

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 ( $\beta$ -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 their 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

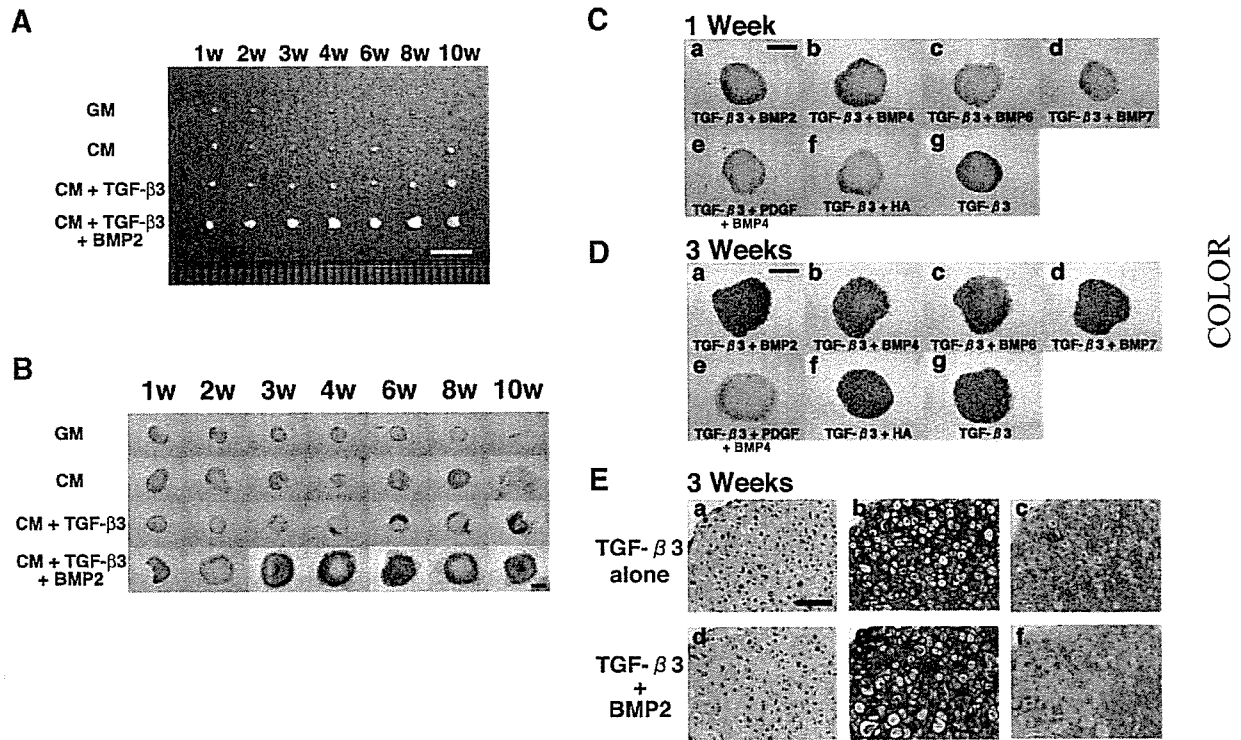
### Pelleted Micromass Culture of KUM5 Cells

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 per-

formed 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)- $\beta$  and bone morphogenetic protein (BMP) are involved in chondrogenesis and osteogenesis [Fujii et al., 1999; Maeda et al., 2004], we used TGF- $\beta$ 3 and BMPs on KUM5 culture. Exposure of the cells to TGF- $\beta$ 3 augmented the metachromatic toluidine blue staining in the KUM5-micromass (Fig. 1A,B). BMP2 dramatically enhanced this TGF- $\beta$ 3-induced differentiation, that is, caused stronger metachromatic staining and enlarged metachromatic area. To determine the effect of other cytokines on the TGF- $\beta$ 3-induced chondrogenic differentiation, we added BMP4, BMP6, BMP7, PDGF, or hyaluronic acid to the CM supplemented with TGF- $\beta$ 3. BMP4, BMP6, and BMP7 enhanced the TGF- $\beta$ 3-induced chondrogenic differentiation in a manner similar to BMP2 (Fig. 1C,D). With exposure to BMP2, the number of the post-mitotic 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- $\beta$ 3 and BMP4-induced differentiation, as determined by toluidine blue staining (Fig. 1C<sub>e</sub>,D<sub>e</sub>). To confirm the chondrogenic 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- $\beta$ 3-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- $\beta$ 3 (**a–c**), or TGF- $\beta$ 3 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  $\mu$ m (**B, C, D**), 100  $\mu$ m (**E**).

et al., 1994]. (<http://1954.jukuin.keio.ac.jp/umezawa/chip/sugiki>) by using the Affymetrix gene chip oligonucleotide arrays (Table I). RNAs were isolated from cell lines cultured in the GM without any induction of differentiation to perform the gene chip analysis. Of the 23,000 genes represented on the gene chip, chondrocyte-specific- or associated-genes such as type II collagen  $\alpha$ 1, 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  $\alpha$ 1, 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.

