MSCs can be a useful source of cells for transplantation for several reasons: they have the ability to proliferate and differentiate into mesodermal tissues, including heart, they entail no ethical or immunological problems, and bone marrow aspiration is an established routine procedure. When placed in appropriate *in vitro* and *in vivo* environments, MSCs can give rise to all major mesenchymal tissues, such as bone, cartilage, muscle, and adipose tissue [18]. Murine MSCs can also differentiate into cardiomyocytes and start to beat synchronously *in vitro* [19], and direct injection of murine MSCs into the heart has been shown to be feasible in murine models of ischemic heart disease and normal mouse heart. Thus far, only endothelial cells have been shown to exhibit '*in vitro* cardiomyogenesis' in humans [20].

Large numbers of cells must be injected into damaged sites in ischemic heart disease to restore cardiac function in humans, and cells need to be injected into the entire heart in cardiomyopathy. Until now, however, there have been no reports of a sufficient number of differentiated human cardiomyocytes ever having been obtained to restore the function of a failing heart. One of the reasons for this is that the life span of human cells *in vitro* is limited. Human cells reach senescence or stop cell growth after a limited number of cell replications [21], and the average number of hMSC population doublings (PDs) has been found to be 38 [22], implying that it would be difficult to obtain enough cells to restore the function of a failing human heart.

To resolve these problems and to establish a model of cell therapy of the failing heart, we attempted to prolong the life span of hMSCs by using the system to infect retrovirus encoding bmi-1, human telomerase reverse transcriptase (TERT), and human papillomavirus E6 and E7 genes. Both Rb/p16INK4a inactivation with E7 and telomerase activation with E6 are required to extend the life span of human epithelial cells [23]. bmi-1, a c-myc cooperating oncogene in murine lymphomas, reduces expression of p16INK4a, stimulates cell proliferation [24], and is required for maintenance of self-renewing hematopoietic stem cells [25,26]. This method was highly efficient in extending the life span of hMSCs. In the present study we investigated whether hMSCs with an extended life span have the ability to differentiate into cardiomyocytes *in vitro*.

Materials and methods

Isolation and cell culture of hMSCs

After obtaining signed informed consent, bone marrow cells were harvested from a 91-year-old human female donor with the approval of the Ethics Committee of Keio University School of Medicine (Tokyo). Cells were resuspended in bone marrow stromal cell culture medium (10% fetal bovine serum in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose [DMEM-HG]) with antibiotic/antimycotic supplements (Gibco), and cultures were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. When the cultures reached subconfluence, the cells were harvested with 0.25% trypsin and 1 mM EDTA, and replated with one half of the harvested cells. After a series of passages, the attached marrow stromal cells were devoid of hematopoietic cells. Several bone marrow stromal cell strains were then generated by the limiting-dilution method, and one of them was designated H4-1. The H4-1 cells were cultured in MSC growth medium (MSCGM) at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

Preparation and infection of recombinant retroviruses

The full-length human bmi-1 cDNA was cloned by RT-PCR using RNA extracted from K562 cells. Thermoscript reverse transcriptase (Invitrogen) and KOD polymerase (TOYOBO, Japan) were used for the RT and PCR reactions, respectively. The forward primer, 5′-ACGCGTCGACCGCCATGCATCGAACAACGAGAAT-3′, and reverse primer, 5′-CGGATCCTCAACCAGAAGAAGTTGCTG-3′, were designed to obtain the coding sequence of human bmi-1 flanked by the *Sal*I site (underlined) and the Kozak consensus sequence at the 5′-end and the *Bam*HI site (underlined) at the 3′-end. The *Sal*I-*Bam*HI segment of the PCR product was cloned between the *Xho*I and *Bgl*II sites of pCLXSN to generate pCLXSN-bmi1. The coding sequence of the cDNA was confirmed to be identical to the published sequence (NCBI ACC# NM_005 180.4). Construction of pCLXSH-hTERT has been described previously [27]. The gateway system (Invitrogen) was used to subclone a deletion mutant of HPV16 E6 (16E6SDD151) that lacked transforming activity to 3Y1 cells [28] into pCMSCVpuro. pCMSCVpuro comprises the CMV/LTR fusion promoter, the packaging signal Psi, and the multicloning sequence from pCLXSN (Imgenex Corp., San Diego, CA, USA) followed by the PGK-puro cassette and the 3′ long terminal repeat of murine embryonic stem cell virus from pMSCVpuro (Clontech). The destination vector pCMSCVpuro-DEST was constructed by inserting a modified cassette containing attR sites and ccdB (Invitrogen) between the *Eco*RI and *Bgl*II sites of pCMSCVpuro. 16E6SDD151 was first recombined into pDONR201 by BP reaction, and then into the destination vector by LR reaction according to the manufacturer's instructions (Invitrogen) to generate pCMSCVpuro-16E6SDD151. Production of recombinant retroviruses has been described previously [29,30]. Briefly, the retroviral vector together with the packaging construct, pCL-10A1, was transfected into 293T cells, and the culture fluid was harvested 48–72 h post-transfection. The preparation of the LXSN-16E7 retrovirus and the infection protocols have been described previously [31], except that FLYA13 [32] was used as the packaging cell line instead of PG13. The titers of the recombinant viruses were greater than 5×10^5 drug-resistant colony-forming units per milliliter on HeLa cells, and 1 ml of the culture fluid was added to the cells in the presence of polybrene (8 µg/ml). Following inoculation with the viruses, hMSCs were grown in the presence of G418 (100 µg/ml), hygromycin B (50 µg/ml), or puromycin (1 µg/ml), and a polyclonal drug-resistant cell line was established and further analyzed. To achieve combinations of retroviral infections, cells were sequentially transduced with LXSN-E7 or LXSN-bmi-1, and LXSH-hTERT, and then MSCVpuro-16E6SDD151, if indicated, and selected with G418, hygromycin B, and puromycin, respectively. The stably transduced cells with an expanded life span were designated UBT-5, UBET-7, UEET-1, UEET-11, and UET-13.

Flow cytometric analysis

Cells were detached and stained for 30 min at 4 °C with primary antibodies and immunofluorescent secondary antibodies. After washing, the cells were analyzed on an EPICS ALTRA analyzer (Beckman Coulter). Antibodies (anti-human CD13, CD14, CD24, CD29, CD31, CD34, CD44, CD45, CD50, CD54, CD55, CD59, CD90, CD105, CD117, CD133, CD140a, CD166, Flk-1) were purchased from Beckman Coulter, Immunotech, Cytotech, and Pharmingen Pharmaceutical, Inc.

Introduction of the GFP and β-galactosidase genes

Recombinant adenovirus expressing β-galactosidase and the green fluorescent protein (GFP) was prepared as described [33]. Cells were infected with these viruses at 10 plaque-forming units/cell. hMSCs were examined cytochemically *in vitro* for expression of the β-galactosidase gene and by fluorescent confocal microscopy for expression of the GFP gene. By 7 days post-infection nearly all the cells expressed β-galactosidase and GFP.

Preparation of murine fetal cardiomyocytes

Fetal cardiomyocytes were obtained from the hearts of day 14 mouse fetuses. 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 for 5 min at 37 °C. After adding DMEM supplemented with 10% fetal bovine serum (FBS), the cardiomyocytes were centrifuged at 1000 rpm for 5 min. The pellet was then resuspended in 10 ml DMEM with 10% FBS and incubated on glass dishes for 1 h to separate the cardiomyocytes from fibroblasts. The floating cardiomyocytes were collected and replated at $1 \times 10^5/\text{cm}^2$.

hMSC and murine fetal cardiomyocyte co-culture system

Human MSCs were plated on dishes at $5 \times 10^4/\text{cm}^2$, and infected with EGFP-expressing adenovirus on the next day. The supernatant was then removed, and the cells were cultured for 2 days in DMEM supplemented with 10% FBS. The cells were then exposed to 10 µM of 5-azacytidine for 24 h to induce cell differentiation. The 5-azacytidine-treated hMSCs were harvested with 0.25% trypsin and 1 mM EDTA and overlaid onto the fetal cardiomyocytes at $5 \times 10^3/\text{cm}^2$. The morphology of the beating hMSCs was evaluated under a fluorescent microscope.

RT-PCR

Total RNA was prepared from co-cultured hMSCs and mouse heart with Isogen (Nippon Gene). Human cardiac RNA was purchased (Clontech). RNA for RT-PCR was converted to cDNA with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotec) according to the manufacturer's recommendations. RT-PCR of the bmi-1, E6, E7, TERT, myosin light chain-2a (MLC-2a), Nkx2.5, and human atrial natriuretic peptide (hANP) genes was performed, and the PCR primers used are listed in Table 1. RT-PCR was performed with PCR primers that can amplify human but not mouse genes. PCR primers of 18S used as a positive control react with both human and murine genes. PCR was performed with TaKaRa Z-Taq (Takara Shuzo Co., Ltd) for 30 cycles, with each cycle consisting of 98 °C for 5 s, 68 °C or 60 °C for 1 s, and 72 °C for 10 s, with an additional 30-s incubation at 72 °C after completion of the final cycle.

Action potential recording and microinjection of dye

An inverted microscope (IX-70, Olympus, Tokyo, Japan) with a fluorescence filter (U-MNIBA2, Olympus) was used for action potential (AP) recording. The microscope was equipped with a recording chamber and a noise-free heating plate (Microwarm Plate, Kitazato Supply, Fujinomiya, Shizuoka, Japan). A 10 mmol/l volume of HEPES was added to the culture medium to stabilize the pH of the perfusate at 7.5–7.6. Standard glass microelectrodes having a DC resistance of 25–35 MΩ when filled with pipette solution were used. Alexa 568 compound was dissolved to a concentration of 0.5 mmol/l in 2 mol/l of KCl solution in order to completely dissolve the Alexa 568 in the pipette solution. The electrodes were positioned with a motor-driven micromanipulator (PCS-5000, Burleigh Instruments, Inc., New York, USA) under optical control. Spontaneously beating GFP-positive cells were selected as targets, and, after the APs of the targeted cells had been recorded, the dye was injected by iontophoresis (−7 nA for 30–60 s). The extent

Table 1. PCR primers used in this study

Gene product	Primer (sense)	Primer (anti-sense)	Annealing temperature (°C)	Product size (bp)
Bmi-1	TCATCCTTCTGCTGATGCTG	GCATCACAGTCATTGCTGCT	60	220
E6	GACCCAGAAAGTTACCACAG	GCAACAAGACATACATCGAC	60	397
E7	ATGACAGCTCAGAGGAGGAG	TCCTAGTGTGCCCATTAACAG	60	178
TERT	CGGAAGAGTGTCTGGAGCAA	GGATGAAGCGGAGTCTGGA	60	144
MLC-2a				
1st	TCGTGATGGCATCATCTGCAAGG	ACAGAGTTTATTGAGGTGCCCC	60	429
2nd	AAGGTGAGTGTCCCAGAGG	ATGGGTGTCAGGGCGAACATC	60	259
NkX2.5				
1st	CTTCAAGCCAGAGGCCTACG	CCGCCTCTGTCTTCTCCAGC	60	233
2nd	CTTCACCGGCCAAGTGTGCGTC	CCGCCTCTGTCTTCTCCAGC	60	152
hANP				
1st	GAACCAGAGGGGAGAGACAGAG	CCCTCAGCTTGCTTTTTAGGAG	60	406
2nd	GTCAGACCAGAGCTAATCCC	ACCTCCATCTCTCTGGGCTG	68	223
18S	GTGGAGCGATTTGTCTGGTT	CGCTGAGCCAGTCAGTGTAG	60	200

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 the microscope with a fluorescence filter (U-MWIG2; Olympus). The recording pipette was connected to a patch-clamp amplifier (Axopatch 200B; Axon Instruments), and the signal was low-pass filtered at 2 kHz and digitized with an A/D converter with sampling frequency of 10 kHz (Digidata 1322A; Axon Instruments) connected to a computer with Pentium4. Signals were monitored, recorded as electric files, and analyzed offline with pCLAMP 8.2 software (Axon Instruments). The rhythm was considered regular if the maximum beating rate minus the minimum beating rate divided by the max beating rate was <0.4.

