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

Tadashi Sugiki,[1,2] Taro Uyama,[1] Masashi Toyoda,[1] Hideo Morioka,[2] Shoen Kume,[3] Kenji Miyado,[1] Kenji Matsumoto,[4] Hirohisa Saito,[4] Noriyuki Tsumaki,[5] Yoriko Takahashi,[6] Yoshiaki Toyama,[2] and Akihiro Umezawa[1]*

[1]Department of Reproductive Biology and Pathology, National Institute for Child and Health Development, Tokyo 157-8535, Japan
[2]Department of Orthopaedic Surgery, Keio University School of Medicine, Tokyo 160-8582, Japan
[3]Division of Stem Cell Biology, Department of Regeneration Medicine, Institute of Molecular Embryology and Genetics, Kumamoto University, Kuhonji, Kumamoto 862-0976, Japan
[4]Department of Allergy & Immunology, National Research Institute for Child Health and Development, Tokyo, Japan
[5]Department of Orthopaedics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[6]Mitsui Knowledge Industry, Co, Ltd, Harmony Tower 21st Floor, 1-32-2 Honcho, Nakano-ku, Tokyo 164-8721, Japan

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. 100: 1240–1254, 2007.

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

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*Correspondence to: Akihiro Umezawa, MD, PhD, Department of Reproductive Biology and Pathology, National Research Institute for Child Health and Development, 2-10-1, Okura, Setagaya, Tokyo 157-8535, Japan.
E-mail: umezawa@1985.jukuin.keio.ac.jp

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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

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, Deckman Coulter, Inc., Fullerton, CA).

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 Japan K.K., Tokyo, Japan) 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, Deckman Coulter, Inc., Fullerton, CA).

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

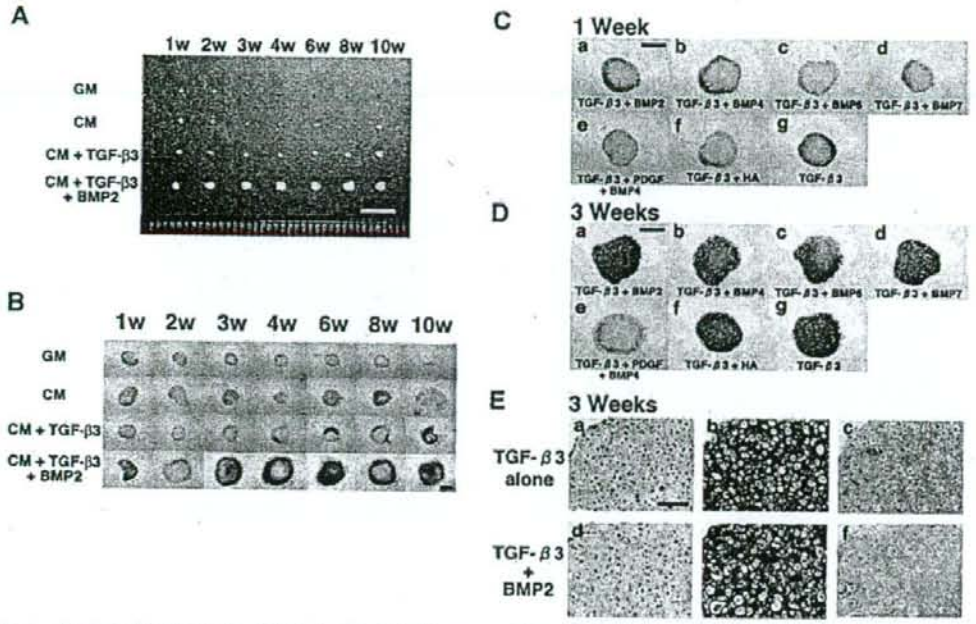

Fig. 1. In vitro chondrogenesis of KUM5 cells. **A,B:** Time-course analysis of growth factors-induced matrix production in KUM5 cells. Macroscopic view of KUM5 chondrogenic nodules which were generated after pellet culture for 1–10 weeks in the GM or the CM supplemented with or without growth factors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

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 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

TABLE I. 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	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	2,551	P	518	Col11a1
1419627_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	Den
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.