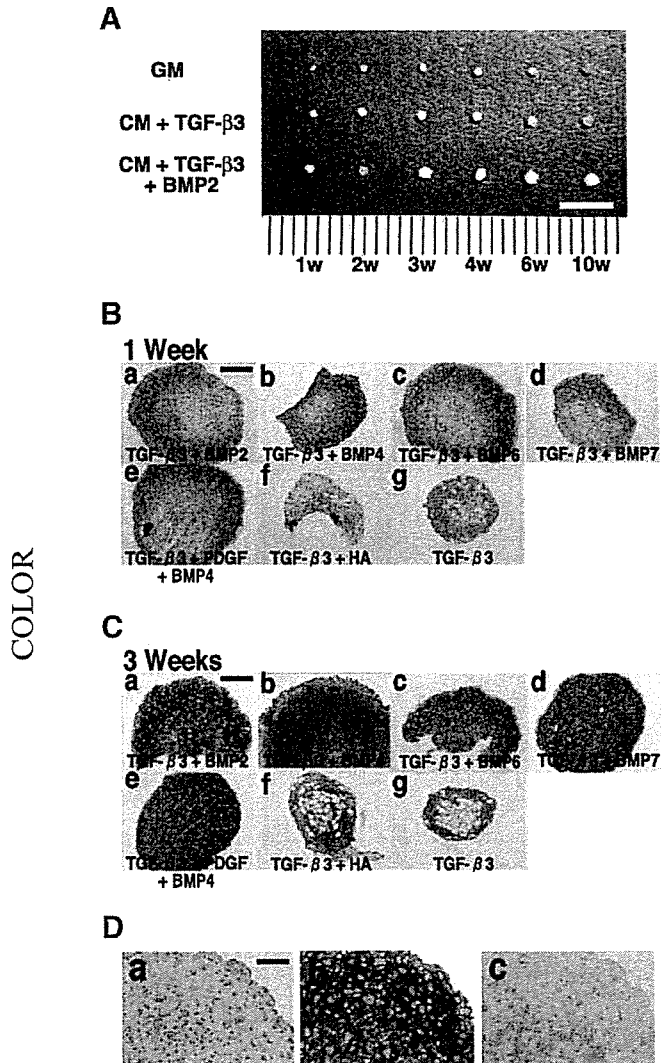
### Pelleted Micromass Culture of OP9 Cells

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- $\beta$ 3, 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- $\beta$ 3 and BMP2 dramatically induced the chondrogenic differentiation (Fig. 2A,Ba,Ca), that is, the pellet cells produced abundant extracellular matrix (Fig. 2D) and caused deeper metachromatic staining and an enlarged metachromatic area (Fig. 2Db). Additionally, we examined the effect of other cytokines on the differentiation of OP9 cells

