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A Comparison of Neural Differentiation and Retinal Transplantation with Bone Marrow-Derived Cells and Retinal Progenitor Cells

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ABSTRACT

Retinal progenitor cells (RPCs) are immature precursors that can differentiate into retinal neurons, including photoreceptors. Recently, it has been reported that bone marrow-derived cells may also be capable of differentiation into cells of central nervous system lineage, including retinal neurons. We compared these two cell types to evaluate their potential as a source of cells for retinal transplantation. Marrow stromal cells (MSCs) and macrophages were isolated from enhanced green fluorescence protein mice. MSCs were cultured with brain-derived neurotrophic factor, nerve growth factor, and basic fibroblast growth factor to induce neuronal differentiation. RPCs were cultured under the same conditions or with 10% fetal bovine serum. Neuronal marker expression was examined and compared between MSCs and RPCs. MSCs, macrophages, and RPCs were also cultured

with explanted retinas from rhodopsin knockout mice to study their potential for retinal integration. MSCs expressed neuronal and retina-specific markers by reverse transcription-polymerase chain reaction and immunocytochemistry. Both types of cells migrated into retinal explants and expressed neurofilament 200, glial fibrillary acidic protein, protein kinase C- α , and recoverin. RPCs expressed rhodopsin, a photoreceptor marker we never detected in MSCs. A majority of bone marrow derived-macrophages differentiated into cells that resembled microglia, rather than neural cells, in the explanted retina. This study shows that RPCs are likely to be a preferred cell type for retinal transplantation studies, compared with MSCs. However, MSCs may remain an attractive candidate for autologous transplantation. *STEM CELLS* 2006;24:2270–2278

INTRODUCTION

Marrow stromal cells (MSCs) are a population of multipotent mesenchymal stem cells distinct from hematopoietic stem cells. MSCs were originally reported to contribute to the microenvironment of bone marrow and to be necessary for the proliferation of hematopoietic stem cells [1]. It has recently been shown that MSCs can differentiate into various cell lineages, including bone [2, 3], muscle [4], fat [5], cartilage [6], cardiomyocytes [7–9], and hepatocytes [10]. Recently, some studies claimed that MSCs could differentiate cells expressing markers of neurons and glia in vitro [11–17]. MSCs also have the capacity to migrate into the uninjured [18] and diseased brain [19, 20] and spinal cord [21, 22]. Interestingly, studies show that MSCs differentiate into cells expressing markers of photoreceptors and glia in the retina [23, 24].

The two major clinical subtypes of retinal degeneration (RD) are retinitis pigmentosa and age-related macular degeneration. A hallmark of these diseases is photoreceptor cell degeneration, resulting in visual loss. No effective restorative treatment exists for either RD subtype. Previously, we reported that brain-derived progenitor cells can migrate and differentiate into cells expressing markers of mature neurons and glia when grafted to the retina of mice and rats with RD [25–29]. Despite incorporation into the host retina and morphological similarities to various retinal cell types, the transplanted cells failed to express retina-specific markers in each of these studies. Recently, the transplantation of stem and progenitor cells isolated from retina has shown promise as a strategy for photoreceptor replacement [26, 28, 30–32]. Many mammalian tissues, including the retina, contain stem or progenitor cells that can be

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isolated, propagated, and grafted into animal models of RD [26, 32]. The goal of retinal transplantation is the replacement of dead or diseased host cells with healthy, functional donor cells. In the present study, we investigated whether MSCs could effectively differentiate into retinal cells by using a cocktail of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and basic fibroblast growth factor (bFGF), which (as we previously reported) induces MSC differentiation into neurons [17]. Because there are reports of the differentiation of microglial cells into neurons [33] and bone marrow-derived macrophages into brain microglia [34, 35], we examined the differentiation of macrophages when grafted into the retina. Here, we compared the potential of retinal progenitor cells (RPCs) and MSCs for use in retinal transplantation studies.

MATERIALS AND METHODS

Experimental Animals

All experiments were performed in adherence with the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research and with the Schepens Eye Research Institute Animal Care and Use Committee (Boston, MA). Rhodopsin knockout mice ($\rho^{-/-}$ mice; C57/Bl6 background, provided by Peter Humphries, University of Dublin, Trinity College, Dublin, Ireland) and postnatal day 1 (P1) enhanced green fluorescence protein (EGFP) mice (C57BL/6 background; Dr. Masaru Okabe, University of Osaka, Osaka, Japan) were euthanized by CO₂ gas.

Isolation of MSCs and Macrophages

Humeri, femurs, and tibiae were obtained from P1 EGFP mice and divided into small pieces. These small pieces were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 with 10% fetal bovine serum (FBS), and the nonadherent cells were removed by replacement of the media. After approximately 2 weeks, the adherent cells became confluent and were incubated with trypsin for 3 minutes and removed from the flask. All cell cultures were maintained at 37°C, 5% CO₂.

After two or three passages, bone marrow-derived adherent cells were incubated with trypsin for 3 minutes to generate a single-cell suspension. Cells (1×10^6) were labeled with phycoerythrin-conjugated antibody against CD11b (1:50, marker for macrophages; BD Biosciences PharMingen, San Diego, <http://www.bdbiosciences.com>) and Cy-5-conjugated antibody against CD45 (1:50, marker for hematopoietic cells; BD Biosciences PharMingen). To isolate MSCs (CD45⁻, CD11b⁻) and macrophages (CD45⁺, CD11b⁺) from bone marrow-derived adherent cells, cell sorting was performed (data not shown). After sorting, the isolated MSCs and macrophages were cultured in 20% FBS for 2–3 days and then used for the subsequent experiments.

RPC Line

RPCs harvested from the retina of P1 EGFP mice were isolated and maintained in culture as previously described [32]. Briefly, retinas were surgically removed. The tissue was finely minced with two scalpel blades (no. 10), these whole retina homogenates were incubated in 0.1% collagenase, and a single-cell suspension was obtained. Dissociated cells were then cultured in

DMEM/F-12 supplemented with B27 (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) and 20 ng/ml of epidermal growth factor (EGF). The neurospheres that were generated could in turn be dissociated and subcultured to generate new spheres [26, 32].

Neural Differentiation and Characterization of MSCs

To examine the differentiation of GFP-expressing MSCs in vitro, MSCs were incubated with trypsin for 3 minutes to generate a single-cell suspension. Cells (1×10^3) were plated on eight-well poly(D-lysine)/laminin-coated chamber slides (BD Biosciences, San Jose, CA, <http://www.bdbiosciences.com>) in DMEM/F-12 medium supplemented with 25 ng/ml BDNF (R&D Systems, Minneapolis, <http://www.rndsystems.com>), 40 ng/ml NGF (R&D Systems), and 20 ng/ml bFGF (R&D Systems) and were fixed with 4% paraformaldehyde (PFA) at 2 weeks after plating. The cells were blocked in 1% bovine serum albumin (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) + 0.2% Triton-100 (Sigma-Aldrich) and then incubated for 2 hours with primary antibody to Ki67 (1:100, cell proliferation marker; Vector Laboratories, Burlingame, CA, <http://www.vectorlabs.com>), nestin (1:1, immature neuronal marker; Developmental Studies Hybridoma Bank, Iowa City, IA, <http://www.uiowa.edu/~dshbwww/>), glial fibrillary acidic protein (GFAP) (1:50, astrocyte marker, Dako), MAP-2 (1:500, neuronal markers; Sigma-Aldrich), anti-protein kinase C (PKC)- α (1:200, bipolar cell marker; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, <http://www.scbt.com>), 2D4 rhodopsin (1:500, rod photoreceptor marker; kind gift of Dr. R. Molday, University of British Columbia, Vancouver, BC, Canada), and recoverin antibodies (1:1,000, photoreceptor and bipolar cell marker; Chemicon International, Temecula, CA, <http://www.chemicon.com>). After rinsing in phosphate-buffered saline (PBS [0.1 M]), samples were incubated in Cy3-conjugated species-specific IgG (1:800) for 1 hour. Samples were rinsed again and then coverslipped in polyvinyl alcohol-1,4-diazabicyclo (2.2.2) octane (PVA-Dabco) with 4',6-diamidino-2-phenylindole (DAPI) and viewed under fluorescent illumination. As a control, the untreated MSCs were fixed with 4% PFA and labeled with the same antibodies.

