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

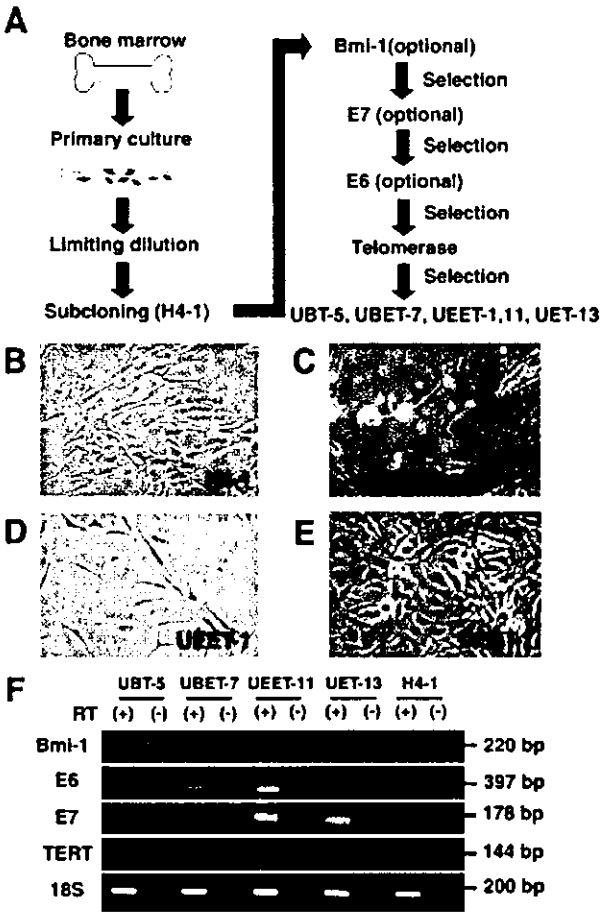

Figure 1. Experimental scheme. (A) Bone marrow stromal cells were obtained from a human donor and subcloned by limiting dilution. One of the cells isolated was designated H4-1 cells, and they were transduced with E6, E7, TERT, or bmi-1 genes to extend their life span. The combinations of genes transferred 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

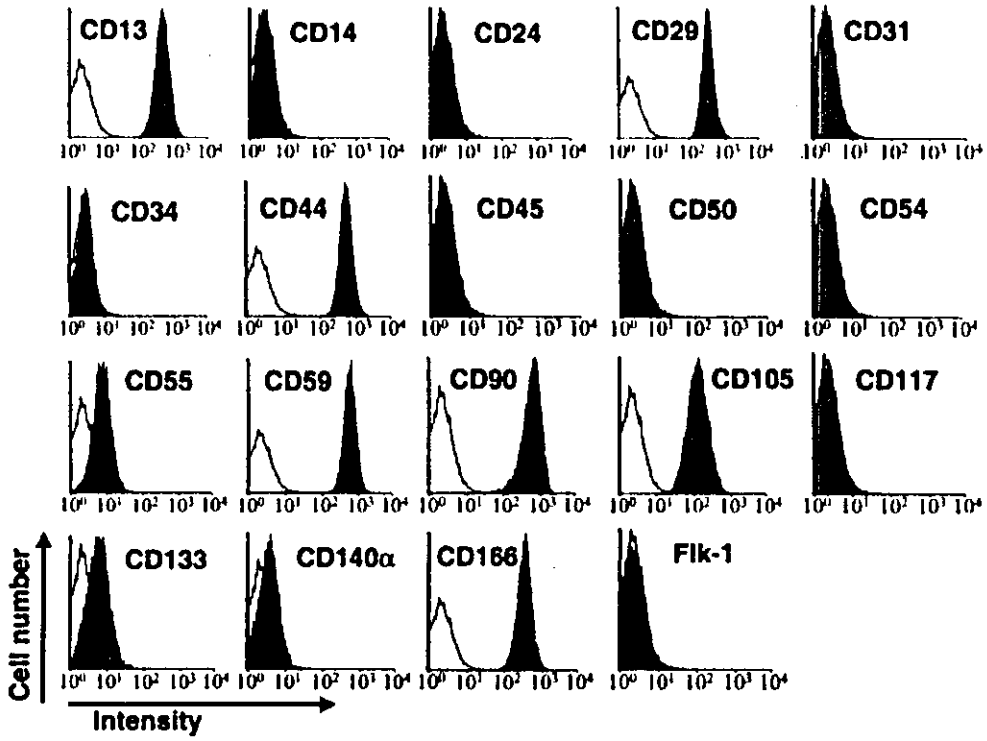

Figure 2. Flow cytometric analysis of UEET-1 cells. UEET-1 cells were labeled with FITC-coupled antibodies against CD13, CD14, CD24, CD29, CD31, CD34, CD44, CD45, CD50, CD54, CD55, CD59, CD90, CD105, CD117, CD133, CD140a, CD166, and Flk-1 and analyzed with an EPICS ALTRA analyzer

