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In Vitro Chondrogenesis of Human Synovium-Derived Mesenchymal Stem Cells: Optimal Condition and Comparison With Bone Marrow-Derived Cells

Shinichi Shirasawa,[1] Ichiro Sekiya,[1]* Yusuke Sakaguchi,[1] Kazuyoshi Yagishita,[2] Shizuko Ichinose,[3] and Takeshi Muneta[1,4]

[1]Section of Orthopaedic Surgery, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan
[2]Department of Orthopaedic Surgery, Tokyo Medical and Dental University, Tokyo, Japan
[3]Instrumental Analysis Research Center, Tokyo Medical and Dental University, Tokyo, Japan
[4]Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental University, Tokyo, Japan

Abstract There are increasing reports that mesenchymal stem cells (MSCs) are present in various tissues other than bone marrow, including synovium. Here we investigated the optimal conditions for in vitro chondrogenesis of human synovium-derived MSCs and compared these cells with bone marrow-derived MSCs, especially in terms of their chondrogenesis potential. Synovium and bone marrow were harvested from six donors during knee operations for ligament injuries. Digested synovium cells or nucleated cells from bone marrow were expanded clonally. A pellet culture system was used for chondrogenesis, and the best combination of up to three cytokines of the seven assessed. Synovium-derived MSCs plated at a lower density expanded more rapidly. Contrary to previous reports, a combination of TGFβ and dexamethasone was not sufficient to induce chondrogenesis. However, addition of BMP2 to TGFβ and dexamethasone dramatically increased cartilage pellet size and the synthesis of cartilage matrix. The cartilage pellets were also analyzed by electron microscopy and immunohistology. DNA content per pellet decreased during chondrogenesis, indicating the pellet increased its size through the accumulation of newly synthesized extracellular matrix. Sequential chondrogenic gene expression was demonstrated by RT-PCR. Synovium-derived MSCs looked similar to the bone marrow-derived MSCs in their surface epitopes and proliferation potential; however, cartilage pellets from synovium were significantly larger than those from bone marrow in patient-matched comparisons. We demonstrated that the combination of TGFβ, dexamethasone, and BMP2 was optimal for in vitro chondrogenesis of synovium-derived MSCs and that the synovium-derived MSCs have a greater chondrogenesis potential than bone marrow-derived MSCs. J. Cell. Biochem. 97: 84–97, 2006. © 2005 Wiley-Liss, Inc.

Key words: synovium; bone marrow; mesenchymal stem cells; proliferation; chondrogenesis; bone morphogenetic protein

*Correspondence to: Ichiro Sekiya, MD, PhD, Section of Orthopaedic Surgery, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail: sekiya.orj@tmd.ac.jp

Articular cartilage has a limited capacity for healing after trauma. Numerous clinical studies have explored various methods of promoting cartilage repair. One of the more promising procedures is the transplantation of autologous cultured chondrocytes [Brittberg et al., 1994]; however, surgical invasion to normal articular cartilage and limited expansion of the chondrocytes are a problem, leading to difficulties in repairing large defects. The tissue engineering of autologous cartilage in vitro with other cell sources for transplantation would be a future alternative.

Mesenchymal stem cells (MSCs) have the potential to differentiate into lineages of mesenchymal tissues including cartilage. Bone marrow-derived MSCs have been investigated

intensively; however, there are increasing reports that MSCs are present in various tissues, one of which is synovium.

Synovium has an ability to heal sufficiently after subtotal surgical synovectomy, indicating its high regenerative potential. Synovial chondromatosis is observed in a pathological state, suggesting a high chondrogenesis potential of synovium. These corroborative evidences suggest that synovium is an excellent source of MSCs for cartilage regeneration.

In vitro chondrogenesis of MSCs with a three-dimensional structure was first described by Johnstone et al. who used pellet culture system with bone marrow-derived MSCs [Johnstone et al., 1998]. Induction of the cartilage phenotype from the undifferentiated progenitors requires various cytokines and hormones [Johnstone et al., 1998; Hanada et al., 2001; Dragoo et al., 2003b]; however, the induction regimen with the greatest chondrogenic potential of MSCs derived from synovium has not been investigated.

We hypothesized that synovium-derived MSC could be a powerful cell source for cartilage regeneration with efficient methods that can induce their chondrogenic differentiation. The main purpose of this study was to investigate the optimal condition for in vitro chondrogenesis of MSCs derived from synovium. Also, we analyzed this system extensively to show its validity as a method of inducing cartilage formation. Furthermore, we compared MSCs from synovium with those from bone marrow, and in particular their chondrogenesis potential under the condition obtained here.

