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

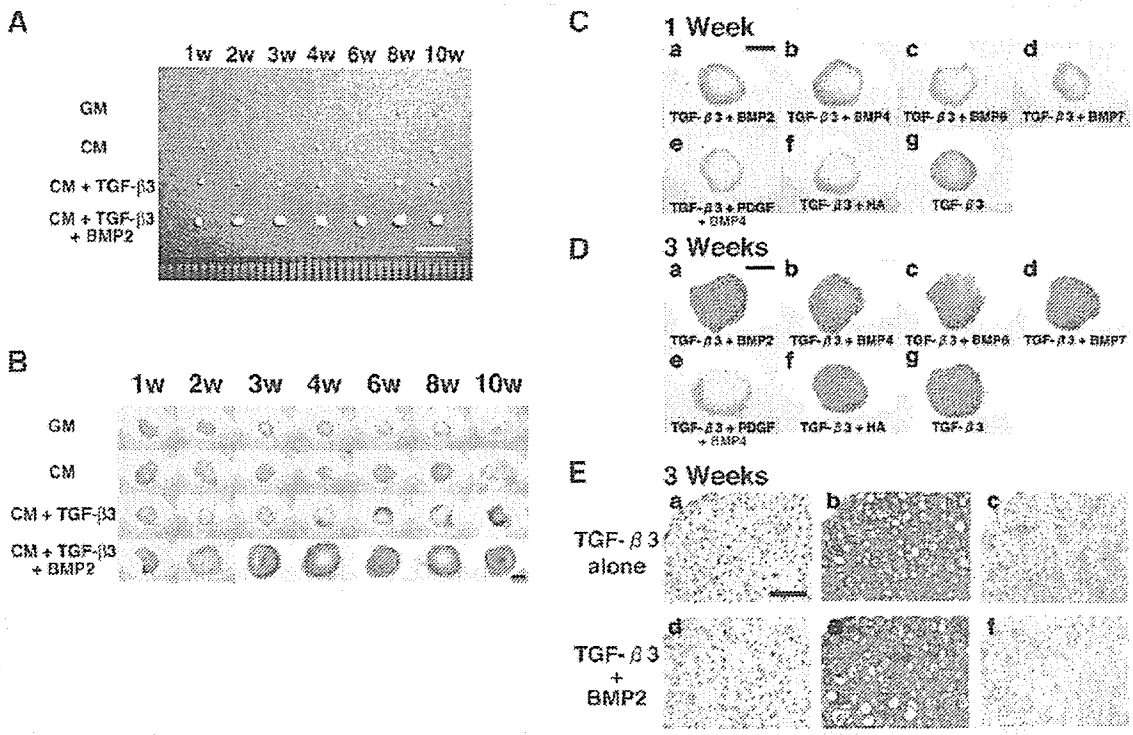

Fig. 1. In vitro chondrogenesis of KUM5 cells. A,B: Time-course analysis of growth factors-induced matrix production in KUM5 cells. Macroscopic view of KUM5 chondrogenic nodules which were generated after pellet culture for 1–10 weeks in the GM or the CM supplemented with or without growth factors as 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).

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

			9–15c		KUSA-O		KUSA-A1		H-1/A		OP9		KUM5		
Probe set	Genbank	Description	Flags	Raw	Flags	Raw	Flags	Raw	Flags	Raw	Flags	Raw	Flags	Raw	Symbol
1450567_a_at	NM_031163	Procollagen, type II, alpha 1	A	28	A	187	A	98	A	46	P	1,7390	P	879	Col2a1
1426571_at	AK004363	Procollagen, type IX, alpha 1	P	85	P	116	P	99	P	57	P	132	P	190	Col9a1
1422252_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	89	P	682	P	4,264	P	5,009	P	2,551	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
1448827_at	NM_007424	Aggrecan 1	A	70	A	116	A	105	A	127	A	94	A	187	Agc1
1418321_s_at	BC019775	Proline arginine-rich end leucine-rich repeat	P	196	A	59	P	899	P	1,092	P	3,169	P	362	Prelp
1415939_at	NM_021355	Fibromodulin	P	368	M	359	P	11,542	P	16,626	A	106	A	320	Fmod
1418745_at	NM_012050	Osteomodulin	P	268	A	50	P	1,849	P	2,186	P	347	P	743	Omd
1415943_at	BC010580	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	4,298	P	605	P	2,039	Sdc2
1420853_at	NM_011520	Syndecan 3	A	382	A	547	P	680	P	902	A	335	P	762	Sdc3
1417654_at	NM_011521	Syndecan 4	P	306	P	281	P	244	P	342	P	305	P	320	Sdc4
1424960_at	BI077717	SRY-box containing gene 9	P	120	A	5	A	59	A	27	P	1,344	P	183	Sox9
1420895_at	BM246342	Transforming growth factor, beta receptor I	P	760	P	703	P	657	P	962	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	7,286	P	1,736	P	2,890	Bmp4
1425492_at	BM246246	Bone morphogenetic protein receptor, type 1A	P	1,486	P	815	P	1,059	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	932	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	883	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.