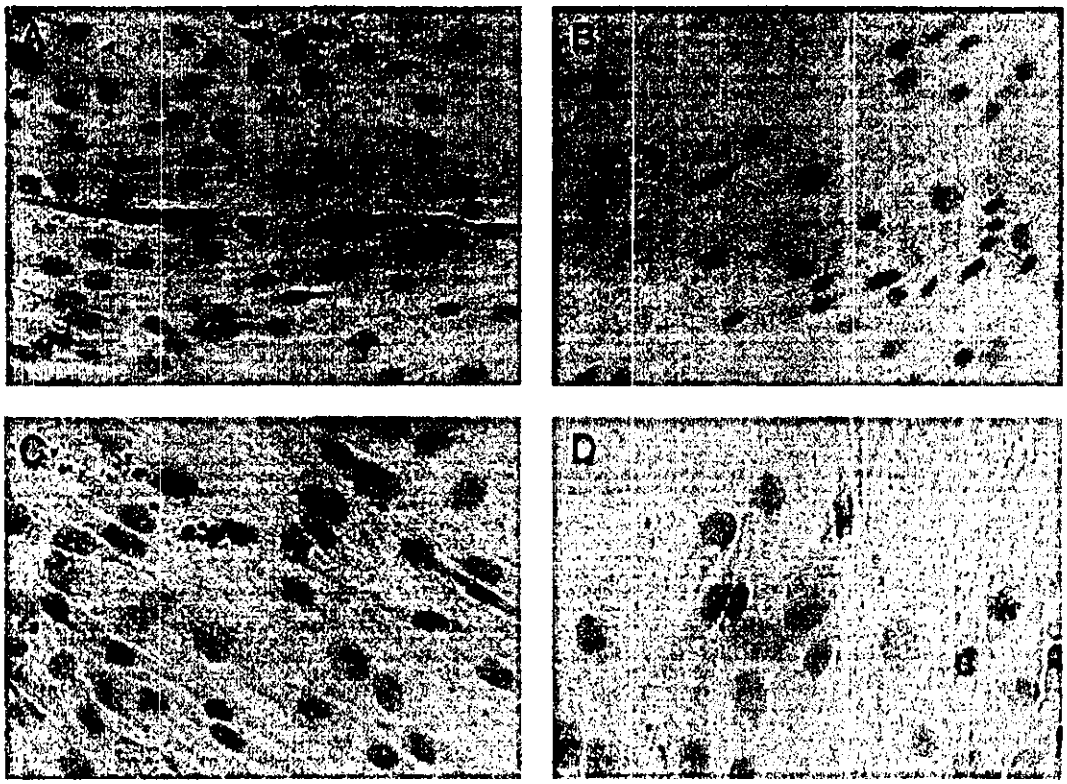

Figure 3. Immunostaining of hMSCs with anti-desmin and anti-troponin-I antibodies after exposure to 5-azacytidine. UBET-7 cells were exposed to 10 μM of 5-azacytidine for 24 h and stained for desmin (A) and cardiac troponin I (B). UBET-7 cells not treated with 5-azacytidine were also stained for desmin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

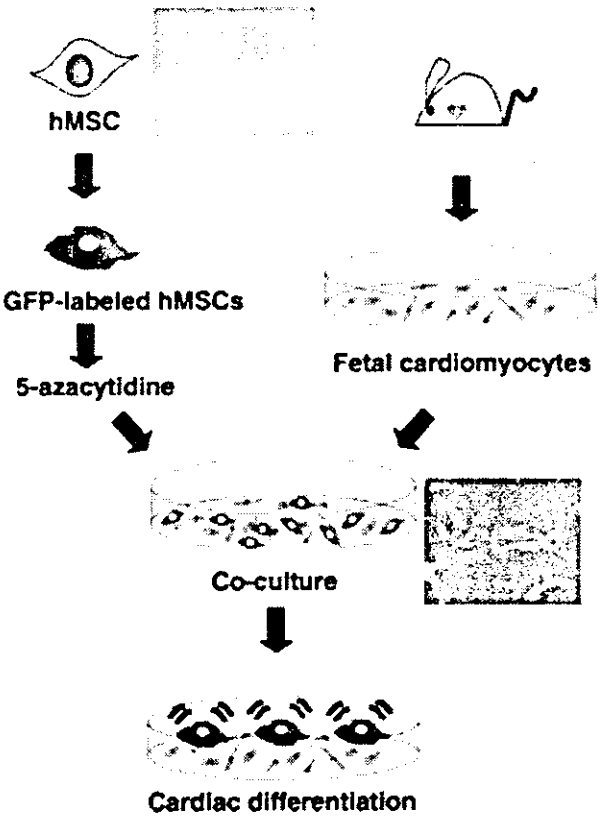

Figure 4. Scheme of the co-culture system. hMSCs infected with adenoviruses carrying the GFP gene were treated with 10 μM of 5-azacytidine for 24 h. hMSCs expressing GFP were then co-cultured with murine fetal cardiomyocytes. hMSCs began beating spontaneously after 7 days of co-culture

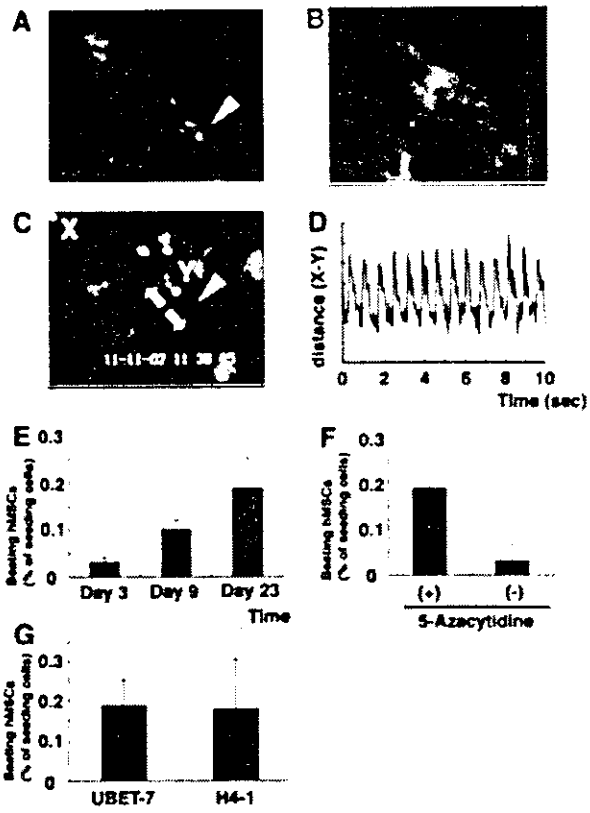

Figure 5. Beating of hMSCs in the *in vitro* co-culture system. (A) 5-Azacytidine-treated GFP-positive UBET-7 cells were co-cultured with murine fetal cardiomyocytes (http://1985.jukuin.keio.ac.jp/umeza/jgm/ubet7). The white arrowhead is pointing to some beating UBET-7 cells whose rhythm was different 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

(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.

