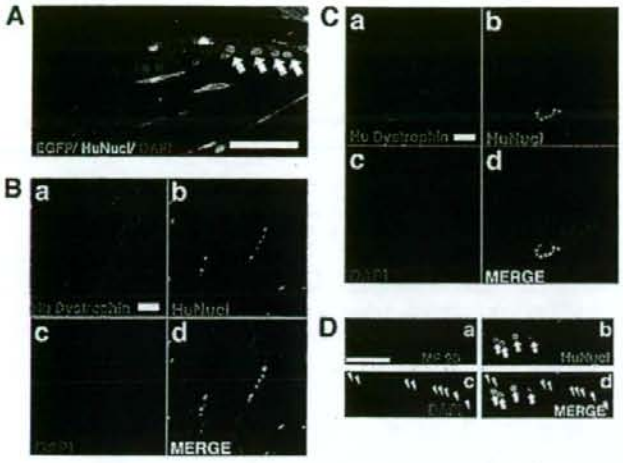

Figure 6. Detection of human endometrial cell contribution to myotubes in an in vitro and in vivo myogenesis model. EGFP-labeled EM-E6/E7/hTERT-2 cells (A) or EM-E6/E7/hTERT-2 cells (B) or menstrual blood–derived cells (C and D) were cocultured with C2C12 myoblasts for 2 d under conditions that favored proliferation. The cultures were then changed to differentiation media for 7 d to induce myogenic fusion. (A) Myotubes were revealed by EGFP (green); human nuclei were detected by antibody specific to human nuclei (HuNucl, red, arrows). (B–D) Myotubes were revealed by specific human dystrophin mAb NCL-DYS3 (B and C, red) or anti-myosin heavy chain mAb MF-20 (D, red). (D) Human nuclei were detected by antibody specific to human nuclei (HuNucl, green, arrows). Total cell nuclei in the culture were stained with DAPI (blue, arrowheads). (B–D) Merge of a–c are shown in d. The cultures were then changed to differentiation media for 7 d to induce myogenic fusion. Scale bars, 100 μm (A–D).

ogeneous populations of cells to cells with the mesenchymal phenotype in our cultivation condition, as determined by cell surface markers (Figure 1, C–E). MyoD-positive cells are present in many fetal chick organs such as brain, lung, intestine, kidney, spleen, heart, and liver (Gerhart *et al.*, 2001), and these cells can differentiate into skeletal muscle in culture. Constitutive expression of MyoD, desmin, and myogenin, all markers for skeletal myogenic differentiation in both immortalized EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells, implies either that most of these cells are myogenic progenitors or that these cells have myogenic potential. Expression of MyoD, one of the basic helix-loop-helix transcription factors that directly regulate myocyte cell specification and differentiation (Edmondson and Olson, 1993), occurs at the early stage of myogenic differentiation, whereas myogenin is expressed later, related to cell fusion and differentiation (Aurade *et al.*, 1994).

Acquisition or recovery of dystrophin expression in dystrophic muscle is attributed to two different mechanisms: 1) myogenic differentiation of implanted or transplanted cells and 2) cell fusion of implanted or transplanted cells with host muscle cells. Recovery of dystrophin-positive cells is explained by muscular differentiation of implanted marrow stromal cells and adipocytes (Dezawa *et al.*, 2005; Rodriguez *et al.*, 2005). In contrast, implantation of normal myoblasts into dystrophin-deficient muscle can create a reservoir of normal myoblasts that are capable of fusing with dystrophic muscle fibers and restoring dystrophin (Mendell *et al.*, 1995; Terada *et al.*, 2002; Wang *et al.*, 2003; Dezawa *et al.*, 2005; Rodriguez *et al.*, 2005). In this study using menstrual blood–derived cells, our findings—that the implantation of immortalized EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells improved the efficiency of muscle regeneration and dystrophin delivery to dystrophic muscle in mice—is explained by both possibilities or the latter possibility alone, because cells expressing human dystrophin had both murine and human nuclei, located in the center and periphery of dystrophic muscular fiber, respectively (Figures 5D, in vivo, and 6, A–D, in vitro).

DMD is a devastating X-linked muscle disease characterized by progressive muscle weakness attributable to a lack of dystrophin expression at the sarcolemma of muscle fibers (Mendell *et al.*, 1995; Rodriguez *et al.*, 2005), and there are no effective therapeutic approaches for muscular dystrophy at present. Human menstrual blood–derived cells are obtained by a simple, safe, and painless procedure and can be expanded efficiently in vitro. In contrast, isolation of mesenchymal stem cells/mesenchymal cells from other sources, such as bone marrow and adipose tissue, is accompanied by a painful and complicated operation. Efficient fusion systems of our immortalized human EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells with host dystrophic myocytes may contribute substantially to a major advance toward eventual cell-based therapies for muscle injury or chronic muscular disease. Finally, we would like to reemphasize that human menstrual blood–derived cells possess high self-renewal capacity, whereas biopsied myoblasts capable of differentiating into muscular cells are poorly expandable in vitro and rapidly undergo senescence (Cossu and Mavilio, 2000).

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Research Article

Single-cell-derived mesenchymal stem cells overexpressing Csx/Nkx2.5 and GATA4 undergo the stochastic cardiomyogenic fate and behave like transient amplifying cells

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ABSTRACT

Bone marrow-derived stromal cells can give rise to cardiomyocytes as well as adipocytes, osteocytes, and chondrocytes in vitro. The existence of mesenchymal stem cells has been proposed, but it remains unclear if a single-cell-derived stem cell stochastically commits toward a cardiac lineage. By single-cell marking, we performed a follow-up study of individual cells during the differentiation of 9-15c mesenchymal stromal cells derived from bone marrow cells. Three types of cells, i.e., cardiac myoblasts, cardiac progenitors and multipotent stem cells were differentiated from a single cell, implying that cardiomyocytes are generated stochastically from a single-cell-derived stem cell. We also demonstrated that overexpression of Csx/Nkx2.5 and GATA4, precardiac mesodermal transcription factors, enhanced cardiomyogenic differentiation of 9-15c cells, and the frequency of cardiomyogenic differentiation was increased by co-culturing with fetal cardiomyocytes. Single-cell-derived mesenchymal stem cells overexpressing Csx/Nkx2.5 and GATA4 behaved like cardiac transient amplifying cells, and still retained their plasticity in vivo.

Introduction

Cell-based therapy is a novel therapeutic strategy, based on the concept of the cell-mediated restoration of damaged or diseased tissue. Candidate cell sources include embryonic stem (ES) cells, hematopoietic stem cells (HSCs), neural stem cells (NSCs), mesenchymal stem cells (MSCs) [1], and so on. Clinical trials with MSCs have been performed in patients with graft-versus-host disease through immunomodulatory effects [2], and osteogenesis imperfecta [3,4], and MSCs are expected to be one of the most available cells. The source of MSCs includes bone marrow [5], adipose tissue [6], umbilical cord [7] and placenta [8].

Bone marrow-derived stromal cells [9] can differentiate into mesenchymal progenitors, including osteoblasts [10], chondroblasts [11], skeletal myoblasts [12], adipoblasts [13],

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and neurons [14,15] when placed in appropriate in vitro and in vivo environments. We have shown that bone marrow-derived stromal cells are also able to differentiate into cardiomyocytes in vitro and in vivo [13,14,16,17]. However, the characteristics of the cells that can differentiate into cardiomyocytes are poorly understood, and how the progeny of multipotent cells adopt one fate among several possible fates remains a fundamental question.

Hematopoietic stem cells are defined as cells that are capable of self-renewal to maintain a long-term supply of progeny and are capable of differentiating into multiple hematopoietic lineages [18]. Retroviral labeling of individual cells is one of the useful clonal assays to monitor lineage commitment at the single cell level [16,17,19]. At present, several models have been proposed in which hematopoietic lineage determination is driven intrinsically [20], extrinsically [21], or both [22]. We therefore performed retroviral labeling experiments of bone marrow-derived stromal cells to investigate whether cardiomyocytes are generated from committed cardiac precursor cells or uncommitted stem cells.

In the present study, we provide evidence that cardiomyocytes are stochastically differentiated from MSCs, and we demonstrate that forced expression of cardiomyocyte-specific transcription factors, i.e., Csx/Nkx2.5 and GATA4, destined these MSCs to a cardiomyocytic lineage.

Materials and methods

Cell culture

9-15c cells were used as a source of uncommitted stem cells in this study [23,24]. 9-15c cells are available through one of the cell banks (JHSF cell bank: http://www.jhsf.or.jp/English/index_gc.html; RIKEN cell bank: http://www.brc.riken.go.jp/lab/cell/english/guide.shtml). 9-15c cells were cultured using methods described previously [25]. The cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum and penicillin (100 μg/ml)/streptomycin (100 μg/ml)/amphotericin B (250 ng/ml) at 33°C with 5% CO_2.

Primary cultures of cardiac myocytes were prepared from the hearts of 16-day-old fetal C3H/HeJ mice (CLEA Japan, Inc., Tokyo, Japan) according to the method of Simpson et al. [26] with minor modifications. In brief, cardiomyocytes were dissociated into single isolated cells by trypsinization and the cells were plated in culture medium (IMDM with 20% fetal bovine serum).

Cloning of Csx/Nkx2.5 and GATA4 cDNAs

The full open reading frames of mouse Csx/Nkx2.5 and GATA4 cDNAs were cloned by RT-PCR from poly(A) RNA obtained from the hearts of fetal mice using the following primers: Csx/Nkx2.5, sense: 5′-TGAAACCTGCGTCGCCACCATGT-3′, antisense: 5′-GGCTCTTTCCCTACCAGGCTCGG-3′; GATA4, sense: 5′-TAGTTCTTGTCTGCCTCGTGCTCA-3′, antisense: 5′-GGCGCTGATTACGCGGTGATTATG-3′. The PCR products were subcloned into pGEM-T vector (Promega). DNA sequencing confirmed that the plasmids contained the full-length fragments of the mouse Csx/Nkx2.5 and GATA4 coding regions.