Differentiation and Characterization of RPCs

To examine the differentiation of GFP-expressing RPCs in vitro, RPC spheres were incubated with trypsin for 1 minute to generate a single-cell suspension. In two separate experiments, cells (1×10^3) were plated on eight-well poly(D-lysine)/laminin-coated chamber slides (BD Biosciences) in DMEM/F-12 medium supplemented either with 10% FBS or with BDNF, NGF, and bFGF (the same growth factors used in MSCs differentiation experiments [17]) and were then fixed with 4% PFA at 1 day and 2 weeks after plating. The cells were then reacted and prepared with the antibodies described for MSCs.

Morphometry of Differentiated Cells

In each of the three culture conditions (MSCs with BDNF, NGF, and bFGF; RPCs with 10% FBS; and RPCs with BDNF, NGF, and bFGF), quantitative morphometry was performed by counting positive cells from a total cell number of at least 200 cells per well in randomly selected wells, selected based on DAPI

labeling ($n = 5$). In this counting study, cells (1×10^3) were plated on eight-well poly(D-lysine)/laminin-coated chamber slides (BD Biosciences). Five of eight wells were randomly chosen (by a masked observer), and all cells in the wells were counted. Nestin-positive cells from RPCs were counted at day 1, and MSCs and RPCs positive for other markers were counted after 2 weeks of treatment.

Reverse Transcription-Polymerase Chain Reaction Analysis of MSCs

For reverse transcription-polymerase chain reaction (RT-PCR) analysis, total RNA was extracted using TRIzol (Invitrogen) from MSCs grown in the presence or absence of BDNF, NGF, and bFGF in poly(D-lysine)/laminin-coated culture dishes (BD Biosciences) and from P1 EGFP mice retina for a positive control. First-strand cDNA was prepared from total RNA by reverse transcriptase using oligo(dT) primers. To detect nestin, β -tubulin class III (BT-III; neuronal marker), Map2, GFAP, PKC- α , recoverin, and rhodopsin, primers were used as described in Table 1.

Retinal Organ Culture

Retinal organ culture was performed as previously described [36–38] with minor modifications. Briefly, eyes were enucleated from rhodopsin knockout ($\rho^{-/-}$) mice and transferred to ice-cold Hanks' balanced salt solution (Invitrogen). The retinas were separated from the retinal pigment epithelium and placed onto Millicell-CM membrane culture inserts (diameter 30 mm, pore size 0.4 μ m; Millipore Corporation, Billerica, MA, <http://www.millipore.com>) with the ganglion cell layer downward. The inserts with neural retina were placed in six-well plates containing approximately 1 ml/well of medium containing DMEM/F-12 supplemented with B27 neural supplement (Invitrogen), 2 mM L-glutamine (Sigma-Aldrich), 2,000 U of nystatin (Invitrogen), and 100 μ l/ml penicillin-streptomycin (Sigma-Aldrich). Organ cultures were maintained at 37°C, 5% CO₂ and fed every 2–3 days.

Explant Coculture

The host retinas were explanted from $\rho^{-/-}$ mice (4–8 weeks of age). Cell suspensions (1 μ l, 5×10^3 cells/ μ l) containing (a) RPCs ($n = 12$); (b) MSCs with ($n = 12$) or without ($n = 6$)

pretreatment with BDNF, NGF, and bFGF for 1 week; and (c) macrophages ($n = 6$) were added to the retinas using a pipette immediately after isolation of recipient retinas. We placed the grafted cells onto the surface of retinal explants using a 200- μ l pipette. The cells were spread out over the entire surface of the explant, confirmed by viewing under fluorescent illumination. The explanted retinas were cultured for 1 week.

Tissue Preparation

After 1 week in explant coculture, the explanted retinas were fixed with 4% PFA, followed by cryoprotection with 20% sucrose. The retinas were sectioned at 12 μ m on a cryostat. Sections were stained with neurofilament (NF) 200 (1:1,000, neuronal marker; Sigma-Aldrich), GFAP, PKC- α , recoverin, and rhodopsin antibodies as described above. After fixation with PFA and sucrose, some whole-mount retinas were stained with biotin-*Griffonia simplicifolia* (GS)-lectin (5 μ g/ml, microglia and macrophages marker; Sigma-Aldrich) for 15 minutes and NF200 antibody for 2 hours. After rinsing in PBS, samples were respectively incubated in Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, <http://www.jacksonimmuno.com>) and Cy3-conjugated species-specific IgG (1:800) for 1 hour. Samples were rinsed again and then coverslipped in PVA-Dabco and viewed under fluorescent illumination.

RESULTS

Characterization of MSCs

When grown on conventional substrates in media supplemented with 10% FBS, GFP-transgenic MSCs exhibited high levels of endogenous green fluorescence (Fig. 1A). The untreated MSCs did not express nestin, Map2, GFAP, PKC- α , recoverin, or rhodopsin (data not shown). To examine differentiation in vitro, medium without 10% FBS was supplemented with BDNF, NGF, and bFGF. After 2 weeks of culture under differentiation conditions, MSCs differentiated into cells with neuronal morphologies and neurite-like processes (Fig. 1B) and also formed spheres (Fig. 1C). Subpopulations of MSCs expressed nestin (Fig. 1D–1F), Map2 (Fig. 1G–1I), GFAP (Fig. 1J–1L), PKC- α (Fig. 1M–1O), and recoverin (Fig. 1P–1R). These markers are consistent, although not conclusive, with differentiation into

Table 1. Primers used for reverse transcription-polymerase chain reaction analysis

Genes	Primer sequences (5'–3')	Product size (bp)	Temperature (°C)
Nestin	F: AACTGGCACACCTCAAGATGT	235	60
	R: TCAAGGGTATTAGGCAAGGGG		
GFAP	F: CACGAACGAGTCCCTAGAGC	234	60
	R: ATGGTGATGCGGTTTTCTFC		
TB-III	F: ACCTCAACCACCTGGTATCG	344	60
	R: TGCTGTTCTTGCTCTGGATG		
Map2	F: CTGGACATCAGCCTCACTCA	164	60
	R: AATAGGTGCCCTGTGACCTG		
PKC- α	F: CCCATTCCAGAAGGAGATGA	212	60
	R: TTCCTGTCAGCAAGCATCAC		
Recoverin	F: ATGGGGAATAGCAAGAGCGG	179	60
	R: GAGTCCGGGAAAACTTGAATA		
Rhodopsin	F: TCACCACCCTCTACACA	216	60
	R: TGATCCAGGTGAAGACCACA		

Abbreviations: bp, base pair; F, forward; GFAP, glial fibrillary acidic protein; PKC, protein kinase C; R, reverse; TB, tubulin.