Immunohistochemistry

The hMSCs co-cultured with fetal cardiomyocytes *in vitro* were fixed with 4% PFA and stained with anti-β2microglobulin antibody at 1 : 1000, mouse monoclonal antibody against troponin I (Hytest, Euro, Finland) at 1 : 200, anti-desmin antibody at 1 : 100, and anti-β-galactosidase antibody (Chemicon) at 1 : 500. hMSCs expressing GFP were fixed with 4% PFA.

Results

Establishment of hMSCs with an extended life span

H4-1 cells were obtained from primary culture by limiting dilution (Figure 1A). The cells proliferated for a limited number of passages and then underwent senescence, as evidenced by the cells assuming a broad and flattened shape (Figures 1B and 1C). To extend the life span of H4-1 cells, and obtain a large number of cells for cardiac transplantation, four different types of cells were obtained by transferring combinations of bmi-1, E6, E7, and/or TERT genes. Cells transduced with bmi-1 and TERT were designated UBT-5 cells; cells transduced with bmi-1, E6, and TERT were named UBET-7 cells; cells transduced with E7 and TERT were designated UET-13 cells; and cells transduced with E6, E7, and TERT were named UEET-1 and UEET-11 cells (Figures 1D, 1E, and 1F). To simplify nomenclature and avoid confusion, we use the name UEET-1 to refer to cells transduced with E6, E7, and TERT although they have recently been reported as ThMSC1 [29]. The cells were subcloned after each gene transfer, and thus were clonal. The UEET-1 cells were spindle-shaped, and longer than the parental H4-1 cells (Figures 1B, 1D, and 1E). Characteristics of cells with a prolonged life span were investigated. UEET-11 and UET-13 proliferated more than 150 PDs in 400 days, and UBET-7 and UBT-5 proliferated more than 50 PDs in 400 days, while H4-1 stopped dividing at 38 PDs (approximately 200 days). The growth rates of UEET-11 and UET-13 were higher than those of UBT-5 and UBET-7. Chromosome analysis revealed parental H4-1 and UET-13 to exhibit normal karyotypes, while the other cells transduced with E6 and E7 showed chromosome aberrations at low frequencies (data not shown). The transduced cells did not generate tumors, at least for the first 60 days after subcutaneous transplantation into immunodeficient mice.

Surface analysis of hMSCs

Surface markers of the UEET-1, UEET-11, UBT-5, UBET-7, and UET-13 cells were evaluated by flow cytometric analysis. The results showed that all of the MSCs were positive for CD13, CD29 (integrin β1), CD44 (Pgp-1/ly-24), CD55, CD59, CD90 (Thy-1), CD105 (endoglin), CD133, CD140a (PDGFRα or PDGFR2), and CD166 (ALCAM), and negative for CD14 (a marker for macrophage and dendritic cells), CD24, CD31 (PECAM-1), CD34, CD45 (leukocyte common antigen), CD50 (ICAM-3), CD54, CD117 (c-kit), and Flk-1 (Figure 2). Parental H4-1 cells had the same pattern of surface markers as UEET-1, UEET-11, UBT-5, and UBET-7 cells, implying that the surface markers were not influenced by

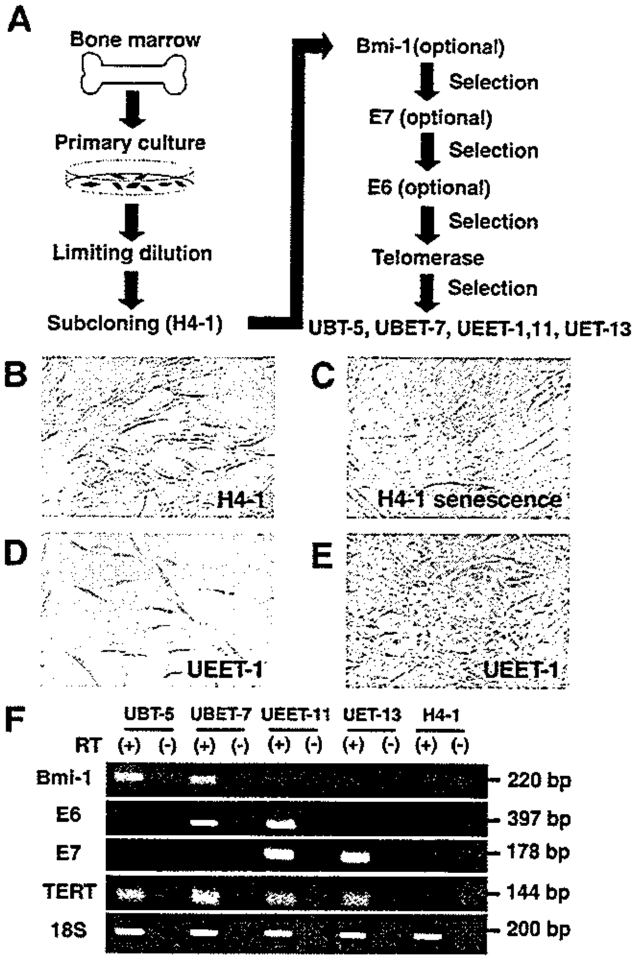

Figure 1. Experimental scheme. (A) Bone marrow stromal cells were obtained from a human donor and subcloned by limiting dilution. One of the cells isolated was designated H4-1 cells, and they were transduced with E6, E7, TERT, or bmi-1 genes to extend their life span. The combinations of genes transferred were: (1) bmi-1 and TERT; (2) bmi-1, E7, and TERT; (3) E7 and TERT; and (4) E6, E7, and TERT. (B) H4-1 cells in the growth phase. (C) H4-1 cells at senescence. The cells showed a broad and flattened shape. (D) H4-1 cells after transfer of E6, E7, and TERT genes were designated UEET-1 cells. (E) UEET-1 cells at confluence. Original magnification, B–E: ×100. (F) The gene expression in each cell line was analyzed using RT-PCR

the exogeneously expressed bmi-1, E6, E7, and/or TERT genes.

Cardiomyogenic differentiation of hMSCs and stably transduced hMSCs

To determine whether H4-1 cells could be induced to undergo cardiomyogenic differentiation, the cells were exposed to 10 µM of 5-azacytidine for 24 h as previously reported in murine stromal cells [19]. All of the transduced hMSCs did not exhibit spontaneous beating despite continuous culturing for up to 3 months. Immunocytochemical analysis revealed the presence of desmin, a myocytic marker, in the hMSCs with an extended life span, i.e., UBT-5 cells and UBET-7 cells (Figure 3A). However, all cells tested were negative for the cardiomyocyte marker troponin-I (Figure 3B).

We employed a co-culture system with fetal cardiomyocytes to induce cardiac differentiation (Figure 4), since *in vitro* simulation of the heart by the environment has been shown to be an efficient means of induced differentiation of human endothelial progenitor cells and murine marrow stromal cells [20,34]. After exposing GFP-labeled UBT-5, UBET-7, UEET-11, and UET-13 cells to 10 µM of 5-azacytidine for 24 h, these cells were co-cultured with fetal cardiomyocytes. On day 3 after the start of co-cultivation, a few GFP-positive UBET-7 cells started to contract (Figure 5A). The contraction was stronger when beating cells were clustered than when scattered (Figure 5B). On day 7, the beating of the UBET-7 cells was synchronous with that of adjacent cells and was independent of that of the surrounding murine cardiomyocytes (Figures 5C and 5D). Repetition of these experiments confirmed the results to be reproducible, and the percentages of UBT-5, UBET-7, UEET-11, and UET-13 cells that underwent cardiomyogenic differentiation were almost the same, implying that cardiomyogenic differentiation is independent of the genes transferred. The number of beating cells increased for up to 3–4 weeks, when the fetal cardiomyocytes spontaneously detached from the dishes (Figure 5E). UBET-7 cells not treated with 5-azacytidine were co-cultured with fetal cardiomyocytes to determine whether environmental factors alone can induce cardiac differentiation, but fewer beating cells were observed (Figure 5F). No significant difference was detected in the number of differentiated cells between parental H4-1 and UBET-7 (Figure 5G).

Expression of cardiomyocyte-specific genes and proteins and the action potential of differentiated hMSCs

We analyzed the co-cultured UBET-7 cells in terms of gene expression and by immunocytochemistry and electrical recording. RT-PCR was performed with primers that react with human cardiomyocyte-specific genes but not with murine orthologues. Differentiated UBET-7 cells expressed MLC-2a, hANP, and the cardiomyocyte-specific transcription factor, Nkx2.5/Csx (Figure 6). Sequence analysis revealed that the cDNAs matched the sequences of the human MLC-2a, hANP, and Nkx2.5/Csx genes.

Action potentials were recorded from spontaneously beating cells. Alexa 568 was injected into cells via a recording microelectrode to stain the cells and confirm that the action potential was generated by GFP-positive UBET-7 cells (Figures 7A and 7B). Since the dye did not diffuse into the murine cardiomyocytes, there were no tight cell-to-cell heterologous connections, i.e., gap junctions. In some experiments, Alexa 568 diffused into the GFP-positive satellite UBET-7 cells, suggesting that a homologous cell-to-cell connection had been established at least 1 week after co-cultivation. The measured parameters of the recorded action potential were averaged

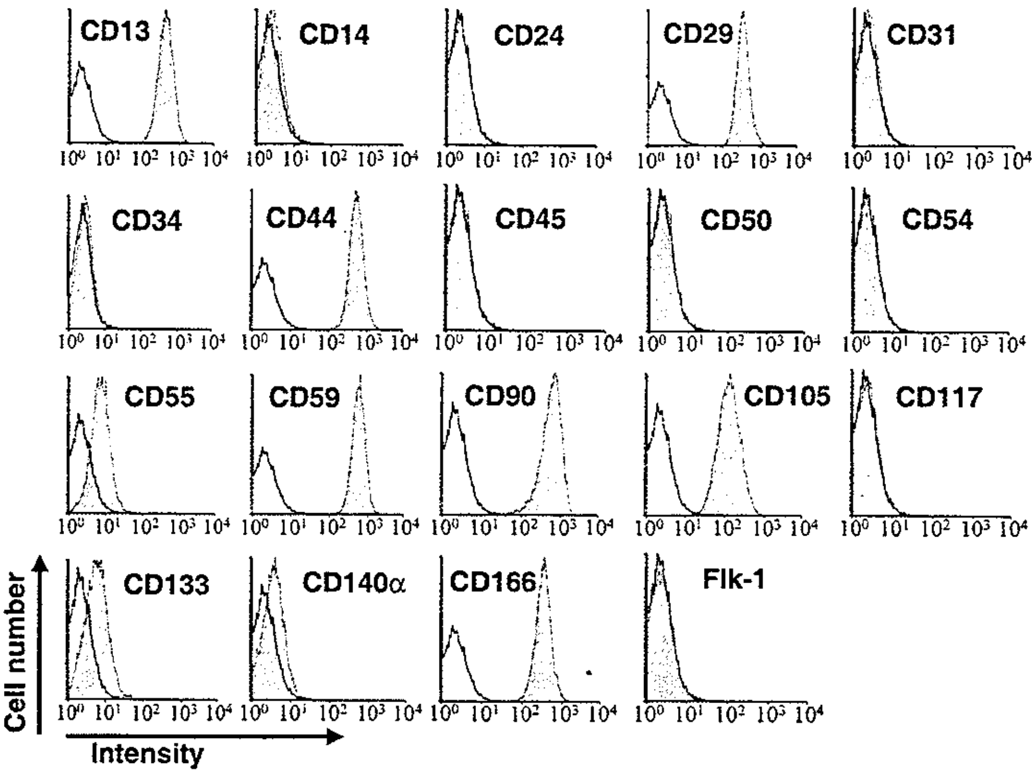

Figure 2. Flow cytometric analysis of UEET-1 cells. UEET-1 cells were labeled with FITC-coupled antibodies against CD13, CD14, CD24, CD29, CD31, CD34, CD44, CD45, CD50, CD54, CD55, CD59, CD90, CD105, CD117, CD133, CD140a, CD166, and Flk-1 and analyzed with an EPICS ALTRA analyzer