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

Probe set	Genbank	Description	9-15c			KUSA-O			KUSA-A1			H-1/A			OP9			KUM5			Symbol
			Flags	Raw	Raw	Flags	Raw	Raw	Flags	Raw	Raw	Flags	Raw	Raw	Flags	Raw	Raw	Flags	Raw		
1450567_a_at	NM_081163	Procollagen, type II, alpha 1	A	28	187	A	98	46	P	1,7390	P	679	Col2a1								
1428571_at	AK004383	Procollagen, type IX, alpha 1	P	85	116	P	99	57	A	132	P	190	Col9a1								
1422253_at	NM_009925	Procollagen, type X, alpha 1	A	13	20	A	15	104	A	218	A	270	Col10a1								
1418599_at	BB336814	Procollagen, type XI, alpha 1	A	69	682	P	4,284	5,009	P	2,551	P	518	Col11a1								
1419527_at	NM_016685	Cartilage oligomeric matrix protein	A	120	111	A	64	167	A	1,892	M	172	Comp								
1448368_at	NM_007883	Decorin	A	176	36	A	223	226	A	85	A	110	Den								
1416405_at	BC019502	Biglycan	P	12,600	11,817	P	11,011	12,932	P	21,954	P	18,640	Bgn								
1449827_at	NM_007424	Aggrecan 1	A	70	118	A	105	127	A	94	A	167	Agcl								
1416321_s_at	BC019775	Proline arginine-rich end leucine-rich repeat	P	196	59	P	899	1,092	P	2,169	P	362	Prplp								
1415939_at	NM_021355	Fibromodulin	P	388	359	M	11,542	16,626	P	1,108	A	320	Fmod								
1418745_at	NM_012050	Osteomodulin	P	288	80	P	1,849	2,185	P	347	P	743	Omd								
1417012_at	AL266824	Syndecan 1	P	1,182	2,449	P	1,388	1,607	P	4,704	P	1,799	Sdc1								
1420853_at	NM_011520	Syndecan 2	P	752	1,256	P	2,940	4,398	P	605	P	2,039	Sdc2								
1417654_at	NM_011521	Syndecan 3	A	382	547	P	680	902	P	385	P	762	Sdc3								
1424950_at	BI077717	SRY-box containing gene 9	P	306	281	P	244	342	P	305	P	320	Sdc4								
1420895_at	BM248342	Transforming growth factor, beta receptor I	P	120	5	A	59	27	P	1,344	P	163	Sox9								
1425444_a_at	S69114	Transforming growth factor, beta receptor II	P	780	703	P	657	862	P	1,595	P	802	Tgfb1								
1425444_b_at	AF039601	Transforming growth factor, beta receptor III	P	552	746	P	1,068	1,189	P	868	P	1,133	Tgfb2								
1425620_at	NM_007554	Bone morphogenetic protein 4	P	448	328	A	275	313	P	855	P	1,015	Tgfb3								
1422912_at	BM248248	Bone morphogenetic protein receptor, type I	P	1,048	646	P	6,470	7,266	P	1,736	P	2,860	Bmp4								
1425482_at	NM_010207	Fibroblast growth factor receptor 2	P	1,486	815	P	1,089	1,164	P	1,189	P	1,123	Empr1a								
1417271_a_at	NM_007832	Endoglin	P	833	656	P	1,664	1,998	P	992	P	3,598	Fgfr2								
1451314_a_at	L08431	Vascular cell adhesion molecule 1	P	247	187	A	40	115	A	232	P	1,371	Eng								
			P	462	39	A	28	92	A	812	P	563	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- $\beta$ 3-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- $\beta$ 3 and BMP2 for 3 weeks. **a:** Hematoxylin and Eosin stain; **b:** Toluidine blue stain; **c:** Alcian blue stain. Scale bars: 5 mm (**A**), 200  $\mu$ m (**B,C**), 100  $\mu$ m (**D**).

with procedures analogous to those used for KUM5 cells. BMP4, BMP6, and BMP7 enhanced the TGF- $\beta$ 3-induced differentiation in a manner similar to BMP2 (Fig. 2B,C). Unlike its effect in KUM5 cells, PDGF did not inhibit TGF- $\beta$ 3- and BMP4-induced differentiation, as determined by toluidine blue staining (Fig. 2Be,Ce). To confirm the chondrogenetic

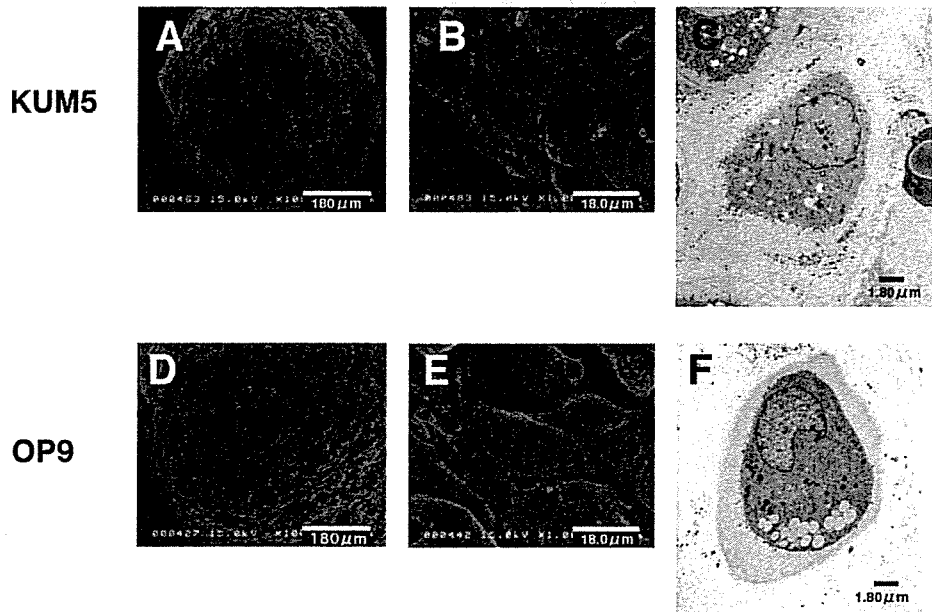
differentiation histologically, we examined the ultrastructural analysis of the cartilaginous micromasses. Extracellular matrix was abundantly deposited over OP9 cells, or the surface of the generated micromass (Fig. 3D). The cells covering the micromass showed a flattened shape (Fig. 3E). The OP9 chondrocytes 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 (PDGFR $\alpha$ ), and negative for c-kit (CD117), Flk-1, CD31 (PECAM-1), CD34, CD144 (VE-cadherin), CD45 (leukocyte common antigen), CD49d (integrin  $\alpha$ 4), 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 sub-categories 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