Discussion

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

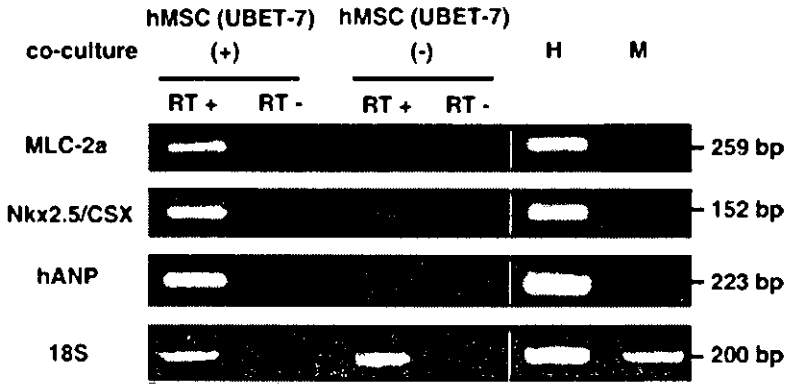

Figure 6. Expression of cardiomyocyte-specific genes in differentiated hMSCs (UBET-7). RT-PCR was performed with PCR primers that react with human genes encoding cardiac proteins (MLC-2a, Nkx2.5, and hANP) but do not with the murine genes. Only the 18S PCR primer used as a positive control reacted with 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)

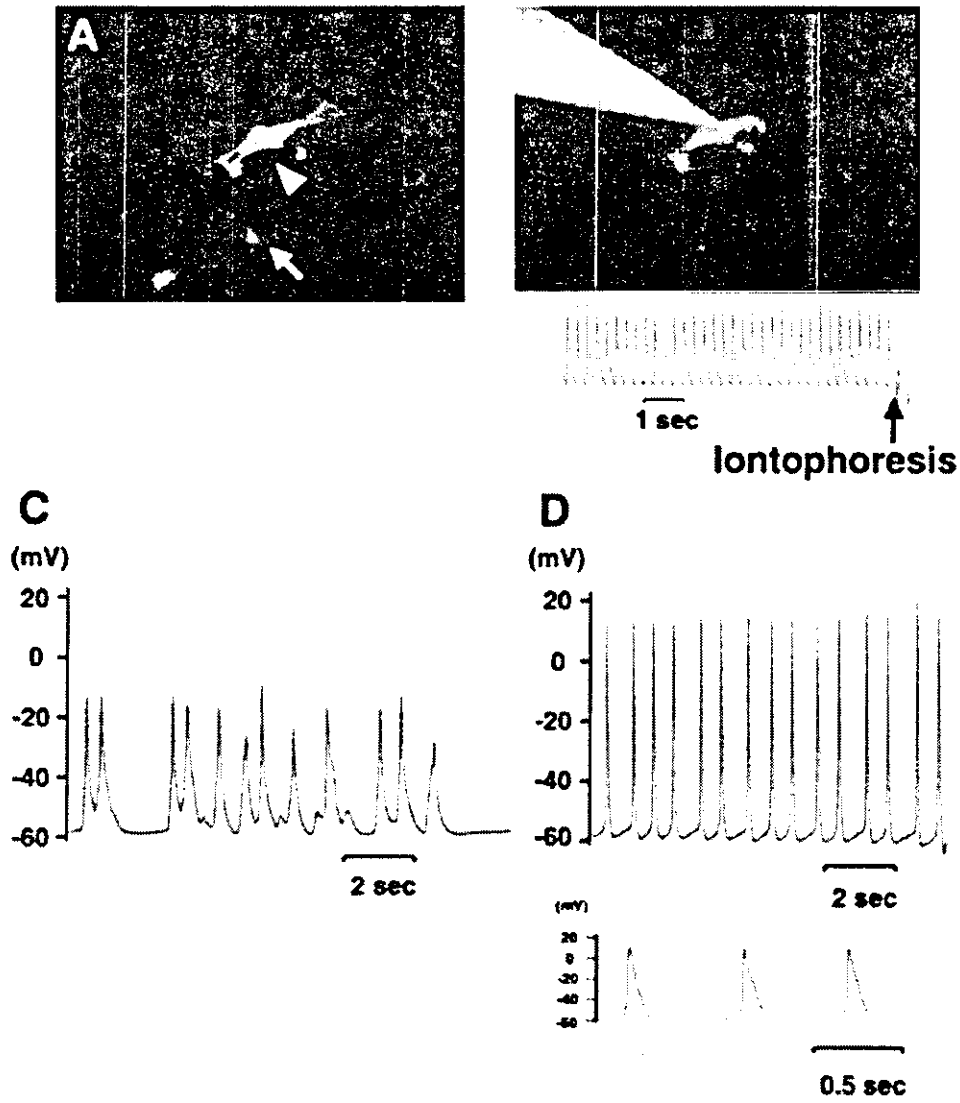

Figure 7. Action potentials of beating hMSCs. GFP-labeled UBET-7 cells (A) were injected with Alexa 568 solution (B) by iontophoresis through a microelectrode. The injected UBET-7 cell and neighboring beating UBET-7 cell are indicated by arrowhead and arrow, respectively. The action potential was recorded (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.