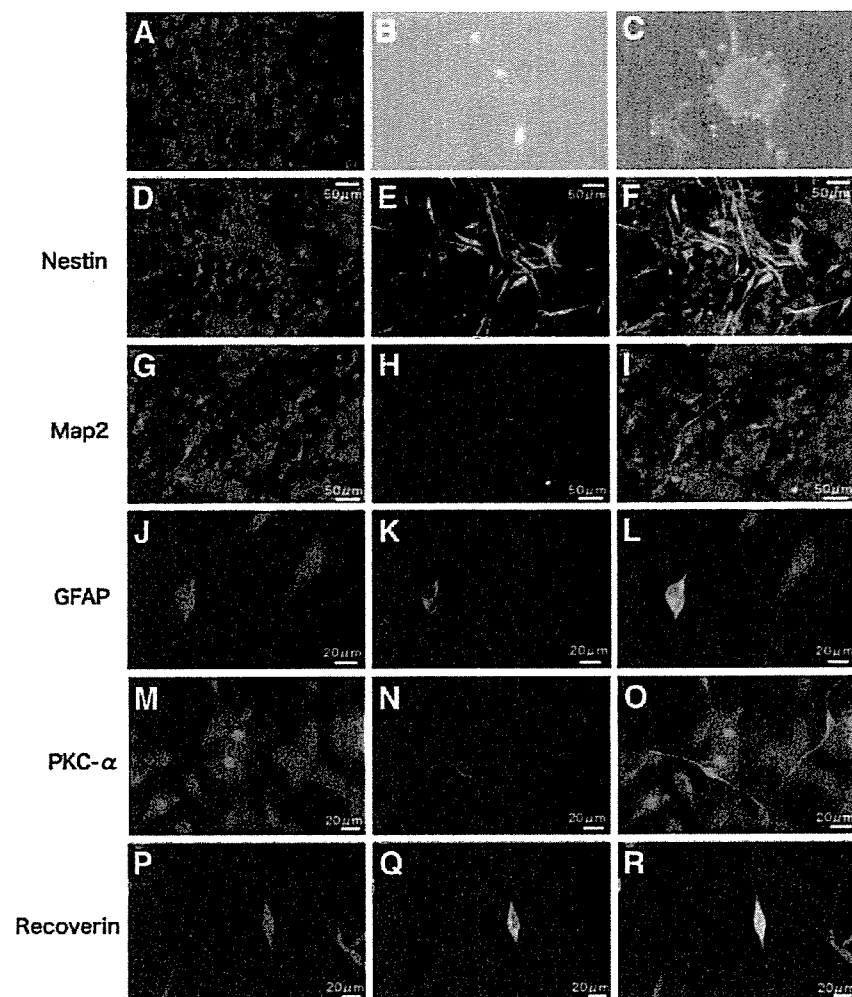


Figure 1. Differentiation and characterization of marrow stromal cell (MSCs) in vitro. Undifferentiated GFP⁺ MSCs grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, viewed under fluorescein isothiocyanate illumination (A). MSCs cultured in serum-free medium with brain-derived neurotrophic factor, nerve growth factor, and basic fibroblast growth factor for 14 days (B–R). After 2 weeks of culture under differentiation conditions, MSCs morphologically differentiated into neuronal shape and had neuronal processes (B) and also formed spheres (C). Constitutive GFP expression (D, G, J, M, P), antibody/cytokeratin-3 immunoreactivity for nestin (E), Map2 (H), GFAP (K), PKC- α (N), and recoverin (Q), and merged images (F, I, L, O, R). Abbreviations: GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; PKC, protein kinase C.

retinal neurons. Interestingly, these immunopositive cells also showed morphological evidence suggestive of differentiation into immature photoreceptors, bipolar cell types, glial cells, and neuronal cells (Fig. 1F, 1I, 1L, 1O, 1R). We could not find any rhodopsin-positive cells from treated MSCs.

Characterization of RPCs

When grown on conventional substrates in medium supplemented with EGF, GFP-transgenic RPCs exhibited high levels of endogenous green fluorescence (Fig. 2A) and maintained an undifferentiated state characterized by ubiquitous Ki67 and nestin immunoreactivity (Fig. 2B, 2C). Cells could be maintained in this state for up to 1 year or 50 passages as neurospheres. To examine differentiation in vitro, medium without EGF was supplemented with 10% FBS. After 2 weeks culture under differentiation conditions, the cells were analyzed immunocytochemically. The number of Ki67⁺ cells markedly decreased (data not shown), and subpopulations expressed GFAP (Fig. 2D), Map2 (Fig. 2E), PKC- α (Fig. 2F), recoverin (Fig. 2G), or rhodopsin (Fig. 2H). These markers are consistent with differentiation into rod photoreceptors, bipolar cells, and Muller glia, all of which are known to be born late in retinogenesis. More-

over, these immunopositive cells also showed morphological evidence suggestive of immature photoreceptor differentiation, as well as of other retinal cell types (Fig. 2D–2H).

Quantitative Evaluation of Differentiated Cell Numbers: MSCs Versus RPCs

To examine the optimal source of cells for retinal transplantation, quantitative evaluation of differentiation into neuronal and retinal cells was carried out using cell counting as previously described [39].

After 2 weeks of BDNF, NGF, and bFGF treatment, the percentages of surviving MSCs expressing nestin, Map2, GFAP, PKC- α , and recoverin were 5.55%, 3.27%, 1.42%, 3.97%, and 13.9%, respectively. The percentages of nestin-, Map2-, GFAP-, PKC- α -, recoverin-, and rhodopsin-positive cells from RPCs treated with 10% FBS were 90.5%, 15.2%, 64.4%, 12.9%, 23.6%, and 3.17%, respectively. The rates of nestin-, Map2-, GFAP-, PKC- α -, recoverin-, and rhodopsin-positive cells from RPCs treated with BDNF, NGF, and bFGF were 89.2%, 29.4%, 10.9%, 28.2%, 22.3%, and 2.25%, respectively (Fig. 3A).

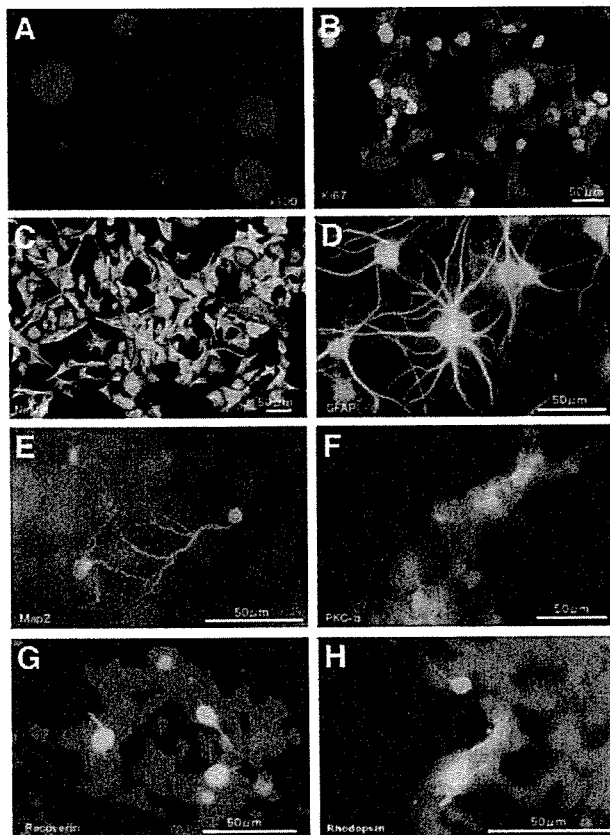


Figure 2. Differentiation and characterization of retinal progenitor cell (RPCs) in vitro. RPCs formed green fluorescent protein-positive neurospheres (A). RPCs cultured in the absence of epidermal growth factor and in the presence of 10% fetal bovine serum for 1 (B, C) or 14 (D–H) days. The cells were stained for Ki67 (B), nestin (C), GFAP (D), Map2 (E), PKC- α (F), recoverin (G), and rhodopsin (H). Abbreviations: GFAP, glial fibrillary acidic protein; MSC, marrow stromal cell; PKC, protein kinase C.

RT-PCR Analysis of BDNF, NGF, and bFGF Treatment

Semiquantitative RT-PCR analysis was carried out to determine the effect of BDNF, NGF, and bFGF on MSCs (Fig. 3B). MSCs without treatment showed only weak recoverin expression. (MSCs without treatment did not express nestin, BT-III, Map2, GFAP, PKC- α , or rhodopsin.) After 2 weeks of BDNF, NGF, and bFGF treatment, MSCs expressed nestin, BT-III, Map2, GFAP, PKC- α , and recoverin. Rhodopsin expression was not found. Recoverin expression was increased in treated MSCs.

Macrophages Differentiated into Microglia After Coculture with Explanted Retinas

After coculture with explanted rho^{-/-} mouse retinas, macrophages were viewed by fluorescent illumination at 3 and 7 days. Macrophages migrated into the retina and assumed morphology very reminiscent of microglial cells (Fig. 4A–4C). The cocultured macrophages also expressed GS-lectin, a marker of microglia (Fig. 4D–4F). There was no evidence of neuronal differentiation upon immunocytochemical and morphological analyses (data not shown).