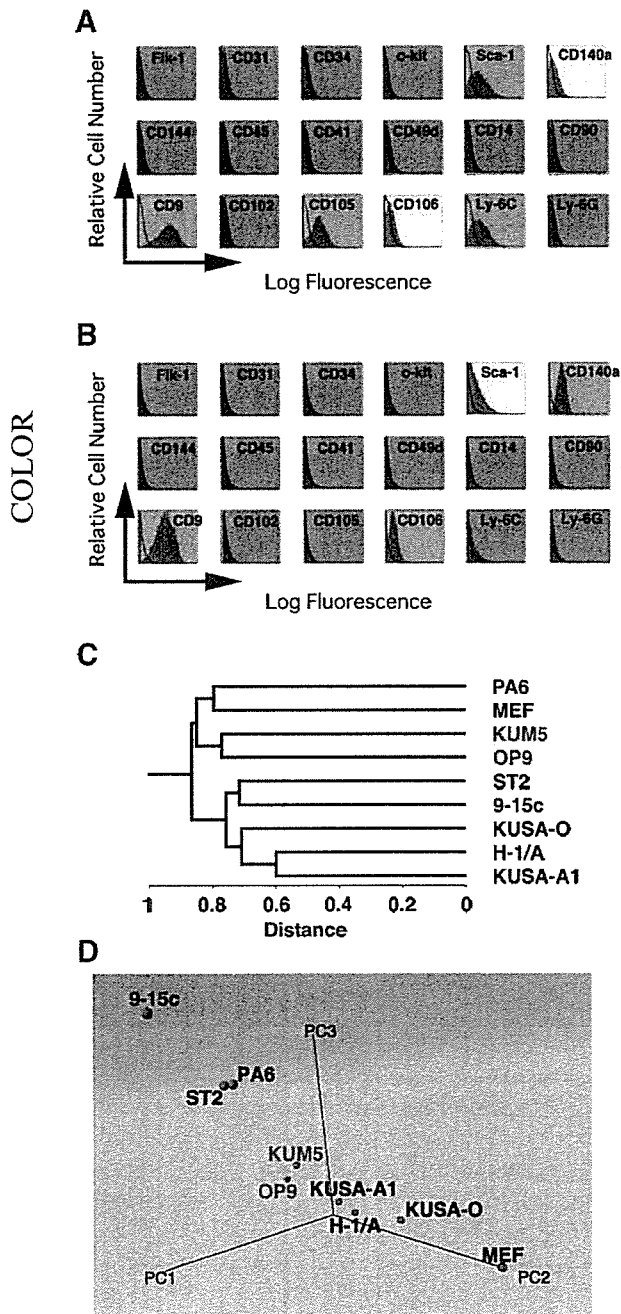
**Fig. 3.** Ultrastructural analysis of the in vitro chondrogenic micromass. Micromasses of KUM5 cells (A–C) and OP9 cells (D–F) were generated by culturing in the CM supplemented with TGF- $\beta$ 3 for 3 weeks. (A,B,D,E), SEM; (C,F), TEM.

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  $\alpha$ 1(II) procollagen genes, and OP9 cells expressed cartilage-specific and -associated genes such as the  $\alpha$ 1(II) procollagen,  $\alpha$ 1(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.