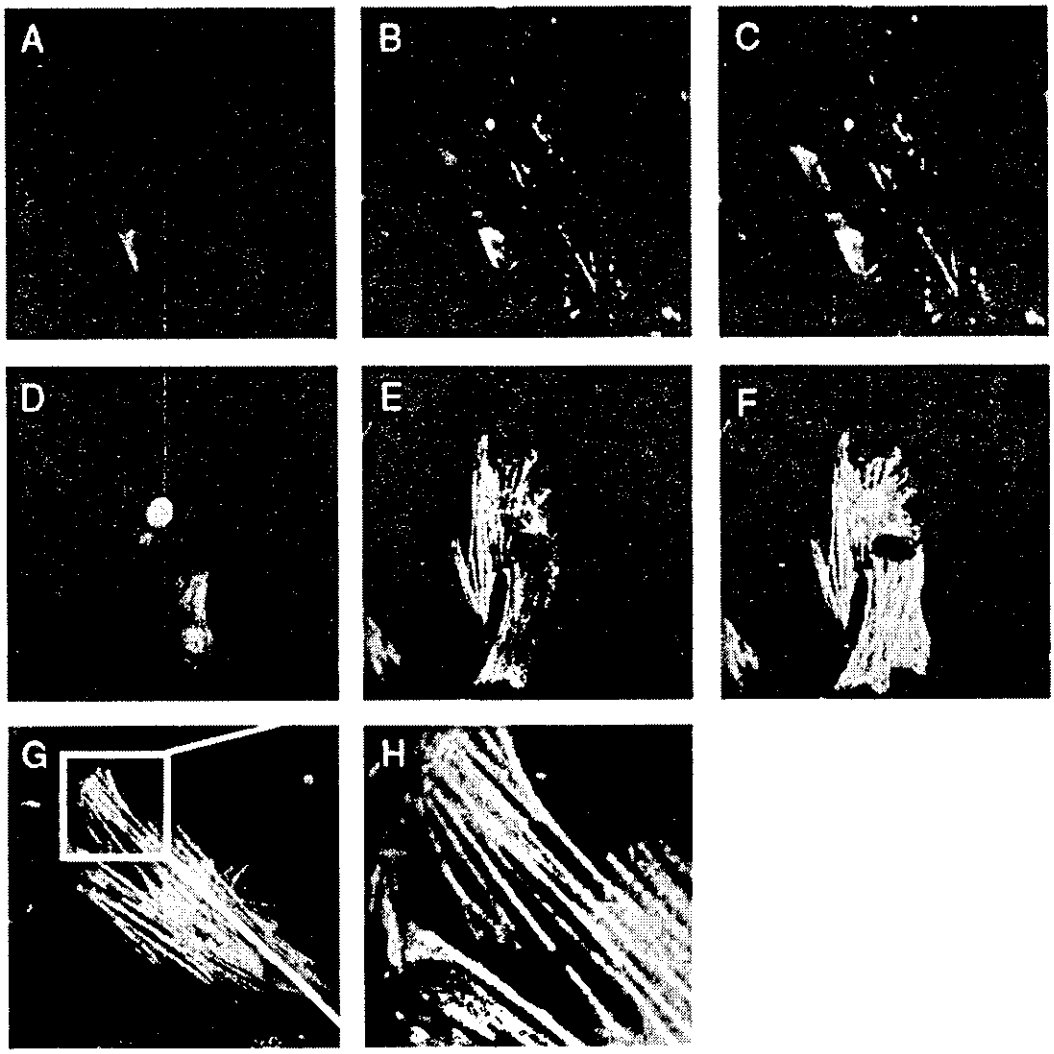

Figure 8. Immunocytochemistry of differentiated hMSCs with anti-desmin and anti-troponin-I antibodies. UBET-7 cells were co-cultured with cardiomyocytes. UBET-7 cells were analyzed for myogenic and cardiac differentiation by immunohistochemistry with desmin and cardiac troponin-I, respectively. Co-cultured 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

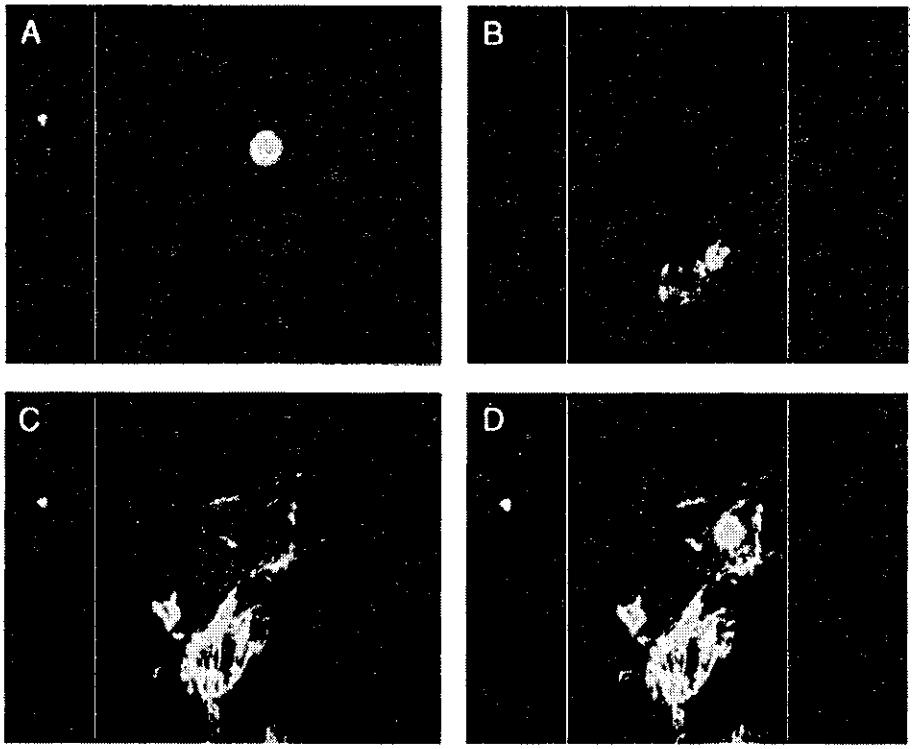

Figure 9. Cardiomyogenic differentiation of hMSCs is not due to cell fusion. We labeled UBET-7 cells with GFP (A) and murine cardiomyocytes with β-galactosidase (B). Cells were tested for the presence of β-galactosidase immunocytochemically (B, Cy5). Differentiation of UBET-7 cells was determined with 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: ×600

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 μW/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 μW/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.

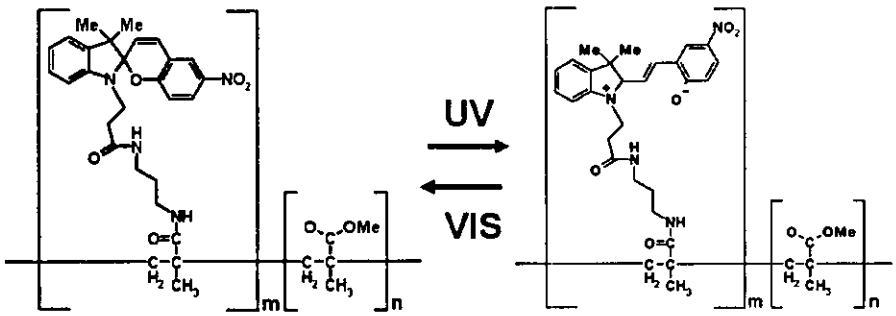

Figure 1. Schematic of transition of poly(NSP-*co*-MMA) upon exposure to UV irradiation.