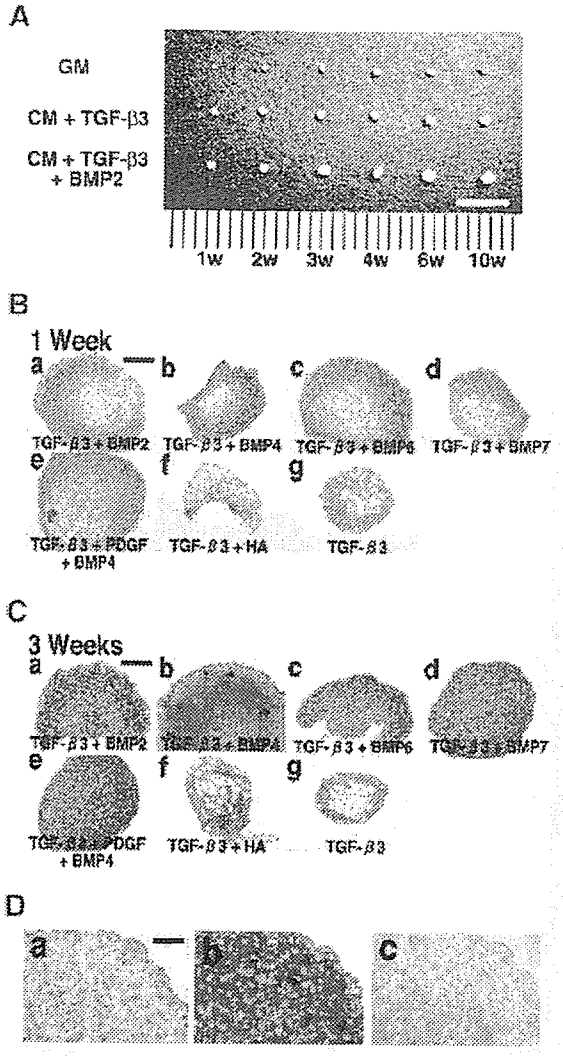

Fig. 2. In vitro chondrogenesis of OP9 cells. A: Time-course analysis of growth factors-induced matrix production in OP9 cells. Macroscopic view of OP9 chondrogenic nodules which were generated after pellet culture for 1–10 weeks in the GM or the CM supplemented with growth factors as indicated. BMP2 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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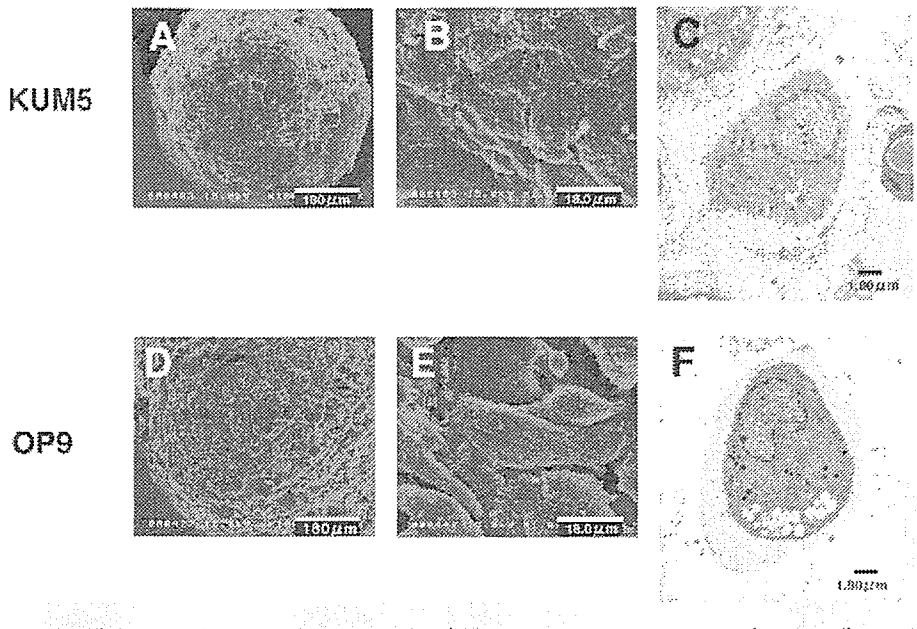

Fig. 3. Ultrastructural analysis of the in vitro chondrogenic micromass. Micromasses of KUM5 cells (A–C) and OP9 cells (D–F) were generated by culturing in the CM supplemented with TGF-β3 for 3 weeks. (A,B,D,E), SEM; (C,F), TEM.

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).

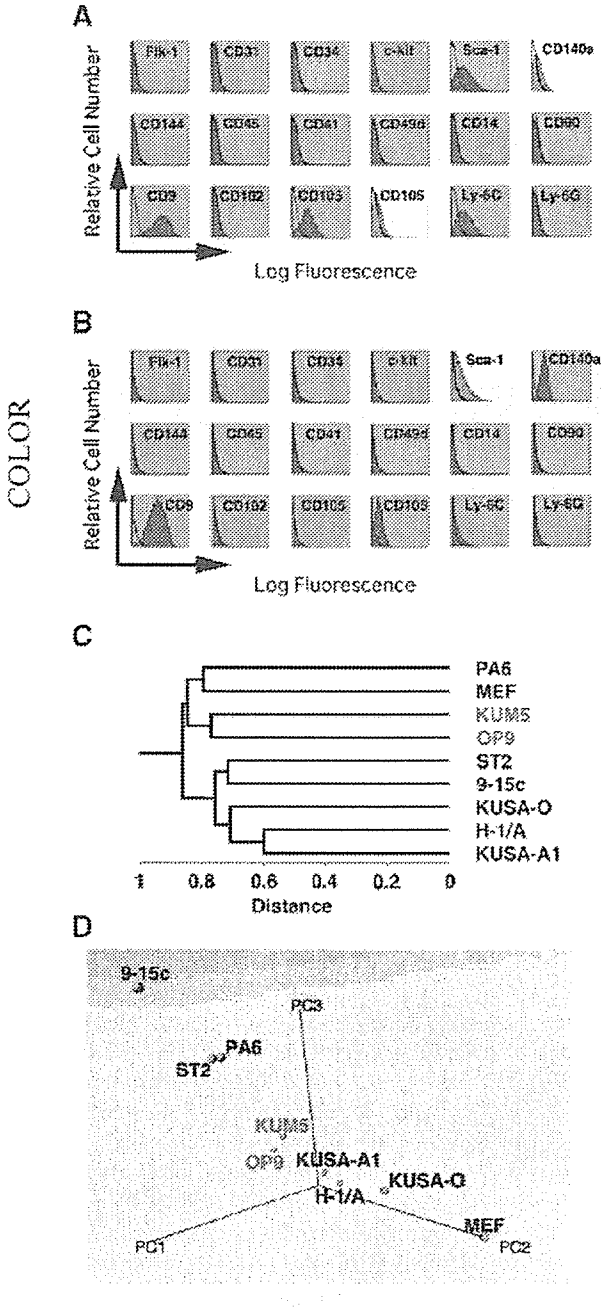

Fig. 4. Expression profiling, hierarchical clustering, and principal component analysis (PCA) of cell surface markers in marrow stromal cells. A,B: Flow cytometric analysis of cell surface markers in KUM5 cells (A) and OP9 cells (B). Red and pink colors indicate positive and marginal expression, respectively, 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.