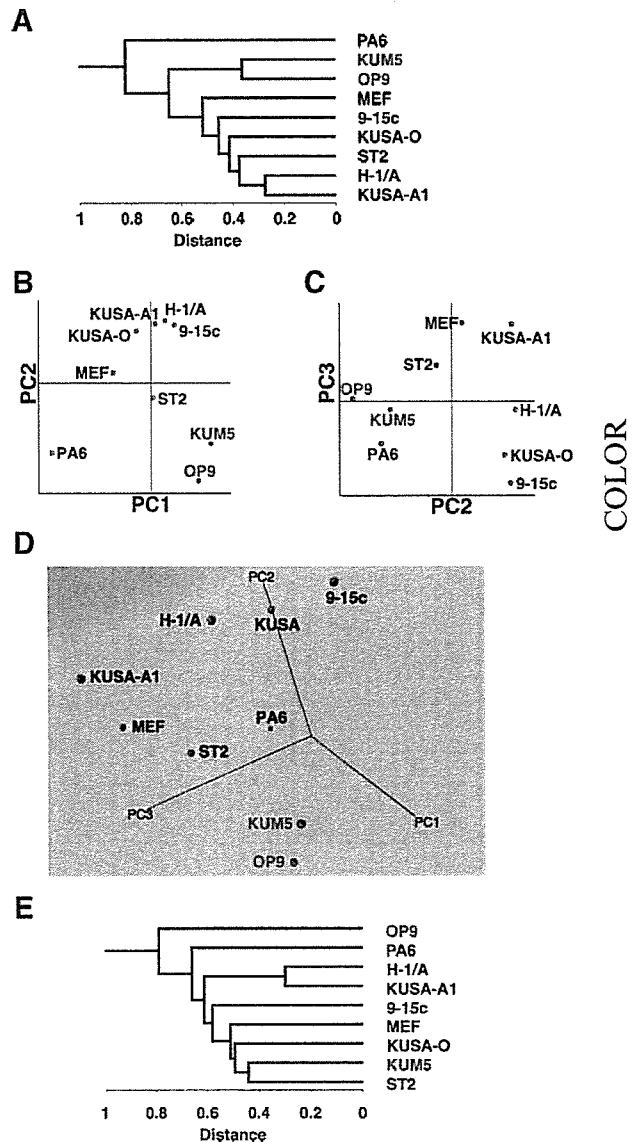
#### In Vivo Chondrogenesis

To examine the chondrogenic activity of KUM5 cells, we injected KUM5 cells at confluence without any treatment (i.e., without TGF- $\beta$ 3 and BMP2 treatment) into mice sub-

cutaneously (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).