Retroviral transduction

The retroviral vectors pCLNCX (Imgenex), pCLPCX and pCLHCX were used. pCLPCX was constructed from pCLNCX by replacing the neomycin resistance gene with a puromycin resistance gene (pPUR; CLONTECH). pCLHCX was constructed from pCLNCX by replacing the neomycin resistance gene with a hygromycin resistance gene (pcDNA3.1/Hygro(+); Invitrogen). Fragments containing the EGFP, Csx/Nkx2.5, and GATA4 genes were cloned into pCLNCX, pCLPCX, or pCLHCX. Each of these DNAs and pCMV-Eco (kindly provided by Nikunj Somia) were transfected into the producer cells (293 gag pol; kindly provided by Nikunj Somia) using TransFast (Promega). Two days after the transfection, the culture supernatant was filtered through a 0.45-μm filter. 9-15c cells were treated with viruses and hexadimethine bromide (polybrene) (Sigma) (8 μg/ml) for 4–6 h. To generate stably expressing cells, 9-15c cells were cultured in the presence of 300 μg/ml G418, 300 ng/ml puromycin or 300 μg/ml hygromycin. The mixtures of drug-resistant clones were used to average the clonal variation of the transfected gene expression.

Cardiomyogenic induction

To induce differentiation, cells were initially plated at a density of 2×10^4 cells/ml. The cells were treated with 3 μM 5-azacytidine (Sigma) for 24 h the next day. In some experiments, PDGF-BB (Peprotech) and retinoic acid (Sigma) were added to the culture dish coated with fibronectin (BD Biosciences) to give a final concentration of 10 ng/ml and 1 nM, respectively, for 6 days. Total number of beating cells was estimated under phase contrast microscopy.

RT-PCR

Total RNA was extracted from adult mouse hearts, skeletal muscles and cultured cells with an RNeasy kit (QIAGEN), and cDNA was made using the SuperScript First-strand Synthesis System (Invitrogen) from 1 μg of total RNA. First-strand cDNA was diluted 20 fold and 1 μl of cDNA was used for each PCR reaction. The following primer sets for cardiomyocyte-associated genes were used: atrial natriuretic peptide (ANP), sense: 5′-TTCCTCGTCTTGGCCTTTTGG-3′, antisense: 5′-GCTGGATCTTCGTAGGCTCCG-3′; cardiac troponin I (cTnI), sense: 5′-GATCCTGTTCTCTGCCTCTGGA-3′, antisense: 5′-TCATCCACTTTGTCCACCCGAG-3′; fast troponin I (fTnI), sense: 5′-GAAGCGCAACAGGGCCATCACG-3′, antisense: 5′-CCACGTCACGCAGGTCCCGTTC-3′; Csx/Nkx2.5, sense: 5′-TGGCGTCTGGGGACCTGTCTG-3′, antisense: 5′-GAGTCTGGTCCTGCCGCTGTC-3′; GATA4, sense: 5′-TACATGGCCGACGTGGGAGCA-3′, antisense: 5′-TGGAGTTACCGCTGGAGGCAC-3′; exogeneous GATA4, sense: 5′-CCAGAAAACGGAAGCCCAAGAA-3′ (the sequence derived from mouse GATA4 gene), antisense: 5′-GCTTGCCAAACCTACAGGTGGG-3′ (the sequence derived from pCLPCX vector); adiponectin, sense: 5′-CTGAAGAGCTAGCTCCTGCTTTG-3′, antisense: 5′-GAAGAGAACGGCCTTGTCCTTC-3′; glyceraldehyde-

3-phosphate dehydrogenase (G3PDH), sense: 5′-CCCATCACCATCTTCCAGGAGC-3′, antisense: 5′-TTCACCACCTTCTTGATGTCATCATA-3′. G3PDH was used as an internal control. PCR was performed with TaKaRa Ex-Taq (TAKARA SHUZO CO., LTD) for 30–35 cycles, with each cycle consisting of 94°C for 1 min, 61–68°C for 1 min, and 72°C for 2 min, with an additional 7 min incubation at 72°C after completion of the final cycle.

RT-PCR samples were electrophoresed through agarose gels and stained with ethidium bromide and visualized through a UV light digital imaging system. Densities of electrophoresis bands were analyzed using ScnImage software (Scion Corporation).

Western blot analyses

Western blots were performed using whole-cell extracts according to the standard protocol [27]. Aliquots (30 μg) of whole-cell extracts were electrophoresed in SDS-polyacrylamide gels and transferred onto Immobilon-P polyvinylidene difluoride membrane (Millipore) by electroblotting. After treatment in blocking buffer, membranes were sequentially probed with the antibodies against Nkx2.5 (sc-8697, Santa Cruz) or Gata4 (sc-9053, Santa Cruz), and then with HRP-conjugated anti-goat or rabbit IgG. The bands were revealed using the ECL Plus standard protocol (Amersham Pharmacia Biotechnology).

Cellular transplantation

Following priming by 5-azacytidine for 24 h, the cells were cultured for an additional 3 days. Then the cells were harvested with 0.05% trypsin and 0.25 mM EDTA, and suspended as single cells at a concentration of 1×10^5 cells/μl with PBS. The cell viability in suspension, determined by 0.05% erythrosine dye exclusion, was 90% to 95%. After general anesthesia of the recipient mice by an intraperitoneal injection of 0.05 mg/g body weight pentobarbitone, cell transplantation was performed into the quadrant muscles of syngeneic adult recipient C3H/HeJ mice (CLEA Japan, Inc., Tokyo, Japan), aged 8 to 10 weeks old at a dose of 1×10^6 and 1×10^8 cells per mouse. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by Keio University School of Medicine and the National Research Institute for Child Health and Development, and the experimental procedures were approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine.

Histological analyses

Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections (6 μm) were mounted on poly-L-lysine-coated slides. After deparaffinization with xylene, tissues were rinsed in acetone or ethanol. Slides were incubated in 0.3% H_2O_2 for 30 min. After washing in PBS, tissues were preblocked for 30 min with 5% normal swine serum. They were incubated overnight at 4°C with mouse monoclonal antibody against recombinant GFP (CLONTECH Laboratories, Inc.) diluted 1:500. After rinsing in PBS, the slides were incubated with horseradish peroxidase-conjugated swine anti-mouse immunoglobulin diluted 1:100 with 1% BSA in PBS, and washed in cold PBS. Staining was developed using a solution containing DAB and 0.01% H_2O_2 in 0.05 M Tris–HCl buffer, pH 6.7. Slides were counterstained with hematoxylin. Slices with positive signals for EGFP were further stained

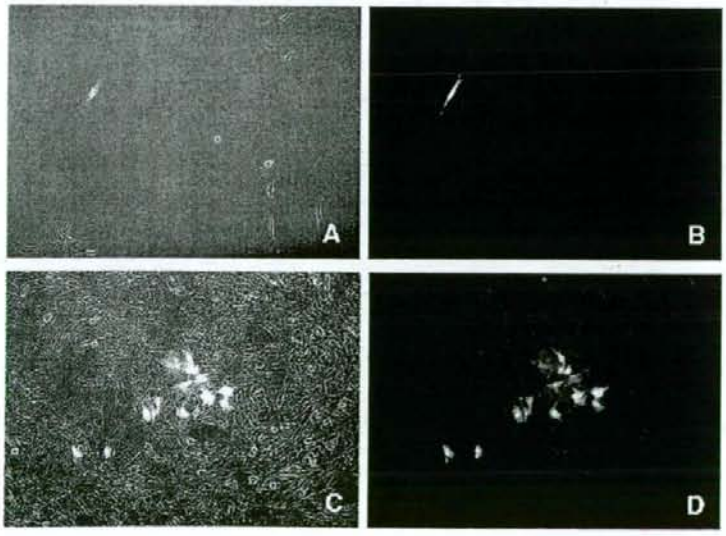

Fig. 1 – Single cell marking by infection of retrovirus carrying EGFP. Phase contrast photomicrograph (A, C) and fluorescent photomicrograph (B, D) of 9-15c cells 1 day (A, B) or 7 days (C, D) after infection with retroviruses carrying EGFP. EGFP-positive single cell-derived cells were clustered.

with anti-CD31 (PECAM-1) antibody (M-20, Santa Cruz Biotechnology, Inc, California, USA).

Frozen sections (6 μm) of the samples were used to detect the donor cells and the differentiation status by examination under a fluorescence microscope. After fixation with acetone and blocking with PBS containing 5% rabbit serum, anti-CD31 or anti-desmin (Bio-Science Products AG, Switzerland) antibodies was used as the first antibody, and rat anti-mouse IgG antibody conjugated with tetramethylrhodamine isothiocyanate (T4280, Sigma, Missouri, USA) and goat anti-mouse IgG antibody conjugated with rhodamine (M116, Leinco Technology, Inc., MO, USA) were used as the second antibody, respectively.