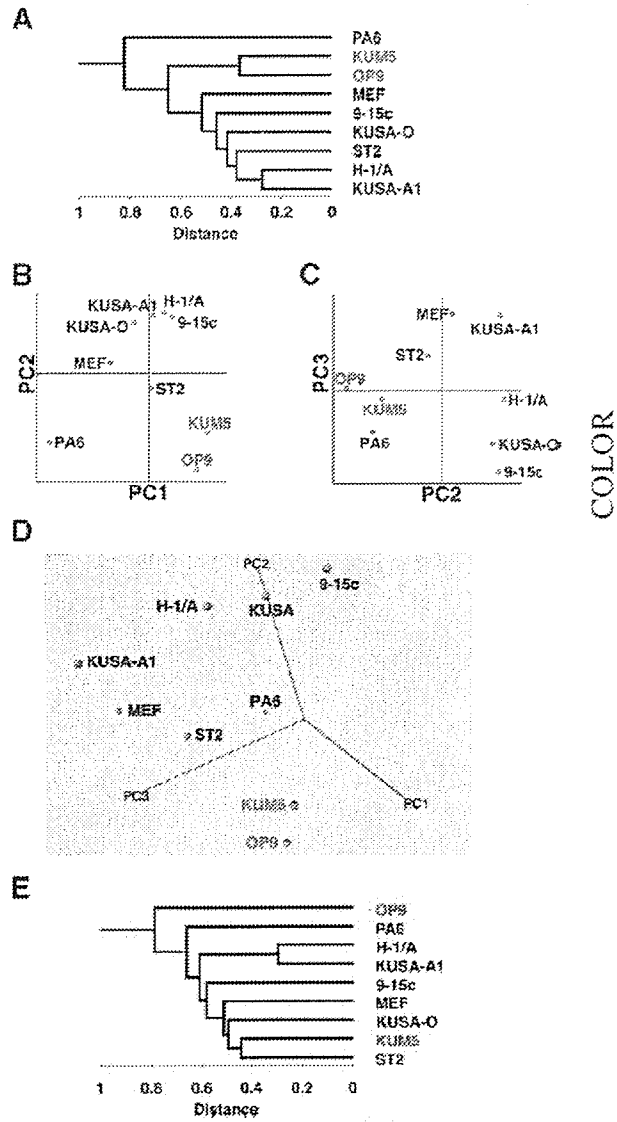

Fig. 5. Hierarchical clustering and PCA of fat- and cartilage-associated gene expression in marrow stromal cells. A. Dendrogram revealing clustering profile of 9 marrow stromal cells using 34 fat-associated genes (Supplementary Table II). B–D. PCA on expression levels of 34 fat-associated genes. The 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

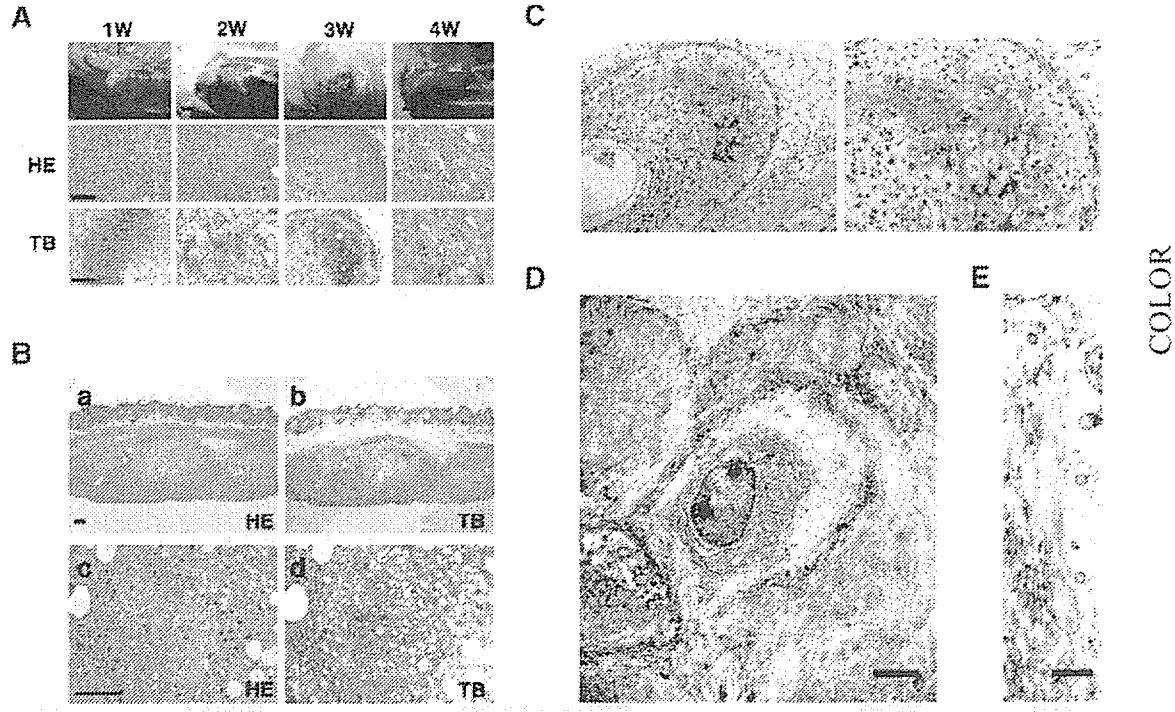

Fig. 6. In vivo chondrogenesis of KUM5 cells. A: Macroscopic view (top), hematoxylin and eosin stain (HE) (middle) and toluidine blue stain (TB) (bottom) analysis at 1, 2, 3, and 4 week (w)-cultivation in vivo after direct injection of KUM5 cells. B: KUM5 chondrogenic nodules, that were generated after pellet culture for 7 days in the CM supplemented with TGF-β3 and BMP2, were implanted just beneath the cutaneous muscle in the subcutaneous tissue and were cultivated in vivo for 3 weeks. Panels c and d are higher magnifications of a and b, respectively. C: Expression of chondrocyte-specific collagen type II. The KUM5 chondrogenic nodules were sectioned after 2 week-in vivo cultivation and stained with collagen type II-specific antibody. D,E: Ultrastructural analysis (TEM) of KUM5 implants. KUM5 cells were implanted into the subcutaneous tissue of Balb/c nu/nu mice, and the generated cartilage was resected 2 weeks after implantation. Scale bars: 2 mm (A, top row), 100 μm (A, middle and bottom row), 100 μm (B), 2 μm (D), 1 μm (E).