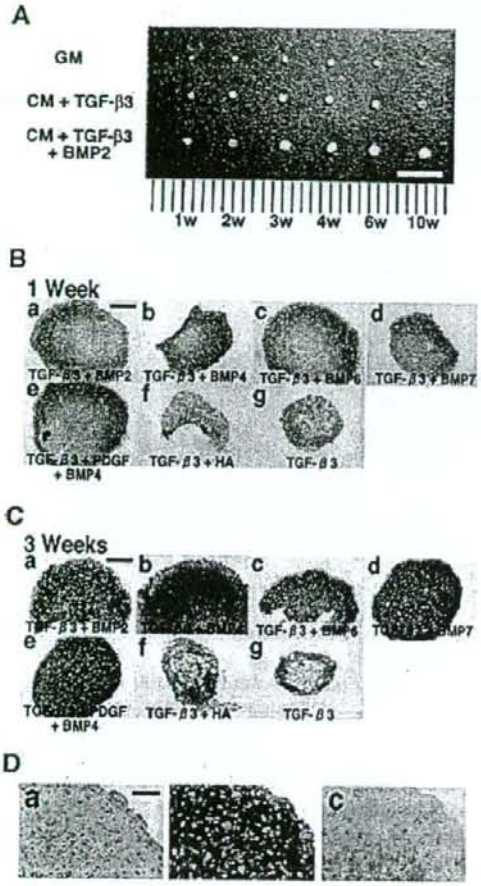

Fig. 2. In vitro chondrogenesis of OP9 cells. A: Time-course analysis of growth factors-induced matrix production in OP9 cells. Macroscopic view of OP9 chondrogenic nodules which were generated after pellet culture for 1–10 weeks in the GM or the CM supplemented with growth factors as indicated. BMP2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

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 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

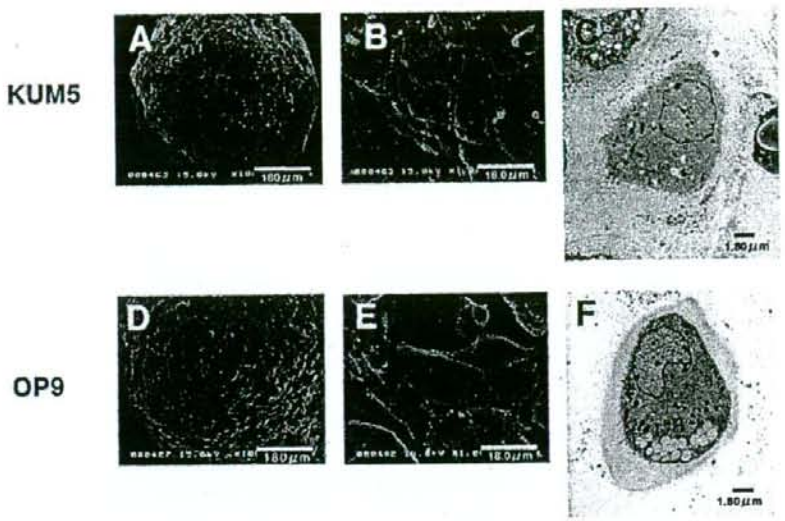

Fig. 3. Ultrastructural analysis of the in vitro chondrogenic micromass. Micromasses of KUM5 cells (**A–C**) and OP9 cells (**D–F**) were generated by culturing in the CM supplemented with TGF-β3 for 3 weeks. (A,B,D,E), SEM; (C,F), TEM.

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 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 type II collagen α1 genes, and OP9 cells expressed cartilage-specific and -associated genes such as the type II collagen α1, type XI collagen α1, 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