Results

Single-cell marking of 9-15c cells

9-15c cells are mesenchymal stem cells [23,24] capable of differentiating into cardiomyocytes in vitro with the use of 5-azacytidine. To determine if cardiomyocytes were generated from committed cardiac precursor cells or uncommitted stem cells during the differentiation of 9-15c cells, we carried out a single-cell marking experiment. Following retrovirus-mediated EGFP gene infection, a single EGFP-labeled cell could be detected at Day 1 after infection (Figs. 1A, B). The fate of

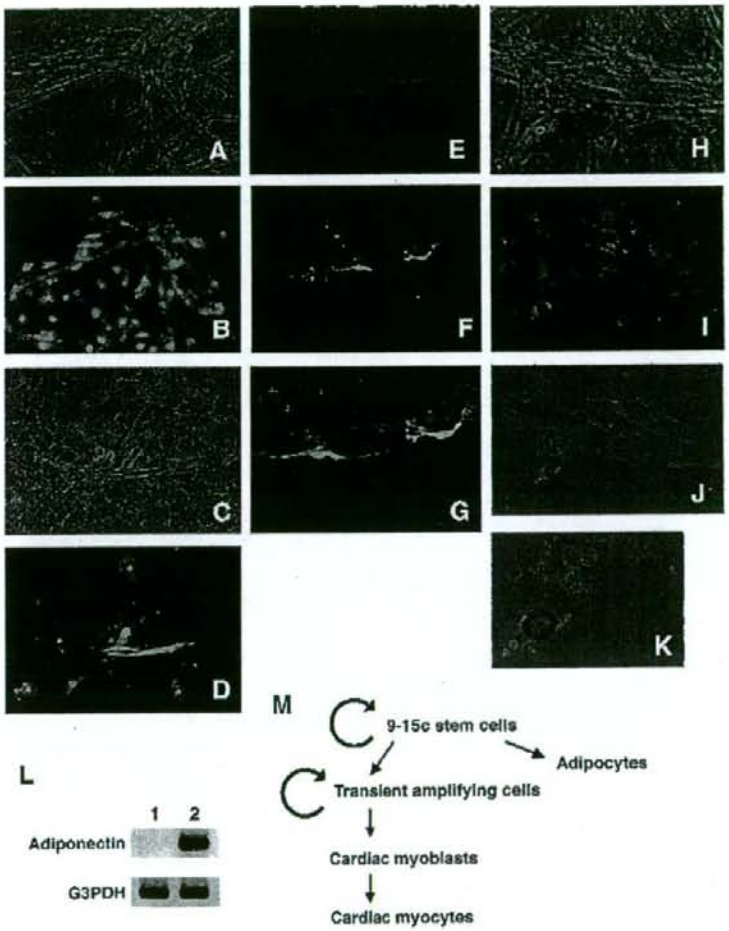

Fig. 2 – Bipotency, i.e., cardiomyogenic and adipogenic differentiation, of single cell-derived cells. Single-cell-derived 9-15c cells marked by EGFP exhibited cardiomyogenic and adipogenic differentiation after exposure to 5-azacytidine. (A–B) Cardiomyogenic and undifferentiated EGFP-marked, single-cell-derived 9-15c cells; (C–G) Cardiomyogenic differentiation of EGFP-marked, single-cell-derived 9-15c cells; (H–J) Cardiomyogenic and adipogenic differentiation of EGFP-marked, single-cell-derived 9-15c cells. (A, C, E, H, J) Phase contrast photomicrographs; (B, D, F, G, I) fluorescent photomicrographs. (K) Enlargement of the panel J. (L) RT-PCR analysis of the adiponectin and G3PDH genes in 9-15c cells at the growing phase without any treatment (lane 1) and 4 weeks after exposure to 5-azacytidine (lane 2). (M) Scheme of 9-15c cell differentiation.

retrovirally tagged 9-15c cells could be traced by monitoring EGFP throughout the differentiation process after exposure to 5-azacytidine. Seven days later, the EGFP-positive, single-cell-derived cells were clustered (Figs. 1C, D). Four weeks after 5-azacytidine treatment, the EGFP-positive cells were examined for differentiated phenotypes. We identified beating cells as cardiomyocytes and oil-red-positive cells as adipocytes. Three kinds of cell populations were observed: a) a cell population in which cardiomyocytes and undifferentiated stem cells were EGFP-positive (Figs. 2A, B); b) a cell population in which all the EGFP-positive cells were cardiomyocytes (Figs. 2C–G); c) a cell population in which cardiomyocytes, adipocytes and undifferentiated stem cells were EGFP-positive (Figs. 2H–K). RT-PCR analysis shows that these cells express adiponectin (Fig. 2L), suggesting the presence of adipocytes among the differentiated population. These results imply that cardiomyocytes are generated from uncommitted stem cells (Fig. 2M).

9-15c multipotent cells were preferentially destined to generate cardiomyocytes by forced expression of transcription factors Csx/Nkx2.5 and GATA4

In order to elucidate the roles of Csx/Nkx2.5 and GATA4 in 9-15c cell differentiation, we infected 9-15c cells with retroviruses carrying Csx/Nkx2.5 and GATA4. We detected Csx/Nkx2.5 and GATA4 gene expression in the infected cell by RT-PCR and Western blotting (Figs. 3A and B). GATA4 gene was originally expressed in 9-15c; we detected the GATA4 transgene with specific primers, but not the endogenous GATA4 gene (Fig. 3A).

Four weeks after the induction of differentiation by 5-azacytidine treatment, we examined the efficiency of cardiomyogenic differentiation or the expression of cardiomyogenic markers. The expression of the ANP and cTnI genes was up-regulated in 9-15c cells overexpressing Csx/Nkx2.5 and GATA4 (9-15c-CG cells) compared to the uninfected 9-15c cells (Fig. 3C, lanes 5 and 9). When 9-15c-CG cells were treated with PDGF and retinoic acid on dishes coated with fibronectin in addition to 5-azacytidine, the expression of the ANP and cTnI gene was further up-regulated (Fig. 3C, lane 10).

Cell implantation into immunodeficient mice

To investigate whether 9-15c-CG cells differentiate in vivo, the cells treated with 10 μM 5-azacytidine for 24 h were injected into immunodeficient mice (Figs. 4A–F). The donor cells clearly formed striated muscles without a branched structure as well as undifferentiated cells 81 days after implantation. The implanted 9-15c-CG cells clearly expressed desmin (Fig. 4G). The grafted cells also generated neovascularization near the injected site 1 month after injection; the EGFP-positive donor cells could be identified as the endothelium of these vessels (Fig. 4H). Immunohistochemistry with an antibody against CD31, a marker for endothelium, confirmed that the donor cells of the newly formed vessels had differentiated into endothelium (Fig. 4Hb). Engrafted donor cells appeared to maintain the characteristics of stem cells, that is, they continued to produce progeny, i.e., differentiated endothelial cells in this case.

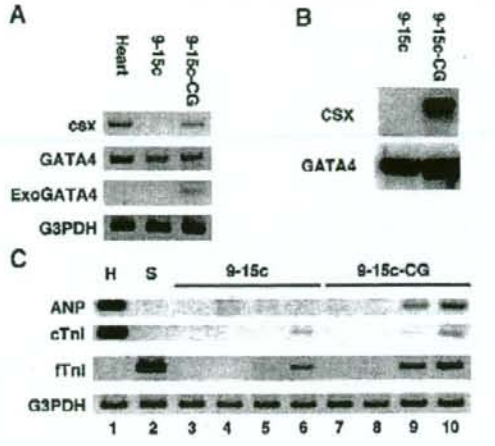

Fig. 3 – Expression of cardiomyocyte-specific or associated genes in 9-15c cells. A: RT-PCR analysis of the Csx, GATA4, exogeneous GATA4 and G3PDH genes (from top to bottom) in adult mouse heart, 9-15c cells and 9-15c cells overexpressing the Csx and GATA4 genes (9-15c-CG cells). B: Western blotting analysis of the Csx and GATA4 proteins in 9-15c cells and 9-15c-CG cells. C: RT-PCR analysis of the ANP, cTnI, and G3PDH genes (from top to bottom) in 9-15c cells (lanes 3–6) and 9-15c-CG cells (lanes 7–10). 9-15c cells (lane 3) and 9-15c-CG cells (lane 7) were cultured without any treatment (lanes 4 and 8) or with exposure to 5-azacytidine alone (lanes 5 and 9), or 5-azacytidine, PDGF, retinoic acid, and fibronectin coating on a dish (lanes 6 and 10) for 4 weeks. Heart (lane 1: H) and skeletal muscle (lane 2: S) served as controls.

Enhancement of cardiomyogenic differentiation by the co-cultivation with cardiomyocytes

We co-cultured EGFP-labeled 9-15c-CG cells with cardiomyocytes of fetal mice in vitro. Four weeks after 5-azacytidine treatment, EGFP-positive beating cardiomyocytes were increased (Figs. 5A, B). To determine whether factors secreted from the cultured cardiomyocytes promoted cardiomyocytic differentiation, 9-15c cells and 9-15c-CG cells were cultured in growth medium supplemented with conditioned medium from cardiomyocyte cultures. The expression of the ANP and cTnI genes was up-regulated in both 9-15c cells and 9-15c-CG cells with exposure to the conditioned medium of cardiomyocyte cultures (Fig. 5C, lanes 3 and 7). Furthermore, treatment with PDGF and retinoic acid, and fibronectin coating on a dish enhanced cardiomyogenic marker expression in both 9-15c cells and 9-15c-CG cells (Fig. 5C, lanes 4 and 8).