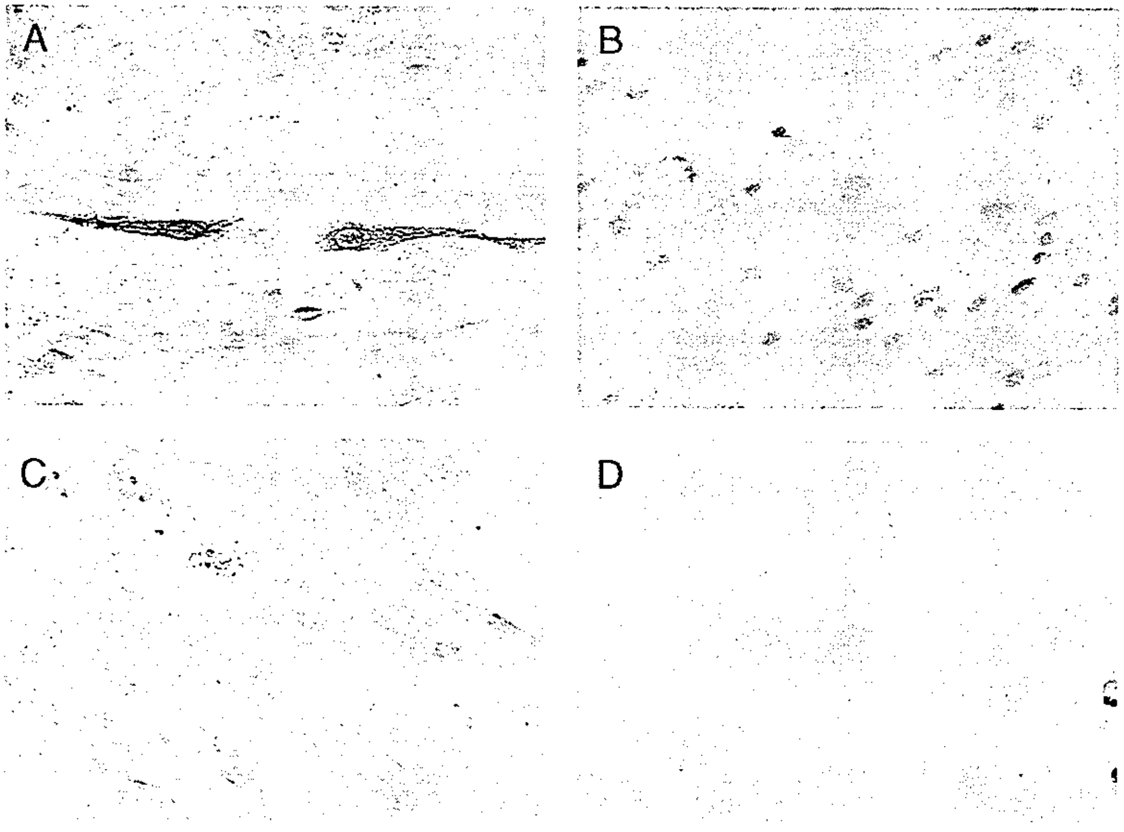

Figure 3. Immunostaining of hMSCs with anti-desmin and anti-troponin-I antibodies after exposure to 5-azacytidine. UBET-7 cells were exposed to 10 μM of 5-azacytidine for 24 h and stained for desmin (A) and cardiac troponin I (B). UBET-7 cells not treated with 5-azacytidine were also stained for desmin (C) and cardiac troponin I (D). Original magnification: ×400

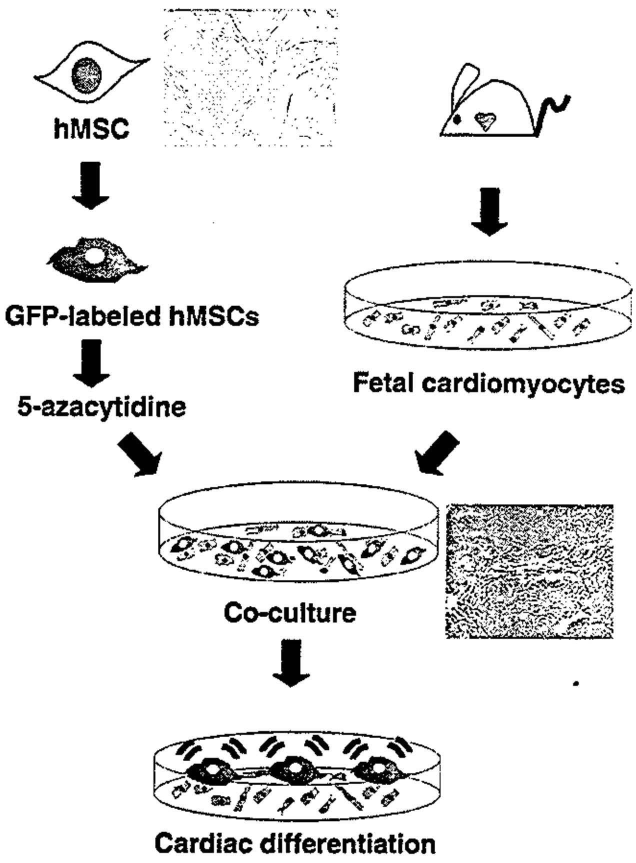

Figure 4. Scheme of the co-culture system. hMSCs infected with adenoviruses carrying the GFP gene were treated with 10 μM of 5-azacytidine for 24 h. hMSCs expressing GFP were then co-cultured with murine fetal cardiomyocytes. hMSCs began beating spontaneously after 7 days of co-culture

(Table 2). The duration of the action potentials of the UBET-7 cells was extremely long, and they were therefore concluded to be action potentials of cardiomyocytes, not of smooth muscle, nerve cells, or skeletal muscle. Time-course analysis of the action potentials revealed shortening of their duration, a gain in amplitude, and stabilization and organization of the spontaneous beating rhythm. Representative action potential recordings are shown in Figures 7C and 7D. The rhythm of some (33%) of the UBET-7 cells was still disorganized at 1 week (Figure 7C), whereas the rhythm of the UBET-7 cells (100%) had become regular and had stabilized at 3 weeks (Figure 7D).

Immunohistochemistry revealed that UBET-7 cells expressing human β2microglobulin and GFP stained positive for desmin (Figures 8A–8C) and cardiac troponin I (Figures 8D–8F) on day 14. Clear striations were observed in the differentiated UBET-7 cells (Figure 8H).

Absence of cell fusion between hMSCs and murine fetal cardiomyocytes

To determine whether the beating cells had fused with the fetal cardiomyocytes, GFP-expressing hMSCs were co-cultured with fetal cardiomyocytes labeled with β-galactosidase. On day 7, when almost 100% of the cardiomyocytes were labeled with β-galactosidase, and almost 100% of the co-cultured-hMSCs expressed GFP, none of the cells were double-stained for GFP and β-galactosidase (Figures 9A–9D). This observation indicates that the cardiomyogenic differentiation of hMSCs is not attributable to cell fusion on day 7.

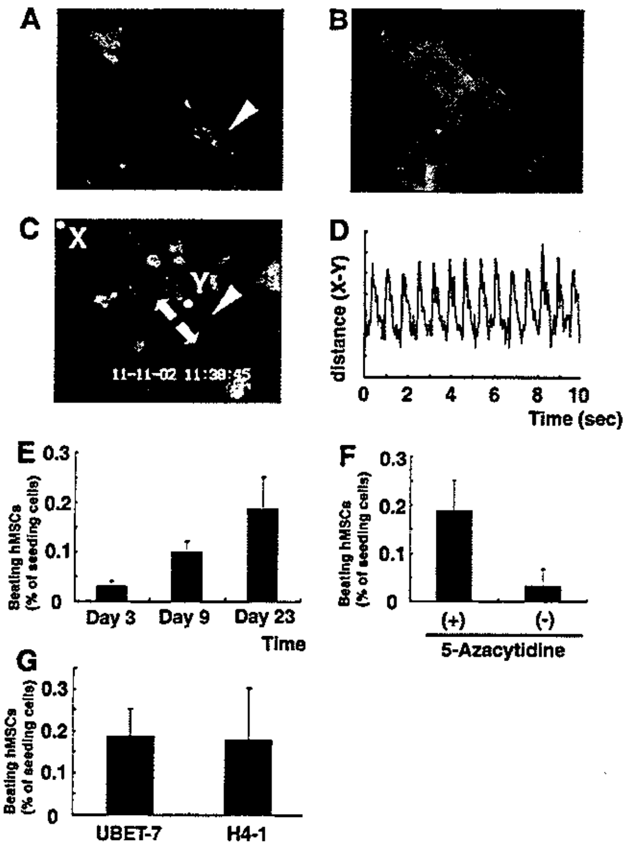

Figure 5. Beating of hMSCs in the *in vitro* co-culture system. (A) 5-Azacytidine-treated GFP-positive UBET-7 cells were co-cultured with murine fetal cardiomyocytes (http://1985.jukuin.keio.ac.jp/umeza/jgm/ubet7). The white arrowhead is pointing to some beating UBET-7 cells whose rhythm was different from that of the fetal cardiomyocytes. (B) More UBET-7 cells tended to contract in areas where they were clustered than in areas where they were scattered. (C) Beating UBET-7 cells were videotaped at 30 flames/s and their contractions were analyzed. Point X in this view was fixed, and point Y was used as a reference point on the differentiated UBET-7 cell (arrowhead). Arrows point in the direction of contraction, and point Y moved with each contraction. Original magnification, A–C: ×150. (D) The distances between points X and Y were measured for a 10-s period and plotted on the graph. The UBET-7 cells contracted regularly at 84 beats/min. (E) The ratio of the number of beating UBET-7 cells to the number of the cells seeded increased for 3 weeks. (F) On day 23, the ratio was higher in the cells exposed to 5-azacytidine than in the cells not exposed to 5-azacytidine. (G) Parental H4-1 was compared with UBET-7 in terms of the number of beating cells

Discussion

This study was conducted to determine whether prolongation of cell life span by cell-cycle-associated molecules

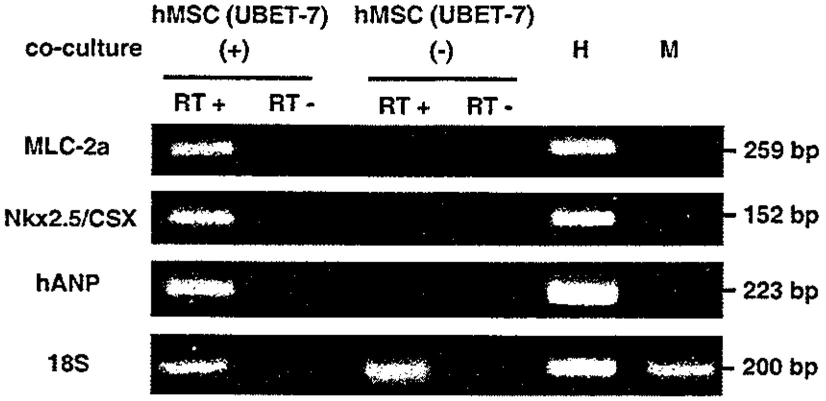

Figure 6. Expression of cardiomyocyte-specific genes in differentiated hMSCs (UBET-7). RT-PCR was performed with PCR primers that react with human genes encoding cardiac proteins (MLC-2a, Nkx2.5, and hANP) but do not with the murine genes. Only the 18S PCR primer used as a positive control reacted with the human and murine genes. Human heart (H) and mouse heart (M) were used as a positive control and negative control, respectively. The human cardiac genes, MLC-2a, Nkx2.5/csx and hANP, were expressed in the co-culturing system, but were not expressed in the undifferentiated state (without feeder cells)