COLOR

micromass after the pellet culture into the subcutaneous tissue just beneath the cutaneous muscle (Fig. 7A,B). The OP9 cartilage was formed at 2 and 4 weeks, and abundant metachromatic matrix was observed with the toluidine blue stain. The immunohistochemical analysis shows that OP9 cartilage stains positive for the chondrocyte-specific type II collagen (Fig. 7C).

Sorting of Chondroblasts by Chondrocyte-Specific Cis-Regulatory Element of the Collagen α2(XI) Gene

Although the KUM5 cells used in this study were derived from a single-cell origin or clone, it could be argued that both cells responsive and non-responsive to chondrogenic induction were present [Ko et al., 1990]. In this sense, KUM5 cells might have been a largely heterogeneous cell population. Even cells derived from a single clone have been shown to be heterogeneous in terms of differentiation capacity and stages [Muraglia et al., 2000]. To validate the chondrogenic differentiation observed here, a homogenous population of committed cell obtained after induction should be isolated. Therefore, for the purpose of sorting chondrogenically committed cells, we transfected KUM5 cells with a Venus-expression vector under the control of the Col α2(XI) promoter, analyzed the transfected cells, and collected Venus-positive cells (Fig. 8A–D). The sorted cells were assessed for in vitro (Fig. 9A–F) and in vivo chondrogenesis (Fig. 9G–I). The cells again showed metachromatic chondrogenic micromasses with toluidine blue staining in vitro (Fig. 9B). Direct injection of the cells resulted in the cartilage formation within 1 week and obvious enchondral ossification at the periphery of the cartilage at 4 weeks (Fig. 9G). Again, ultrastructural analysis revealed that KUM5 chondrocytes implanted into the subcutaneous

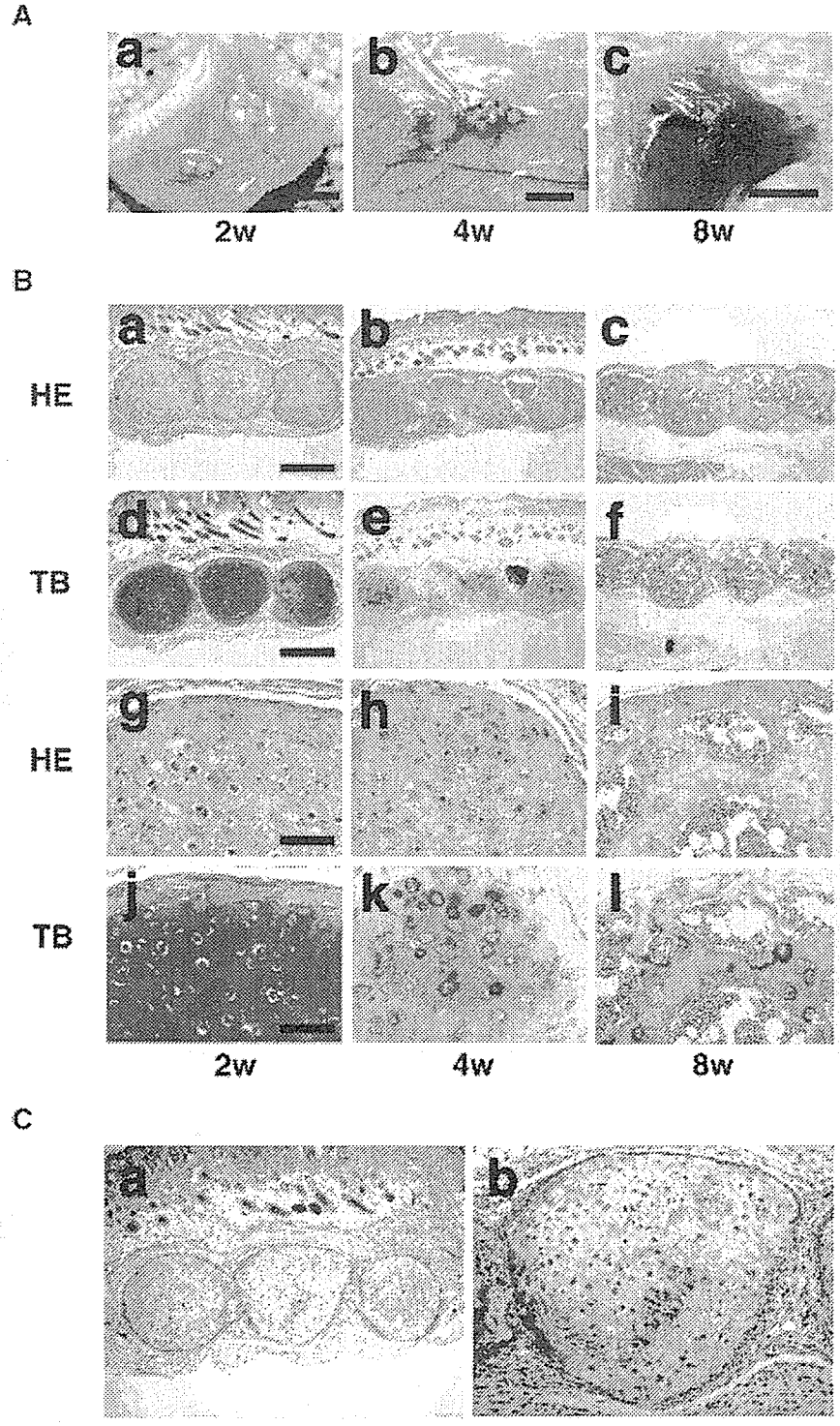

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Fig. 7. In vivo chondrogenesis of OP9 cells. In vivo chondrogenesis was examined by implantation of OP9 chondrogenic nodules. OP9 chondrogenic nodules, which were generated after pellet culture for 7 days in the CM supplemented with TGF-β3 and BMP2, were implanted just beneath the cutaneous muscle in the subcutaneous tissue and were cultivated in vivo for the number of weeks indicated. A: Macroscopic view of OP9 cartilage after 2 (a), 4 (b), and 8 (c)-week-in vivo cultivation. B: Histological analysis of OP9 cartilage after 2 (a,d,g,j), 4 (b,e,h,k), and 8 (c,f,i,l)-week-in vivo cultivation. (a,b,c,g,h,i). HE stain; (d,e,f,j,k,l), TB stain. Panels g–l are higher magnifications of a–f, respectively. C: Immunohistochemical analysis of the in vivo OP9 chondrogenic nodules. The OP9 chondrogenic nodules after 2-week-in vivo cultivation stained positive for collagen type II. Scale bars: 2 mm (A), 500 μm (Ba–f), 100 μm (Bg–l).