Discussion

Different models arise from different conceptions of the MSCs as in hematopoietic stem cells' differentiation [28,29]. A hierarchical model of MSCs has been proposed based on the in vitro differentiation potential of human MSCs as observed

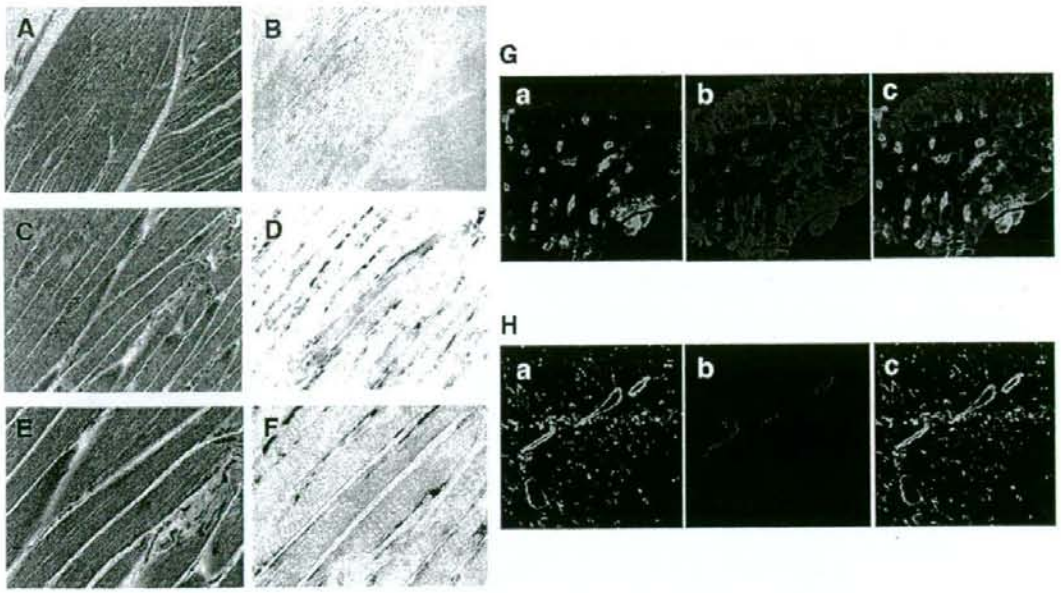

Fig. 4 – Myogenic differentiation of the EGFP-labeled 9-15c-CG cells into the quadriceps femoris muscle. EGFP-labeled 9-15c-CG cells could be recognized morphologically as the skeletal myocytes in the quadriceps femoris muscle 3 months after transplantation (A, C, E: HE staining; B, D, F: immunohistochemistry using anti-GFP antibody). The EGFP-positive donor cells exhibited skeletal myocyte-specific features such as multiple nuclei in the periphery of the cells and striation. Generation of myocytes (G) and endothelial cells (H) by the EGFP-labeled 9-15c cells. The injected donor 9-15c cells labeled with EGFP were detected by green fluorescence. (Ga, Ha) Green fluorescence of EGFP-labeled donor cells. (Gb, Hb) Immunohistochemistry for desmin (Gb, red) or CD31 (Hb, red). (Gc, Hc) The merged images of green fluorescence of injected 9-15c cells and rhodamine of desmin or CD31 clearly demonstrated that 9-15c cells differentiated into myocytes or endothelium. A–F: Longitudinal section; G, H: Cross section.

by clonal analysis [30]. In the present study using single-cell marking, we found that 9-15c cells in culture consisted of a mixture of at least three types of cells, i.e., cardiac myoblasts, cardiac progenitors and multipotent stem cells. Cardiac myoblasts are defined as cells which can differentiate into only cardiac myocytes and have low proliferative potential; cardiac progenitors have proliferative capability and the ability to become cardiomyocytes; multipotent stem cells have both proliferative capability and multipotency. The results obtained in the present study suggest that 9-15c cells are stochastically committed toward the cardiac lineage, and that following this commitment they proliferate as transient amplifying cells and differentiate into cardiac myocytes through the differentiation process, and the hierarchical model applies in the case of 9-15c multipotent cells.

In the present study, we used 5-azacytidine to induce differentiation. 5-azacytidine is a cytosine analog that causes extensive demethylation. The demethylation is attributable to covalent binding of DNA methyltransferase to 5-azacytidine in the DNA [31], with the subsequent reduction of enzyme activity in cells resulting in random loss of methylation at many sites in the genome. Previously, it has been thought that 5-azacytidine activates cardiomyogenic master genes, such as Nkx2.5/Csx, GATA4, and MEF-2C, leading to stochastic transdifferentiation of MSCs into cardiomyocytes [32,33]. This concept is difficult to account for the existence of cardiac progenitors and multipotent stem cells we identified, and we propose two possibilities how 5-azacytidine works. First, treatment of 5-azacytidine modulates heterochromatin remodeling and leads to dedifferentiation of 9-15c cells. Second, 9-15c cells are stochastically committed toward the cardiac lineage, being independent of treatment of 5-azacytidine. At this time we cannot conclude which is feasible, but it is certain cardiomyocytes are not only transdifferentiated by treatment of 5-azacytidine.

Csx/Nkx2.5 and GATA4 are two cardiac-enriched transcription factors that are expressed in precardiac mesoderm from the very early developmental stage [34,35]. In the present study, increased frequency of cardiomyogenic differentiation of 9-15c cells was successfully achieved in vitro by forced expression of Csx/Nkx2.5 and GATA4. These results are consistent with a report showing that both Csx/Nkx2.5 and GATA4 are required for the cardiac differentiation of P19CL6 cells derived from embryonic teratocarcinoma cells [36]. Cardiomyogenic differentiation, however, could proceed only after treatment with 5-azacytidine in our experimental setting, implying that Csx/Nkx2.5 and GATA4 are required but not sufficient for cardiac differentiation. Unknown factors

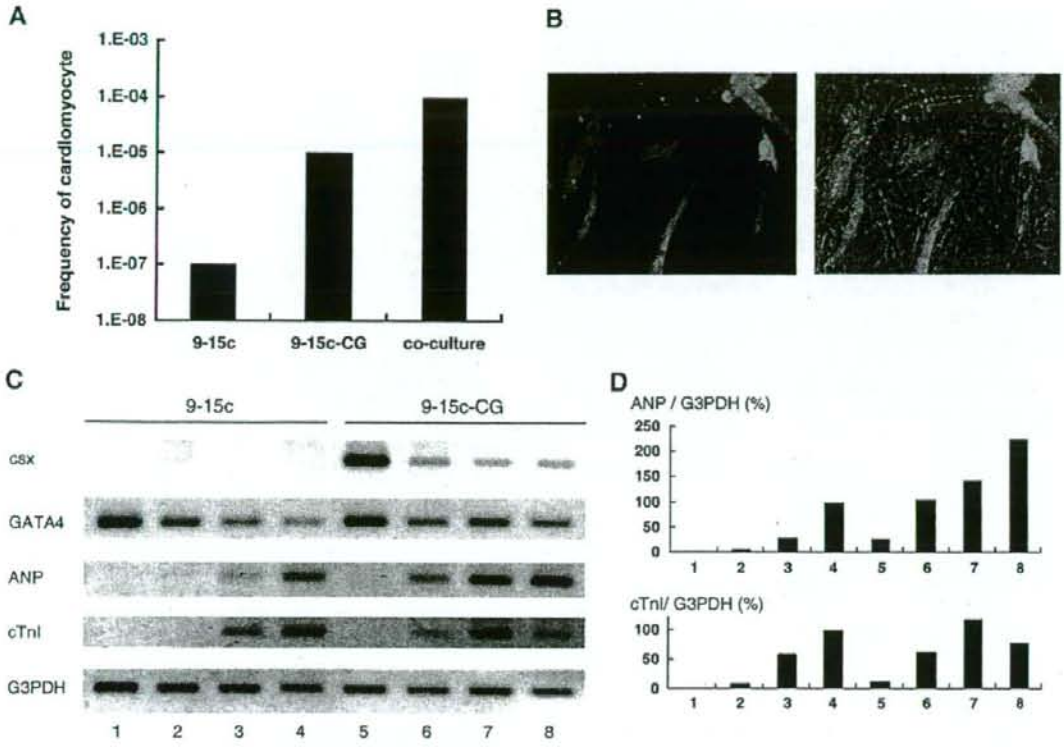

Fig. 5 – Enhancement of cardiomyogenic differentiation of 9-15c cells by co-cultivation with murine fetal cardiomyocytes. A: Frequencies of cardiomyogenic differentiation in 9-15c cells, 9-15c cells overexpressing the Csx and GATA4 genes (9-15c-CG cells), and 9-15c-CG cells co-cultured with murine fetal cardiomyocytes. B: Cardiomyogenic differentiation of EGFP-positive 9-15c-CG cells co-cultured with murine fetal cardiomyocytes. Left: Green fluorescence of EGFP-positive 9-15c-CG cells. Right: Same field visualized by phase-contrast microscopy merged with fluorescence image. C: RT-PCR analysis of the Csx, GATA4, ANP, cTnI and G3PDH genes in 9-15c cells (lanes 1–4) and 9-15c-CG cells (lanes 5–8). 9-15c cells (lane 1) and 9-15c-CG cells (lane 5) were cultured with exposure to 5-azacytidine alone (lanes 2 and 6) or 5-azacytidine and conditioned medium of cardiomyocyte cultures (lanes 3 and 7), or 5-azacytidine, conditioned medium of cardiomyocyte cultures, PDGF, retinoic acid, and fibronectin coating on a dish (lanes 4 and 8) for 4 weeks. D: Ratio mRNA expression level of ANP and cTnI to G3PDH in C. The mRNA level of 9-15c cells (lane 4) was regarded as equal to 100%.

induced by 5-azacytidine or microRNAs, whose key roles in stem cell biology are just emerging [37], also seem to be needed.