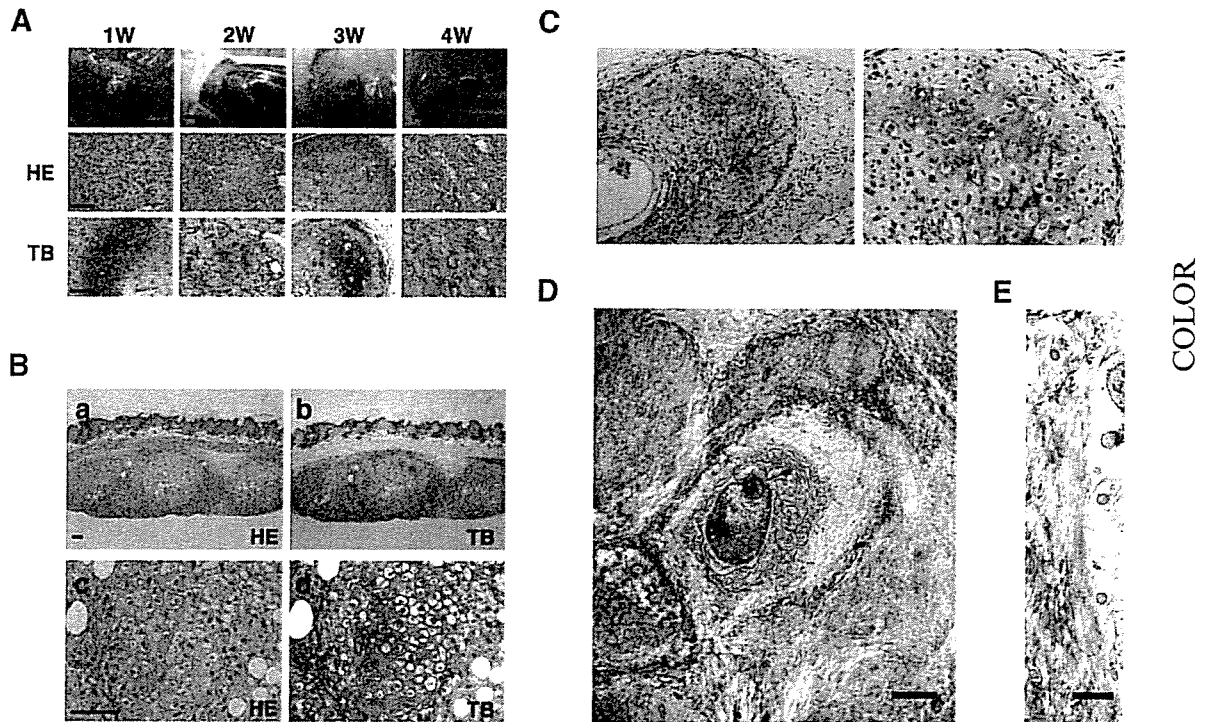


**Fig. 4.** Expression profiling, hierarchical clustering, and principal component analysis (PCA) of cell surface markers in marrow stromal cells. **A,B:** Flow cytometric analysis of cell surface markers in KUM5 cells (A) and OP9 cells (B). Red and pink colors indicate positive and marginal expression, 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- $\beta$ 3 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.

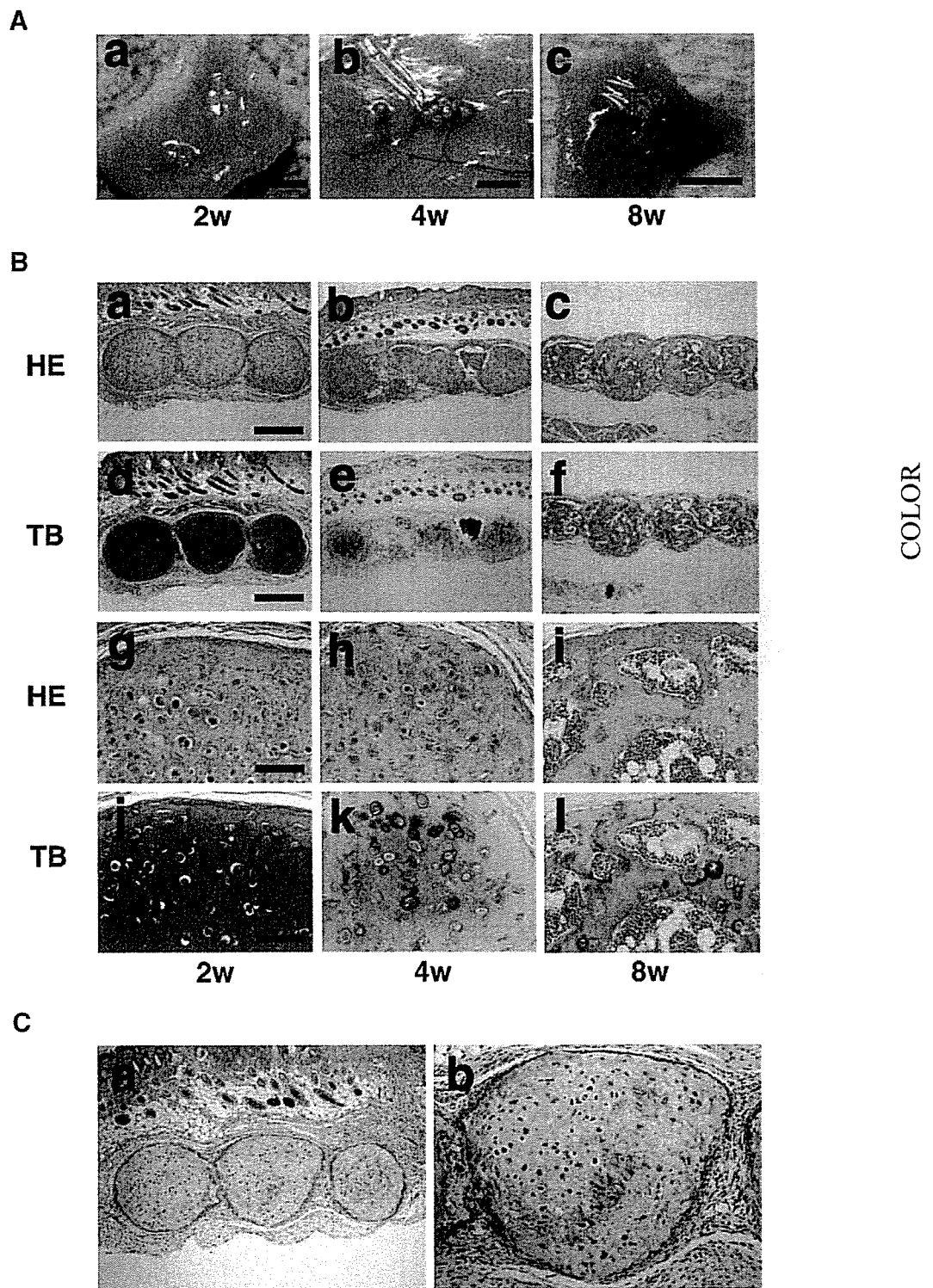
**C:** Expression of chondrocyte-specific collagen type II. The KUM5 chondrogenic nodules were sectioned after 2 week-in vivo cultivation and stained with collagen type II-specific antibody. **D,E:** Ultrastructural analysis (TEM) of KUM5 implants. KUM5 cells were implanted into the subcutaneous tissue of Balb/c nu/nu mice, and the generated cartilage was resected 2 weeks after implantation. Scale bars: 2 mm (A, top row), 100  $\mu$ m (A, middle and bottom row), 100  $\mu$ m (B), 2  $\mu$ m (D), 1  $\mu$ m (E).