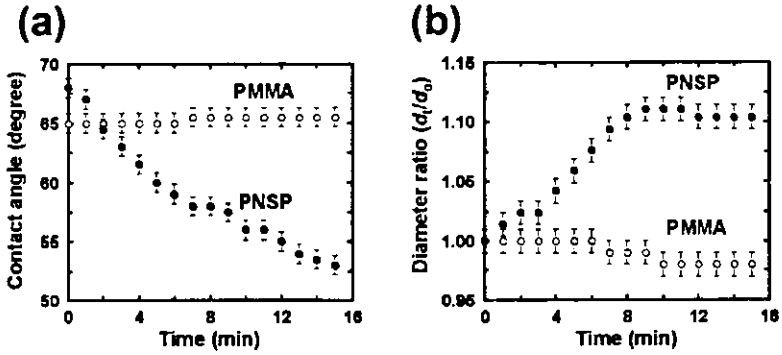

Figure 2. Hydrophobicity–hydrophilicity change on poly(NSP-*co*-MMA)-coated and PMMA-coated glass plates induced by UV irradiation. (a) Time dependence of water contact angle of water drop on poly(NSP-*co*-MMA)-coated and PMMA-coated glass plates. (b) Time dependence of diameter ratio (d_t/d_0) of water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

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

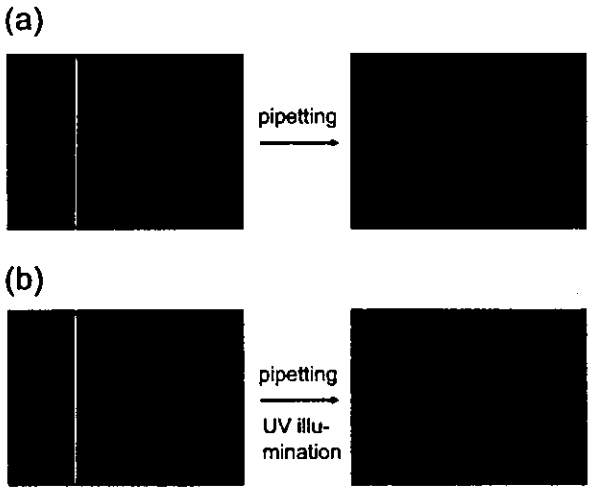

Figure 3. Light-induced detachment of KUSA-A1 cells. (a) KUSA-A1 cells on poly(NSP-*co*-MMA)-coated glass plates. (b) KUSA-A1 cells on PMMA-coated glass plates.

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.

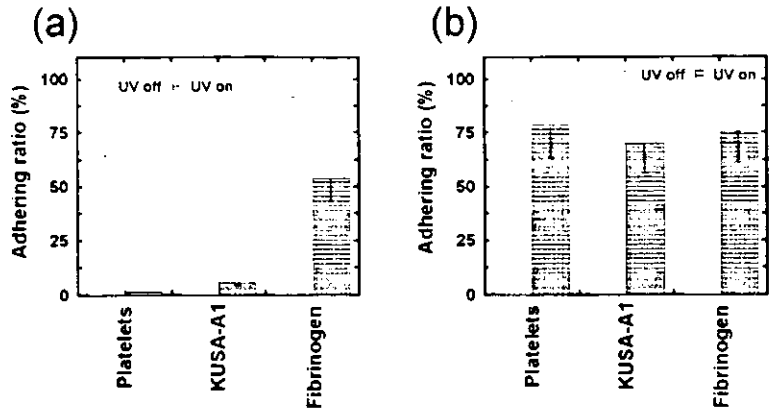

Figure 4. Light-induced detachment of platelets, KUSA-A1 cells and fibrinogen. (a) Platelets, KUSA-A1 cells, and fibrinogen on poly(NSP-*co*-MMA)-coated glass plates. (b) Platelets, KUSA-A1 cells, and fibrinogen on PMMA-coated glass plates.

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 $\mu g/cm^2$, whereas poly(NSP-*co*-MMA) coated glass plates had NSP consisting of nonionic spiropyran adsorbed at 5.0 ± 0.4 $\mu g/cm^2$. 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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第50回昭和医学会総会特別講演

ヒト幹細胞を用いた再生医療

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はじめに

再生医療（または再生医学）という新しい医療分野がマスコミをにぎわせている．ここ数年，この分野が劇的ともいえる進展をみせているのは，幹細胞（stem cell）に関する学問が大きく進んだこと，生分解性プラスチックなど細胞を生体に移植する際の足場になる材料としてよりよいものができてきたこと，増殖因子やマトリックスなど補助的材料も容易に入手できるようになったことなどが大きな促進要因になっていると思われる．

再生というのは欠損した臓器あるいは組織が自分の力で元に戻る現象だが，もともと臓器の中には再生しやすいものと，再生しにくいものとがある．一方，現在の再生医療は自己の再生能力を高めるというよりは，外から細胞を移植してやり，増殖因子やマトリックスを入れてやることによって元の臓器と同じ形で同様な機能をもつものをつくるという戦略をとっている．

では再生しやすい臓器は何かというと，最も代表的なものは肝臓である．プロメテウスが火を盗んだ罪によって，毎日ワシに肝臓をついばまれる罰をゼウスから課せられたというギリシャ神話の中の有名な寓話からもわかるように，肝臓の再生能力がきわめて高いことは非常に古くから知られていた．実際に，肝臓がんの手術で肝臓全体の2/3を大きく切除したような場合でも，失われた部分がきちんと再生して，元の機能を取り戻すので患者にとってはそれほど大きなダメージにはならない．

一方，再生しにくい臓器の代表は心臓である．私が経験した心筋梗塞で亡くなった患者の例を示すと，左心房の前壁に大きな梗塞巣がみられる．この梗塞巣は心筋が死んでしまった名残で，これは肝臓とは異なり元に戻ることは決してない．そこには膠原線維がたまる一方で，収縮能を完全に失い事実上心臓としての機能が完全に消失している．また，その梗塞巣がぺらぺらに薄くなって破れ，心破裂を引き起こすという場合もある．腎臓も同様に再生能力は弱い．

胚性幹細胞（ES細胞）と体性幹細胞

さて幹細胞には大きく分けて2つの種類がある．1つは有名なES細胞（embryonicstem cell）で，もう1つは全身の組織・臓器にある幹細胞である．ES細胞は日本語では胚性幹細胞または胎児性幹細胞，全身の臓器にある幹細胞は成体幹細胞あるいは体性幹細胞と呼ばれる．

ではES細胞とは何か．受精卵は卵割を繰り返し，桑実胚を経て胚盤胞になるが，その胚盤胞の内部細胞塊由来の細胞株として樹立されたものがES細胞である．ES細胞はマスコミなどでよく「万能細胞」として報じられているように，さまざまな細胞に分化し得る多分化能（pluripotency）を有しており，受精卵と同等の分化全能性をもつという意味で，toti-potencyという言葉も用いられている．このES細胞を筋肉，肝臓，血液，骨，心臓，神経などの細胞に分化させて患者に移植し，失われた機能の回復を図るというのが，現在研究が進められている再生医療の1つの戦略であると理解していただきたい．