Adipogenic 3T3-L1 [38], osteogenic MC3T3-E1 [39], and chondrogenic ATDC5 cells [40] have been isolated from stem cells with a mesenchymal nature. In addition, cardiomyogenic precursors may be obtained from stem cells such as cardiac stem cells, embryonic stem cells, and mesenchymal stem cells. Fetal cardiomyocytes are differentiated cardiomyocytes, but not stem cells that can proliferate in vitro. Recently, cardiac stem cells capable of clonogenically self-renewing have been isolated from the adult heart [41–43]. Some cardiac stem cells also retain plasticity. The retention of plasticity, i.e., the ability to transdifferentiate into skeletal myocytes and endothelium, of 9-15c cells overexpressing Csx/Nkx2.5 and GATA4 supports the idea that these cells may be considered cardiac stem or amplifying cells in terms of differentiation and self-renewal. On the other hand, Csx/Nkx2.5 inhibits the myogenic differentiation of C2C12 cells and promotes neuronal differentiation [44]. This unexpected effect of Csx/Nkx2.5 may be due to differential effects of the gene in different cell types, or of transient versus constitutive expression of the infected gene; dependency of the differentiated phenotypes on the gene expression period is observed for the Notch gene [45,46] and noggin gene [47].

Cell transplantation has been attempted to improve cardiac function in severe heart failure; MSCs have been transplanted to functionally restore damaged or diseased tissue in animal models, and mononuclear cells or myoblasts have been injected into ischemic hearts clinically. MSCs are capable of differentiating into many types of cells, and 'cardiomyogenic master genes' are able to enhance the commitment or determine the path to cardiomyogenic differentiation of these MSCs. The stemness of MSCs determined by single-cell

marking in this study needs to be taken into consideration when we are considering mesenchymal stem cell-based therapy: we should pay attention to the possible unexpected differentiation of donor MSCs such as osteogenesis or chondrogenesis in the implanted heart.

In conclusion, we demonstrated that cardiomyocytes were stochastically differentiated from MSCs and that forced expression of Csx/Nkx2.5 and GATA4 enhanced the commitment or determined the path to cardiogenic differentiation of these MSCs. Our findings suggest that single-cell-derived MSCs overexpressing Csx/Nkx2.5 and GATA4 behave like cardiac transient amplifying cells and that Csx/Nkx2.5 and GATA4 could be interesting target molecules for enhancing cardiogenesis of MSCs.

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Hyaline Cartilage Formation and Enchondral Ossification Modeled With KUM5 and OP9 Chondroblasts

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Abstract What is it that defines a bone marrow-derived chondrocyte? We attempted to identify marrow-derived cells with chondrogenic nature and immortality without transformation, defining "immortality" simply as indefinite cell division. KUM5 mesenchymal cells, a marrow stromal cell line, generated hyaline cartilage in vivo and exhibited enchondral ossification at a later stage after implantation. Selection of KUM5 chondroblasts based on the activity of the chondrocyte-specific cis-regulatory element of the collagen α2(XI) gene resulted in enhancement of their chondrogenic nature. Gene chip analysis revealed that OP9 cells, another marrow stromal cell line, derived from macrophage colony-stimulating factor-deficient osteopetrotic mice and also known to be niche-constituting cells for hematopoietic stem cells expressed chondrocyte-specific or -associated genes such as type II collagen α1, Sox9, and cartilage oligomeric matrix protein at an extremely high level, as did KUM5 cells. After cultured OP9 micromasses exposed to TGF-β3 and BMP2 were implanted in mice, they produced abundant metachromatic matrix with the toluidine blue stain and formed type II collagen-positive hyaline cartilage within 2 weeks in vivo. Hierarchical clustering and principal component analysis based on microarray data of the expression of cell surface markers and cell-type-specific genes resulted in grouping of KUM5 and OP9 cells into the same subcategory of "chondroblast," that is, a distinct cell type group. We here show that these two cell lines exhibit the unique characteristics of hyaline cartilage formation and enchondral ossification in vitro and in vivo. J. Cell. Biochem. 9999: 1–15, 2006.

Key words: Hyaline cartilage; chondroblasts; enchondral[Q2]ossification; bioinformatics; gene chip

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The concept of regenerative medicine refers to the cell-mediated restoration of damaged or diseased tissue, and practically, regeneration of bone and cartilage may be one of the most accessible approaches. Candidate cell sources for regeneration of tissue include embryonic stem cells, fetal cells, or adult cells such as marrow stromal cells [Bianco and Robey, 2000], each of which has both benefits and drawbacks. Multipotent mesenchymal stem cells proliferate extensively, and to maintain the ability to differentiate into multiple cell types such as osteoblasts, chondrocytes, cardiomyocytes, adipocytes, and myoblasts in vitro [Umezawa et al., 1992; Pittenger et al., 1999; Bianco and Robey, 2000]. Marrow-derived stromal cells are also able to generate cardiomyocytes and endothelial cells [Makino et al., 1999], neuronal cells [Kohyama et al., 2001], and adipocytes [Umezawa et al., 1991]. Thus, marrow stromal cells are expected to be a good source of cell therapy in addition to embryonic stem cells and fetal cells [Pittenger et al., 1999].

In adults, chondrocytes maintain the extracellular matrix that gives cartilage its unique mechanical properties. Chondrocytes are long-lived and the development of new cells that are capable of producing cartilage de novo (i.e., chondroblasts) is not a normal part of adult cartilage physiology. A better understanding of the molecular mechanisms that regulate postnatal chondroblast differentiation would have a high impact on the design of strategies for cartilage repair. Cultures are commonly made from suspensions of cells dissociated from cartilage. Cartilage-derived cells in primary cultures can be removed from the culture dish and made to proliferate to form a large number of so-called secondary cultures: in this way, these cells may be repeatedly subcultured for weeks or months. Such cells often display many of the differentiated properties appropriate to their origin: the phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of cartilage-specific extracellular matrix molecules, including type II collagen and aggrecan [Archer et al., 1990; Hauselmann et al., 1994; Reginato et al., 1994]. The phenotype of differentiated chondrocytes is unstable in culture and is rapidly lost during serial monolayer subculturing [Benya and Shaffer, 1982; Lefebvre et al., 1990; Bonaventure et al., 1994]. This process is referred to as "dedifferentiation" and is a major impediment to the use of mass cell populations for cell therapy or tissue engineering of damaged cartilage. However, when cultured three-dimensionally in a scaffold such as agarose, collagen, or alginate, redifferentiated chondrocytes start to reexpress the chondrocytic differentiation phenotype.

This study was undertaken to obtain bone marrow-derived chondroblastic cell lines that retain critical in vivo cell functions. Previous studies showed that it was possible to obtain lines of bone marrow-derived mesenchymal stem cells, mammary gland epithelial cells, skin keratinocytes, and pigmented epithelial cells that retained critical in vivo cell functions. By implanting cells into immunodeficient mice, we identified a newly isolated KUM5 chondroblastic cell line capable of in vivo hyaline-type chondrogenesis and serendipitously found that OP9 cells derived from osteopetrotic mice and also known as a niche-constituting cells for hematopoietic stem cells had chondrogenic potential.

MATERIALS AND METHODS

Cell Culture and Chondrogenic Differentiation

The cells were cultured in the growth medium (GM): Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% fetal bovine serum for KUM5 cells; α-MEM supplemented with 10% serum (BIOWEST, lot number: S03400S1820) for OP9 cells. For chondrogenic induction of pellet culture [Johnstone et al., 1998], both KUM5 and OP9 cells were cultured in the chondrogenic medium (CM): DMEM-high glucose containing 0.1 μM dexamethasone, 1 mM sodium pyruvate, 0.17 mM ascorbic acid-2-phosphate, 0.35 mM proline, 6.25 μg/ml bovine insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenous acid, 5.33 μg/ml linoleic acid, and 1.25 mg/ml BSA (BioWhittaker). In the chondrogenic differentiation, the combination of one or several growth factors was added to the CM: TGF-β3 10 ng/ml, BMP2 50 ng/ml, BMP4 50 ng/ml, BMP6 50 ng/ml, BMP7 50 ng/ml, PDGF 50 ng/ml, hyaluronic acid 250 ng/ml. The cells and the pellets were maintained at 37°C with 5% CO_2.

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

The pelleted micromasses were examined by SEM and TEM. The micromasses were coated

with gold using a Sputter Coater (Sanyu Denshi Co., Tokyo, Japan) for SEM. The gas pressure was set at 50 mtorr, the current was 5 mA, and the coating time was 180 s. The samples were examined with a scanning electron microscope (JSM-6400Fs; JEOL, Ltd., Tokyo, Japan) operated at a voltage of 3 kV. For TEM, the micromasses and cell implants were initially fixed in PBS containing 2.5% glutaraldehyde for 24 h, and were embedded in epoxy resin. Ultrathin sections were double stained with uranyl acetate and lead citrate and were viewed under a JEM-1200EX transmission electron microscope (JEOL, Ltd.).

Flow Cytometric Analysis

Flow cytometric analysis was performed as previously described [Ochi et al., 2003; Mori et al., 2005; Terai et al., 2005].

Preparation and Transfection of Plasmid

The Venus gene (gift from Miyawaki) was obtained by BamHI and NotI digestion of Venus/pCS2 [Nagai et al., 2002]. The Venus gene was then cloned between the BamHI and NotI sites of pBluescriptII SK (−), excised by SalI and NotI digestion, and inserted between the XhoI and NotI sites of the p742-LacZ plasmid [Tsumaki et al., 1996], from which the LacZ gene was excised by XhoI and NotI digestion. This was named p742-Venus-Int plasmid. Transfection was performed using LipofectAmine 2000 (Invitrogen) according to the manufacturer's instructions.

Isolation of KUM5 Chondroblast

Cells were transfected with p742-Venus-Int plasmid and were cultured for 72 h. Venus-positive cells were sorted using the cell sorter (EPICS ALTRA, Beckman Courter).