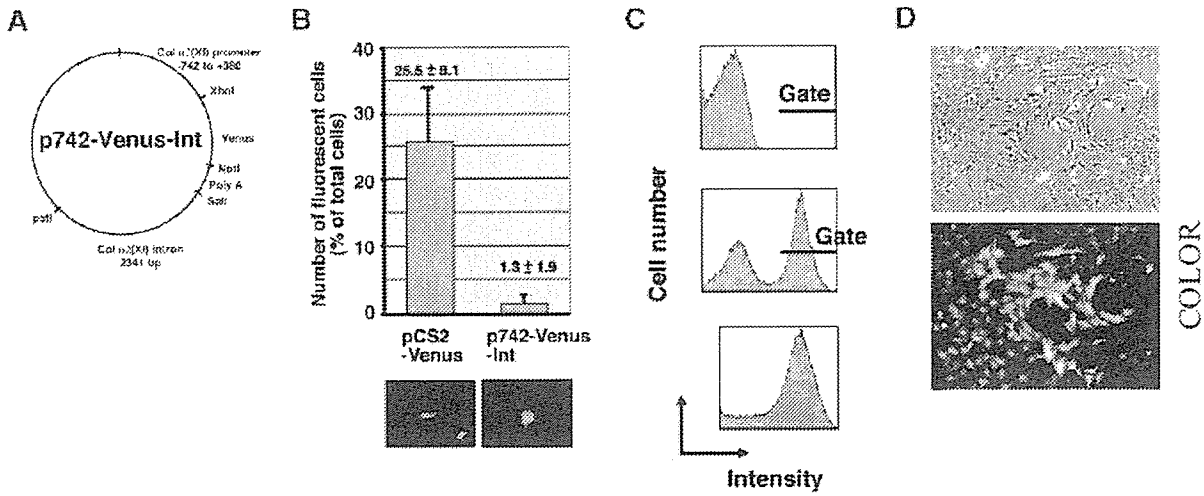

Fig. 8. Isolation of KUM5 chondroblasts using the chondroblast-specific cis-regulatory element. A: The p742-Venus-Int plasmid containing the fluorescent Venus gene driven by the cis-regulatory elements of the α2(XI) collagen gene. B: The number of fluorescent KUM5 cells (upper) after transfection with the p742-Venus-Int plasmid or pCS2-Venus containing the Venus gene driven by the CMV-promoter. Fluorescent photomicrograph of KUM5 cells after the first sorting (lower). C: Flowcytometric analysis of KUM5 cells after transfection with the p742-Venus-Int plasmid (top); The fluorescence-positive cells were sorted, propagated, and analyzed (middle). Again, the propagated fluorescence-positive cells were sorted, propagated, and analyzed (bottom). The "gate" for sorting is shown by the horizontal bar in the upper and middle panels. More than 80% of cells became positive after the final sorting. D: Phase contrast micrograph (upper) and fluorescent photomicrograph (lower) of the finally sorted cells (the lower panel of C).

tissue of nude mice were embedded in the hypertrophic chondrocytes and had abundant endoplasmic reticulum and a small number of mitochondria (Fig. 9H,I). The post-mitotic daughter cells in the cell nest, which are often observed in cartilage, were also detected (Fig. 9I).

DISCUSSION

In this study, we focus on the chondrogenic differentiation in vitro and in vivo using the two cell lines, KUM5 and OP9. The chondrogenic process is determined by the sequential expression of matrix component, and the differential response of differentiating cells to the growth factors may be attributed to the differentiating stages that depend on the expression patterns of the gene set as is the case for hematopoietic cells. The process of the chondrogenic differentiation is influenced by a number of growth factors including TGF-β and/or BMPs. Three isoforms of TGF-β have been known to have the ability to induce the chondrogenic differentiation. Both TGF-β2 and -β3 are more effective than TGF-β1 in promoting chondrogenesis, and TGF-β3 accelerates production of cartilaginous extracellular matrix in differentiating mesenchymal stem cells [Barry et al., 2001].

This study was undertaken to obtain mesenchymal stem cells with chondrogenic potential that retain critical in vivo cell functions, as do mammary gland epithelial cells, skin keratinocytes, and pigmented epithelial cells. To achieve this, we attempted to identify marrow-derived cells with chondrogenic nature and immortality without transformation among the cells obtained by the limiting-dilution method [Umezawa et al., 1992], defining "immortality" simply as indefinite cell division.

OP9 cells are known to serve as a niche or a specific microenvironment for the regulation of self-renewal and differentiation of stem cells [Nakano, 1996], and the question is raised of whether marrow stromal cells or marrow-derived mesenchymal cells with chondrogenic potential are capable of constituting a microenvironment for stem cells. It is inconceivable that cartilage can form a niche for cells in the living body based on structural and morphological considerations; however, a cell with chondrogenic or adipo-chondrogenic potential may serve as a niche not only in the case of OP9 cells but also as a general concept, at least in vitro.