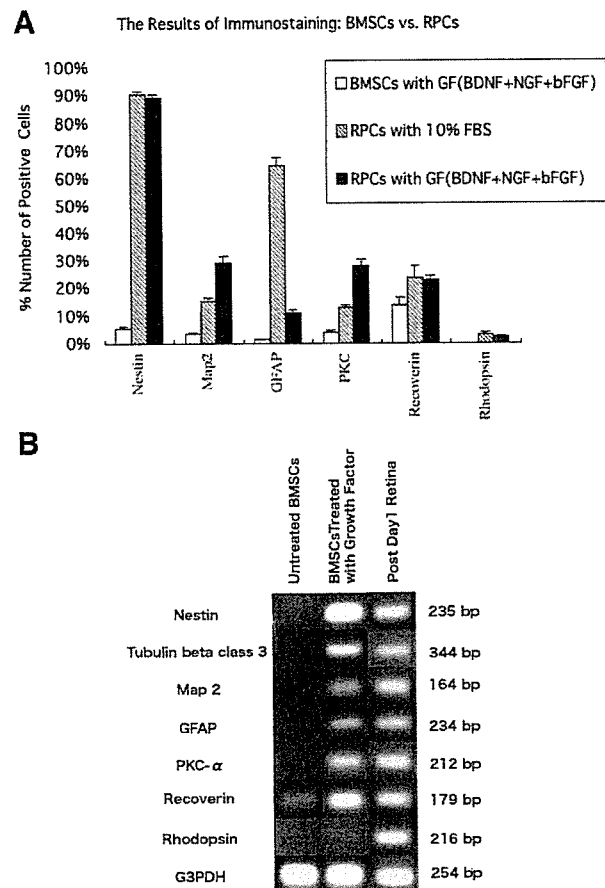


Figure 3. Comparison of BMSCs and RPCs. (A): The number of cells differentiated into retinal cells: comparison of marrow stromal cell (MSCs) and RPCs. In this study, nestin-positive cells were counted at day 1, and other markers cells were counted at 2 weeks after treatment. (B): Effect of BDNF, NGF, and bFGF on transcription of retinal cell markers. Semiquantitative reverse transcription-polymerase chain reaction analysis was carried out to determine the effect of BDNF, NGF, and bFGF on MSCs. MSCs without treatment showed only weak recoverin expression. (MSCs without treatment did not express nestin, BT-III, Map2, GFAP, PKC- α , and rhodopsin completely.) After 2 weeks of BDNF, NGF, and bFGF treatment, treated MSCs expressed nestin, BT-III, Map2, GFAP, PKC- α , and recoverin; however, rhodopsin expression was not found. Recoverin expression was increased in treated MSCs. Abbreviations: BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; BMSC, bone marrow stromal cell; bp, base pair; BT-III, β -tubulin class III; FBS, fetal bovine serum; GF, growth factor; GFAP, glial fibrillary acidic protein; NGF, nerve growth factor; PKC, protein kinase C; RPC, retinal progenitor cell.

Migration and Differentiation of MSCs

At 1 week in coculture, MSCs with and without pretreatment of BDNF, NGF, and bFGF migrated into explanted rho^{-/-} retina (Fig. 5A). MSCs without pretreatment did not show morphological or immunocytochemical evidence of neural differentiation (data not shown). On the other hand, pretreated MSCs showed morphological and immunocytochemical evidence of neuronal differentiation. Pretreated MSCs migrated into explanted retinas (Fig. 5A) and expressed NF200 (Fig. 5B–5G), GFAP (Fig. 5H–5J), PKC- α (Fig. 5K–5M), and recoverin (Fig.

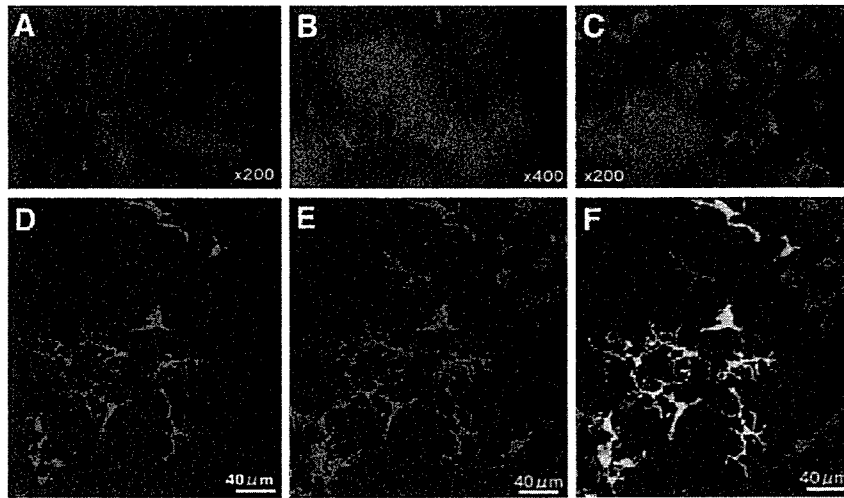


Figure 4. Macrophages differentiated into microglia after transplantation to explanted retinas. *Rho*^{-/-} mice retina at 3 (A) and 7 (B, C) days. Macrophages migrated into retina and morphologically changed their shape to that resembling microglia (A–C). Confocal (D–F) images seen at 1 week after grafting; constitutive green fluorescent protein expression (D), macrophage/microglia antibody/cytokeratin-3 immunoreactivity (E), and merged images (F).

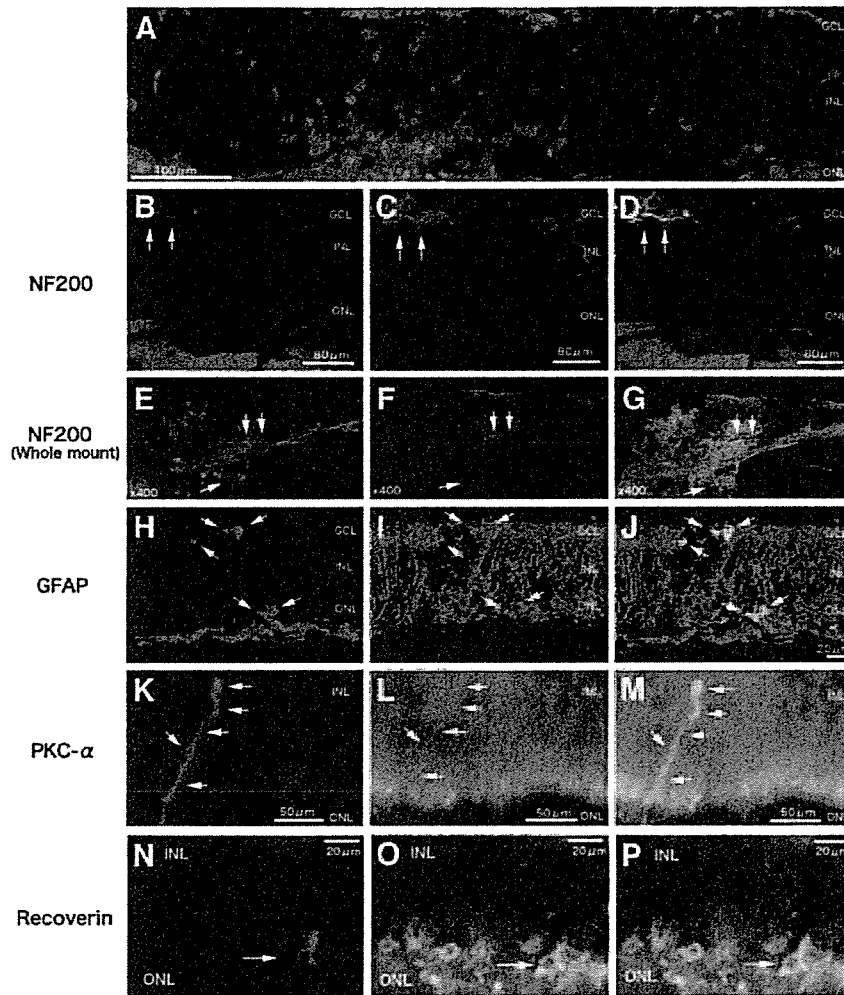


Figure 5. Migration and differentiation of pretreated marrow stromal cell (MSCs) into explanted retinas of *rho*^{-/-} mice. A large number of MSCs migrated into explanted retinas of *rho*^{-/-} mice (A). Epi-fluorescent (K–P) and confocal (B–J) images of the expression of neural and photoreceptor markers by pretreated MSCs that were grafted onto explanted retinas from *rho*^{-/-} mice, seen at 1 week after grafting; constitutive green fluorescent protein expression (B, E, H, K, N), antibody/cytokeratin-3 immunoreactivity for NF200 (C, F) (whole mount), GFAP (I), PKC- α (L), recoverin (O), and merged images (D, G, J, M, P). Abbreviations: GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; INL, inner nuclear layer; NF, neurofilament; ONL, outer nuclear layer; PKC, protein kinase C.

5N–5P). We also found morphological evidence of neuronal differentiation (Fig. 5B–5P). However, we could not find any rhodopsin-positive cells among coculture, pretreated MSCs.