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 $\alpha$ 2(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

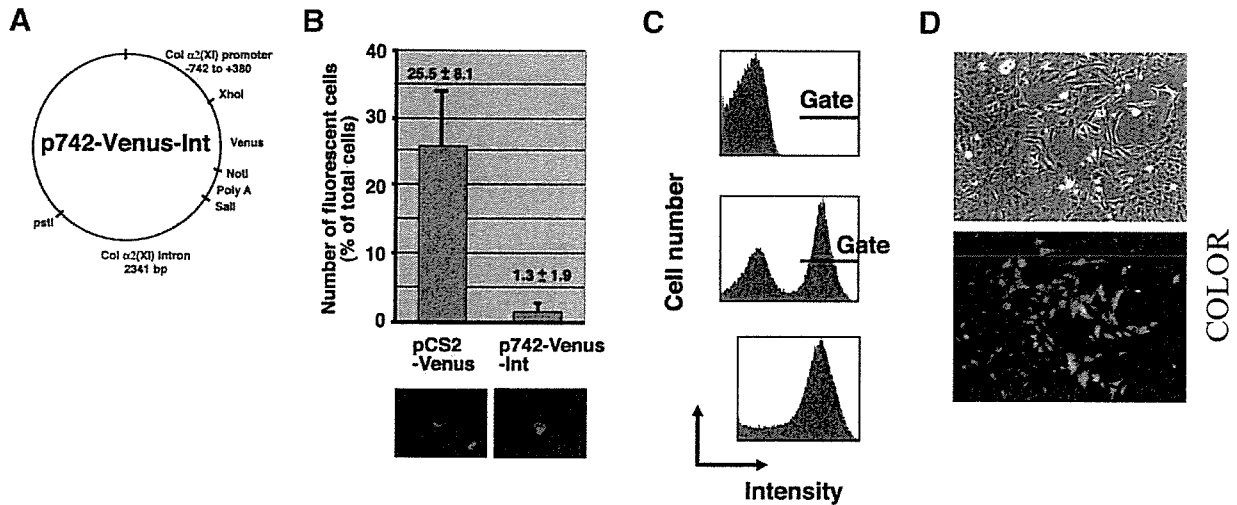
terms of differentiation capacity and stages [Muraglia et al., 2000]. To validate the chondrogenic differentiation observed here, a homogeneous population of committed cell obtained after induction should be isolated. Therefore, for the purpose of sorting chondrogenically committed cells, we transfected KUM5 cells with a Venus-expression vector under the control of the Col  $\alpha$ 2(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 micro-masses 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



**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- $\beta$ 3 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  $\mu$ m (Ba–f), 100  $\mu$ m (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  $\alpha 2(XI)$  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).