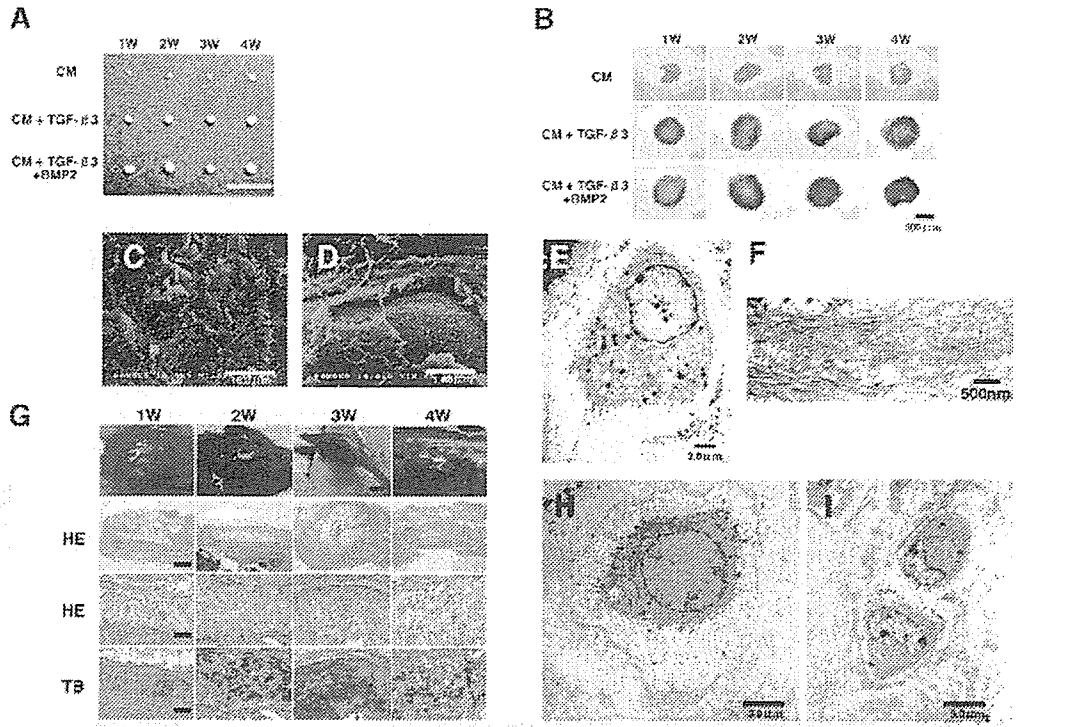

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Fig. 9. In vitro and in vivo chondrogenesis of KUM5 cells sorted according to the activity of the chondrocyte-specific cis-regulatory element. A,B: Macroscopic view of the chondrogenic nodules which were generated after pellet culture of the finally sorted KUM5 cells for 1–4 weeks in the CM supplemented with growth factors as indicated (A) and toluidine blue stained section (B). C–F: Ultrastructural analysis of the micromasses of KUM5 cells sorted according to the activity of the Col α2(XI) cis-regulatory element (KUM5-Venus) after culturing in the CM supplemented with TGF-β3 for 3 weeks. (C,D), SEM; (E,F), TEM. G: In vivo chondrogenesis was examined 1–4 weeks after direct injection of the finally sorted KUM5 cells. From top to bottom: Macroscopic view, scale bars: 2 mm; histological analysis, scale bar: 600 μm, HE stain; histological analysis, scale bar: 120 μm, HE stain; histological analysis, scale bar: 120 μm, TB stain. H,I: Ultrastructural analysis (TEM) of the sorted KUM5 cartilage. The sorted KUM5 cells were implanted into the subcutaneous tissue of Balb/c nu/nu mice, and the generated cartilage was resected 2 weeks after implantation. Scale bars: 5 mm (A), 500 μm (B), 2 mm (G, **top row**), 500 μm (G, **2nd row**), 100 μm (G, **3rd** and **bottom row**).

The sequence of enchondral or perichondral ossification by KUM5 and OP9 cells was as follows: deposition of homogeneous matrix surrounding the small nests of the injected cells that subsequently became positive for type II collagen and exhibited metachromasia with toluidine blue staining, trapping them in the secreted homogeneous matrix, and the appearance of small nests of isogenous chondrocytes that probably resulted from repeated cell division. At a later stage, that is, 4–8 weeks after injection, the peripheral region of the generated cartilage became ossified. Importantly, the chondrogenesis by KUM5 and OP9 cells was irreversible and reproducible, and the implanted cells never transformed into malignant cells, formed any abnormal extracellular matrices, or induced any significant inflammatory reactions. It is again noteworthy that the osteogenesis by these two different lines of cells was mediated by chondrogenesis, and it was therefore considered to be chondral ossification. Thus, the unique characteristics of these two cell lines provide an opportunity to analyze the process of enchondral or perichondral ossification in an experimental system in detail.

In fetal life, primary ossification centers form by one of two processes: enchondral ossification or membranous ossification. Enchondral ossification refers to bony replacement of cartilage and is the mode of formation of the long bones. During membranous ossification mesenchymal cells form membranes within which ossification occurs and this is the mode of formation of the scapula and skull and, in part, of the clavicle and pelvis. After birth, bone growth continues by both enchondral and membranous ossification. Further enchondral ossification occurs in

the physes and results in continuous longitudinal growth of the long bones until skeletal maturity. KUM5 and OP9 cells were obtained from long bone and calvaria, respectively, and showed enchondral ossification. We have also reported that KUSA-A1 cells form bone by membranous ossification in vivo, and thus we have three different types of cells showing distinctive in vivo characteristics. The process of chondrogenesis or enchondral ossification may also serve as a model for chondromatosis and osteochondromatosis in a joint cavity.

The expression pattern of chondrocyte-specific genes in OP9 and KUM5 cells is different from that in ATDC5 cells, which are a mouse embryonal carcinoma-derived chondrogenic cell line. ATDC5 cells exhibit a multistep differentiation process encompassing the stages from chondrogenesis to enchondral ossification [Shukunami et al., 1996]. Early-phase differentiation is characterized by the expression of type II collagen, followed by induction of the aggrecan gene. Late stage differentiation is characterized by the start of expression of short-chain collagen type X genes. By contrast, marrow-derived mesenchymal stem cells express the aggrecan genes at an early stage and then type II collagen during chondrogenic differentiation [Pittenger et al., 1999]. Surprisingly, gene expression pattern determined by the gene chip analysis was consistent with protein levels of cell surface molecules; this consistency indicates that the expression profiling is valid. Expression of "structural proteins" on Gene Ontology, including the extracellular matrix, was much higher by OP9 and KUM5 cells than by non-chondrogenic cells such as KUSA-A1 osteoblasts, H-1/A preadipocytes, and 9-15c mesenchymal stem cells, implying that the OP9 and KUM5 cells are mainly engaged in synthesizing extracellular matrix.

Can we inhibit enchondral or perichondral ossification after the completion of chondrogenesis? This is a challenge for the future, probably the not-too-distant future. We could not prevent the generated hyaline cartilage from ossifying at present even after selection based on the chondrocyte-specific cis-regulatory element of the collagen α2(XI) gene, probably due to the inability to inhibit vasculogenesis from the neighboring connective tissue. However, these established murine marrow-derived mesenchymal cells with in vivo chondrogenic activity and expression profiles provide a powerful model for studies of chondrogenic differentiation and our further understanding of cartilage regeneration. Bone marrow-derived chondroblasts with chondrogenic potential are useful candidate cell sources in addition to dedifferentiated chondrocytes obtained from cartilage for transplantation in osteoarthritis and rheumatoid arthritis.