Migration and Differentiation of RPCs

At 1 week in coculture, RPCs migrated into all retinal lamina adjacent to the graft after addition to the outer retina and showed

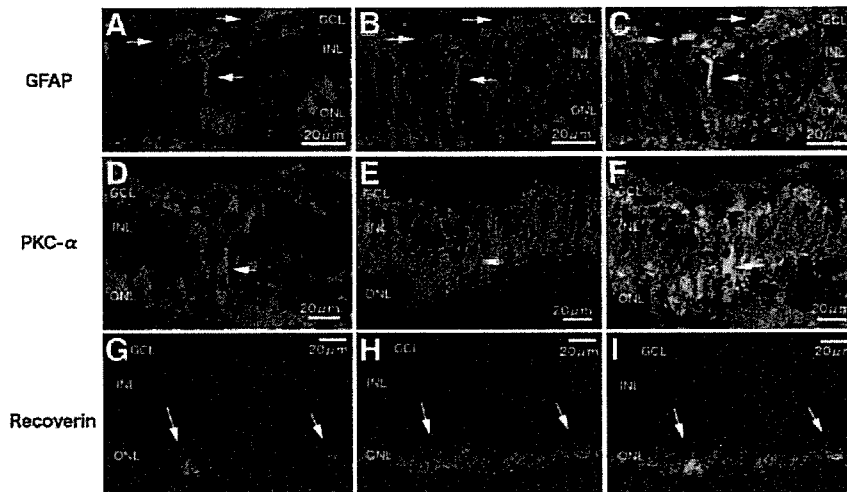


Figure 6. Migration and differentiation of pretreated retinal progenitor cells (RPCs) into explanted retinas of $\rho^{-/-}$ mice. Confocal images of the expression of neural and photoreceptor markers by RPCs grafting to explanted retinas of $\rho^{-/-}$ mice, seen at 1 week after grafting; constitutive green fluorescent protein expression (A, D, G), antibody/cytokeratin-3 immunoreactivity for GFAP (B), PKC- α (E), recoverin (H), and merged images (C, F, I). Abbreviations: GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; INL, inner nuclear layer; MSC, marrow stromal cell; ONL, outer nuclear layer; PKC, protein kinase C.

morphological evidence of neuronal differentiation (Fig. 6D–6I). GFP⁺ donor cells coexpressed a number of markers indicative of phenotypic maturation, including GFAP (Fig. 6A–6C), PKC- α (Fig. 6D–6F), and recoverin (Fig. 6G–6I). In the $\rho^{-/-}$ mice, the rod marker rhodopsin was not detected in either grafted RPCs or the host outer nuclear layer.

DISCUSSION

The results presented here demonstrate that MSCs treated with BDNF, NGF, and bFGF can differentiate into retinal cells expressing Map2, BT-III, GFAP, PKC- α , and recoverin by immunocytochemistry and RT-PCR. In the explanted retina, pretreated MSCs showed differentiation into retinal cells expressing NF200, GFAP, PKC- α , and recoverin, although nonpretreated MSCs did not show any evidence of differentiation into retinal cells. This shows that treatment with growth factors (as in our previous report [17]) is very important for neural induction of MSCs. Moreover, our data show that using growth factors promoted neuronal differentiation over glial differentiation in RPCs (Fig. 3A). In the present study, RPCs clearly showed a higher level of differentiation into retinal cells compared with MSCs. Induced MSCs expressed neuronal and glial markers and morphologically differentiated into neuron- and glia-like cells; however, RPCs showed better morphological differentiation and also expressed rhodopsin (Figs. 1, 2). Although a subpopulation of MSCs differentiated morphologically into neuronal-like cells and expressed neuronal markers, the majority remained undifferentiated both in terms of morphology and marker expression during the time course examined. The lack of rhodopsin expression *in vivo* and *in vitro* by MSCs may be an impediment to their use in photoreceptor replacement. One must be cognizant of the fact that the absence of evidence is not evidence of absence. The lack of differentiation *in vitro* indicates that the optimal conditions have yet to be determined. This is especially true in the case of RPC photoreceptor differentiation, which we have shown to be dependent upon specific conditions *in vivo*. The fact that RPCs failed to express rhodopsin after migration into explants is not surprising, considering that our previous studies found no evidence for rhodopsin among RPCs transplanted to $\rho^{-/-}$

mice *in vivo* [32]. The same study showed that RPCs expressed rhodopsin in another mouse strain with RD, the C3H mouse [32].

As with previous studies in the brain [34, 35], our results showed that macrophages migrated into explanted retina and appeared to differentiate into microglia. Although a previous report showed that microglia have potential for neuronal differentiation [33], we did not find evidence of differentiation into neuronal or glial cells in our explant study. Further studies will be needed to determine the neuronal potential of macrophages and microglia.

From a clinical perspective, MSCs are a good source for stem cell transplantation. Bone marrow cell transplantation is already an approved therapy for some kinds of hematological diseases and has the advantage of the possibility of autologous cell transplantation. Moreover, because recent reports have shown that MSCs have the capacity to modulate allogeneic cellular immunity [40, 41], MSCs may be useful for allogeneic transplantation.

Cell fusion has recently been proposed as the underlying explanation for the apparent plasticity and “transdifferentiation” of stem cells, including MSCs. This raises questions about the mechanisms of transdifferentiation *in vitro* and *in vivo* [42, 43]. Evidence against cell fusion has begun to mount; recent studies reported that MSCs can undergo transdifferentiation into various organ cell types, including neurons, without fusion [10, 44, 45]. We believe that our results cannot be attributed to cell fusion; this study shows that MSC differentiation into post-mitotic neuronal and retinal cells occurred in a controlled culture environment. Recent studies have shown that MSCs have a potential of transdifferentiation as cultured MSCs express mesodermal, endodermal, ectodermal, and germline genes, suggesting the potential to differentiate into all these cell types [46–48]. Moreover, our previous study [17], using the same methods for neuronal induction as this study, showed neuroectodermal induction, neural differentiation, and calcium uptake in response to a depolarizing stimulus from human MSCs. It has also been reported that neuroectodermal induction and electrophysiological characteristics of midbrain dopaminergic, serotonergic, and GABA-ergic neurons arise from treated MSCs [16].

CONCLUSION

The present study shows that RPCs have clear advantages over MSCs in potential retinal transplantation applications. First, no evidence was found for MSC differentiation into rod photoreceptors. Second, RPCs showed more complete differentiation into retinal cell subtypes than did MSCs, and this occurred at a significantly higher rate. Finally, we have previously reported that neuronal progenitor cells (NPCs) have inherent immune privilege, suggesting increased resistance of allogeneic NPC grafts to host rejection [49, 50]. Such findings suggest the possibility that RPCs possess immune privilege properties as well. MSCs also have significant therapeutic potential in transplantation medicine because they can be readily obtained through a well-established clinical procedure. They are relatively easy to isolate and expand

for autologous transplantation without the need for immunosuppression or the risk of rejection. In this comparison study, we submit that RPCs possess significant advantages for differentiation into retinal cells compared with MSCs.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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

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Abstract What is it that defines a bone marrow-derived chondrocyte? We attempted to identify marrow-derived cells with chondrogenic nature and immortality without transformation, defining "immortality" simply as indefinite cell division. KUM5 mesenchymal cells, a marrow stromal cell line, generated hyaline cartilage *in vivo* and exhibited enchondral ossification at a later stage after implantation. Selection of KUM5 chondroblasts based on the activity of the chondrocyte-specific cis-regulatory element of the collagen $\alpha 2(XI)$ 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 $\alpha 1$, Sox9, and cartilage oligomeric matrix protein at an extremely high level, as did KUM5 cells. After cultured OP9 micromasses exposed to TGF- $\beta 3$ and BMP2 were implanted in mice, they produced abundant metachromatic matrix with the toluidine blue stain and formed type II collagen-positive hyaline cartilage within 2 weeks *in vivo*. Hierarchical clustering and principal component analysis based on microarray data of the expression of cell surface markers and cell-type-specific genes resulted in grouping of KUM5 and OP9 cells into the same subcategory of "chondroblast," that is, a distinct cell type group. We here show that these two cell lines exhibit the unique characteristics of hyaline cartilage formation and enchondral ossification *in vitro* and *in vivo*. *J. Cell. Biochem.* 9999: 1–15, 2006. © 2006 Wiley-Liss, Inc.