In Vivo Cell Implantation Assay

To determine the ability of cultured cells to differentiate in vivo, freshly scraped cells ($2–3 \times 10^7$ cells) were subcutaneously inoculated into Balb/c nu/nu mice (Sankyo Laboratory, Hamamatsu, Japan) as previously described [Umezawa et al., 1992]. Animals were sacrificed by cervical dislocation between 1 and 8 weeks after inoculation. The subcutaneous specimens were dissected at various times after implantation and fixed and decalcified for 1 week in 10% EDTA (pH 8.0) solution. After dehydration in ascending concentrations of ethanol and xylene, the implants were embedded in paraffin. The paraffin sections were then deparaffinized, hydrated, and stained with hematoxylin and eosin, alcian blue, or toluidine blue. Paraffin sections were immunohistochemically stained with anti-type II collagen antibodies (Daiichi Fine Chemical Co., Ltd., Tokyo, Japan, Product No. F-57).

All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the US National Institutes of Health (NIH Publication No. 86–23, revised 1985). The operation protocols were accepted by the Laboratory Animal Care and Use Committee of the Research Institute for Child and Health Development (2003–002).

Gene Chip Expression Analysis

Mouse-genome-wide gene expression was examined with the Mouse Genome MOE430A Probe array (GeneChip, Affymetrix), which contains the oligonucleotide probe set for approximately 23,000 full-length genes and expressed sequence tags (ESTs), according to the manufacturer's protocol (Expression Analysis Technical Manual and GeneChip small sample target labeling Assay Version 2 technical note. http://www.affymetrix.com/support/technical/index.affx). Total RNA was isolated with an RNeasy mini-kit (Qiagen, Chatsworth, CA). Double-stranded cDNA was synthesized, and the cDNA was subjected to in vitro transcription in the presence of biotinylated nucleoside triphosphates. The biotinylated cRNA was hybridized with a probe array for 16 h at 45°C, and the hybridized biotinylated cRNA was stained with streptavidin-PE and scanned with a Hewlett–Packard Gene Array Scanner. The fluorescence intensity of each probe was quantified by using the GeneChip Analysis Suite 5.0 computer program (Affymetrix). The expression level of a single mRNA was determined as the average fluorescence intensity among the intensities obtained with 11 paired (perfect matched and single nucleotide-mismatched) probes consisting of 25-mer oligonucleotides. If the intensities of mismatched probes was very high, gene expression was judged to be absent (A), even if high average fluorescence was obtained with the GeneChip Analysis Suite 5.0 program. The

level of gene expression was determined with the GeneChip software as the average difference (AD). Specific AD levels were then calculated as percentages of the mean AD level of six probe sets for housekeeping genes (β-actin and GAPDH). Further data analysis was performed with the Genespring software version 5 (Silicon Genetics, San Carlos, CA). To normalize the staining intensity variations among chips, the AD values for all genes on a given chip were divided by the median of all measurements on that chip. To eliminate changes within the range of background noise and to select the most differentially expressed genes, data were used only if the raw data values were less than 100 AD and gene expression was judged to be present by the Affymetrix data analysis.

Hierarchical Clustering and Principal Component Analysis

To analyze the gene expression data in an unsupervised manner by gene chip array, we used agglomerative hierarchical clustering and principal component analysis (PCA) (http://lgsun.grc.nia.nih.gov/ANOVA/). The hierarchical clustering techniques classify data by similarity and there results are represented by dendrogram. PCA is a multivariate analysis technique which finds major pattern in data variability. Hierarchical clustering and PCA were performed to group mesenchymal cells obtained from bone marrow into subcategories. Expression data of 244 cell surface marker genes (Supplementary Table I), 34 fat-associated genes (Supplementary Table II), 36 cartilage-associated genes (Supplementary Table III) dotted onto the gene chip were used for analysis.

RESULTS

Pelleted Micromass Culture of KUM5 Cells

KUM5 cells, one of the cloned lines of cells, were found to exhibit chondrogenesis in vivo within 4 weeks after direct injection. This possible chondrogenic cell line was subcloned by the limiting dilution method to obtain a cell line capable of forming elastic, fibrous or hyaline cartilage. When cultured in monolayer, KUM5 cells had a fibroblast-like morphology, and their doubling time was approximately 29.7 h. After reaching confluence, the cells had larger nucleus and cytoplasm, and generated so-called "chondrogenic nodules." We performed the micromass culture of KUM5 cells in the GM or the CM, and continued the pelleted micromass culture for up to 10 weeks (Fig. 1A). The cells were equally embedded in the extracellular matrix, and the extracellular matrix of the KUM5 pellet culture did not show metachromasia with toluidine blue staining in the GM and the CM. Since transforming growth factor (TGF)-β and bone morphogeneic protein (BMP) are involved in chodrogenesis and osteogenesis [Fujii et al., 1999; Maeda et al., 2004], we used TGF-β3 and BMPs on KUM5 culture. Exposure of the cells to TGF-β3 augmented the metachromatic toluidine blue staining in the KUM5-micromass (Fig. 1A,B). BMP2 dramatically enhanced this TGF-β3-induced differentiation, that is, caused stronger metachromatic staining and enlarged metachromatic area. To determine the effect of other cytokines on the TGF-β3-induced chondrogenic differentiation, we added BMP4, BMP6, BMP7, PDGF, or hyaluronic acid to the CM supplemented with TGF-β3. BMP4, BMP6, and BMP7 enhanced the TGF-β3-induced chondrogenic differentiation in a manner similar to BMP2 (Fig. 1C,D). With exposure to BMP2, the number of the postmitotic daughter cells in the cell nest increased, matrix became more abundant, and hypertrophic chondrocytes became larger at higher magnification (Fig. 1E). In contrast, PDGF inhibited the TGF-β3 and BMP4-induced differentiation, as determined by toluidine blue staining (Fig. 1Ce,De). To confirm the chondrogenetic differentiation histologically, we examined the ultrastructural analysis of the cartilaginous micromasses. Extracellular matrix was abundantly deposited over KUM5 cells, or the surface of the generated micromass. The cells covering the micromass showed a flattened shape (Fig. 3A,B). The KUM5 chondrocytes inside the micromass showed an oval or round structure, had cellular processes, and were embedded in the hypertrophic chondrocytes. Abundant rough endoplasmic reticulum and a small number of mitochondria were observed in the KUM5 chondrocytes (Fig. 3C).

Gene Chip Analysis of the KUM5 and OP9 Chondroblasts

To clarify the specific gene expression profile of marrow stromal cells, we compared the expression levels of approximately 23,000 genes in the KUM5, 9-15c, KUSA-O, KUSA-A1, H-1/A, and OP9 cells [Umezawa et al., 1992; Nakano

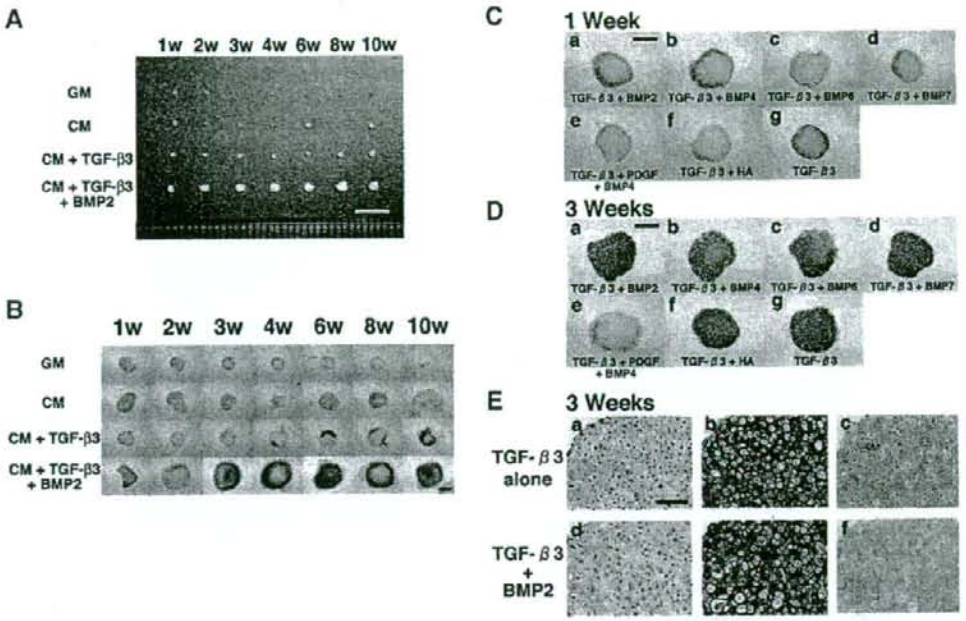

Fig. 1. In vitro chondrogenesis of KUM5 cells. **A,B:** Time-course analysis of growth factors-induced matrix production in KUM5 cells. Macroscopic view of KUM5 chondrogenic nodules which were generated after pellet culture for 1–10 weeks in the GM or the CM supplemented with or without growth factors as indicated (see "Cell culture" Section in Materials and Methods) (A) and Toluidine blue stained section (B). BMP2 drastically enhanced TGF-β3-induced matrix production of KUM5 cells. **C,D:** Toluidine blue stained section of KUM5 chondrogenic nodules in the pellet culture exposed to growth factors as indicated for 1 week (C) or 3 weeks (D). **E:** Higher magnification of KUM5 chondrogenic pellet exposed to TGF-β3 (**a–c**), or TGF-β3 and BMP2 (**d–f**) for 3 weeks. **a,d:** Hematoxylin and Eosin stain; **b,e:** Toluidine blue stain; **c,f:** Alcian blue stain. Scale bars: 5 mm (A), 500 μm (B, C, D), 100 μm (E).

et al., 1994]. (http://1954.jukuin.keio.ac.jp/umezawa/chip/sugiki) by using the Affymetrix gene chip oligonucleotide arrays (Table I). RNAs were isolated from cell lines cultured in the GM without any induction of differentiation to perform the gene chip analysis. Of the 23,000 genes represented on the gene chip, chondrocyte-specific- or associated-genes such as type II collagen α1, Sox9, and cartilage oligomeric matrix protein were more strongly expressed in KUM5 cells than in other marrow-derived mesenchymal cells. Surprisingly, OP9 cells [Nakano, 1996] also expressed these chondrocyte-specific or -associated genes at higher levels: the type II collagen α1, and cartilage oligomeric matrix protein genes were expressed in OP9 cells at more than tenfold higher levels than in 9–15c mesenchymal stem cells, KUSA-O osteo-adipogenic progenitor cells, H-1/A pre-adipocytes, or even KUM5 chondroblasts. These results implied that KUM5 and OP9 cells have increased chondrogenic potential.