ACKNOWLEDGMENTS

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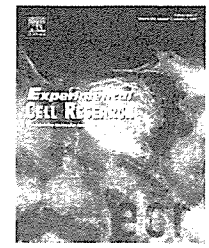

Research Article

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

Yoji Yamada[a], *Kazuhiro Sakurada*[a,1], *Yukiji Takeda*[b,2], *Satoshi Gojo*[b,3], *Akihiro Umezawa*[b,*]

[a]*BioFrontier Laboratories, Kyowa Hakko Kogyo Co. Ltd., 3-6-6 Asahi-machi, Machida-shi, Tokyo 194-8533, Japan*
[b]*National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan*

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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],

* *Corresponding author.*
E-mail address: umezawa@1985.jukuin.keio.ac.jp (A. Umezawa).
[1] Present address: Research Center, Nihon Schering K.K., 1-5-5 Minatojima-minamicho, Chuo-ku, Kobe-shi, Hyogo 650-0047, Japan.
[2] Present address: Department of General Medicine and Clinical Investigation, Nara Medical University, 840 Shijo-cho, Kashihara-city, Nara 634-8522, Japan.
[3] Present address: Department of Cardiovascular Surgery, Saitama Medical Center, 1981 Kamoda, Kawagoe, Saitama 350-8550, Japan.

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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′-CCCATCAC-CATCTTCCAGGAGC-3′, antisense: 5′-TTCACCACCTTCTT-GATGTCATCATA-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

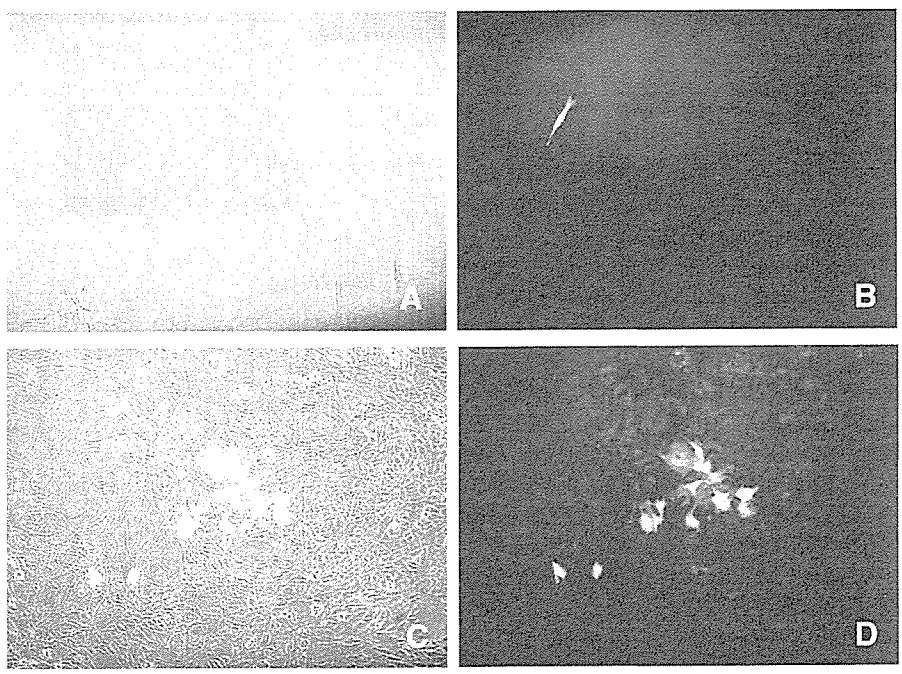

Fig. 1 – Single cell marking by infection of retrovirus carrying EGFP. Phase contrast photomicrograph (A, C) and fluorescent photomicrograph (B, D) of 9-15c cells 1 day (A, B) or 7 days (C, D) after infection with retroviruses carrying EGFP. EGFP-positive single cell-derived cells were clustered.