Key words: Hyaline cartilage; chondroblasts; enchondral^{Q2}ossification; bioinformatics; gene chip

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

In adults, chondrocytes maintain the extracellular matrix that gives cartilage its unique mechanical properties. Chondrocytes are long-lived and the development of new cells that are capable of producing cartilage *de novo* (i.e., chondroblasts) is not a normal part of adult cartilage physiology. A better understanding of the molecular mechanisms that regulate post-natal 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 μ M dexamethasone, 1 mM sodium pyruvate, 0.17 mM ascorbic acid-2-phosphate, 0.35 mM proline, 6.25 μ g/ml bovine insulin, 6.25 μ g/ml transferrin, 6.25 μ g/ml selenous acid, 5.33 μ g/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- β 3 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% CO₂.

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

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

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

Flow Cytometric Analysis

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

Preparation and Transfection of Plasmid

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

Isolation of KUM5 Chondroblast

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

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 (Daichi 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 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)- β and bone morphogenetic protein (BMP) are involved in chondrogenesis and osteogenesis [Fujii et al., 1999; Maeda et al., 2004], we used TGF- β 3 and BMPs on KUM5 culture. Exposure of the cells to TGF- β 3 augmented the metachromatic toluidine blue staining in the KUM5-micromass (Fig. 1A,B). BMP2 dramatically enhanced this TGF- β 3-induced differentiation, that is, caused stronger metachromatic staining and enlarged metachromatic area. To determine the effect of other cytokines on the TGF- β 3-induced chondrogenic differentiation, we added BMP4, BMP6, BMP7, PDGF, or hyaluronic acid to the CM supplemented with TGF- β 3. BMP4, BMP6, and BMP7 enhanced the TGF- β 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- β 3 and BMP4-induced differentiation, as determined by toluidine blue staining (Fig. 1C_e,D_e). 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

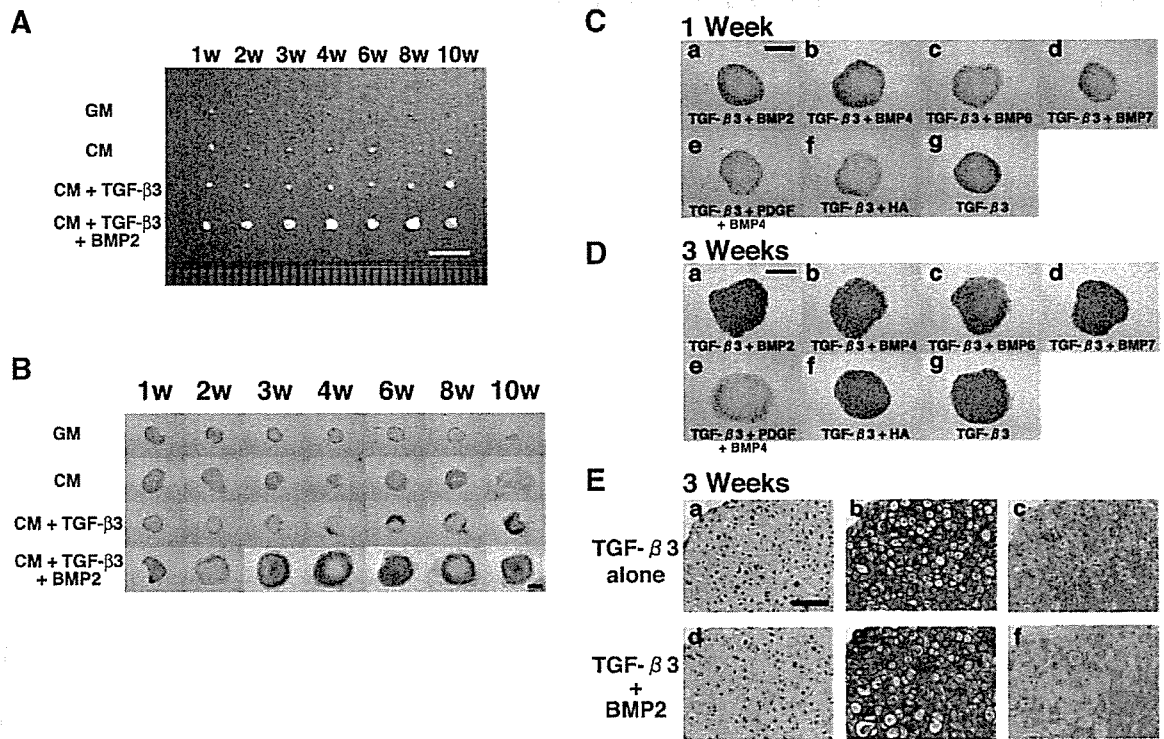


Fig. 1. In vitro chondrogenesis of KUM5 cells. **A,B:** Time-course analysis of growth factors-induced matrix production in KUM5 cells. Macroscopic view of KUM5 chondrogenic nodules which were generated after pellet culture for 1–10 weeks in the GM or the CM supplemented with or without growth factors as indicated (see “Cell culture” Section in Materials and Methods) (A) and Toluidine blue stained section (B). BMP2 drastically enhanced TGF- β 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- β 3 (a–c), or TGF- β 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 μ m (B, C, D), 100 μ 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 α 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 α 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- β 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- β 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

COLOR

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

Probe set	Genbank	Description	9-15c				KUSA-O				KUSA-A1				H-1/A				OP9				KUM5				Symbol
			Flags		Raw		Flags		Raw		Flags		Raw		Flags		Raw		Flags		Raw		Flags		Raw		
1450567_a_at	NM_031163	Procollagen, type II, alpha 1	A	28	A	187	A	98	A	223	A	226	A	46	A	1,7390	P	85	A	110	A	110	P	679	P	679	Col2a1
1428571_at	AK004383	Procollagen, type IX, alpha 1	P	86	P	116	P	99	P	99	P	57	P	57	P	132	P	192	P	186,640	P	186,640	P	190	P	190	Col9a1
1422253_at	NM_009925	Procollagen, type X, alpha 1	A	13	A	20	A	15	A	104	A	104	A	104	A	218	A	218	A	362	A	362	P	270	P	270	Col10a1
1418599_at	BB336814	Procollagen, type XI, alpha 1	A	69	A	682	A	4,284	A	5,009	A	5,009	A	5,009	A	2,551	A	2,551	A	320	A	320	P	518	P	518	Col11a1
1419527_at	NM_016685	Cartilage oligomeric matrix protein	A	120	A	111	A	64	A	167	A	167	A	167	A	1,892	P	1,892	M	172	M	172	M	172	M	172	Comp
1449368_at	NM_007833	Decorin	A	176	A	36	A	223	A	223	A	226	A	226	A	85	A	85	A	110	A	110	A	110	A	110	Den
1416405_at	BC019902	Biglycan	P	12,600	P	11,817	P	11,011	P	12,932	P	12,932	P	12,932	P	21,954	P	21,954	P	18,640	P	18,640	P	18,640	P	18,640	Bgn
1449827_at	NM_007424	Aggrecan 1	A	70	A	118	A	105	A	105	A	127	A	127	A	94	A	94	A	167	A	167	A	167	A	167	Agc1
1416321_s_at	BC019775	Proline arginine-rich end leucine-rich repeat	P	196	P	59	P	899	P	899	P	1,092	P	1,092	P	2,169	P	2,169	P	352	P	352	P	352	P	352	Prelp
1415939_at	NM_021355	Fibromodulin	P	388	M	359	P	11,642	P	16,626	P	16,626	P	16,626	P	108	A	108	A	320	A	320	A	320	A	320	Fmod
1418745_at	NM_012050	Osteomodulin	P	288	A	50	P	1,849	P	1,849	P	2,185	P	2,185	P	347	P	347	P	743	P	743	P	743	P	743	Omd
1415943_at	BC010560	Syndecan 1	P	1,182	P	2,449	P	1,358	P	1,607	P	1,607	P	1,607	P	4,704	P	4,704	P	1,799	P	1,799	P	1,799	P	1,799	Sdc1
1417012_at	A1266824	Syndecan 2	P	752	P	1,256	P	2,940	P	4,398	P	4,398	P	4,398	P	605	P	605	P	2,089	P	2,089	P	2,089	P	2,089	Sdc2
1420853_at	NM_011520	Syndecan 3	A	382	A	547	A	680	A	902	A	902	A	902	A	385	A	385	A	762	A	762	A	762	A	762	Sdc3
1417654_at	NM_011521	Syndecan 4	P	306	P	281	P	244	P	342	P	342	P	342	P	305	P	305	P	320	P	320	P	320	P	320	Sdc4
1424950_at	B1077717	SRY-box containing gene 9	P	120	A	5	P	59	A	97	A	97	A	97	A	1,944	P	1,944	P	183	P	183	P	183	P	183	Sox9
1420895_at	BM248342	Transforming growth factor, beta receptor I	P	780	P	703	P	657	P	862	P	862	P	862	P	1,595	P	1,595	P	802	P	802	P	802	P	802	Tgfb1
1425444_a_at	S69114	Transforming growth factor, beta receptor II	P	552	P	746	P	1,068	P	1,189	P	1,189	P	1,189	P	865	P	865	P	1,133	P	1,133	P	1,133	P	1,133	Tgfb2
1425620_at	AF039601	Transforming growth factor, beta receptor III	P	448	A	328	A	275	A	313	A	313	A	313	A	565	P	565	P	1,015	P	1,015	P	1,015	P	1,015	Tgfb3
1422912_at	NM_007554	Bone morphogenetic protein 4	P	1,048	P	646	P	6,470	P	7,266	P	7,266	P	7,266	P	1,736	P	1,736	P	2,890	P	2,890	P	2,890	P	2,890	Bmp4
1425492_at	BM248248	Bone morphogenetic protein 4	P	1,486	P	815	P	1,089	P	1,164	P	1,164	P	1,164	P	1,189	P	1,189	P	1,123	P	1,123	P	1,123	P	1,123	Bmp4
1420847_a_at	NM_010207	Fibroblast growth factor receptor 2	P	833	P	656	P	1,664	P	1,998	P	1,998	P	1,998	P	992	P	992	P	3,598	P	3,598	P	3,598	P	3,598	Fgfr2
1417271_a_at	NM_007932	Endoglin	A	247	A	187	A	40	A	115	A	115	A	115	A	222	A	222	A	1,371	A	1,371	A	1,371	A	1,371	Eng
1451314_a_at	L08431	Vascular cell adhesion molecule 1	P	462	P	39	A	28	A	92	A	92	A	92	A	512	P	512	P	583	P	583	P	583	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.

