, Hata J. Multipotent marrow stromal cell line is able to induce hematopoiesis in vivo. *J Cell Physiol.* 1992;151:197-205.
5. Owen M. Marrow stromal stem cells. *J Cell Sci Suppl.* 1988;10:63-76.
6. Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem.* 1997;64:295-312.
7. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999;284:143-147.
8. Sekiya I, Larson BL, Vuoristo JT, Cui JG, Prockop DJ. Adipogenic differentiation of human adult stem cells from bone marrow stroma (MSCs). *J Bone Miner Res.* 2004;19:256-264.
9. Horwitz EM. Mesenchymal cells: a basic review. 2002. Available at: http://www.celltherapysociety.org/files/documents/2005_Information_Booklet_-_Mesenchymal.doc. Accessed May 8, 2007.
10. Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood.* 2004;103:1669-1675.
11. 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.
12. Terai M, Uyama T, Sugiki T, Li XK, Umezawa A, Kiyono T. Immortalization of human fetal cells: the life span of umbilical cord blood-derived cells can be prolonged without manipulating $p16^{INK4a}$/RB braking pathway. *Mol Biol Cell.* 2005;16:1491-1499.
13. Lennon DP, Haynesworth SE, Young RG, Dennis JE, Caplan AI. A chemically defined medium supports in vitro proliferation and maintains the osteochondral potential of rat marrow-derived mesenchymal stem cells. *Exp Cell Res.* 1995;219:211-222.
14. Wiles MV, Johansson BM. Embryonic stem cell development in a chemically defined medium. *Exp Cell Res.* 1999;247:241-248.
15. Yao CL, Chu IM, Hsieh TB, Hwang SM. A systematic strategy to optimize ex vivo expansion medium for human hematopoietic stem cells derived from umbilical cord blood mononuclear cells. *Exp Hematol.* 2004;32:720-727.
16. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res.* 1961;25:585-621.
17. Campisi J. From cells to organisms: can we learn about aging from cells in culture? *Exp Gerontol.* 2001;36:607-618.
18. Wei W, Sedivy JM. Differentiation between senescence (M1) and crisis (M2) in human fibroblast cultures. *Exp Cell Res.* 1999;253:519-522.
19. Allsopp RC, Vaziri H, Patterson C, et al. Telomere length predicts replicative capacity of human fibroblasts. *Proc Natl Acad Sci U S A.* 1992;89:10114-10118.
20. Wright WE, Shay JW. The two-stage mechanism controlling cellular senescence and immortalization. *Exp Gerontol.* 1992;27:383-389.
21. Bodnar AG, Ouellette M, Frolkis M, et al. Extension of life-span by introduction of telomerase into normal human cells. *Science.* 1998;279:349-352.
22. Mori T, Kiyono T, Imabayashi H, et al. Combination of hTERT and *bmi-1*, E6, or E7 induces prolongation of the life span of bone marrow stromal cells from an elderly donor without affecting their neurogenic potential. *Mol Cell Biol.* 2005;25:5183-5195.
23. Okamoto T, Aoyama T, Nakayama T, et al. Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. *Biochem Biophys Res Commun.* 2002;295:354-361.
24. Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D, Barrett JC. Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proc Natl Acad Sci U S A.* 1996;93:13742-13747.
25. Haga K, Ohno S, Yugawa T, et al. Efficient immortalization of primary human cells by $p16^{INK4a}$-specific short hairpin RNA or Bmi-1, combined with introduction of hTERT. *Cancer Sci.* 2007;98:147-154.
26. Kim RH, Kang MK, Shin KH, et al. Bmi-1 cooperates with human papillomavirus type 16 E6 to immortalize normal human oral keratinocytes. *Exp Cell Res.* 2007;313:462-472.
27. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science.* 2003;302:415-419.
28. Sherr CJ, DePinho RA. Cellular senescence: mitotic clock or culture shock? *Cell.* 2000;102:407-410.
29. Toussaint O, Medrano EE, von Zglinicki T. Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. *Exp Gerontol.* 2000;35:927-945.
30. Ramirez RD, Morales CP, Herbert BS, et al. Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions. *Genes Dev.* 2001;15:398-403.
31. Raucci A, Laplantine E, Mansukhani A, Basilico C. Activation of the ERK1/2 and p38 mitogen-activated protein kinase pathways mediates fibroblast growth factor-induced growth arrest of chondrocytes. *J Biol Chem.* 2004;279:1747-1756.
32. Morrison SJ, Wandycz AM, Akashi K, Globerson A, Weissman IL. The aging of hematopoietic stem cells. *Nat Med.* 1996;2:1011-1016.
33. Harrison DE, Zhong RK, Jordan CT, Lemischka IR, Astle CM. Relative to adult marrow, fetal liver repopulates nearly five times more effectively long-term than short-term. *Exp Hematol.* 1997;25:293-297.
34. Kim M, Moon HB, Spangrude GJ. Major age-related changes of mouse hematopoietic stem/progenitor cells. *Ann N Y Acad Sci.* 2003;996:195-208.
35. Henckaerts E, Langer JC, Snoeck HW. Quantitative genetic variation in the hematopoietic stem cell and progenitor cell compartment and in lifespan are closely linked at multiple loci in BXD recombinant inbred mice. *Blood.* 2004;104:374-379.
36. Liang Y, Van Zant G, Szilvassy SJ. Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. *Blood.* 2005;106:1479-1487.
37. Bandyopadhyay D, Medrano EE. The emerging role of epigenetics in cellular and organismal aging. *Exp Gerontol.* 2003;38:1299-1307.
38. Oakley EJ, Van Zant G. Unraveling the complex regulation of stem cells: implications for aging and cancer. *Leukemia.* 2007;21:612-621.