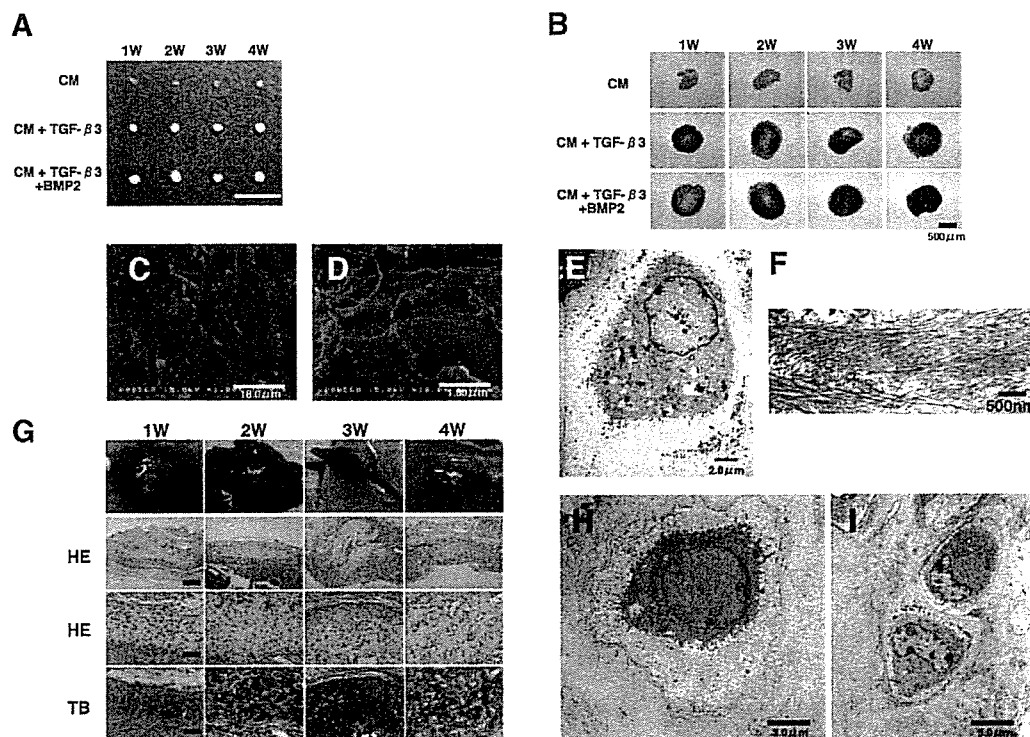
## DISCUSSION

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- $\beta$  and/or BMPs. Three isoforms of TGF- $\beta$  have been known to have the ability to induce the chondrogenic differentiation. Both TGF- $\beta 2$  and - $\beta 3$  are more effective than TGF- $\beta 1$  in promoting chondrogenesis, and TGF- $\beta 3$  accelerates production of cartilagi-

nous extracellular matrix in differentiating mesenchymal stem cells [Barry et al., 2001].

This study was undertaken to obtain mesenchymal stem cells with chondrogenic potential that remain 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 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  $\alpha 2(XI)$  cis-regulatory element (KUM5-Venus) after culturing in the CM supplemented with TGF- $\beta 3$  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  $\mu$ m, HE stain; histological analysis, scale bar: 120  $\mu$ m, HE stain; histological analysis, scale bar: 120  $\mu$ m, 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  $\mu$ m (B), 2 mm (G, top row), 500  $\mu$ m (G, 2nd row), 100  $\mu$ m (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  $\alpha 2(XI)$  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

We would like to express our sincere thanks to Shin-ichiro Takayama, Yasushi Nakao, Hiroyasu Ikegami, and Toshiyasu Nakamura for support throughout the work, Atsushi Miyawaki for the Venus/pCS2 plasmid, Kayoko Saito for secretarial assistance, and Toshihiro Nagai and Yoshie Hashimoto for providing expert technical assistance. This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan, the Health; Labour Sciences Research Grants, and the Pharmaceuticals and Medical Devices Agency; by Research on Health Science focusing on Drug Innovation (KH71064) from the Japan Health Science Foundation; by the program for promotion of fundamental Studies in Health Science of the Pharmaceuticals and Medical Devices Agency (PMDA); by the research Grant for Cardiovascular Disease (H16C-6) from the ministry of Health, Labour and Welfare; by supported by a Grant for Child Health and Development (H15C-2) from the Ministry of Health, Labour and Welfare. The raw data from the gene chip analysis is available at our laboratory's web site (<http://1954.jukuin.keio.ac.jp/omezawa/chip/sugiki/index.html>). The photomicrographs of the pelleted micromasses examined by SEM and TEM were available at <http://1954.jukuin.keio.ac.jp/omezawa/sugiki/EM/index.html>. The wrl files of the 3D-representation of PCA are available at <http://1954.jukuin.keio.ac.jp/omezawa/sugiki/pca/index.html>.

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