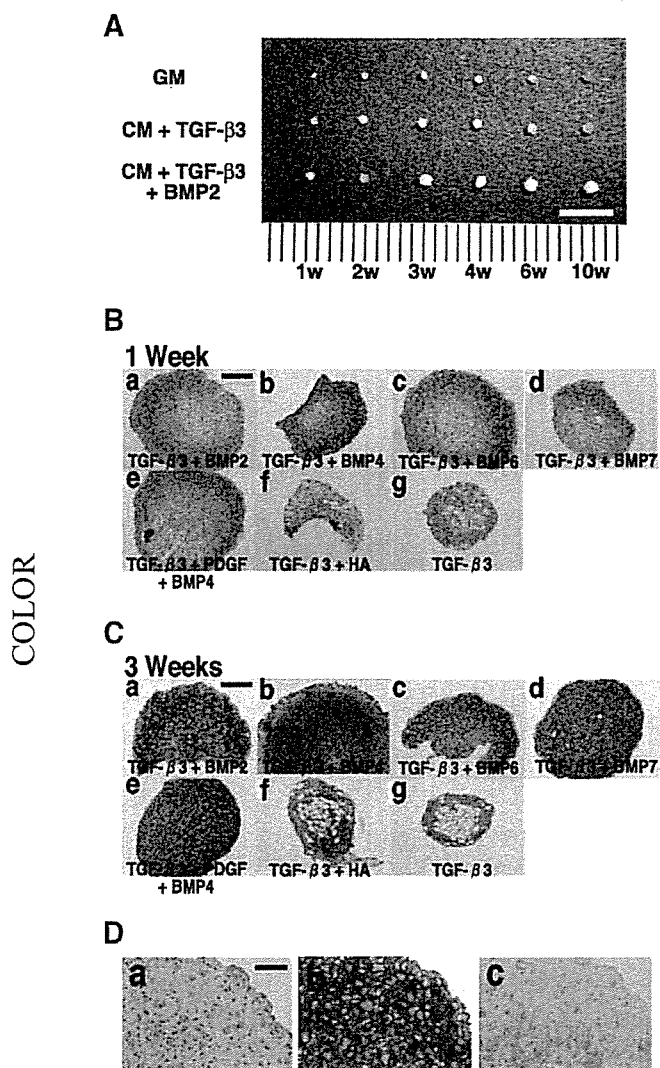


Fig. 2. In vitro chondrogenesis of OP9 cells. **A:** Time-course analysis of growth factors-induced matrix production in OP9 cells. Macroscopic view of OP9 chondrogenic nodules which were generated after pellet culture for 1–10 weeks in the GM or the CM supplemented with growth factors as indicated. BMP2 drastically enhanced TGF- β 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- β 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 μ m (**B,C**), 100 μ m (**D**).

with procedures analogous to those used for KUM5 cells. BMP4, BMP6, and BMP7 enhanced the TGF- β 3-induced differentiation in a manner similar to BMP2 (Fig. 2B,C). Unlike its effect in KUM5 cells, PDGF did not inhibit TGF- β 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 α), and negative for c-kit (CD117), Flk-1, CD31 (PECAM-1), CD34, CD144 (VE-cadherin), CD45 (leukocyte common antigen), CD49d (integrin α 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

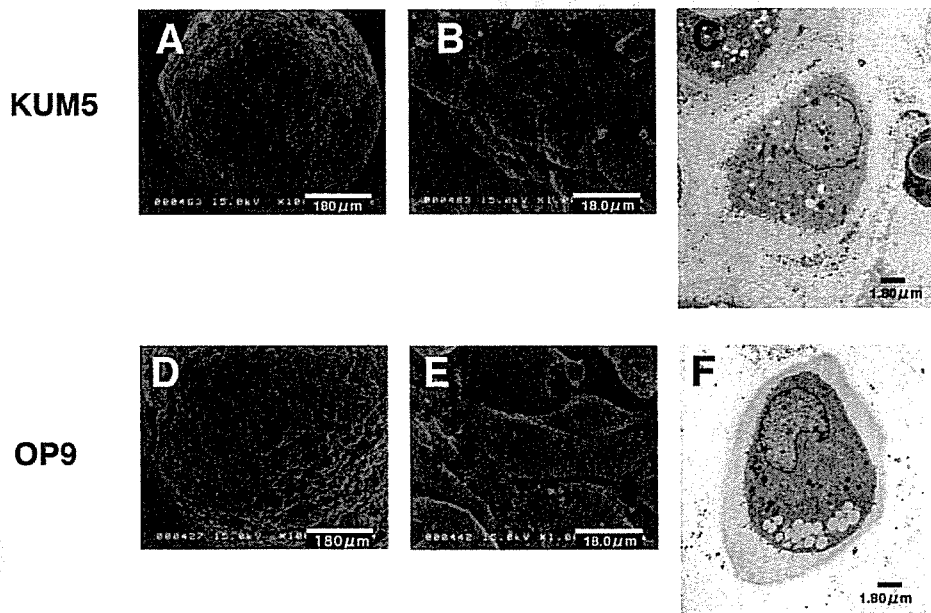


Fig. 3. Ultrastructural analysis of the in vitro chondrogenic micromass. Micromasses of KUM5 cells (A–C) and OP9 cells (D–F) were generated by culturing in the CM supplemented with TGF- β 3 for 3 weeks. (A,B,D,E), SEM; (C,F), TEM.

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 α 1(II) procollagen genes, and OP9 cells expressed cartilage-specific and -associated genes such as the α 1(II) procollagen, α 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- β 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).