MATERIALS AND METHODS

Isolation of Synovium- and Bone Marrow-Derived Cells

The study was approved by an Institutional Review Board and informed consents were obtained from all study subjects. Human synovium and bone marrow were harvested from six donors (four males and two females, 22 ± 4 years old) under spinal anesthesia during knee operations to reconstruct the anterior cruciate ligament after a ligament injury. Synovium was minced, digested with 3 mg/ml Collagenase D (Roche Diagnostics, Mannheim, Germany) for 3 h, and separated with a 70 mm nylon filter (Becton-Dickinson, Franklin Lakes, NJ). Nucleated cells from bone marrow were isolated with a density gradient (Ficoll-Paque, Pharmacia Biosystems, Uppsala, Sweden). The cells were plated at a clonal density in a 60-cm^2 culture dish (Nalge Nunc International, Rochester, NY) in 10 ml complete culture medium: αMEM containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA; lot selected for rapid growth of MSCs derived from bone marrow), 100 units/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 250 ng/ml amphotericin B (Invitrogen). Then the cells were expanded for 14 days (Passage 0) and replated at 50 cells/cm^2 in a 145-cm^2 culture dish, incubated for 14 days, and harvested (Passage 1). To cryopreserve the cells, they were resuspended at a concentration of 1×10^6 cells per ml in αMEM with 5% dimethylsulfoxide (Wako, Osaka, Japan) and 20% FBS (Passage 1 cells). Aliquots of 1 ml were slowly frozen and cryopreserved in liquid nitrogen. A frozen vial of the cells was thawed, plated in a 60-cm^2 culture dish, and incubated for 4 days in the recovery plate (passage 2).

Adipogenesis and Calcification

For adipogenic differentiation, one hundred cells were plated in a 60 cm^2 dish and cultured in complete culture medium for 7 days. The medium was replated with adipogenic medium, which was complete culture medium supplemented with 0.5 μM dexamethasone (Sigma, St. Louis, MO), 0.5 μM isobutylmethylxanthine (Sigma), and 50 μM indomethacin (Sigma), and the cells were cultured for an additional 21 days. The adipogenic cultures were fixed in 10% formalin for over 1 h and stained with fresh Oil Red-O solution (Sigma) for 2 h [Sekiya et al., 2002b]. For calcification, 100 cells were plated in a 60 cm^2-dish and cultured in complete culture medium for 14 days. They were then incubated in calcification medium, which was complete culture medium supplemented with 10 nM dexamethasone, 200 μM ascorbic acid, and 10 mM β-glycerolphosphate for 21 days. Cultures were stained with 40 mM Alizarin Red (pH 4.1; Sigma) [Sakaguchi et al., 2004].

Optimal Combination of Cytokines for In Vitro Chondrogenesis

Two hundred thousand cells were centrifuged in 15-ml polypropylene tube and the cell pellets were cultured at 37°C with 5% CO$_2$ in chondrogenic basal media that consisted of DMEM

supplemented with 50 µg/ml ascorbate-2-phosphate (Sigma), 40 µg/ml proline (Sigma), 100 µg/ml pyruvate (Sigma), and 50 mg/ml ITS+ Premix (Becton-Deckinson: 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 µg/ml selenious acid, 1.25 mg/ml bovine serum albumin, 5.35 mg/ml linoleic acid). For the identification of optimal conditions, we added 10 ng/ml transforming growth factor β-3 (TGFβ3), 100 nM dexamethasone (DEX), 1 nM retinoic acid (RA), 300 ng/ml insulin like growth factor-1 (IGF1), 10 µg/ml insulin, 2 ng/ml fibroblast growth factor-2 (FGF2), and 500 ng/ml BMP2 independently, binary, or trinary. To investigate the optimal initial cell numbers for pellet culture, 200×10^3, 400×10^3, 800×10^3, $1,600 \times 10^3$, $2,000 \times 10^3$ cells were centrifuged in 15-ml polypropylene tube and cultured with chondrogenic medium that consisted of DMEM supplemented with TGFβ3, DEX, and BMP2. The cell pellets were embedded in paraffin, cut into 5 µm sections, and stained with Toluidine Blue.