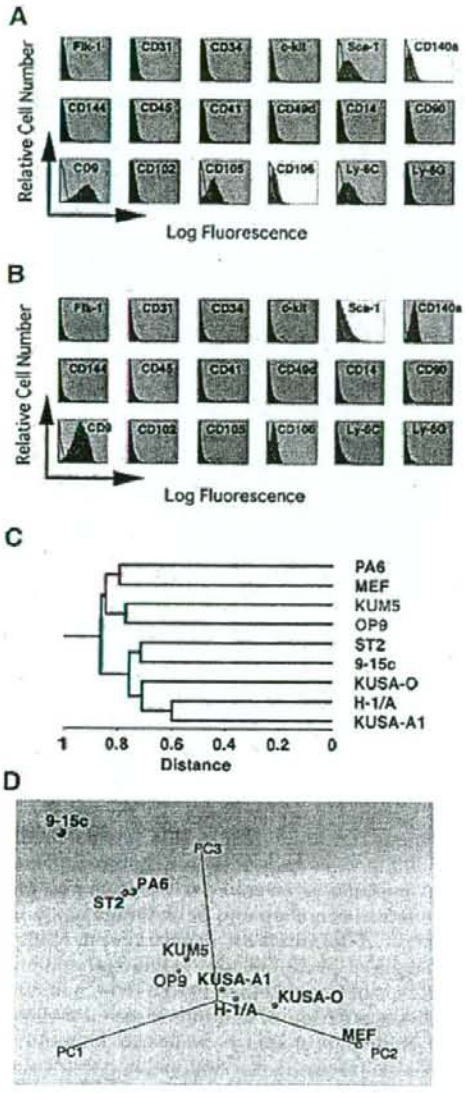

Fig. 4. Expression profiling, hierarchical clustering, and principal component analysis (PCA) of cell surface markers in marrow stromal cells. **A,B:** Flow cytometric analysis of cell surface markers in KUM5 cells (A) and OP9 cells (B). Red and pink colors indicate positive and marginal expression,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

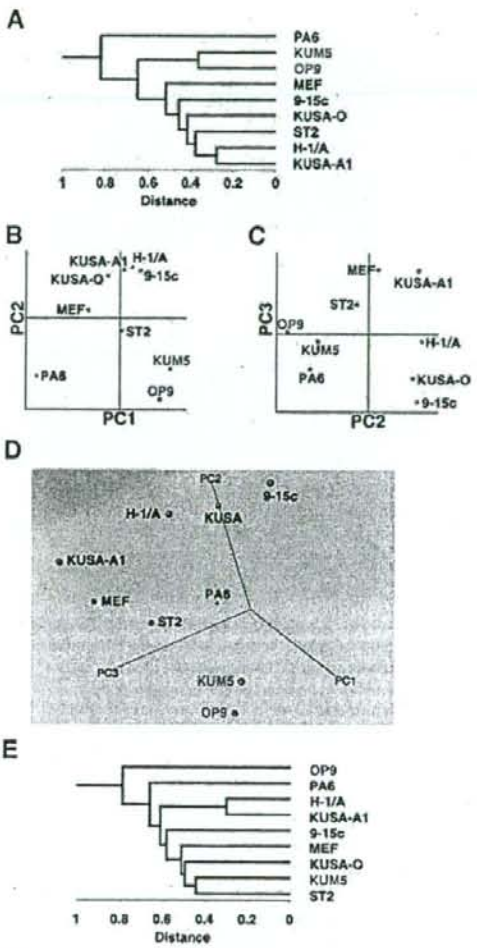

Fig. 5. Hierarchical clustering and PCA of fat- and cartilage-associated gene expression in marrow stromal cells. **A.** Dendrogram revealing clustering profile of 9 marrow stromal cells using 34 fat-associated genes (Supplementary Table II). **B–D.** PCA on expression levels of 34 fat-associated genes.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

abundant endoplasmic reticulum and a small number of mitochondria (Fig. 6D), and collagen fibers were produced around the lacunae cavity of the KUM5 chondrocytes (Fig. 6E), as is the case of the in vitro conditions.

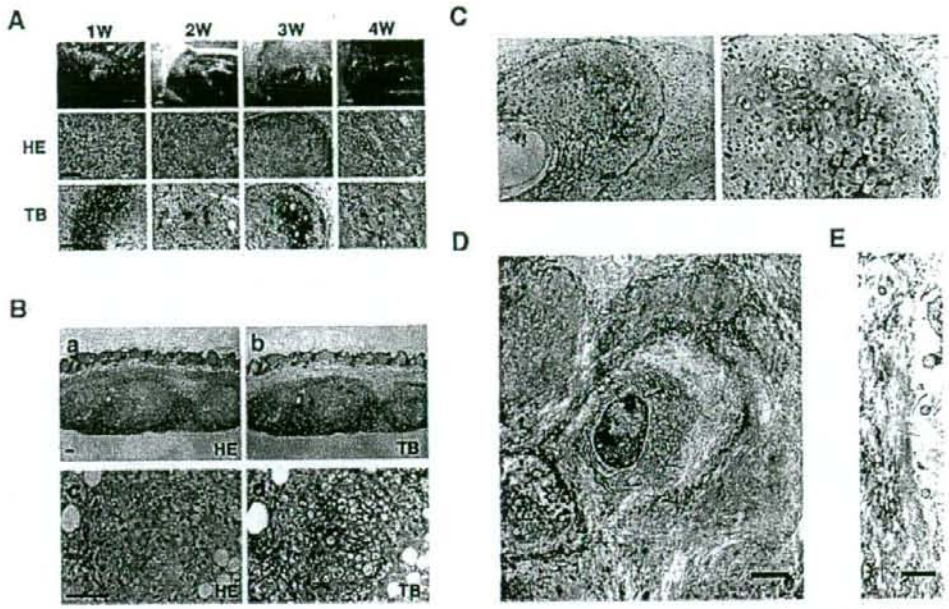

Fig. 6. In vivo chondrogenesis of KUM5 cells. **A:** Macroscopic view (**top**), hematoxylin and eosin stain (HE) (**middle**) and toluidine blue stain (TB) (**bottom**) analysis at 1, 2, 3, and 4 week (w)-cultivation in vivo after direct injection of KUM5 cells. **B:** KUM5 chondrogenic nodules, that 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 type II collagen. 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).