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

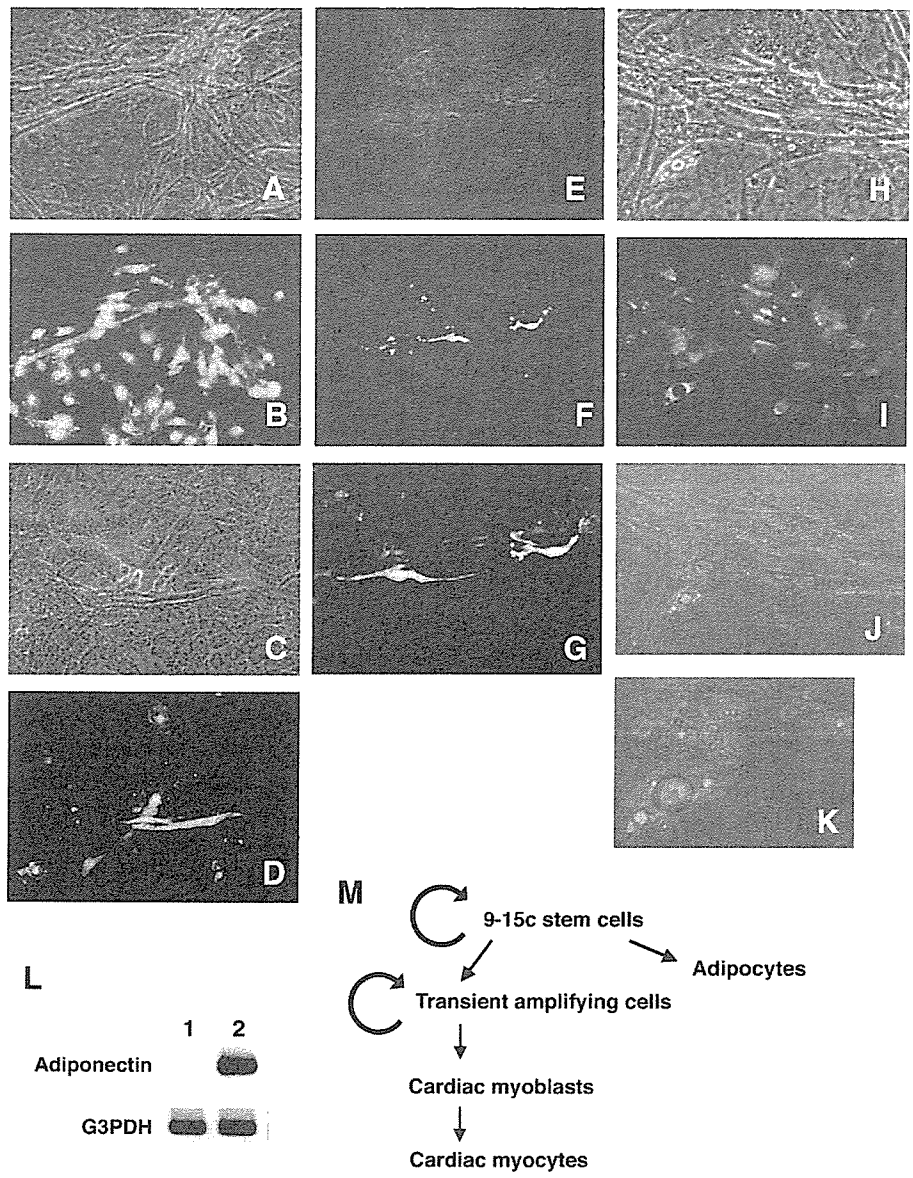

Fig. 2 – Bipotency, i.e., cardiomyogenic and adipogenic differentiation, of single cell-derived cells. Single-cell-derived 9-15c cells marked by EGFP exhibited cardiomyogenic and adipogenic differentiation after exposure to 5-azacytidine. (A–B) Cardiomyogenic and undifferentiated EGFP-marked, single-cell-derived 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.

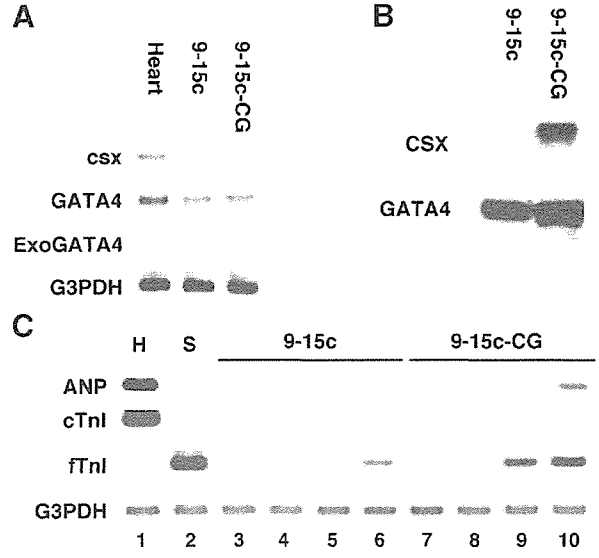

Fig. 3 – Expression of cardiomyocyte-specific or associated genes in 9-15c cells. A: RT-PCR analysis of the Csx, GATA4, exogeneous GATA4 and G3PDH genes (from top to bottom) in adult mouse heart, 9-15c cells and 9-15c cells overexpressing the Csx and GATA4 genes (9-15c-CG cells). B: Western blotting 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

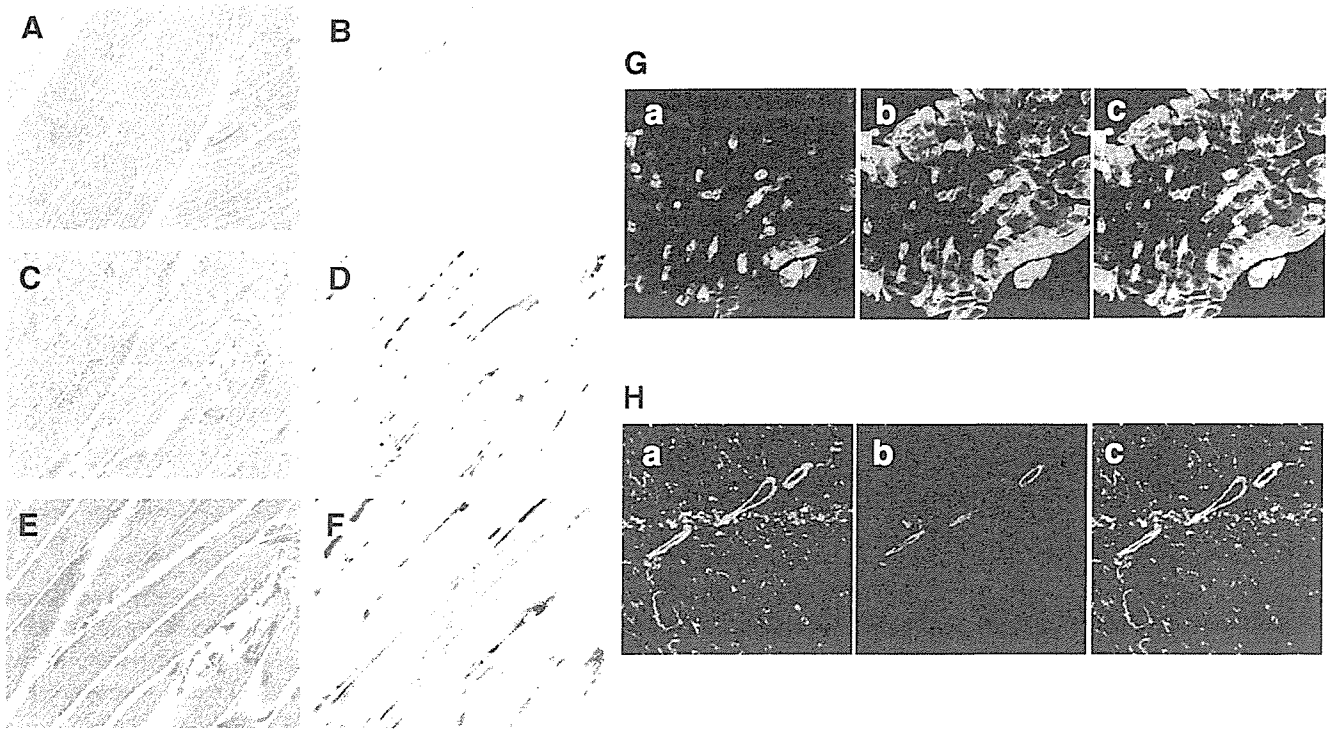

Fig. 4 – Myogenic differentiation of the EGFP-labeled 9-15c-CG cells into the quadriceps femoris muscle. EGFP-labeled 9-15c-CG cells could be recognized morphologically as the skeletal myocytes in the quadriceps femoris muscle 3 months after transplantation (A, C, E: HE staining; B, D, F: immunohistochemistry 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