一方，体性幹細胞は，由来する体細胞によってごく限られた範囲の細胞にしか分化できないものから，かなり広範囲な細胞に分化し得るものまでさまざまであり，それぞれの分化能に応じてbi-potencyとかoligo-potency，multi-potencyといった言葉が使われる．というわけで一口に幹細胞といっても，いろいろなレベルのものがあることになるが，胚性幹細胞と体性幹細胞を区別する考え方は医療の現場にとっては意味のあることである．

腫瘍由来のEC細胞

実はES細胞や体細胞性幹細胞の研究に先駆けてマウ

スの初期胚に由来する胚性（胎児性）腫瘍細胞（embryonalcarcinomacell＝EC 細胞）に多分化能があり，このEC細胞を胚盤胞に入れると全身の臓器・組織になるという実験結果がキメラマウスなどの研究で有名なB・ミンツ博士らによって報告されていた．そしてヒトの場合でも，このようなEC細胞を精巣や卵巣の腫瘍から分離することができ，ヒト初期発生の実験モデルとして使えるということもわかっていた．

われわれも32歳の男性の精巣にできた胎児性腫瘍からEC細胞株を樹立し，これにG3という名前をつけた．このG3はビタミンAを処理すると神経細胞，筋肉，胎盤などに分化し得ることが，それぞれの細胞に特有のマーカー（たとえば胎盤ではhCG＝ヒト絨毛ゴナドトロピン）を検出することによって明らかになった．つまり，この細胞はかなり広い分化能をもっているということになるわけだが，この分化はかなり確率的でこれをコントロールできたことはない．

骨髄間質細胞を用いる戦略

以上を前置きにして，本日の主題，骨髄中の幹細胞である間質細胞の話に移ることにしたい．この細胞は本来骨髄における造血機能を補助するという役割を担っている．電子顕微鏡で観察すると，一種の網目構造をとっていることがわかるが，この網目のところに血液が生み出されてくる．どちらかというと二次的な役割しかない細胞ということになるが，本日の話ではこれが主役である．われわれはこの細胞を大腿骨の骨髄から採取して用いている．

従来，細胞を移植する医療で最も成功しているのは疑いもなく骨髄移植だと思われる．骨髄移植の場合は，ドナーの腰骨から500ccの血液を採取し，その日のうちに患者に静注して全身的に造血を促す．われわれがやる細胞移植では採取した細胞を体外で1回培養して必要な量に増やしたり，適当な処理によって変身させたりして，骨や心臓などの目的の臓器に局注する．この培養することと目的の臓器に直接打ち込むという点が，骨髄移植と大きく違っている．そして変身させるための細工に関してはいままでに得られたサイエンスの知見をできる限り利用するという戦略をとる．

骨，軟骨，脂肪などへ変身させる

さて，われわれはこの骨髄間質細胞から骨，軟骨，脂肪，骨格筋，心筋，神経などの細胞に分化させることに成功した．順を追って簡単に紹介していくことにする．

骨髄間質細胞は通常の条件で培養すると，線維芽細胞とほとんど区別がつかない．これに分化誘導処理を施してマウスの背中の皮下に移植すると，2週間で早くも骨が形成されてくる．移植後4週間の時点で標本をつくって顕微鏡で観察すると，新たに形成された骨の中に宿主由来の顆粒球，赤血球，巨核球という3系統の細胞が浸潤している様子が見られる．つまり移植した細胞はきちんと骨をつくることが確認できたわけである．

そこで，次にこの細胞の骨をつくる能力を治療に利用できるかどうか調べてみた．マウスの大腿骨（全長約1cm）の骨幹部を5mmにわたって大きく切除し6週間後に観察すると，患部は肉芽が形成されただけで骨軟骨形成がみられず全く治癒していない．これに対して先の骨髄間質細胞を移植すると，4週間後には移植した細胞から新しい密な骨ができ，それと残っていたマウスの骨が完全に結合してほとんど治癒している所見が得られた．

ただし，当初の実験ではできた骨があまりにも歪であったので，次に目的の形の骨をつくることを試みた．産業技術総合研究所の専門家にお願いして，生分解プラスチックのポリ乳酸を四角い形のスポンジ状に加工してもらい，そのスポンジの穴に間質細胞を入れて，マウスに移植すると，こんどはちゃんと四角い骨ができたこのように工学分野の専門家の協力と指導によって適当な生体適合性の材料を使えば，目的の形の骨をつくることが可能になる．われわれは単に細胞だけでなく，そのようなことまで視野に入れて研究を進めているということを強調しておきたい．

次は軟骨である．実は患者自身の軟骨細胞を培養して移植し，損傷した軟骨を部分的に回復させるという治療法はすでに実地に行なわれている．しかし，われわれは骨髄間質細胞を使っているので，この細胞から軟骨をつくることを行っている．間質細胞を軟骨になるような処理を行った上で，マウスの皮下に移植するとそこに軟骨が形成されたことが肉眼でも観察できるが，その際軟骨に特異的なマーカーであるコラーゲン・タイプⅠとアグリカンの遺伝子の発現がみられ，この点からも確かに軟骨が形成されたことが証明できる．

われわれがなぜ骨髄間質細胞に固執しているかというと，病院の外来で簡単に採取できるという便利さがあるからである．その際，痛みを感じるかどうかは人によってまちまちだが，いずれにしろ簡単に採取でき傷跡も残らない．これは医療の現場にとってはかなり大きなメリッ

トである．

次は脂肪だが，実は骨髄間質細胞は非常に脂肪になりやすい．血清中から脂肪を取り込んでくるのではなく，この細胞自身が脂肪を合成するのである．ただ骨や軟骨にくらべて脂肪の移植に対する需要が少ないために，あまり利用されていない．乳房再建術に脂肪が使われることもあるが，ダイエットをすると形が崩れてしまうことがあるので，筋肉を使うほうが良いと言われている．

われわれは間質細胞をTNFα（腫瘍壊死因子α）で処理して脂肪細胞への分化を阻害し，脂肪へ分化するときには造血機能が失われるので，それを顆粒球コロニー刺激因子（G-CSF）やマクロファージコロニー刺激因子（M-CSF）などのコロニー刺激因子などによって復活させるといったコントロール方法を開発した．何故そういうことを考えたかというと，骨髄の中に脂肪髄ができると造血機能が全くなくなり，そのことと骨粗鬆症が密接に相関していることが知られているからである．たとえば，寝たきりの人とか，閉経後の女性，ダイエットをやりすぎた人，特殊なケースとしては宇宙飛行をした人などでは，脂肪髄ができて造血機能が衰えたために骨粗鬆症になりやすい．それを防ぐ意味でこのようなコントロールが重要になるというのが，われわれの考え方である．