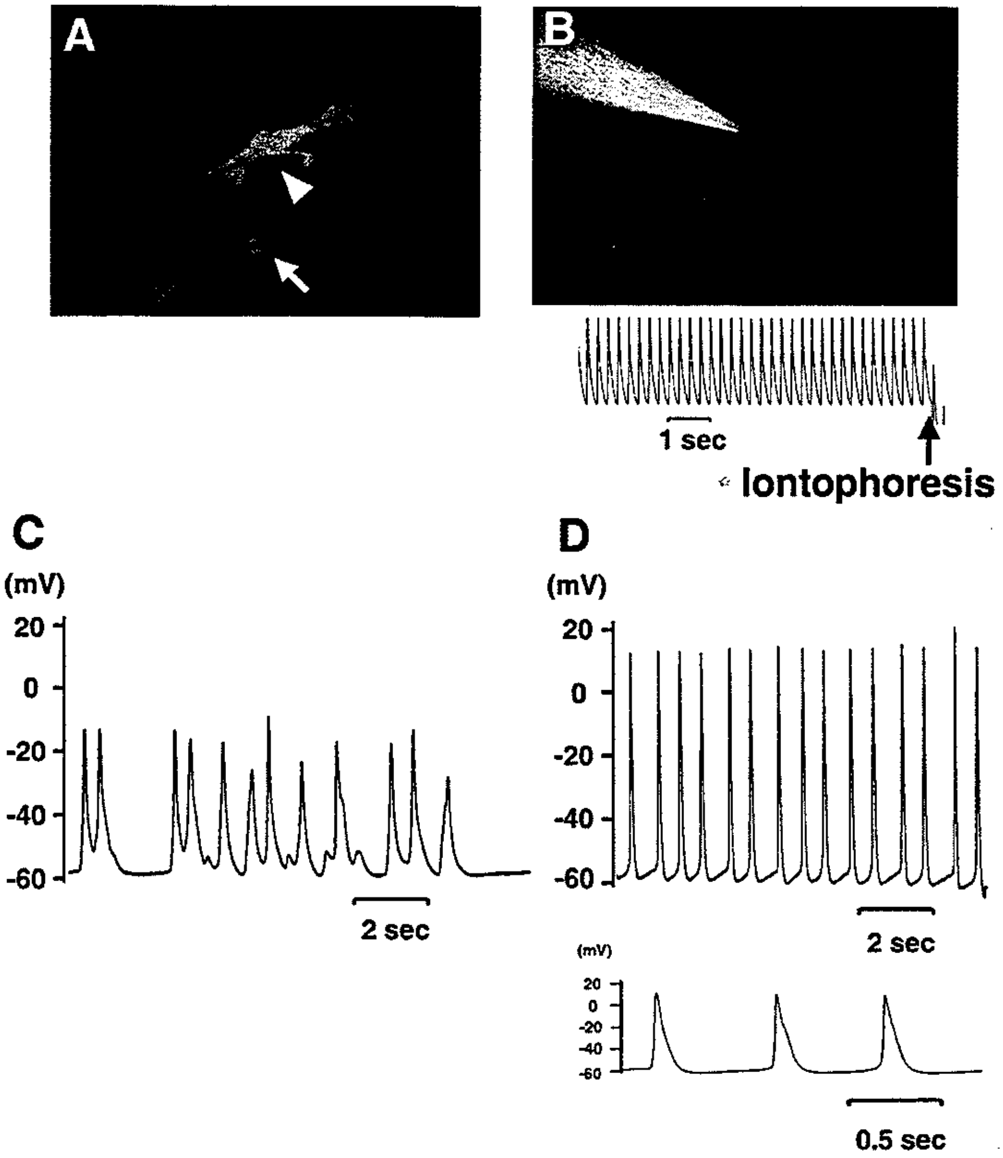

Figure 7. Action potentials of beating hMSCs. GFP-labeled UBET-7 cells (A) were injected with Alexa 568 solution (B) by iontophoresis through a microelectrode. The injected UBET-7 cell and neighboring beating UBET-7 cell are indicated by arrowhead and arrow, respectively. The action potential was recorded (B, lower panel). Some of the rhythms recorded at 1 week of co-cultivation were irregular (C), but the rhythm became regular at 3 weeks (D); top: large scale (2 s), bottom: small scale (0.5 s). Original magnification, A, B: ×400

Table 2. Action potential parameters in human mesenchymal stem cell derived cardiomyocytes

Time of co-culture	n	Ratio of regular rate	Beating rate (beats/min)	MDP (mV)	Amplitude (mV)	APD_{90} (ms)
1 week	12	67%	70.6 ± 12.8	−49.9 ± 1.6	47.2 ± 2.9	345.8 ± 21.4
2 weeks	9	67%	65.9 ± 12.7	−50.3 ± 2.3	60.2 ± 4.5	169.7 ± 13.8
3 weeks	9	100%	68.2 ± 12.1	−45.1 ± 1.4	63.7 ± 3.0	163.4 ± 16.5

The values are shown as mean ± S.E. The ratio of regular rate: regular beating rhythm/irregular beating rhythm. MDP: maximum diastolic potential. APD: action potential duration.

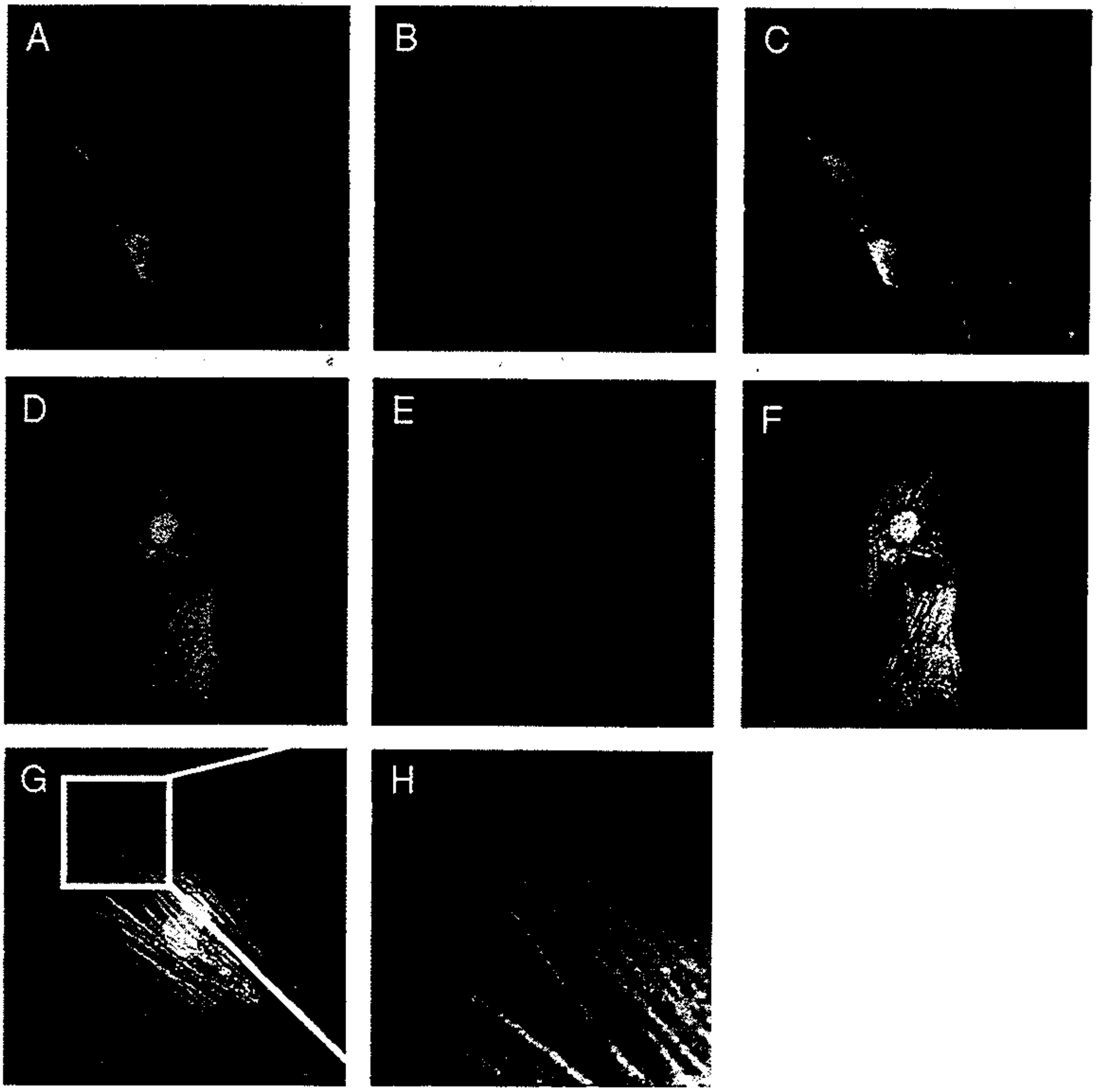

Figure 8. Immunocytochemistry of differentiated hMSCs with anti-desmin and anti-troponin-I antibodies. UBET-7 cells were co-cultured with cardiomyocytes. UBET-7 cells were analyzed for myogenic and cardiac differentiation by immunohistochemistry with desmin and cardiac troponin-I, respectively. Co-cultured UBET-7 cells were stained with anti-β2microglobulin antibody (A) and anti-desmin antibody (B). A superimposed image ('Merge') of A and B is shown in C. GFP-expressing UBET-7 cells (D) were stained with anti-troponin-I antibody (E). 'Merge' is shown in F. A differentiated UBET-7 cell is shown at higher magnification (G). The differentiated UBET-7 cells have striations in their cytoplasm (H). Original magnification, A–G: ×600, H: ×2000

would predominate over cardiomyogenic differentiation of marrow stromal cells *in vitro*. The primary findings of the present study were: (1) the life span of hMSCs was extended by bmi-1, E6, E7, and TERT; (2) hMSCs exposed to 5-azacytidine and cultured with fetal cardiomyocytes underwent cardiomyogenic differentiation as manifested by their morphology, gene expression, and electrophysiology, and started to beat spontaneously (automaticity); and (3) cardiomyogenic differentiation of the hMSCs was not attributable to cell fusion.