Pelleted Micromass Culture of OP9 Cells

We performed the pellet culture of OP9 cells in the GM and continued the culture for up to 10 weeks (Fig. 2A). The cells were equally embedded in the extracellular matrix and the extracellular matrix of the OP9 pellet culture did not show metachromasie with the toluidine blue stain in the GM (data not shown). With exposure to TGF-β3, the cells in the peripheral zone generated cartilage and exhibited adipocyte-like morphology in the center (Fig. 2Bg,Cg). Next, we investigated the effect of BMP2 in the pellet culture of OP9 cells. The CM with TGF-β3 and BMP2 dramatically induced the chondrogenic differentiation (Fig. 2A,Ba,Ca), that is, the pellet cells produced abundant extracellular matrix (Fig. 2D) and caused deeper metachromatic staining and an enlarged metachromatic area (Fig. 2Db). Additionally, we examined the effect of other cytokines on the differentiation of OP9 cells

TABLE I. Cartilage-Associated Genes Expressed in KUM5 and OP9 Cells in Comparison With Other Marrow Stromal Cells

Probe set	Genbank	Description	9–15c		KUSA-O		KUSA-A1		H-1/A		OP9		KUM5		Symbol
			Flags	Raw	Flags	Raw	Flags	Raw	Flags	Raw	Flags	Raw	Flags	Raw	
1450567_a_at	NM_031163	Procollagen, type II, alpha 1	A	28	A	187	A	98	A	46	P	1,7390	P	679	Col2a1
1428571_at	AK004383	Procollagen, type IX, alpha 1	P	85	P	116	P	99	P	57	P	132	P	190	Col9a1
1422253_at	NM_009925	Procollagen, type X, alpha 1	A	13	A	20	A	15	A	104	A	218	A	270	Col10a1
1418599_at	BB836814	Procollagen, type XI, alpha 1	A	69	P	682	P	4,284	P	5,009	P	2,561	P	518	Col11a1
1419527_at	NM_016685	Cartilage oligomeric matrix protein	A	120	A	111	A	64	A	167	P	1,892	M	172	Comp
1449368_at	NM_007833	Decorin	A	176	A	36	A	223	A	226	A	85	A	110	Dcn
1416405_at	BC019502	Biglycan	P	12,600	P	11,817	P	11,011	P	12,932	P	21,954	P	18,640	Bgn
1449827_at	NM_007424	Aggrecan 1	A	70	A	118	A	105	A	127	A	94	A	167	Agc1
1416321_s_at	BC019775	Proline arginine-rich end leucine-rich repeat	P	196	A	59	P	899	P	1,092	P	2,169	P	362	Prelp
1415939_at	NM_021355	Fibromodulin	P	388	M	359	P	11,542	P	16,626	A	108	A	320	Fmod
1418745_at	NM_012050	Osteomodulin	P	288	A	50	P	1,849	P	2,185	P	347	P	743	Omd
1415943_at	BC010560	Syndecan 1	P	1,182	P	2,449	P	1,358	P	1,607	P	4,704	P	1,799	Sdc1
1417012_at	AI266824	Syndecan 2	P	752	P	1,256	P	2,940	P	4,398	P	605	P	2,039	Sdc2
1420853_at	NM_011520	Syndecan 3	A	382	A	547	P	680	P	902	A	385	P	762	Sdc3
1417654_at	NM_011521	Syndecan 4	P	306	P	281	P	244	P	342	P	305	P	320	Sdc4
1424950_at	BI077717	SRY-box containing gene 9	P	120	A	5	A	59	A	27	P	1,344	P	183	Sox9
1420895_at	BM248342	Transforming growth factor, beta receptor I	P	780	P	703	P	657	P	862	P	1,595	P	802	Tgfbr1
1425444_a_at	S69114	Transforming growth factor, beta receptor II	P	552	P	746	P	1,068	P	1,189	P	868	P	1,133	Tgfbr2
1425620_at	AF039601	Transforming growth factor, beta receptor III	P	448	A	328	A	275	P	313	P	855	P	1,015	Tgfbr3
1422912_at	NM_007554	Bone morphogenetic protein 4	P	1,048	P	646	P	6,470	P	7,266	P	1,736	P	2,890	Bmp4
1425492_at	BM248248	Bone morphogenetic protein receptor, type 1A	P	1,486	P	815	P	1,089	P	1,164	P	1,189	P	1,123	Bmpr1a
1420847_a_at	NM_010207	Fibroblast growth factor receptor 2	P	833	P	656	P	1,664	P	1,998	P	992	P	3,598	Fgfr2
1417271_a_at	NM_007932	Endoglin	A	247	A	187	A	40	A	115	A	222	P	1,371	Eng
1451314_a_at	L08431	Vascular cell adhesion molecule 1	P	462	A	39	A	28	A	92	P	812	P	583	Vcam1

The raw data from the gene chip analysis are available at our laboratory's web site (http://1954.jukuin.keio.ac.jp/umezawa/chip/sugiki). Flag indicates the presence or absence of gene expression determined by presence/absence call (Affymetrix).
P (presence): gene is expressed. M (marginal): gene is marginally expressed. A (absence): gene is not expressed.

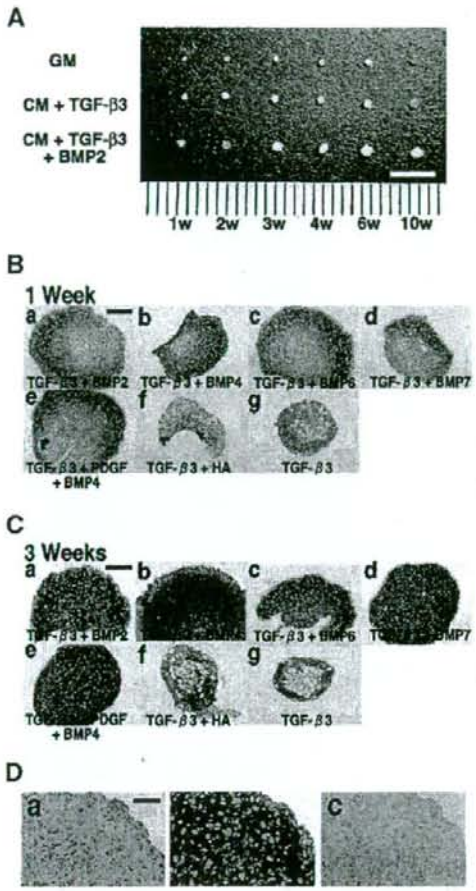

Fig. 2. In vitro chondrogenesis of OP9 cells. **A**: Time-course analysis of growth factors-induced matrix production in OP9 cells. Macroscopic view of OP9 chondrogenic nodules which were generated after pellet culture for 1–10 weeks in the GM or the CM supplemented with growth factors as indicated. BMP2 drastically enhanced TGF-β3-induced matrix production of OP9 cells. **B,C**: Microscopic view of OP9 chondrogenic nodules in the pellet culture exposed to growth factors as indicated for 1 week (B) or 3 weeks (C). **D**: OP9 chondrogenic pellet exposed to TGF-β3 and BMP2 for 3 weeks. **a**: Hematoxylin and Eosin stain; **b**: Toluidine blue stain; **c**: Alcian blue stain. Scale bars: 5 mm (A), 200 μm (B,C), 100 μm (D).

with procedures analogous to those used for KUM5 cells. BMP4, BMP6, and BMP7 enhanced the TGF-β3-induced differentiation in a manner similar to BMP2 (Fig. 2B,C). Unlike its effect in KUM5 cells, PDGF did not inhibit TGF-β3- and BMP4-induced differentiation, as determined by toluidine blue staining (Fig. 2Be,Ce). To confirm the chondrogenetic differentiation histologically, we examined the ultrastructural analysis of the cartilaginous micromasses. Extracellular matrix was abundantly deposited over OP9 cells, or the surface of the generated micromass (Fig. 3D). The cells covering the micromass showed a flattened shape (Fig. 3E). The OP9 chondrocytes inside the micromass showed an oval or round structure, had cellular processes, and were embedded in the hypertrophic chondrocytes. Abundant rough endoplasmic reticulum and a small number of mitochondria were observed in the OP9 chondrocytes (Fig. 3F).

Cell Surface Markers in KUM5 and OP9 Cells

To characterize the KUM5 and OP9 cells, we analyzed the cell surface markers by using flowcytometry. KUM5 cells were positive (more than tenfold compared to the isotype control) for CD9, CD105 (endoglin), Sca-1 and Ly-6C, marginal for CD106 (VCAM-1) and CD140a (PDGFRα), and negative for c-kit (CD117), Flk-1, CD31 (PECAM-1), CD34, CD144 (VE-cadherin), CD45 (leukocyte common antigen), CD49d (integrin α4), CD90 (Thy-1), CD102, CD14, Ly-6G, and CD41 (Fig. 4A). OP9 cells were strongly positive for CD140a, CD106, and CD9, weakly positive for Sca-1, and negative for CD105, c-kit, Flk-1, CD31, CD34, CD144, CD45, CD49d, CD90, CD102, CD14, Ly-6C, Ly-6G, and CD41 (Fig. 4B). Next, we performed hierarchical clustering by analyzing the global gene expression pattern for cell type classification and cell function prediction. When 244 cell surface marker genes are used for analysis, KUM5 and OP9 formed one cluster independent of seven other marrow stromal cells (Fig. 4C, Supplementary Table I, http://1954.jukuin.keio.ac.jp/umezawa/sugiki/pca). We then performed PCA to determine whether it is possible to discriminate OP9 and KUM5 from other cells in three-dimensional expression space. Using the same gene sets for clustering analysis, KUM5 and OP9 cells can clearly separated from the other seven cell lines (Fig. 4D). The similarity of the in vitro phenotype of KUM5 and OP9 cells was supported by the results of grouping the marrow stromal cells into subcategories in terms of cell surface markers.