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 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

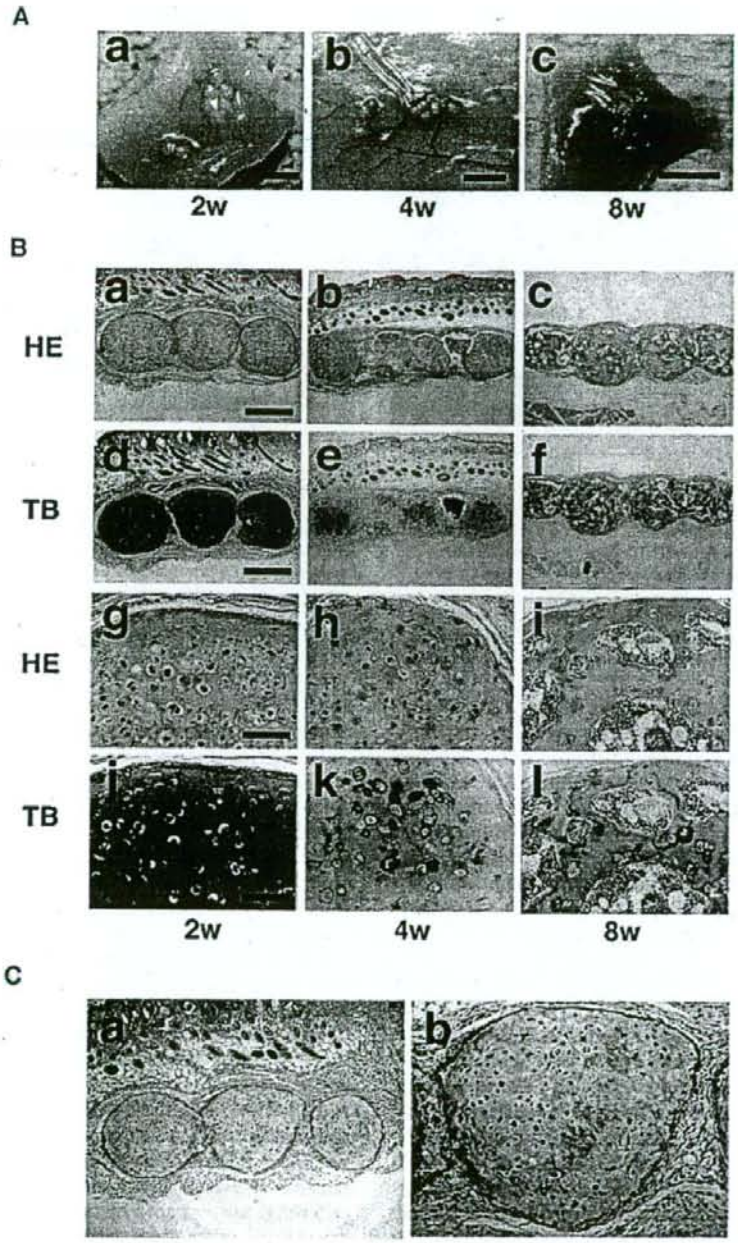

Fig. 7. In vivo chondrogenesis of OP9 cells. In vivo chondrogenesis was examined by implantation of OP9 chondrogenic nodules. OP9 chondrogenic nodules, which were generated after pellet culture for 7 days in the CM supplemented with TGF-β3 and BMP2, were implanted just beneath the cutaneous muscle in 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 type II collagen. Scale bars: 2 mm (A), 500 μm (Ba–f), 100 μm (Bg–l).