MSCs are pluripotent cells capable of differentiating into many cell types, such as neurons [35], myocytes, cardiomyocytes, chondrocytes, and adipocytes [36]. Multipotent adult progenitor cells (MAPCs) have recently

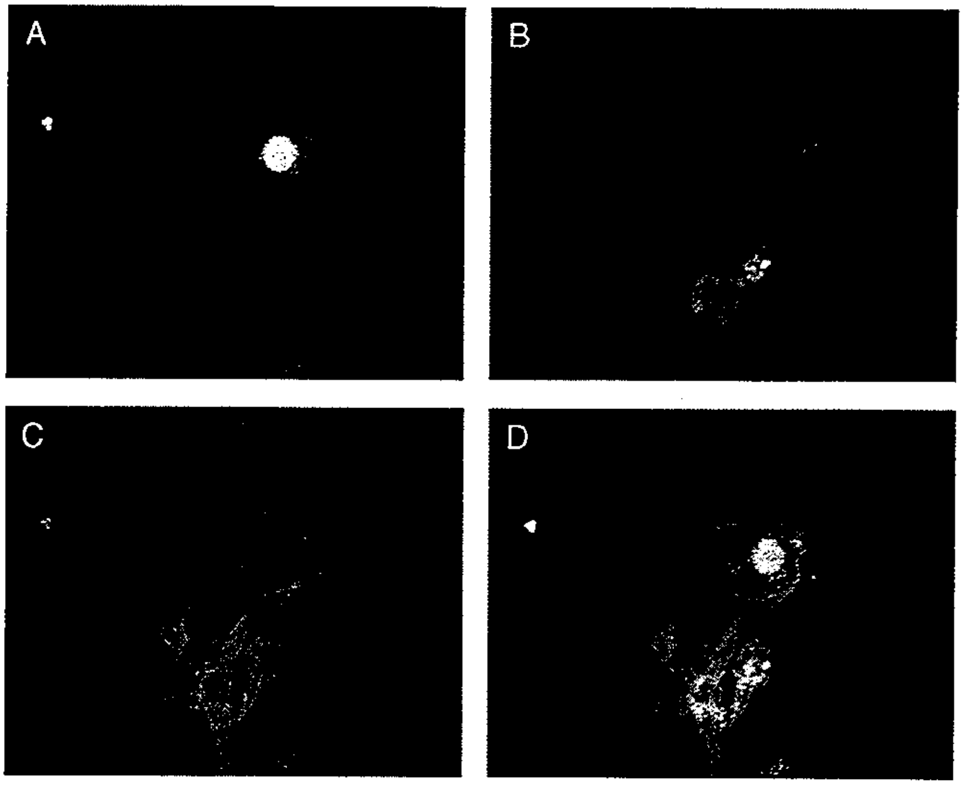

Figure 9. Cardiomyogenic differentiation of hMSCs is not due to cell fusion. We labeled UBET-7 cells with GFP (A) and murine cardiomyocytes with β-galactosidase (B). Cells were tested for the presence of β-galactosidase immunocytochemically (B, Cy5). Differentiation of UBET-7 cells was determined with anti-troponin I antibody (C, Rhodamine). 'Merge' of A, B, and C is shown in D. The differentiated GFP-expressing UBET-7 cells had not fused with murine feeder cells labeled with β-galactosidase. Original magnification: x600

been shown to differentiate, at single cell level, not only into mesenchymal cells, but also into cells with characteristics of visceral mesoderm, neuroectoderm, and endoderm [37]. MAPCs have the ability to proliferate extensively without any clear evidence of senescence or loss of differentiation potential, and they are thought to be extremely similar to embryonic stem (ES) cells. MSCs and MAPCs also have the advantages of the absence of ethical and immunological problems, and undesired differentiation is infrequent. Thus they are one of the most promising sources of cells for cell therapy.

However, there are two major problems with hMSCs: (1) a large enough number of cells to regenerate tissue by cell transplantation is difficult to obtain, and (2) detailed investigation has been limited by their finite life span. A system that allows human cells to escape senescence by using cell-cycle-associated molecules may be used to obtain sources of cell therapy and to overcome these problems and establish a good model of cell transplantation to the failing heart [23,38]. Both inactivation of the Rb/p16INK4a pathway and activation of telomerase are required for immortalization of human epithelial cells such as mammary epithelial cells and skin keratinocytes. Human papillomavirus E7 can inactivate pRb, and bmi-1 can repress p16INK4a expression. Inactivation of the p53 pathway is also beneficial, even if not essential, to extension of the life span [39]. Based on the above notion, we transferred E7 or bmi-1 plus hTERT in combination with or without E6 into hMSCs and obtained several hMSC strains with an extended life span: UEET-1, UBT-5, UBET-7, UEET-11, and UET-13 cells. The cells with the extended life span continued growing *in vitro* for over 150 PDs, and their differentiation potential was maintained. Transfer of TERT alone was insufficient to prolong the life span of hMSCs in the present study, despite TERT having been reported to extend the life span of cells beyond senescence without affecting their differentiation ability [40]. The characteristics of the cells with an extended life span were unchanged after transfer of bmi-1, E6, E7, and TERT genes, and this finding is consistent with flow cytometric analysis showing that the surface markers of H4-1 cells and stably transduced cells (UEET-1, UBT-5, UBET-7, UEET-11, and UET-13) are identical [29].

In this study, we used the demethylating agent 5-azacytidine as an inducer, the same as in murine marrow stromal cells [19], and clearly showed that co-cultivation with fetal cardiomyocytes is necessary to induce cardiomyogenic differentiation which could be further enhanced by pretreatment of cells with 5-azacytidine. 5-Azacytidine is a cytosine analog that has a remarkable effect on transdifferentiation of cells and has been shown to induce differentiation of mesenchymal cells into cardiomyocytes, skeletal myocytes, adipocytes, and chondrocytes [19,41]. The effect of this low-molecular substance is not surprising, since 5-azacytidine is incorporated into DNA and has been shown to cause extensive demethylation. The demethylation is attributable to covalent binding of DNA methyltransferase to 5-azacytidine in the DNA [42], with the subsequent reduction of enzyme activity in cells resulting in dilution-out and random loss of methylation at many sites in the genome. This may in turn account for the reactivation of cardiomyogenic 'master' genes, such as MEF-2C, GATA4, dHAND, and Nkx2.5/Csx, leading to stochastic transdifferentiation of MSCs into cardiomyocytes. Use of 5-azacytidine is beneficial, but since it may have drawbacks, i.e., gene activation leading to oncogenesis and undesired differentiation, care must be exercised before using it to simply induce cells to differentiate into target phenotype(s) because of its stochastic nature.

Thus, it may be necessary to find alternative humoral factors essential for cardiomyogenic differentiation to prepare cells for cell therapy in humans. In addition to demethylating agents, environmental factors promote cardiomyocyte differentiation. A co-culture system has recently been used to induce cardiac differentiation, and human endothelial progenitor cells have been found to transdifferentiate into cardiomyocytes with this system [20]. Murine MSCs have been shown to differentiate into a cardiomyogenic lineage [43], but to our knowledge the present study is the first to report spontaneous beating by human MSCs and exhibition of '*in vitro*' automaticity without cell fusion. These results simply imply that MSCs may have the ability to transdifferentiate into cardiomyocytes in response to demethylation of the genome, in addition to environmental factors.

Co-cultivation makes it difficult to investigate two different types of cells in detail because they are present on the same dishes, and the target cells are difficult to isolate. There was also the question of whether the presence of beating cells means differentiation to cardiomyocytes, or merely fusion with fetal cardiomyocytes. Since marrow cells spontaneously fuse with co-cultured ES cells *in vitro* [44], controversy has arisen as to whether regenerated myocardium represents transplanted cells fused with native cardiomyocytes instead of differentiated donor cells. Cell fusion did not occur in our study, however, because cardiomyogenic differentiation was demonstrated by the double-labeling of two types of co-cultured cells *in vitro*.

The co-culture of the target cells, i.e., the marrow stroma, in this study, on appropriate feeder cells may provide a good system for generating a source of cells for therapy. hMSCs have the ability to form tight cell-to-cell couplings, i.e., gap junctions, with adjacent hMSCs, suggesting that the grafted hMSCs are capable of generating electrical coupling and may function coordinately in the recipient human heart. On the other hand, the disorganization of the spontaneous beating rhythm in the early stage may result in arrhythmogenesis when grafted into the recipient. Early afterdepolarization, which triggers arrhythmias, has been reported in cardiomyocytes generated by ES cells or embryonal carcinoma cells [45]. By contrast, the absence of early afterdepolarization in hMSCs may be beneficial in terms of not leading to arrhythmias when the cells are transplanted *in vivo*. The rhythm of the hMSCs that underwent premature differentiation became regular after complete differentiation in the present study, and culture for a certain period therefore seems necessary before cell transplantation. It is noteworthy that the risk of lethal arrhythmia can be reduced by promoting electrical maturation of hMSCs *in vitro* when this co-culture system is used for therapy clinically.

It remains unresolved how co-cultured hMSCs start beating spontaneously and what the key factor(s) in cardiac differentiation are. Several factors promote differentiation into cardiomyocytes, such as gap junctions, humoral factors, electrical and mechanical stimulation, and cell-to-cell contact. Gap junctions have been shown to be necessary for differentiation of endothelial progenitor cells to cardiomyocytes [20], but the lack of gap junctional communication between hMSCs and feeder cells in our study indicates that gap junctions are not prerequisite for differentiation. In addition, separation of hMSCs and fetal cardiomyocytes in a co-culture system with a membrane that is permeable to humoral factors but not to cells resulted in loss of capacity for cardiac differentiation (data not shown), implying that humoral factors alone do not induce cardiac differentiation of hMSCs, and direct interactions, such as with cell-membrane bound molecules and extracellular matrix, seem to be essential. Cadherins, for example, have been reported to mediate calcium-dependent cell-to-cell contact and affect the differentiation of cardiac muscle cells [46]. Moreover, the decrease in number of beating hMSCs after the feeder cells stopped beating implies that mechanical stimulation in addition to cell-to-cell contact might be indispensable to cardiac differentiation and maintenance of the differentiated state.

Many clinical trials of regeneration therapy using mononuclear cells for the failing heart have been performed [13–16], but many more basic studies are needed. hMSCs with an expanded life span cannot be transplanted clinically, because they have been transduced with human papillomavirus E6 and E7 genes. The present results and others have shown that these molecules do not elicit cell transformation *in vitro*, at least during the period observed. This contrasts with human stromal cells being transformed during immortalization by SV40 large T antigen [47]. Based on the results of this study and the mechanism of cell life span extension, we are now developing a novel strategy to eliminate the possibility of transformation. Thus, cells that undergo reproducible cardiomyogenic differentiation and have a prolonged life span can be used as a good model of cell transplantation.

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The cell names are summarized at http://1985.jukuin.keio.ac.jp/umezawa/cells/name.html. MPEG video stream of UBET-7 is available at http://1985.jukuin.keio.ac.jp/umezawa/jgm/ubet7.

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Photon-Modulated Changes of Cell Attachments on Poly(spiropyran-*co*-methyl methacrylate) Membranes

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Spiropyran is a photoresponsive molecule, and nonionic spiropyran is reversibly changed by UV irradiation to a hydrophilic polar, zwitterionic merocyanine isomer, and back again by visible light irradiation. A copolymer of nitrobenzospiropyran and methyl methacrylate, poly(NSP-*co*-MMA) was used as a material with a photosensitive surface. UV irradiation of the photosensitive surface of poly(NSP-*co*-MMA)-coated glass plates decreased the water contact angles (11 ± 1°) and increased diameter of a water drop relative to the unexposed surface. Light-induced detachment of platelets and mesenchymal stem (KUSA-A1) cells on poly(NSP-*co*-MMA)-coated glass plates was observed upon simple- and patterned-light irradiation, whereas no light-induced detachment of platelets and mesenchymal stem cells was observed on poly(methyl methacrylate)-coated glass plates. This is a result of the change from a closed nonpolar spiropyran to the polar zwitterionic merocyanine isomer induced by UV irradiation. Light-induced detachment of fibrinogen adsorbed on poly(NSP-*co*-MMA) coated glass plates was also observed in this investigation.