Transmission Electron Microscopy

Pellets were fixed with 2.5% glutaraldehyde in 0.1 M PBS for 2 h, washed overnight at 4°C in the same buffer, and post-fixed with 1% OsO4 buffered with 0.1 M PBS for 2 h. The pellets were dehydrated in a graded series of ethanol, and embedded in Epon 812. 100 nm-ultrathin sections were double-stained with Uranyl Acetate and Lead Citrate, and examined with a transmission electron microscope H-7100 (HITACHI, Hitachinaka, Japan).

Immunohistochemistry

Five micrometer thick sections were deparaffinized and treated with 20 µg/ml protenase K (DakoCytomation, Copenhagen, Denmark) for 15 min. After washing with PBS, endogenous peroxidase was blocked in 3% H_2O_2/MeOH for 5 min. The sections were washed again with PBS and treated with blocking reagent (VECTOR, Burlingame, CA). Primary Anti-human type II Collagen IgG (Fujiyakuhin, Toyama, Japan) were applied to the sections and incubated at room temperature for 1 h, rinsed again with PBS repeatedly, and then the sections were incubated with biotinylated secondary antibody (VECTOR) for 30 min. Antibodies were visualized by treating with Avidin-Biotinylated enzyme complex (VECTOR), then peroxidase substrate solution for 2 min. They were counter stained with Mayer's Hematoxylin for 5 min.

RT-PCR

Pellets were digested with 3 mg/ml Collagenase D for about 3 h at 37°C. Total RNA was extracted from the pellets using the RNAqueous kit (Ambion, Austin, TX). RNA was converted to cDNA and amplified by the Titan One Tube RT-PCR System (Roche Diagnostics), according to the manufacturer's recommendations. RT was performed by a 30-min incubation at 50°C, followed by a 2-min incubation at 94°C to inactivate the reverse transcriptase. PCR amplification of the resulting cDNAs was performed by 35 cycles of 94°C for 30 s, 58°C for 45 s, and 68°C for 45 s; this last step at 68°C was increased by 5 s every cycle after 10 cycles. The reaction products were resolved by electrophoresis on a 1% agarose gel and visualized with ethidium bromide. PCR primers are shown in Table I.

Real-Time PCR Analysis

Quantitative PCRs were performed in a LightCycler instrument (Roche Diagnostics). cDNA was synthesized with oligo-dT primer from total RNA using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. cDNAs were mixed with LightCycler FastStart DNAMaster SYBR Green I and LightCycler Primer Set: Col2a1 and β-actin (Search-LC, Heidelberg, Germany). After an initial denaturation step (95°C for 10 min), amplification was performed for 35 cycles (95°C for 1 s, 56°C for 30 s, and 72°C for 30 s).

DNA Assays

Eight hundred thousand synovium-derived MSCs were used for single chondrogenic pellet. DNA was prepared from 2.4 million undifferentiated synovium-derived MSCs as day 0, from 4 pellets at 7 days, from 6 pellets at 14, and 21 days. The pellets were digested with 3 mg/ml collagenase for 3 h at 37°C. Then, the cells were digested with 1 mg/ml protease K (Sigma) at 50°C for 24 h. DNA was extracted with phenol/chloroform/isoamyl alcohol. The concentrations of DNA were calculated from the absorbance at 260 nm measured by a spectrophotometer (Beckman Coulter, Fullerton, CA).

TABLE I. Primers used for RT-PCR Analysis and Expected Sizes of PCR Products

	Forward	Reverse	Size, bp
COL2A1	TTCAGCTATGGAGATGACAATC	AGAGTCCTAGAGTGACTGAG	472
COL10A1	CACCAGGCATTCCAGGATTCC	AGGTTTGTTGGTCTGATAGCTC	825
Link Protein	CCTATGATGAAGCGGTGC	TTGTGCTTGTGGAACCTG	618
SOX4	CAAACCAACAATGCCGAGAAC	CTCTTTTTCTGCGCCGGTTTG	584
SOX5	AGCCAGAGTTAGCACAATAGG	CATGATTGCCTTGTATTC	619
SOX6	ACTGTGGCTGAAGCACGAGTC	TCCGCCATCTGTCTTCATACC	562
SOX9	GAACGCACATCAAGACGGAG	TCTCGTTGATTTCGCTGCTC	631
BMP2	CAGAGACCCACCCCCAGCA	CTGTTTGTGTTTGGCTTGAC	688
BMP4	ATTCCTGGTAACCGAATGCTG	TTCGTGGTGGAAGCTCCTCAC	366
BMPR1B	ACCACCTAACACTCGAGTTG	GCAGGACGATGTTCAAGGCT	527
BMPR2	AGACATGCCTTCCGTTTGGAG	CTAGATCAAGAGAGGGTTCGG	872
RUNX2	AGGCAGTTCCCAAGCATTTC	GGTCGCCAAACAGATTCATC	440
Osterix	TGCAGCAAATTTGGTGGCTC	AGCAAAGTCAGATGGGTAGG	540
BSP	CAGTAGTGACTCATCCGAAG	GGAGAGGTTGTTGTCTTCGA	507
Osteocalcin	ACCGAGACACCATGAGAGCC	GAAGAGGAAAGAAGGGTGCC	383
β-Actin	CCAAGGCCAACCGCGAGAAGATGAC	AGGGTACATGGTGGTGCCGCCAGAC	587