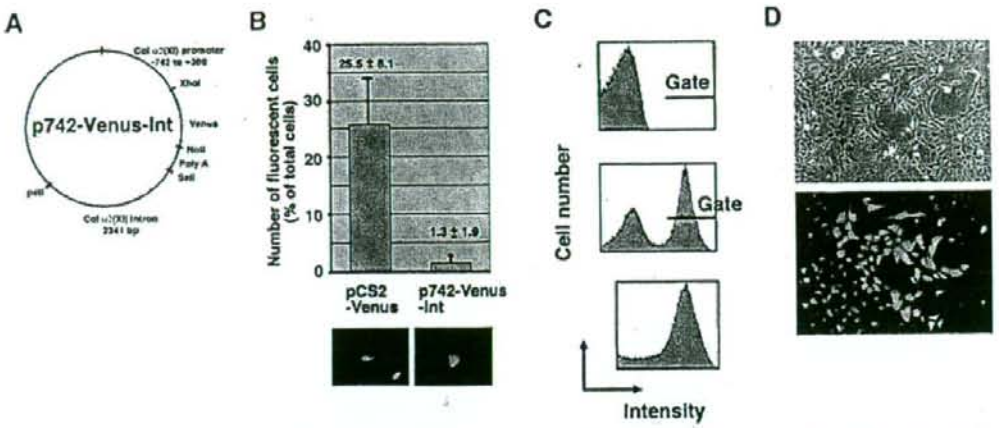

Fig. 8. Isolation of KUM5 chondroblasts using the chondroblast-specific cis-regulatory element. **A**: The p742-Venus-Int plasmid containing the fluorescent Venus gene driven by the cis-regulatory elements of the α2(XI) collagen gene. **B**: The number of fluorescent KUM5 cells (**upper**) after transfection 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).

of the cartilage at 4 weeks (Fig. 9G). Again, ultrastructural analysis revealed that KUM5 chondrocytes implanted into the subcutaneous 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.

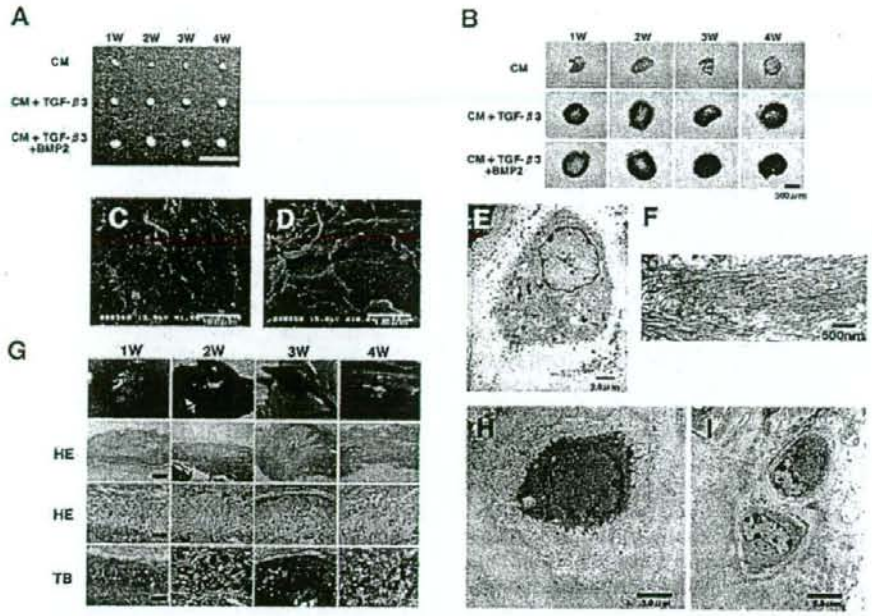

Fig. 9. In vitro and in vivo chondrogenesis of KUM5 cells sorted according to the activity of the chondrocyte-specific cis-regulatory element. **A,B:** Macroscopic view of the chondrogenic nodules which were generated after pellet culture of the finally sorted KUM5 cells for 1–4 weeks in the CM supplemented 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, histological analysis, HE stain; histological analysis, HE stain; histological analysis, 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.

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REGULAR ARTICLE

Establishment of immortalized dental follicle cells for generating periodontal ligament in vivo

T. Yokoi · M. Saito · T. Kiyono · S. Iseki · K. Kosaka · E. Nishida · T. Tsubakimoto · H. Harada · K. Eto · T. Noguchi · T. Teranaka