Introduction

In an effort to induce or control surface wetting by liquids, a number of researchers have proposed the use of a variety of means of changing the interfacial properties of materials including electrical potentials and fields,[1,2] temperature,[3,4] light,[5–7] and chemical means.[8] The focus of these studies has been on the manipulation of microchannels, micro-total analysis systems (μ-TAS),[9,10] and the capillary surface using external stimuli. Rosario et al. investigated the microfluidic actuation of water in capillary tubes coated with a photosensitive layer. Water in the capillary tubes was observed to rise on the order of 2.8 mm for a 500 μm diameter capillary, when the wavelength of incident light was switched from the visible region to the UV region. This is thought to be because the relatively nonpolar spiropyran can be reversibly switched to a polar, zwitterionic merocyanine isomer with a much larger dipole moment upon UV light irradiation, and back to the nonpolar spiropyran isomer again upon visible light irradiation.[7]

Surface control of hydrophilic/hydrophobic properties by external stimuli such as temperature change was also reported to develop thermo-responsive culture dishes for cells.[11,12] Hirose and Okano et al. developed designed shape cell sheets for tissue engineering in which human aortic endothelial cells were cultured and proliferated on tissue culture polystyrene dishes grafted with poly(*N*-isopropylacrylamide) (PIPAAm) and poly(*N*,*N*′-dimethylacrylamide) for thermosensitive response of cell adhesive and cell nonadhesive domains.[11] When the culture temperature was reduced below 32 °C (LCST), PIPAAm changed to hydrophilic and the cell sheets detached from PIPAAm-grafted surfaces in the absence of an enzyme such as trypsin.[11]

In this study, we investigated light-induced detachment of platelets and mesenchymal stem (KUSA-A1) cells on poly(NSP-*co*-MMA)-coated glass plates upon simple- and patterned-light irradiation as well as the change of physical properties on the photosensitive surface of poly(NSP-*co*-MMA)-coated glass plates, i.e., hydrophobicity–hydrophilicity change and change of amount of protein adsorption induced by UV irradiation.

Experimental Section

Preparation of Membranes. Poly(NSP-*co*-MMA) was synthesized by a conventional route according to the literature[17,18] and was donated by M. Kameda, K. Sumaru, and T. Kanamori (AIST).[19] Poly(NSP-*co*-MMA), 0.1 wt % in dichloroethane, was cast onto glass plates in flat Petri dishes. PMMA-coated glass plates were also prepared for use as controls.

Physical Characteristic Measurements. The water contact angles of the poly(NSP-*co*-MMA)-coated and PMMA-coated glass plates were measured at 25 °C and 85% relative humidity by the sessile drop method using ultrapure water.[20] The water contact angles were monitored and recorded with a CCD camera (DCR–PC100, Sony Corporation). At least four readings ($n = 4$) were taken at 2 min after placing water droplets (6–7 mm diameter) on different parts of the glass plates, and the values were averaged.

Fibrinogen Adsorption Assay. Fibrinogen adsorption from human plasma on the surface of the poly(NSP-*co*-

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MMA)-coated and PMMA-coated glass plates was directly evaluated using the method based on the antigen–antibody reaction using enzyme-immunoglobulin conjugate (ELISA assay).[13] Briefly, the poly(NSP-*co*-MMA)-coated and PMMA-coated glass plates were immersed in 50% platelet-poor plasma diluted with phosphate buffer solution (PBS, 0.02M, pH 7.4) containing 0.15 mol/L NaCl for 180 min at 37 °C. After the poly(NSP-*co*-MMA)-coated and PMMA-coated glass plates were rinsed with sufficient PBS and were shifted into another new 24-well tissue culture flask, the poly(NSP-*co*-MMA)-coated and PMMA-coated glass plates were incubated with the primary antibody (i.e., mouse monoclonal anti-human fibrinogen (F4639, Sigma-Aldrich, Inc.)) diluted with Block Ace (UK–B80, Funakoshi Co.) solution for 1 h at 37 °C. Thereafter, the primary antibody was blocked with Block Ace solution after rinsing the glass plates with PBS containing 0.05 wt % Tween 20. The poly(NSP-*co*-MMA)-coated and PMMA-coated glass plates were subsequently incubated with the secondary antibody, rabbit H+L anti-mouse immunoglobulin peroxidase conjugate antibody (014–17611, Wako Pure Chemical Industries, Ltd.) for 60 min at 37 °C.

After sufficiently rinsing the poly(NSP-*co*-MMA)-coated and PMMA-coated glass plates with PBS containing 0.05 wt % Tween 20, 0.6 mL of a H_2O_2 solution containing the substrate for horseradish peroxidase, 3,3′,5,5′-tetramethylbenzidine (TMB Microwell Peroxidase Substrate System, 50–76–00, Kirkegaard & Perry Laboratories), was added to the 24-well tissue culture flask containing the glass plates. The absorbance of the solution was measured at 450 nm after 15 min of the enzyme reaction when the stop solution (1 mole/l H_3PO_4 solution) of the reaction was injected into the 24-well tissue culture flask. These measurements were carried out four times for each glass plates.

Light-Induced Detachment of Platelets. The poly(NSP-*co*-MMA)-coated glass plates in a 24-well tissue culture flask were equilibrated in saline solution at 37 °C for 1 h. The saline solution was removed, and 1 mL of fresh platelet-rich plasma (PRP)[13] was subsequently introduced into each well. The poly(NSP-*co*-MMA)-coated glass plates were incubated with PRP at 37 °C for 30 min. Then, the poly(NSP-*co*-MMA)-coated glass plates were illuminated with UV irradiation using a handy UV lamp (UVGL-25, 365 nm, 950 $\mu W/cm^2$, UVP, Inc.) from the bottom of the 24-well tissue culture flask for 4 min at 37 °C. Control experiments in which the poly(NSP-*co*-MMA)-coated glass plates were not illuminated by UV irradiation were also performed. After 4 min, PRP was removed using a Pasteur pipet and 1 mL of PBS was pipetted into each well of the flask. The platelet numbers on both the poly(NSP-*co*-MMA)-coated glass plates, exposed to UV irradiation and not exposed to UV irradiation, were counted before and after UV irradiation using inverted phase-contrast microscopy (IX70, Olympus Corporation) equipped with a CCD video camera (CS230, Olympus Corporation). Light-induced detachment of platelets on PMMA-coated glass plates was also performed using the same procedures as a control. These measurements were carried out four times for each membrane.

Light-Induced Detachment of KUSA-A1 Cells. Mesenchymal stem cell line, KUSA-A1, derived from the bone marrow of female C3H/He mice (A. U.)[21] was used in this study. The poly(NSP-*co*-MMA)-coated glass plates in the 24-well tissue culture flask were equilibrated with saline solution at 37 °C for 1 h. The saline solution was removed, and 1 mL of KUSA-A1 cell suspension supplemented with DMEM media and 10% fetal bovine serum was subsequently introduced into each well. The poly(NSP-*co*-MMA)-coated glass plates were incubated with KUSA-A1 cells in a constant 5% CO_2 atmosphere at 37 °C for 30 min. Then, the poly(NSP-*co*-MMA)-coated glass plates were illuminated by UV light using the handy UV lamp (UVGL-25, 365 nm, 950 $\mu W/cm^2$, UVP, Inc.) from the bottom of the 24-well tissue culture flask for 4 min. When the patterned light irradiation was performed, a striped pattern mask (width; 2.5 mm), made from a transparency sheet (CG3410, 3M), was placed under the well. Additionally, control experiments in which the poly(NSP-*co*-MMA)-coated glass plates were not illuminated by UV irradiation were also performed. After 4 min, the DMEM media was removed using a Pasteur pipet and 1 mL of DMEM media was inserted into each well of the flask. The cell numbers of KUSA-A1 cells on both the poly(NSP-*co*-MMA)-coated glass plates, illuminated and not illuminated by UV irradiation, were counted before and after UV irradiation using inverted phase-contrast microscopy with CCD video camera. Light-induced detachment of KUSA-A1 cells on the PMMA-coated glass plates was also performed as a control according to the same procedure. These measurements were carried out four times for each membrane.

Results and Discussion

Hydrophobicity–Hydrophilicity Change. Hydrophilicity–hydrophobicity change induced by UV irradiation was investigated on poly(NSP-*co*-MMA) (see Figure 1) and poly(methyl methacrylate) (PMMA) coated glass plates. Figure 2 shows the time dependence of the water contact angle and the diameter ratio (d_t/d_0) of the water drop on poly(NSP-*co*-MMA)-coated and PMMA-coated glass plates where d_0 and d_t are the diameter of water drop at time = 0 and t min after UV (375 nm) irradiation, respectively.

UV irradiation of the control surface of PMMA-coated glass plates demonstrated no change in the water contact angles within the experimental error and only a slight decrease in the diameter of the water droplet. The slight decrease of d_t/d_0 on the PMMA-coated glass plates was attributed to evaporation of water drop during the measurements. The photosensitive surface of poly(NSP-*co*-MMA)-coated glass plates under UV irradiation resulted in decreased water contact angles as well as an increased diameter of water droplet relative to that on the surface before UV light irradiation. The large change in the dipole moment between the two isomeric states of the spiropyran was determined to induce changes in the energy at the surface of poly(NSP-*co*-MMA). Spiropyran-coated photosensitive surfaces were determined to change in the surface energy solely upon light irradiation, as measured by the water contact angle.

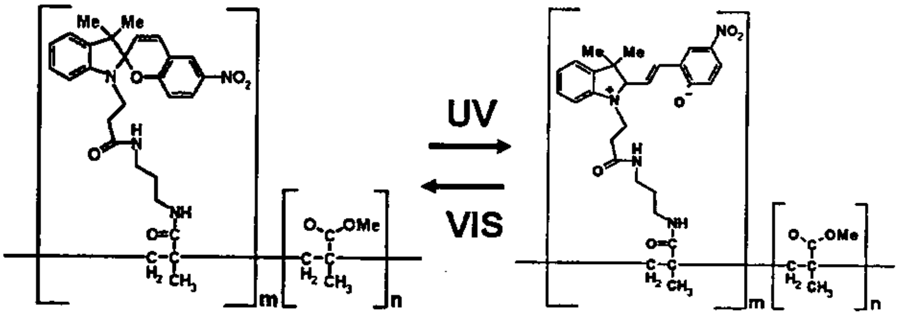

Figure 1. Schematic of transition of poly(NSP-*co*-MMA) upon exposure to UV irradiation.

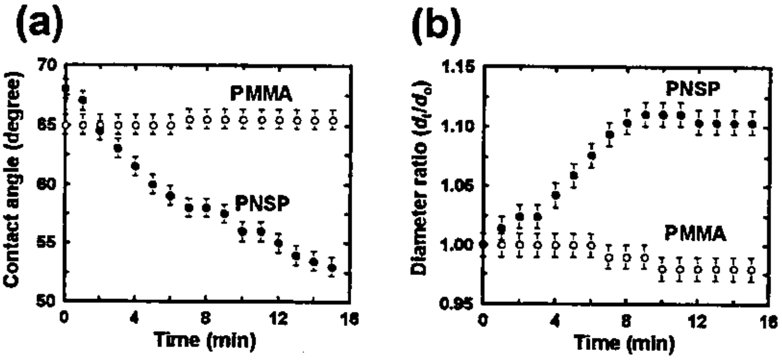

Figure 2. Hydrophobicity–hydrophilicity change on poly(NSP-*co*-MMA)-coated and PMMA-coated glass plates induced by UV irradiation. (a) Time dependence of water contact angle of water drop on poly(NSP-*co*-MMA)-coated and PMMA-coated glass plates. (b) Time dependence of diameter ratio (d/d_0) of water drop on poly(NSP-*co*-MMA) and PMMA coated glass plates. PNSP indicates poly(NSP-*co*-MMA)-coated glass plates.

After the water contact angles on poly(NSP-*co*-MMA)-coated glass plates were measured at first time, the poly(NSP-*co*-MMA)-coated glass plates were dried under vacuum and in the dark place at ambient temperature for 24 h. After this treatment, the color of poly(NSP-*co*-MMA)-coated glass plates changed from purple color to colorless, which indicated the spiropyran in poly(NSP-*co*-MMA)-coated glass plates returned to the first form of nonionic spiropyran. The water contact angles of the poly(NSP-*co*-MMA)-coated glass plates were again measured. Exactly the same results to Figure 2 were obtained within the experimental error.