BMPR1B, bone morphogenetic protein receptor type 1B; BMPR2, bone morphogenetic protein receptor type 2; RUNX2, Runt-related transcription factor 2; BSP, bone sialoprotein.

Analysis of Surface Epitopes

Synovium-derived MSCs and bone marrow-derived MSCs at Passage 0 were harvested 14 days after plating. One million cells were suspended in 500 μl PBS containing 20 μg/ml of antibody. After incubation for 30 min at 4°C, the cells were washed with PBS, and suspended in 1 ml of PBS for the analysis. Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-coupled antibodies against CD34, CD45, CD 90, CD147,and the anti-nerve growth factor receptor antibody (NGFR) were from Becton Dickinson; CD31, CD44, CD54, CD106, CD117 were from eBioscience (San Diego, CA); CD105, CD166 were from Ancell Corporation (Bayport, MN), Flk-1 (VEGF Receptor 2) was from Genzyme-Techne (Minneapolis, MN), STRO-1 was from VECTOR, and CD10 was from DakoCytomation. For isotype control, FITC- or PE-coupled nonspecific mouse IgG (Becton Dickinson) was substituted for the primary antibody. Cell fluorescence was evaluated by flow cytometry using a FACSCalibur instrument (Becton Dickinson) and data analyzed using CellQuest software (Becton Dickinson). Positive expression was defined as the level of fluorescence greater than 99% of the corresponding isotype-matched control antibodies.

RESULTS

Synovium-Derived Cells as Mesenchymal Stem Cells

To evaluate proliferation ability, Passage 2 synovium-derived cells were plated at densities of 10, 100, or 1,000 cells/cm^2 and cultured for 14 days with the complete culture medium. The synovium-derived cells increased very rapidly and reached approximately 800-fold, 140-fold, 17-fold in 14 days, respectively (Fig. 1A). This indicates that the proliferation of synovium-derived cells is affected by the initial cell density, and a lower cell density is recommended to maximize cell proliferation. The cells plated at 10 cells/cm^2 formed single cell-derived colonies and most of the cells kept their fibroblastic spindle shape (Fig. 1B-i). However, large and flat cells were also observed (Fig. 1B-ii). Synovium-derived cells differentiated into adipocytes (Fig. 1C-i) and calcified in vitro (Fig. 1C-ii) when cultured in the appropriate medium. These results indicate that synovium-derived cells have characteristics of MSCs.