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Abstract The dental follicle is a mesenchymal tissue that surrounds the developing tooth germ. During tooth root formation, periodontal components, viz., cementum, periodontal ligament (PDL), and alveolar bone, are created by dental follicle progenitors. Here, we report the presence of PDL progenitors in mouse dental follicle (MDF) cells. MDF cells were obtained from mouse incisor tooth germs and immortalized by the expression of a mutant human papilloma virus type 16 *E6* gene lacking the PDZ-domain-binding motif. MDF cells expressing the mutant *E6* gene ($MDF^{E6\text{-}EGFP}$ cells) had an extended life span, beyond 150 population doublings (PD). In contrast, normal MDF cells failed to proliferate beyond 10 PD. $MDF^{E6\text{-}EGFP}$ cells expressed tendon/ligament phenotype-related genes such as *Scleraxis* (*Scx*), *growth and differentiation factor-5*, *EphA4*, *Six-1*, and *type I collagen*. In addition, the expression of *periostin* was observed. To elucidate the differentiation capacity of $MDF^{E6\text{-}EGFP}$ cells in vivo, the cells were transplanted into severe combined immunodeficiency mice. At 4 weeks, $MDF^{E6\text{-}EGFP}$ cell transplants had the capacity to generate a PDL-like tissue that expressed *periostin*, *Scx*, and *type XII collagen* and the fibrillar assembly of type I collagen. Our findings suggest that $MDF^{E6\text{-}EGFP}$ cells can act as PDL progenitors, and that these cells may be a useful research tool for studying PDL formation and for developing regeneration therapies.

Keywords Dental follicle · Progenitor · Development · Immortalization · Periodontal ligament · Mouse (ICR)

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T. Yokoi · M. Saito (✉) · K. Kosaka · E. Nishida · T. Tsubakimoto · T. Teranaka
Department of Medicine,
Division of Operative Dentistry and Endodontics,
Kanagawa Dental College,
82 Inaoka-cho,
Yokosuka, Kanagawa 238-8580, Japan
e-mail: saitohms@kdcnet.ac.jp

T. Yokoi · E. Nishida · T. Noguchi
Department of Periodontology, School of Dentistry,
Aichi-gakuin University,
Nagoya, Aichi, Japan

M. Saito
Oral Health Science Research Center, Kanagawa Dental College,
Yokosuka, Kanagawa, Japan

T. Kiyono
Virology Division, National Cancer Research Institute,
Tokyo, Japan

S. Iseki · K. Eto
Department of Molecular Craniofacial Embryology,
Graduate School, Tokyo Medical and Dental University,
Tokyo, Japan

H. Harada
Department of Oral Anatomy and Developmental Biology,
Osaka University Graduate School of Dentistry,
Suita, Osaka, Japan

Introduction

The periodontal ligament (PDL), which surrounds the tooth root, absorbs occlusal forces and functions as a sense organ (Ten Cate 1994). In periodontitis, a chronic inflammatory disease, the PDL is irreversibly damaged. Despite a number of novel approaches, no one has yet succeeded in reliably forming PDL (D'Errico et al. 1999).

Hence, there is considerable interest in the developmental mechanisms of PDL.

The PDL originates from dental follicle cells formed during the cap stage of tooth germ development by an ectomesenchymal progenitor cell population originating from cranial neural crest cells (Chai et al. 2000). Progenitors in the dental follicle are thought to contribute to the formation of all periodontal tissues, namely cementum, PDL, and alveolar bone (Bosshardt and Schroeder 1996). After the formation of tooth root dentin, cementoblast progenitors in the dental follicle migrate onto the tooth root surface and differentiate into cementoblasts (Bosshardt and Schroeder 1996). Almost simultaneously, PDL progenitors within the dental follicle cells differentiate into PDL cells. Finally, both bone- and PDL-derived fibers coalesce in the PDL to form the intermediate plexus. Although a specific marker for PDL is not available, tendon/ligament phenotype-related genes are thought to be involved in the differentiation of PDL progenitors. Growth and differentiation factors (GDFs)-5, 6, and 7 are members of the bone morphogenetic protein family that regulate tendon/ligament formation (Wolfman et al. 1997) and have been shown to be expressed by both dental follicle and PDL cells (Morotome et al. 1998; Nakamura et al. 2003; Sena et al. 2003). Screlaxis (*Scx*), a basic helix-loop-helix transcription factor that serves as a tendon progenitor marker gene, has also been found to be expressed by PDL stem cells (Brent et al. 2003; Seo et al. 2004). Non-collagenous extracellular matrix has been shown to be involved in the formation of PDL (Matias et al. 2003). *Periostin* is a marker for preosteoblasts but is also found in the periosteum and PDL (Horiuchi et al. 1999). During tooth germ development, *periostin* is initially expressed in the dental follicle cells and is then restricted to postnatal PDL cells during tooth root formation (Kruzynska-Frejtag et al. 2004). *Periostin-/-* mice develop a periodontal-disease-like phenotype within 3 months of birth, suggesting that this protein plays a critical role in maintenance of the PDL (Rios et al. 2005). These findings suggest that both tendon/ligament phenotype-related genes and extracellular matrices, which are highly expressed in PDL, are involved in PDL formation and maintenance. However, details of the mechanisms involved in PDL formation are yet to be clarified, because of the scarcity of PDL progenitor culture systems.