Therefore, the reversibility between the form of nonionic spiropyran and that of zwitterionic merocyanine isomer in the poly(NSP-*co*-MMA)-coated glass plates was observed in this study.

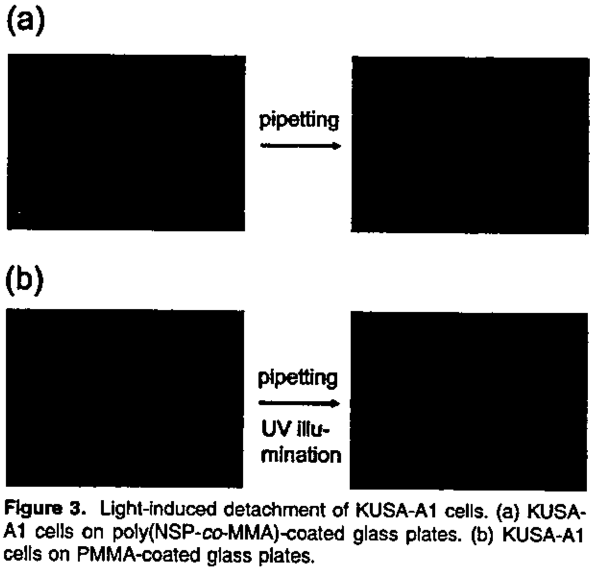

Figure 3. Light-induced detachment of KUSA-A1 cells. (a) KUSA-A1 cells on poly(NSP-*co*-MMA)-coated glass plates. (b) KUSA-A1 cells on PMMA-coated glass plates.

Light-Induced Detachment of Cells. Light-induced detachment of platelets and mesenchymal stem (KUSA-A1) cells was also examined. Figure 3 shows KUSA-A1 cells on poly(NSP-*co*-MMA)-coated glass plates before and after UV irradiation. After UV irradiation to the poly(NSP-*co*-MMA)-coated glass plates, KUSA-A1 cells were rarely observed on the surface of poly(NSP-*co*-MMA)-coated glass plates. On the other hand, KUSA-A1 cells remained attached to the surface of poly(NSP-*co*-MMA)-coated glass plates submitted to the same procedures, but not to be exposed to UV irradiation. The cell density of KUSA-A1 cells and platelets on the surface of poly(NSP-*co*-MMA)-coated glass plates before and after UV irradiation was examined and summarized in Figure 4a. In addition, light-induced detachment of KUSA-A1 cells and platelets was examined on PMMA-coated glass plates, as a nonphotosensitive surface (Figure 4b). Light-induced detachment of KUSA-A1 cells was clearly not observed on PMMA-coated glass plates, indicating that the cell detachment on poly(NSP-*co*-MMA)-coated glass plates upon UV irradiation is not due to the stimulation of the cells by UV light irradiation. Thus, it is thought to be caused by the change in the surface energy and/or the change in the switching movement of closed nonpolar spiropyran to the polar zwitterionic merocyanine isomer upon UV irradiation.

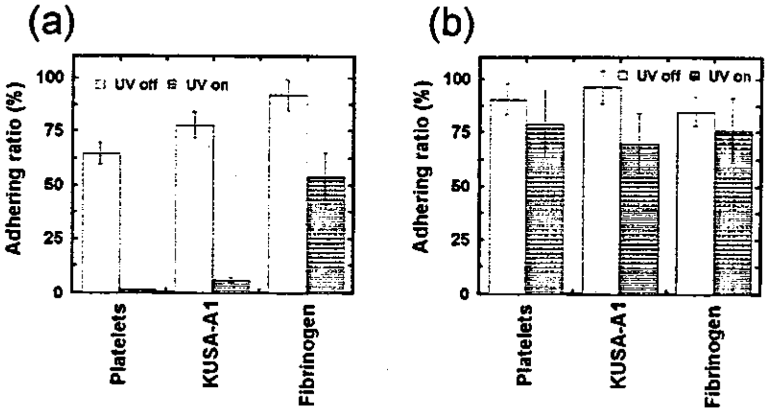

Figure 4. Light-induced detachment of platelets, KUSA-A1 cells and fibrinogen. (a) Platelets, KUSA-A1 cells, and fibrinogen on poly(NSP-*co*-MMA)-coated glass plates. (b) Platelets, KUSA-A1 cells, and fibrinogen on PMMA-coated glass plates.

The cell viability of the detached cells by UV irradiation was found to be more than 98% based on the trypan blue exclusion test.

Light-Induced Detachment of Fibrinogen. Suppression of platelet adhesion is generally believed to be due to a reduction of protein adsorption, particularly fibrinogen, which binds to the platelet membrane glycoprotein GP IIb-IIIa.[13,14] Light-induced detachment of fibrinogen adsorbed on poly(NSP-*co*-MMA) coated glass plates was, therefore, examined (Figure 4a). After the poly(NSP-*co*-MMA) coated glass plates were immersed in platelet-poor plasma (PPP) solution for 30 min, UV light was illuminated on the surface for 4 min, and the fibrinogen adsorbed on the surface was directly measured by an enzyme-immunoglobulin conjugate assay (ELISA).[13] The adsorbed fibrinogen was determined to decrease on the surface of poly(NSP-*co*-MMA)-coated glass plates compared to that on the surface not exposed to UV irradiation.

The following experiments were also performed: The adsorbed amount of fibrinogen was measured on the poly(NSP-*co*-MMA)-coated glass plates where UV light was irradiated for 4 min before the poly(NSP-*co*-MMA)-coated glass plates were immersed into PPP solution (NSP was already in the form of the zwitterionic merocyanine isomer when the poly(NSP-*co*-MMA)-coated glass plates made contact with PPP). The poly(NSP-*co*-MMA) coated glass plates had NSP consisting of zwitterionic merocyanine isomer adsorbed fibrinogen at 6.2 ± 0.5 μg/cm^2, whereas poly(NSP-*co*-MMA) coated glass plates had NSP consisting of nonionic spiropyran adsorbed at 5.0 ± 0.4 μg/cm^2. These findings indicate that the amount of adsorbed fibrinogen on the glass plates with the zwitterionic merocyanine isomer NSP was 1.2 times higher than that with the nonionic spiropyran NSP. These finding were not in agreement with the results shown in Figure 4a. This contradiction suggests that the fibrinogen was detached by means of the switching movement of closed nonpolar spiropyran to the polar zwitterionic merocyanine isomer, and the surface energy (hydrophobicity–hydrophilicity) does not directly contribute to the amount of adsorbed fibrinogen on the poly(NSP-*co*-MMA) coated glass plates. Light-induced detachment of fibrinogen was also examined on the PMMA-coated glass plates, as a nonphotosensitive surface and is shown in Figure 4b. No light-induced detachment of fibrinogen was observed on the PMMA-coated glass plates. This further indicates that fibrinogen detachment by UV irradiation is not due to the stimulation of fibrinogen by UV light, but a result of the change in the switching movement of closed nonpolar spiropyran to the polar zwitterionic merocyanine isomer upon UV irradiation.

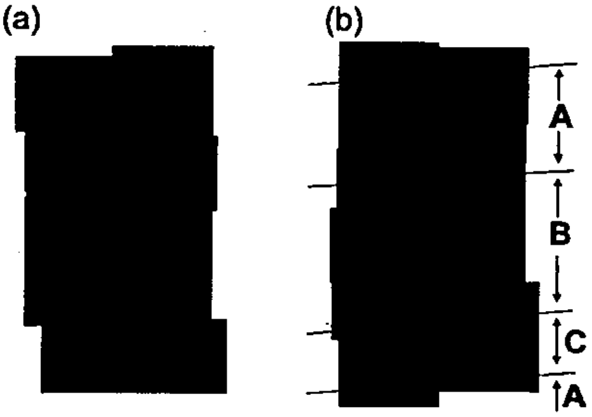

Figure 5. Light-induced detachment of KUSA-A1 cells with patterned light irradiation using striped pattern mask with 2.5 mm widths. (a) KUSA-A1 cells on poly(NSP-*co*-MMA)-coated glass plates before UV irradiation. (b) KUSA-A1 cells on poly(NSP-*co*-MMA)-coated glass plates after striped pattern UV irradiation for 4 min. Regions A and B indicate the area under non-UV irradiation (region A) and UV irradiation (region B), respectively. Region C indicates the border region.

Cell Detachment by Patterned Light Irradiation. Light-induced detachment of KUSA-A1 cells was achieved by simple patterned light irradiation using a striped pattern mask (width; 2.5 mm). Figure 5 shows KUSA-A1 cells on poly(NSP-*co*-MMA)-coated glass plates before and after UV irradiation with the striped pattern. KUSA-A1 cells were clearly observed to detach in the region exposed to UV irradiation (region B), whereas the cells remained attached in the masked area, not exposed to UV irradiation (region A). Therefore, patterned light detachment of KUSA-A1 cells was successively performed by the patterned light irradiation using the striped pattern mask.

Conclusion

KUSA-A1 cells, platelets, and the fibrinogen were detached by means of the switching movement of closed nonpolar spiropyran to the polar zwitterionic merocyanine isomer, and the surface energy (hydrophobicity–hydrophilicity) does not directly contribute to the amount of adsorbed fibrinogen on the poly(NSP-*co*-MMA)-coated glass plates.

Light-induced detachment of cells on poly(NSP-*co*-MMA) surfaces will lead to mild isolation of cells. Furthermore, this will be a powerful tool for surface marker analysis using flow cytometry. This is because this method does not require the addition of trypsin for cell detachment, which degrades the extracellular matrix and cell adhering molecules between cells and the culture flask. Furthermore, patterned light detachment of nerve cells will provide an alternative method to create micro-patterned neuronal networks,[15] which are typically performed using a micro-contact printing method.[16]

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Development of Novel Monoclonal Antibody 4G8 against Swine Leukocyte Antigen Class I α Chain

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ABSTRACT

A mouse monoclonal antibody (MAb) was generated against swine leukocyte antigen (SLA) class I α chain. A newly developed series of MAb clones that react with pan leukocytes were selected and tested by immunohistochemistry using SLA class I α chain expressing Cos-7 cells. Among them, MAb 4G8 was characterized by the following features: (1) 4G8 reacted with Cos-7 cells transfected with SLA class I α chain from the *d* haplotype, (2) 4G8 recognized epitopes that were different from those of commercially available anti-SLA class I MAbs 74-11-10 and PT85A, and (3) 4G8 could be used to immunostain frozen sections of thymus, spleen, lymph node, kidney, and liver tissues with good results.

INTRODUCTION

THE PORCINE SYSTEM has received much attention as a suitable model for transplantation medicine. Therefore, an accurate understanding of human immune responses to porcine tissues has become increasingly important. However, the details of the porcine immune system, especially those features that are novel to the pig, remain unclear. We thus attempted to develop new MAbs that could be used to analyze the porcine immune system.

The immune response to foreign antigens is determined by the expression of specific major histocompatibility complex (MHC) molecules that can bind and present peptide fragments of that protein to T cells. There are two different types of MHC gene products, termed Class I and Class II MHC molecules, and any given T cell recognizes foreign antigens bound to only one Class I or Class II MHC molecule. Antigens associated with Class I molecules are recognized by $CD8^+$ cytolytic T cells, whereas class II–associated antigens are recognized by $CD4^+$ helper T cells. Class I molecules are located on every nucleated cell surface, except those of neurons and trophoblasts. In contrast, the expression of Class II molecules is limited to certain cell types. In pigs, MHC molecules are known as swine leukocyte antigens (SLA). All SLA class I molecules contain two separate polypeptide chains: an MHC-encoded α chain of 45 kD and a non–MHC-encoded β chain of 12 kD.