遺伝子発現調節による分化の誘導

以上，骨，軟骨，脂肪と紹介してきたが，間質細胞がこれらの細胞に分化するのは，ある程度は当然のことだともいえる．そこで次は，これをもっと別の細胞に分化させることができないかということになる．

ここで私自身が米国の研究所で行った研究を簡単に紹介しておく．ケラチン18の遺伝子は1万塩基対からなるかなり長大な遺伝子であるが，そのエンハンサー領域はGCGGATG……という48塩基の配列からなっており，この配列はヒトとマウスで完全に保存されている．私はこのエンハンサー領域がメチル化されると，ケラチン18遺伝子の発現が抑えられ，メチル化されていなければ発現するという現象がヒトでもマウスでも共通に見られることに気付いた．そして，このエンハンサー領域にメチル化されないような変異を入れてやるとマウスの全身の臓器でケラチン18遺伝子が発現することを証明した．このことから転写調節領域のメチル化の有無によって，遺伝子の発現をコントロールできることに気づいた．

その後，UCSDにおいてコラーゲンα1（1）遺伝子の研究を行った．この遺伝子の場合はその3′末端領域に強力なエンハンサー活性があるが，5′末端のメチル化によってその活性は消失する．つまりメチル化のほうがエンハンサー活性より強いということを見出だしたわけである．さらに慶應に帰ってから，Beckwith-Wiedemann症候群という小児科領域の病気の研究を行い，この病気の患者ではH19という遺伝子の転写調節領域がメチル化されているために，肝臓の一部に本来の肝細胞よりはるかに幼若な，または別の細胞に分化する細胞が生じるということを明らかにした．

以上の3つの研究データから，私は遺伝子のメチル化の状態を変えてやれば，ある細胞を別の細胞に変身させることができるはずだという考えに行きついた．

骨格筋，血管，心筋への分化

この考えのもとに，骨髄間質細胞に対して脱メチル化剤である5-azacytidineを処理したところ，通常は線維芽細胞と同様の形をした細胞が長く伸び，収縮はしないが横紋構造が見られるようになった．この細胞をマウスの大腿四頭筋中に移植すると，横紋構造をもった骨格筋がきれいに形成されることがクラゲのGFPと抗体を用いた免疫組織学的検査などから証明された．この結果にはわれわれはかなり満足している．

次は血管である．前と同様の処理をした間質細胞をマウスの皮下に移植すると血管腔をもつ血管が形成されるが，われわれが一番うれしく思ったのは，この血管の中に赤血球が見られたという点である．われわれの間質細胞は赤血球には分化できない．したがって，新たに形成された血管中に赤血球が見られたということは，詳しい機序は不明だがこの血管にマウス本来の血管が吻合して血液が流れはじめたことを示している．

次は骨髄間質細胞を心臓に分化させた．実はわれわれはかなり早い段階で骨髄間質細胞が1分間に60から100回の割合でピクピクと動くことを観察していた．培養しはじめの頃は線維芽細胞と同じ形だが，分化誘導剤を処理して2週間たち，細胞が丸くなって伸びて分岐を生じて全体が3ミリぐらいの塊になると動きだすのである．そして最初は不整脈のような乱れが生じるが最終的には一定のリズムを刻んで拍動するようになる．後に，この拍動する細胞は心筋に特異的にみられるマーカーや転写因子が陽性になることがわかり，われわれは骨髄間質細胞が確かに心筋に変身したと結論づけた．

そこで次に実際にこの拍動する細胞を心臓に移植した

いと思い，心臓外科のプロに頼んでマウスの心臓に打ち込んでもらった．β-ガラクトシダーゼで青くラベルした細胞を，32ゲージという細い針でマウスの心臓に移植したところ，この細胞は右心室の中隔前1/3から前壁にかけてきれいに分布して植え付けられていた．ではというので，こんどは分化誘導剤処理をしたばかりの骨髄間質細胞を同様に心臓外科の手を借りてマウスの心臓に移植してもらった．すると骨格筋とは完全に違って，核が真ん中にあって分岐構造をもつ心筋細胞ができることがわかった．これによって，骨髄間質細胞は試験管の中でも心臓の細胞になるし，生体内でも心臓に変身させることができることが確かめられたわけである．

神経細胞への変身も

さいごの例として，骨髄間質細胞を神経に変身させる試みを紹介する．あるとき，必要があって手持ちの間質細胞の細胞株を横一線に並べて，それらがどのような細胞に分化しやすいかを調べていったところ，移植すると骨になる細胞株がときとして長い突起を出すことがわかってきた．これを報告した研究者は，これは神経細胞になったという意見だったが，骨髄間質細胞は中胚葉由来であり，神経は外胚葉由来であるので，そのような胚葉を超えた変身は有り得ないのではないかと私は考えていた．しかし，彼があまり強く主張するので，3人の神経発生学者に鑑定してもらったところ，全員がこれは神経細胞であるという判定を下したのである．

そこで，神経への分化をより確実なものにするために，この細胞のプロファイリングを米国のNIHに依頼した．その結果にもとづいて改良を加え，さらに神経細胞との接着活性のあるファイブロネクチンをコートした培養皿にこの細胞を移してやったところ，全ての細胞が非常に長い突起を伸ばしてその突起の先端が三角形になる様子が観察できた．そしてニューロンのマーカーであるMAP2やTuj1が陽性であり，グリア細胞を構成するアストロサイト（星状膠細胞）とオリゴデンドロサイト（稀突起膠細胞）のマーカーはほとんど陰性であった．つまり，われわれの骨髄間質細胞はまさしくニューロンに変身したということである．別の実験で，この神経細胞に変身した細胞が神経伝達物質であるグルタミン酸に反応しカルシウムの流入が高まることも明らかになった．

骨髄間質細胞の変身についての話は，大体以上で終りであるが，われわれがこのような成績について学会などで発表すると決まって出てくる質問がある．

第1は，骨髄間質細胞から骨ができたのも，また神経細胞ができたのも発表のとおりだろう．しかし，この実験に使われた細胞にはもともと神経になる幹細胞がコンタミしていて，それが分化しただけではないか．