Recently, we have demonstrated the presence of cementoblast progenitors in bovine dental follicle cells (Handa et al. 2002). A cementoblast progenitor cell line, designated as BCPb8, has the capacity to form PDL- and cementum-like tissue when transplanted into severe combine immunodeficiency (SCID) mice (Saito et al. 2005). Although BCPb8 is a clonal cell line useful for the study of dental follicle progenitors, the use of these cells derived from a bovine species is restricted, because the cDNA database is inadequate, and because antibodies are limited. Thus, little is known about the biological properties of dental follicle progenitors or the mechanisms that regulate their differentiation. Investigations directed at addressing these key questions are essential if we are to understand the developmental mechanisms of PDL. In this study, we have attempted to establish immortalized mouse dental follicle (MDF) cells for the study of progenitors in dental follicle cells.

Materials and methods

Tissue culture

MDF cells were isolated from the dental follicle tissue of the incisor tooth germs of 1-day postnatal (P1) ICR mice. Briefly, mouse dental follicle tissue was mechanically stripped from the lingual posterior region of mouse incisor and placed onto a 24 multi-well plate. The tissues were then incubated with α-minimum essential medium (α-MEM: Sigma, St. Louis, USA) containing 10% fetal bovine serum (FBS; BioWhittaker, Maryland, USA), 50 μg/ml ascorbic acid, 100 U/ml streptomycin and penicillin, in a humidified atmosphere of 5% CO_2 at 37°C. When the cells reached approximately 80% confluence, they were passaged with 0.25% trypsin/1 mM EDTA and maintained as MDF cells. These cells were plated into six wells at a density of 3×10^4 cells/ml, and the medium was changed every 2 days.

Infection of retrovirus constructs and establishment of $MDF^{E6\text{-}EGFP}$ cells

The construction of pCLXSN-$16E6^{\Delta146\text{-}151}$ and the production of LXSN-$16E6^{\Delta146\text{-}151}$ retrovirus have been described previously (Kyo et al. 2003). An aliquot of 1 ml producer cell culture fluid was added to MDF cells (passage 1) in the presence of polybrene (8 μg/ml), and the cells were subsequently selected in the presence of G418 (100 μg/ml). Transduced cells were maintained in the medium described above. Following infection with LXSN-$16E6^{\Delta146\text{-}151}$, MDF cells were transduced with EGFP lentivirus under the control of a CMV promoter to obtain stably expressed EGFP ($MDF^{E6\text{-}EGFP}$).

Osteogenic differentiation

Cells were plated into six wells at a density of 3×10^4 cells/ml and cultured in the medium described above supplemented with 100 nM dexamethasone, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate. The culture medium was

replaced every 2 days, and the cells were maintained for 3 weeks.

Alkaline phosphatase activity and alizarin red staining

To evaluate alkaline phosphatase (ALP) activity, $MDF^{E6\text{-}EGFP}$ cells were fixed with 4% paraformaldehyde for 20 min at 21°C. After being washed with PBS, the cells were incubated for 20 min in a mixture of 0.1 mg/ml naphthol AS-MX phosphate (Sigma), 0.5% N-N dimethyl formamide (Sigma), 2 mM $MgCl_2$, 0.6 mg/ml Fast Blue BB salt (Sigma) in 0.1 M TRIS-HCl (pH 8.5) at room temperature. Calcium accumulation was detected by staining preparations with 2% alizarin red S (pH 6.4) (Sigma). MC3T3E1 (purchased from RIKEN BioResource Center, Japan) and NIH3T3 were used as controls.