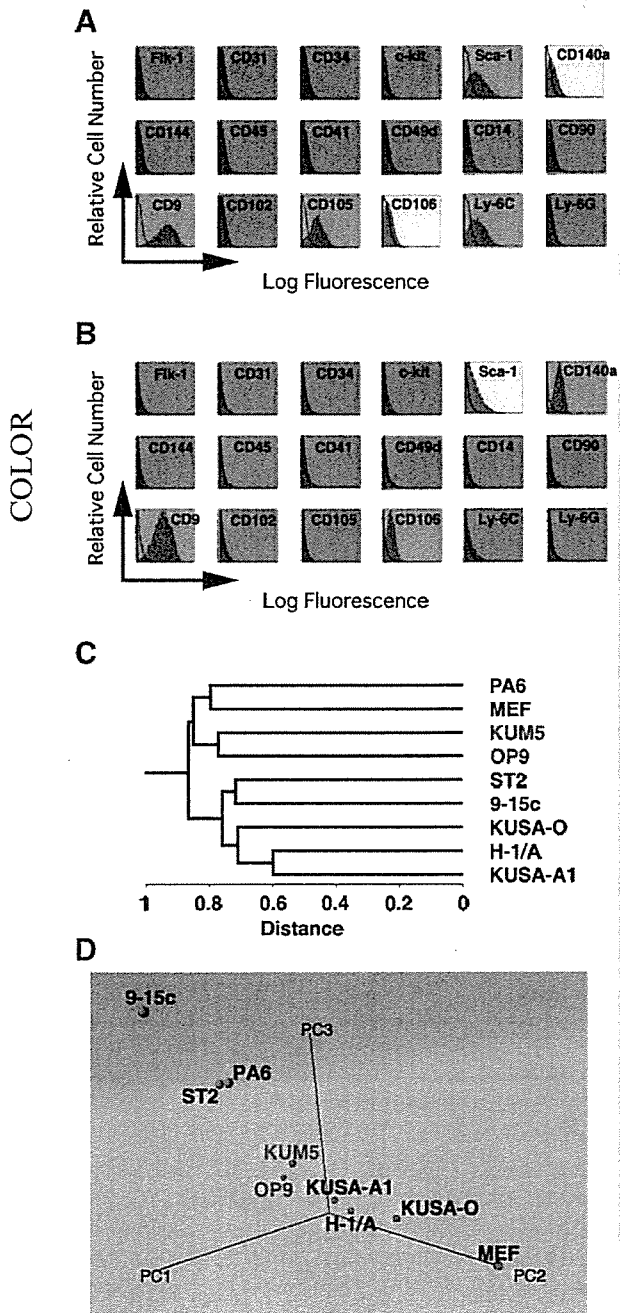


Fig. 4. Expression profiling, hierarchical clustering, and principal component analysis (PCA) of cell surface markers in marrow stromal cells. A,B: Flow cytometric analysis of cell surface markers in KUM5 cells (A) and OP9 cells (B). Red and pink colors indicate positive and marginal expression, respectively,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

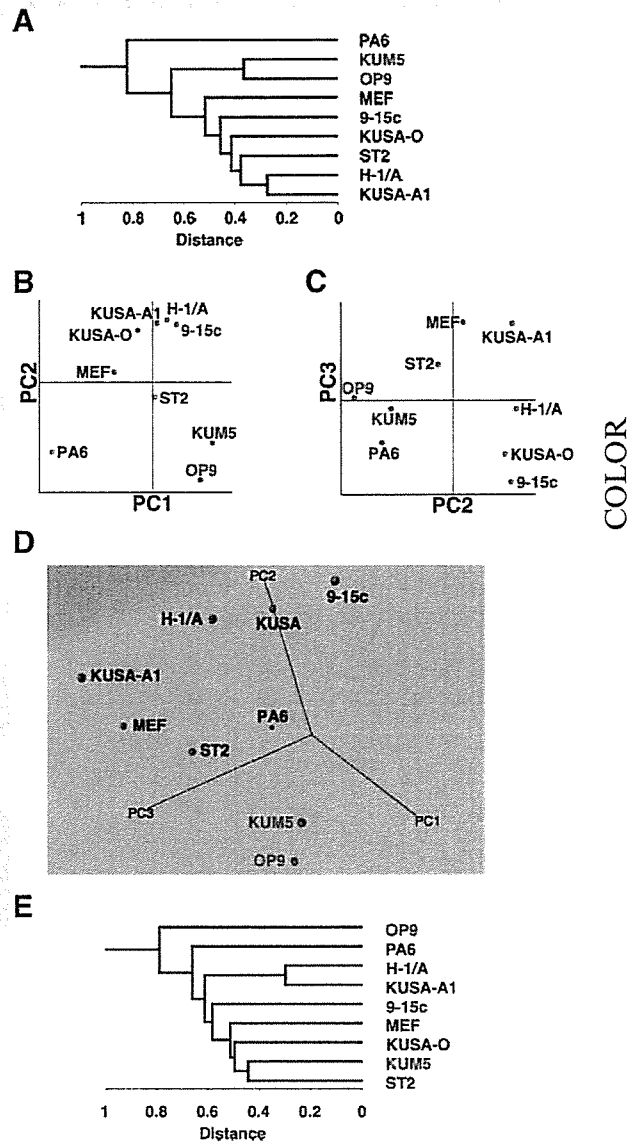


Fig. 5. Hierarchical clustering and PCA of fat- and cartilage-associated gene expression in marrow stromal cells. A. Dendrogram revealing clustering profile of 9 marrow stromal cells using 34 fat-associated genes (Supplementary Table II). B–D. PCA on expression levels of 34 fat-associated genes. The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

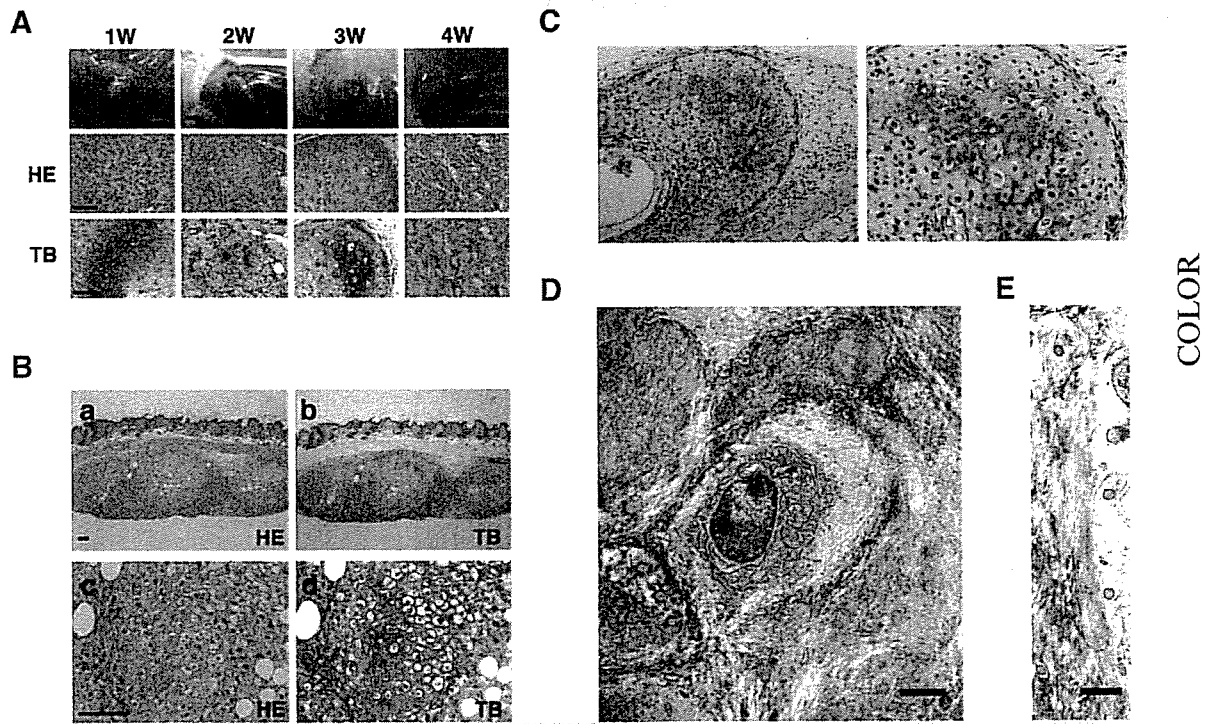


Fig. 6. In vivo chondrogenesis of KUM5 cells. **A:** Macroscopic view (top), hematoxylin and eosin stain (HE) (middle) and toluidine blue stain (TB) (bottom) analysis at 1, 2, 3, and 4 week (w)-cultivation in vivo after direct injection of KUM5 cells. **B:** KUM5 chondrogenic nodules, that were generated after pellet culture for 7 days in the CM supplemented with TGF- β 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 μ m (A, middle and bottom row), 100 μ m (B), 2 μ m (D), 1 μ 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 α 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 α 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 micromasses with toluidine blue staining in vitro (Fig. 9B). Direct injection of the cells resulted in the cartilage formation within 1 week and obvious enchondral ossification at the periphery of the cartilage at 4 weeks (Fig. 9G). Again, ultrastructural analysis revealed that KUM5 chondrocytes implanted into the subcutaneous