Global Outlook by Hierarchical Clustering and PCA by Fat- and Cartilage-Associated Genes

We also performed hierarchical clustering and PCA on the expression pattern of fat- and

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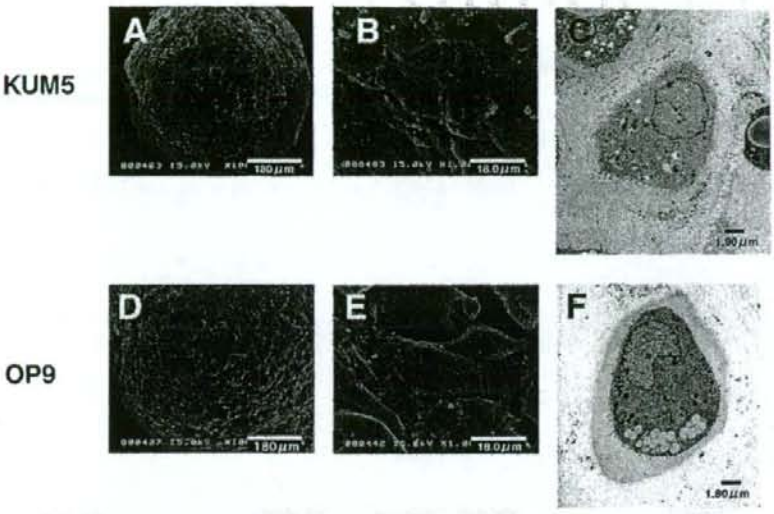

Fig. 3. Ultrastructural analysis of the in vitro chondrogenic micromass. Micromasses of KUM5 cells (A–C) and OP9 cells (D–F) were generated by culturing in the CM supplemented with TGF-β3 for 3 weeks. (A,B,D,E), SEM; (C,F), TEM.

cartilage-associated genes. Using 34 fat-associated genes (Supplementary Table II), KUM5 and OP9 were separated and show smaller distance by both hierarchical clustering and PCA, implying that the KUM5 and OP9 cells have similar characteristics compared with other seven marrow stromal cells (Fig. 5A–D). In contrast, the analysis of 36 cartilage-associated gene expression data (Fig. 5E, Supplementary Table III) demonstrated that these two cell lines were not grouped into the same subcategory. Both cells showed "P: positive" expression in sox9 and α1(II) procollagen genes, and OP9 cells expressed cartilage-specific and -associated genes such as the α1(II) procollagen, α1(XI) procollagen, cartilage oligomeric matrix proteins, and proline arginine-rich end leucine-rich repeat genes at higher levels, when compared to KUM5 cells (Table I). These results imply that OP9 cells are differentiated chondrocytes as a default state while KUM5 cells are oligopotent mesenchymal cells that have a tendency to differentiate into chondrocytes.

In Vivo Chondrogenesis

To examine the chondrogenic activity of KUM5 cells, we injected KUM5 cells at confluence without any treatment (i.e., without TGF-β3 and BMP2 treatment) into mice subcutaneously (Fig. 6A). KUM5 cells generated cartilage-like structures within 1 week and complete cartilage at 3 weeks, and the generated cartilage exhibited metachromasia with toluidine blue staining. Interestingly, the cartilage generated by KUM5 cells showed enchondral ossification at 4 weeks. We then implanted the KUM5 chondrogenic micromass after pellet culture into the subcutaneous tissue just beneath the cutaneous muscle (Fig. 6B). The KUM5 cartilage was formed within 1 week and it exhibited typical chondrogenic structures: post-mitotic daughter cells in the cell nest, hypertrophic chondrocytes, and abundant metachromatic matrix with toluidine blue staining. The immunohistochemical analysis showed that KUM5 cartilage stained positive for chondrocyte-specific type II collagen (Fig. 6C), while only a slight amount of type II collagen was detected in the in vitro pelleted micromass culture. Ultrastructural analysis revealed that KUM5 chondrocytes implanted into the subcutaneous tissue of nude mice were embedded in the lacunae cavities and had abundant endoplasmic reticulum and a small number of mitochondria (Fig. 6D), and collagen fibers were produced around the lacunae cavity of the KUM5 chondrocytes, as is the case of the in vitro conditions (Fig. 6E).

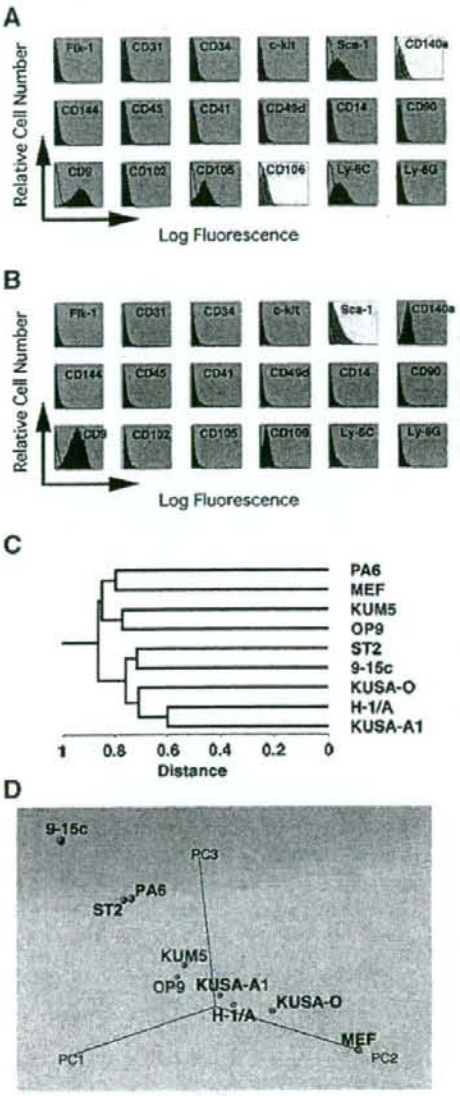

Fig. 4. Expression profiling, hierarchical clustering, and principal component analysis (PCA) of cell surface markers in marrow stromal cells. **A**,**B**: Flow cytometric analysis of cell surface markers in KUM5 cells (A) and OP9 cells (B). Red and pink colors indicate positive and marginal expression, respectively, and blue color indicates negative expression. **C**: Dendrogram revealing clustering profile of nine marrow stromal cells using 244 surface marker genes (Supplementary Table I). **D**: The rotated and dimensionally reduced gene expression data. Nine marrow stromal cells are plotted onto the 1st, 2nd, and 3rd principal component using 244 surface marker genes. These results indicate that KUM5 and OP9 cells were grouped into the same subcategory.

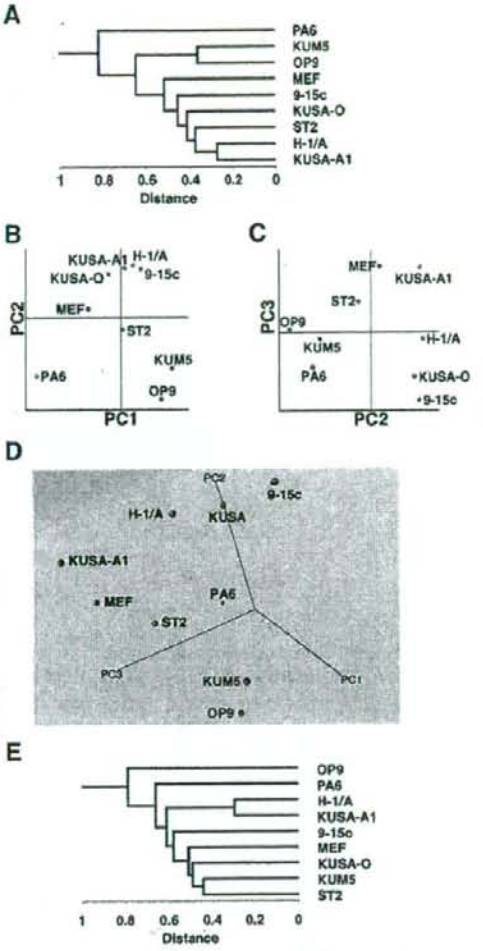

Fig. 5. Hierarchical clustering and PCA of fat- and cartilage-associated gene expression in marrow stromal cells. **A**. Dendrogram revealing clustering profile of 9 marrow stromal cells using 34 fat-associated genes (Supplementary Table II). **B**–**D**. PCA on expression levels of 34 fat-associated genes. The gene expression data from 9 marrow stromal cells were analyzed. Nine marrow stromal cells are plotted onto 2D-representation, PC1 and PC2 axes (B) or PC2 and PC3 axes (C), and 3D-representation (D). These results indicate that KUM5 and OP9 cells were grouped into the same subcategory. **E**. Dendrogram revealing clustering profile of 9 marrow stromal cells using 36 cartilage-associated genes (Supplementary Table III).

To determine the chondrogenic activity of OP9 cells in vivo, we directly injected them into the subcutaneous tissue. The OP9 cells without any induction did not generate cartilage. We then implanted the OP9 chondrogenic