Probes for in situ hybridization

The cDNA of mouse *osteopontin* open reading frame (885 bp), mouse *Scx* 3′ untranslated region (UTR; 291 bp), or mouse *type XII collagen* (500 bp) region was amplified by reverse transcription/polymerase chain reaction (RT-PCR) by using the following primers: *osteopontin*, 5′-ATGAGATTGGCAGTGATTTG-3′ and 5′-GTTGACCT CAGAAGATGAAC-3′; *Scx*, 5′-AAGAGGT GATGCCAC TAGTG-3′and 5′-TATACAAAATTTCCA GACTTTAT ATTATCAT-3′; *type XII collagen*, 5′-TCCCCATCAAA GAACAGACC-3′ and 5′-TGACTGCTGGATGA CAAAGG-3′. The amplicons for *osteopontin* and *Scx* were subsequently cloned into the pCRII vector (Invitrogen, Carlsbad, Calif., USA), and those for *type XII collagen* were cloned into pCR 4-TOPO (Invitrogen). A 623-bp mouse *periostin* cDNA fragment was isolated by RT-PCR with partial T7 and T3 promoter-containing primers (below, in italics) at the 5′ end and the 3′ end, respectively: T7: 5′-*CACTATAGGGC*GGCTGAA GATGGTTCCTCTC-3′, and T3: 5′-*CACTAAAGGG*C CATGTGGCTGTGTAAGG CATTC-3′. These cDNA fragments were further amplified by using the following adaptor primers to install full T7 and T3 promoter sequences into the fragments: 5′-GTAATAC GACTCAC TATAGGGC-3′ for T7, and 5′-AAT TAACCCTCAC TAAAGG-3′ for T3.

In situ hybridization

To generate antisense and sense digoxygenin-labeled riboprobes, linearized *osteopontin*, *Scx*, and *type XII collagen* plasmids and *periostin* cDNA fragments (see previous section) were transcribed by T7, T3, or Sp6 RNA polymerase as described elsewhere (Wilkinson 1995). Heads of P1 C57BL/6 mice were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), and 10-μm-thick sagittal sections were cut. Mandibles of P35 mice were fixed in 4% paraformaldehyde at 4°C overnight, decalcified in 12.5% EDTA containing 2.5% paraformaldehyde for 6 weeks, and then embedded in OCT compound. In situ hybridization was carried out on these sections as previously described with some modification (Iseki et al. 1999). Polyvinyl alcohol was used as buffer during the color reaction.

RNA preparation and RT-PCR

Total RNA was isolated from cells by using ISOGEN (Nippon Gene, Tokyo, Japan) as described previously (Handa et al. 2002). cDNAs were synthesized from 1 μg total RNA in a 20-μl reaction volume containing 10× reaction buffer, 1 mM dNTP mixture, 1 U/μl RNase inhibitor, 0.25 U/μl reverse transcriptase (M-MLV reverse transcriptase; Invitrogen), and 0.125 μM random 9-mers (Takara, Tokyo, Japan). Amplification was performed in a PCR Thermal Cycler SP (Takara, Tokyo, Japan) for 25 cycles with the following reaction profile: 94°C for 1 min, 60°C for 30 s, and 72°C for 30 s. Synthesized cDNA served as a template for subsequent PCR amplification with mouse-specific primers: *bone sialoprotein* (*BSP*): sense 5′-AGGGAACTGACCAGTGTTGG-3′, antisense 5′-TCGTTGCCTGTTTGTTCGTA-3′; *osteocalcin* (*OC*): sense 5′-CATGAGGACCCTCTCTCTGC-3′, antisense 5′-GCCGGAGTCTGTTCACTACC-3′; *osteopontin*: sense 5′-TGCACCCAGATCCTATGACC-3′, antisense 5′-TGTGGTCATGGCTTTCATTG-3′; *periostin*: sense 5′-TTGAAGGTGTCCCGSCTTC-3′, antisense 5′-TGAT TCGTTCTTCCCGAGTC-3′; *Scx*: sense 5′-CTTTCTTC CACAAGCGGTCGT-3′, antisense 5′-TGTCACGGTCTT TGCTCAAC-3′; and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*): sense 5′-TGTTCCTACCCCCAA TTGTGT-3′, antisense 5′-AGGAGACAACCTGGTCC TCA-3′. Specific primers for *six-1*, *EphA4*, *GDF-5*, *collagen I* (*Coll*), and *osterix* have been described previously (Salingcamboriboon et al. 2003).

In vivo differentiation assay

The differentiation potential of $MDF^{E6\text{-}EGFP}$ cells was assessed by transplantation of the cells into SCID mice as described previously (Handa et al. 2002). Briefly, cells were inoculated subcutaneously into 5-week-old male CB-17 scid/scid (SCID) mice (Nihoncrea, Tokyo, Japan) after incubation of 1.5×10^6 cells in a mixture of 40 mg hydroxyapatite powder (Osferion, Olympus, Tokyo, Japan) and fibrin clot (mixture of mouse fibrinogen and thrombin: both from Sigma). Transplantation analysis was carried out three times, and three transplants were prepared per group. The mice were sacrificed after 4 weeks and subjected to