第2は，間質細胞が1回，元の始原的な細胞に戻ってから，神経に分化したのではないか．第3は，第1と第2ではなく，間質細胞が完全に神経に変身したのか．この3点である．

第1の質問については，10数年にわたって何度もサブクローニングを繰返していて，分化誘導処理をしなければ骨にしかならない細胞であることを確認しており，すべての細胞には骨のマーカーが現れていることから，神経の幹細胞がコンタミしている可能性は絶対にない．したがってこの質問に対してはいつも「そんなことはあり得ません」という一言で片付けている．第2の質問については，われわれはこれまで中間体の細胞を観察したことがないので，私の考えは否定的である．そしてわれわれいつも第3の変身説をとっている．つまり，骨の細胞が一気に神経になったという考え方である．

胚葉を超えた分化は例外的

最近この細胞はいろいろなことですぐに神経になってしまうので，本来は神経の細胞で骨形成因子を出して骨の細胞として振る舞っているのは，実は仮の姿ではないかという考えをもっている．といってもとくに証拠があるわけではないので，あくまでも1つのアイディアであるが……．

本日紹介した骨髄間質細胞は中葉胚由来の細胞であり，骨，軟骨，脂肪，骨格筋，心臓いずれも中葉胚由来である．神経だけは外葉胚由来であり，その意味ではこの細胞は胚葉を超えて変身することもあり得ることになるが，これはあくまでも例外だと考えている．内胚葉系の細胞に変身させられるかという質問を受けることがよくあるが，何回となく試みてもそういうことはできない．肝臓はつくれないし，皮膚もできないし，髪の毛も眼もできたことはない．逆に言うとほとんどのものはできないということで，私たちはできた少数の例だけを皆さんに紹介しているのだと，理解していただきたい．

間質細胞の変身できる範囲が狭いならそれをもっと広げる方法はないか，というのでわれわれはクローン技術を利用して変身能力を上げることを試みている．日本では体細胞のクローン牛の研究や生産が進んでいるが，その専門家にお願いして骨髄間質細胞の核を除核卵細胞に

移植して胚盤胞をつくり，その内部細胞塊由来の細胞を使って変身能力が上るかどうか調べている．

実際にやってみると，まずこの内部細胞塊由来の細胞を培養すると，はじめ線維芽細胞様だったものが丸くなって塊をつくって増殖するようになる，つまり先に示した胎児性がん細胞（EC細胞）と同じような未分化の細胞になることがわかった．このEC細胞様の細胞を使って最初に試みたのは，これを胎盤に変身させることである．胎盤をつくって具体的に何をしようという気はないのだが，とにかく胎盤ができれば骨髄間質細胞の変身ぶりを示すことができるだろうという科学的興味から行っている．マウスに移植してみると，骨とか軟骨に分化するという様子は全くみられず，多核の巨細胞が生じてくる．もしかしたら胎盤ができたのではないかと考えているが，まだ確実ではない．

体性幹細胞移植による再生医療の問題点

体性幹細胞移植による再生医療の問題点あるいは今後の課題について若干考えてみたい．まず，この方法は自己の細胞を使うことができ，しかも細胞の採取も外来で簡単に行うことができるという大きな利点がある．ただし，いったん体外で培養する必要があるので，この間たとえば骨折した患者をそのまま待たせるわけにはいかず，医療の現場では最初は他家移植ということになる．

そこで問題点の第1は拒絶反応で，この問題は普通の臓器移植と同様に，再生医療とか細胞移植の現場でもやはり困ることである．

第2は，移植した細胞が予期せぬ分化をしないかという問題．これは今後より詳細に検討する必要がある．

第3には腫瘍化の問題がある．これまではあえて申し述べなかったが，われわれがこれまで行った実験では全く腫瘍化は観察されていない．ただいったん体外で培養するので，この間に何かが起きないかという懸念がないわけではない．その可能性は全く否定することはできない，というのがわれわれの見解である．

実際に移植したマウスでは，1年間経過しても腫瘍は発生していないことを確かめているが，ヒトでは，とくに移植がうまくいった場合，その人は30年，40年と生きるわけで，その間に腫瘍化しないかどうかは全く予想もつかない．もう1つ，われわれは移植用の細胞数を確保するために，とくにヒトの場合にはテロメラーゼを使って細胞の寿命を伸ばすことをやっている．このような処理を行ったために，移植してすぐに腫瘍はできないにしても，長い間にはLow grade malignacy，つまり悪性腫瘍の中でも非常に悪性度が低い腫瘍ができる可能性はないかという問題があるが，これも実験的に証明できない．また，テロメラーゼを用いることについて社会の理解が得られるかどうかは，現在のところ全く不明である．

以上のように幹細胞移植による再生医療には，まだいろいろな問題点があることは確かだが，何が利益で，またどういう不利益があるかを絶えず意識しながら，この医療を進めることが非常に大事だと思う．つまり，たいしたことのない病気の治療に再生医療を適用する必要はないということである．現在のわが国の移植医療では臓器不足が深刻で，移植に適した臓器を待ち切れずに亡くなる方が非常に多く，私は病理解剖を通じてそのような現実を日常的に肌で感じている．このような中で利益と不利益を明確にし，一定の手続きをきちんととった上で，何をやってその結果がどうなったかが公開されて社会的に検証され認知されるという過程をふんで，この再生医療とその基盤となるサイエンスを進めることができれば，祝福された形で世に送り出せるのではないかと考えている．

福田恵一博士（慶應義塾大学），戸山芳昭博士（慶應義塾大学），今林英明先生（慶應義塾大学），桜田一洋博士（協和発酵工業株式会社），岡野栄之博士（慶應義塾大学）との骨髄間質細胞の分化に関する共同研究で多くの示唆を受け，多くの世界的な潮流について最新情報の供与を受けました．草刈悟氏，阿部仁氏は，魅力的な骨髄間質細胞をたくさん樹立し，数々の発見は彼らに帰属するべきものが多い．時間のある方は，骨髄間質細胞の心筋への分化アニメーションをウェブサイトで見て貰えると嬉しいです．

司会　どうも面白いお話，ありがとうございました．（拍手）

質疑応答

司会　最先端の話を非常にわかりやすく，ウイットに富んだ話をしていただきました．梅澤先生のほうから，「質問を4つ5つ受けていいぞ」というご許可いただいていますのでご質問などございましたら，はい，それでは阪本先生，どうぞ．

阪本　僕は整形外科の阪本ですが，貴重な大変すばら