Recently, the profound involvement of SLA Class I molecules in human anti-porcine cell reactions has been described. Several studies have shown that human T cells can directly recognize porcine MHC molecules and that such recognition can lead to the killing of the porcine cells. Porcine cells have recently been shown, moreover, to be targets for human NK cells. Since human MHC class I molecules deliver a negative signal to human NK cells, protecting syngeneic cells from lysis, we surmised that differences in the gene sequences of porcine MHC class I molecules may be responsible for the lack of recognition by human NK cell receptors and subsequent cytolysis of the porcine cells. In addition, it was reported that a single treatment with a monoclonal antibody (MAb) directed against the SLA class I provides an attractive approach to the induction of T cell tolerance, possibly enabling long-term graft survival in porcine-to-human cell transplantations.(1) These studies indicate that SLA class I molecules play critical roles in transplantation medicine.

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Here, we report a novel MAb 4G8 against the SLA class I α chain that was proven to be different from four commercially available anti-SLA class I MAbs. The utilization of 4G8 in tissue sections was also examined.

MATERIALS AND METHODS

Animals and tissues

Landrace or (Landrace × Large White) F1 pigs were used in this study. Peripheral blood (PB) and tissues were obtained from anesthetized animals and were processed. PB was collected in acid citric buffer to avoid coagulation. Tissues were immediately snap frozen and kept in the deep freezer until use.

Monoclonal antibodies

PB leukocytes were treated using RBC lysis with NH_4Cl lysis buffer followed by centrifugation at 1,500 rpm for 10 min. After washing twice in phosphate-buffered saline (PBS), approximately 1×10^8 cells were injected into the abdominal cavity of 8-week-old female Balb/c mice. Boost injections were performed twice at 2-week intervals. At 4 days after the last boost, splenocytes were fused with P3U1 mouse myeloma cells and incubated in hypoxanthine and thymidine (HAT) medium. Supernatants of growing hybridomas were screened on porcine PB leukocytes by flow cytometry and clones secreting antibodies reactive with porcine PB leukocytes were subcloned twice by limiting dilution. Clones were grown in the abdominal cavity of Pristane-treated Balb/c mice, and ascites were obtained. Purification of MAbs was performed by Protein-A or Protein-G column (Bio-Rad Laboratories, Hercules, CA). After purification, MAb was fluorescence isothiocyanate (FITC) conjugated as described previously.(2) Commercially available MAbs against SLA class I 74-11-10, PT85A, H17A*, H58A* (* indicates known as cross-reactive with pig and other species) were obtained from Veterinary Medical Research and Development (Pullman, WA).

Flowcytometry and immunohistochemistry

Flowcytometrical analysis of MAbs was carried out as follows. Briefly, aliquot of porcine PB was incubated with appropriate amount of MAb for 30 min at 4°C. After washing with PBS, cells were incubated with FITC-conjugated (Jackson Laboratory, West Grove, PA) for 30 min at 4°C. Cells were washed with PBS and analyzed by EPICS XL analyzer (Beckman/Coulter, Westbrook, MA).

Reactivity of MAbs on tissues were analyzed by immunohistochemistry on frozen sections. Briefly, porcine tissues were snap-frozen in optimal cutting temperature (OCT) compounds and frozen sections were made by cryostat apparatus. Sections were fixed by acetone for 15 min at 4°C. After washing in PBS and blocked with normal rabbit serum, sections were incubated with MAbs at appropriate dilutions for 30 min at room temperature. Sections were then washed with PBS and incubated with horseradish peroxidase (HRP)–conjugated rabbit anti-mouse antibodies (Jackson Laboratory) for 30 min at room temperature. After washing with PBS, color development was done in diaminobenzidine solution (10 mM in 0.05 M Tris-HCl, PH 7.5) with 0.003% H_2O_2.

Binding competition assay

Binding competition assay was carried out as follows. Briefly, after aliquot of porcine PB leukocytes were incubated with 2ug saturated amount of commercially available MAbs for 30 min at 4°C. The cells saturated with these commercially available MAbs were stained with FITC-4G8 for 30 min at 4°C. Then, PB leukocytes were treated using RBC lysis with NH_4Cl lysis buffer followed by centrifugation at 1,500 rpm for 10 min. FITC-mouse immunoglobulin (MsIg) was used as control antibody. Cells were washed with PBS and analyzed by EPICS XL analyzer (Beckman/Coulter).

Cloning and expression of porcine cDNA library

As another purpose for analysis of $\gamma\delta$TCR against MAb (7G3) and CD8 against MAb (6F10), cDNA libraries of 7G3-positive as well as 6F10-positive PB leukocytes were first constructed. A brief description is shown below. Porcine PB labeled with FITC-7G3 antibody was incubated with magnetic-activated cell sorting (MACS) beads conjugated with anti-FITC antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) and was loaded onto AutoMACS cell separator (Miltenyi Biotec). 7G3-positive cells were positively selected and a cDNA library was constructed using the oligo-capping method(3) and plasmid vector pME18S-FL3, which contains the SR-α promoter for expression in mammalian cells. To 7G3-negative pass-through fractions, FITC-6F10 was added and labeled. These cells were also positively selected by AutoMACS and used for the cDNA library construction. Out of several thousand clones sequenced from both cDNA libraries, one clone was selected which exhibited homologies to known porcine MHC class I sequences from d haplotype and contained full-length open reading frames.

Complementary DNA coding for porcine MHC class I under SRα promoter was introduced into COS7 cells by lipofec-

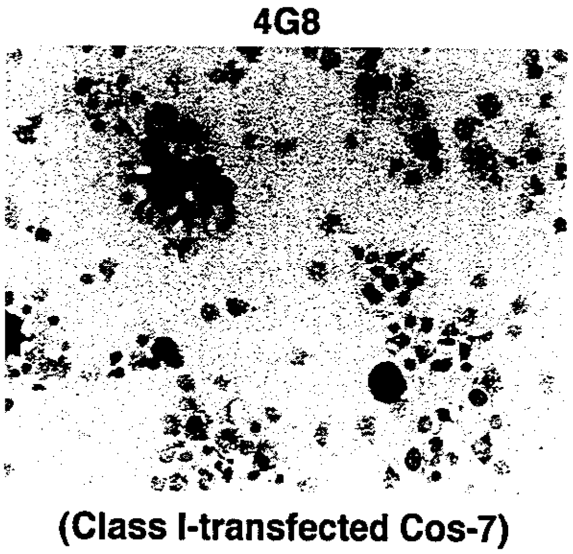

FIG. 1. Reactivity of 4G8 on Cos-7 cells transfected with SLA class I PD1. Mammalian expression vectors containing SLA class I PD1 were introduced into Cos-7 cells, and the cells were stained with 4G8 using immunohistochemistry.

tion (LIPOFECTAMIN, Invitrogen, Groningen, Netherlands) and cells were stained with 4G8 MAb after 3 days.

RESULTS AND DISCUSSION

Anti-SLA Class I MAb 4G8 recognizes a distinct epitope from those of commercially available antibodies

From one hybridization experiment, 45 hybridoma clones were established. The MAbs produced by these clones reacted differently to the porcine PB leukocytes, as revealed by flow cytometry (data not shown). To determine whether a MAb against SLA class I α chain was included among these clones, MAb clones that reacted with pan leukocytes were selected and tested by immunohistochemistry using SLA class I α chain expressing Cos-7 cells. As shown in Figure 1, when a mammalian expression vector of SLA class I PD1 from d haplotype was transfected into Cos-7 cells, clone 4G8 was found to stain the cells, whereas control MsIg failed to stain the cells (data not shown). Therefore, 4G8 was considered to recognize the SLA class I α chain, including the d haplotype.

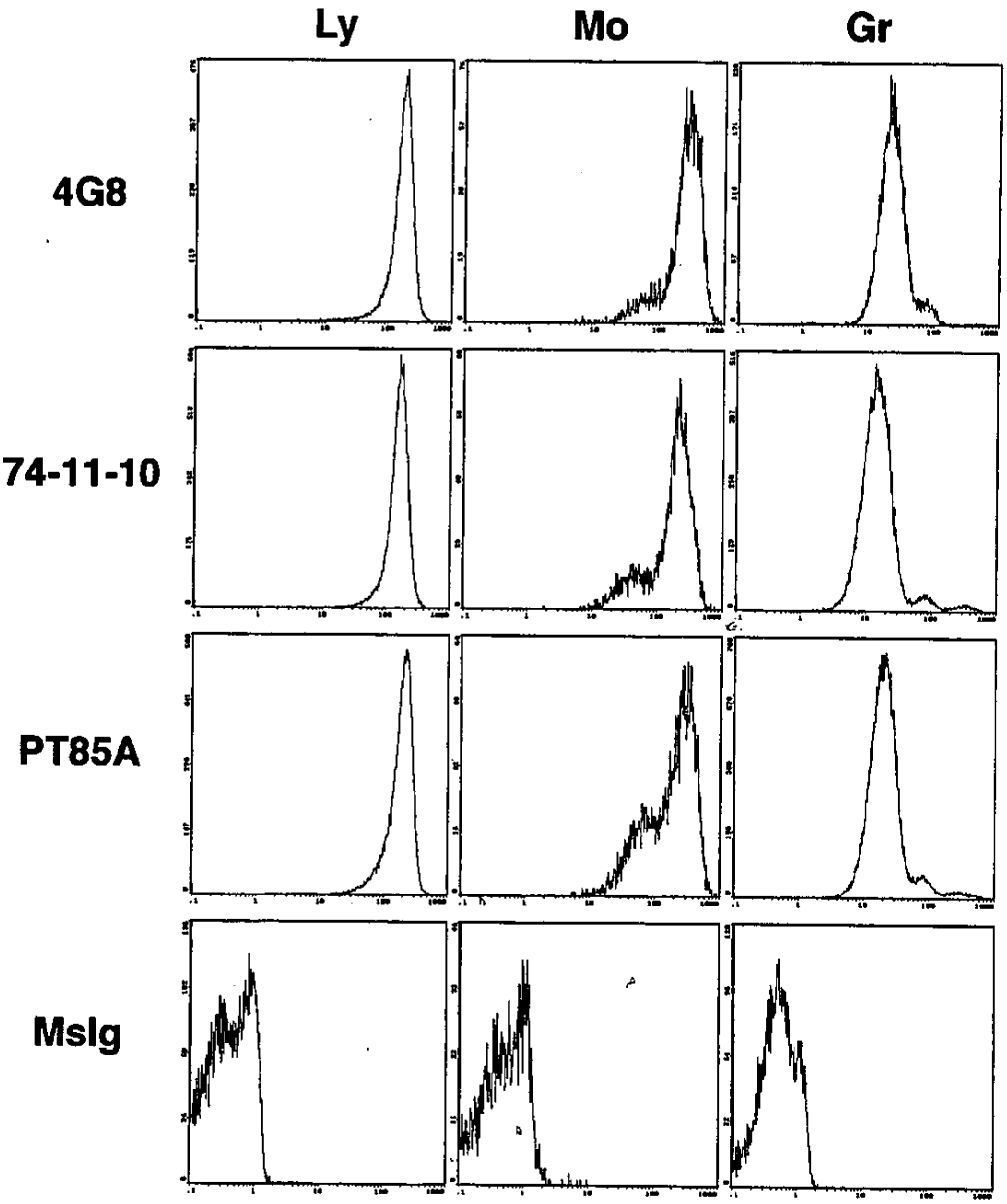

FIG. 2. Comparison of reactivity profiles of 4G8 and commercially available anti-SLA class I monoclonal antibodies. Porcine PB leukocytes were stained with 4G8 and commercially available anti-SLA class I MAbs, 74-11-10, and PT85A, using flow cytometry. MsIg was used as a control antibody.