Effect of Cytokines on Chondrogenesis of Synovium-derived MSCs

For in vitro chondrogenesis of synovium-derived MSCs, we examined various cytokines and hormones with chondrogenic potential. As shown in Figure 2A, RA, IGF1, FGF2, insulin, or BMP2 independently did not increase pellet size. Additionally, TGFβ3 or DEX alone failed to induce chondrogenesis (data not shown). Glycosaminoglycans (GAGs) accumulations were not observed in these cell pellets treated with a single cytokine (data not shown). The addition of both TGFβ3 and DEX increased pellet size; however, contrary to previous reports [Nishimura et al., 1999; De Bari et al., 2001], significant accumulation of GAGs were not observed (Fig. 2B). In the presence of either TGFβ and DEX together, or IGF1, FGF2, or insulin alone, some proteoglycan production
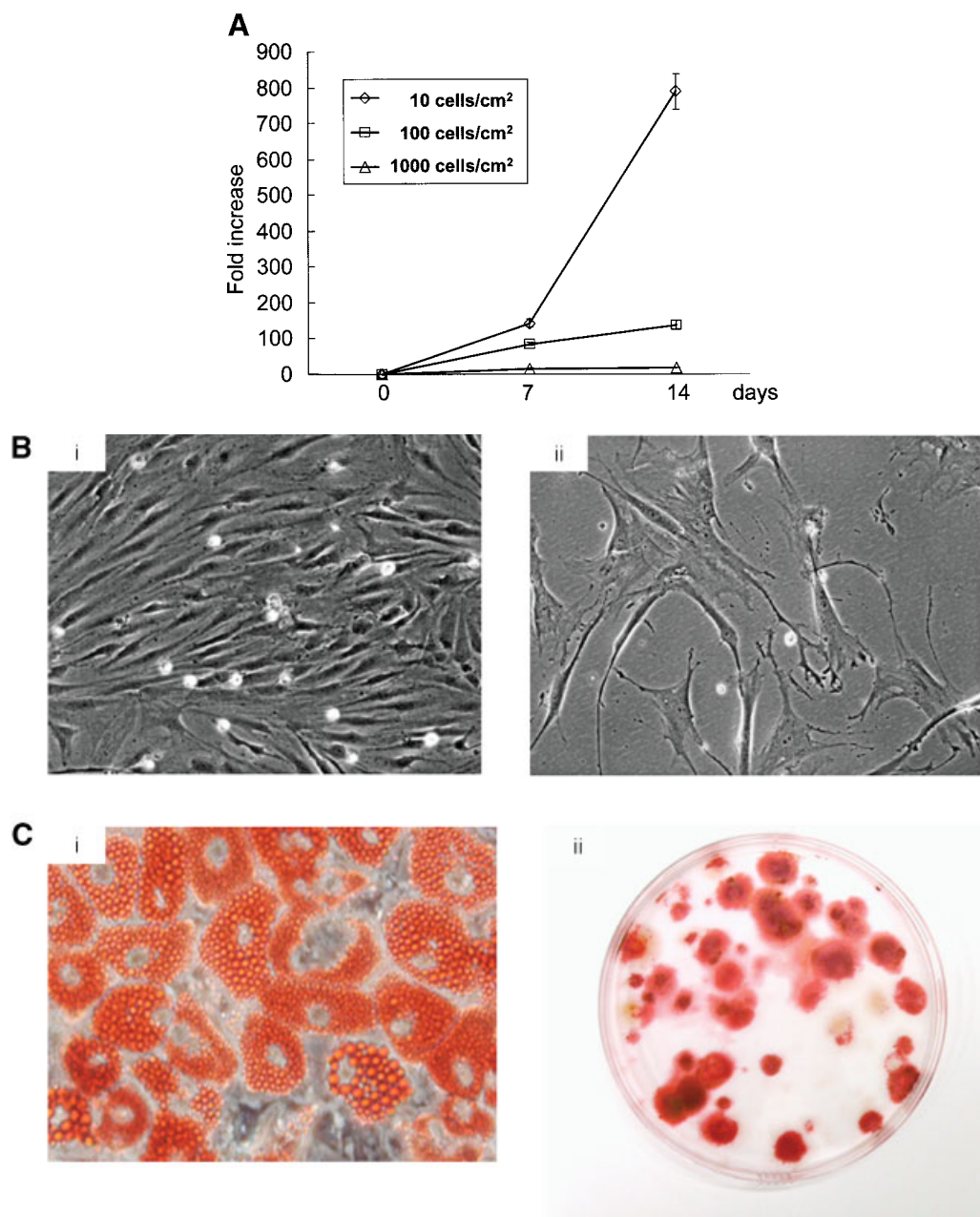
Fig. 1. Proliferation and multiple differentiation of synovium-derived MSCs. **A**: Passage 2 cells were plated at 10, 100, 1,000 cells/cm^2 and harvested at 7 and 14 days. Average fold increase is shown (n = 3). Error bars indicate standard deviations. **B**: Morphology of synovium-derived MSCs in monolayer culture. The cells were plated at 10 cells/cm^2, cultured for 14 days to produce single cell-derived colonies. (i) Most of the cells kept their fibroblastic spindle shape. (ii) Large and flat cells were also observed. **C**: (i) Adipogenesis of synovium-derived MSCs. The cells were cultured in adipogenesis medium for 21 days and stained with Oil Red-O. Representative lipid vacuoles are shown. (ii) Calcification of synovium-derived MSCs. One hundred cells were plated in a 60 cm^2-dish, cultured for 14 days in complete medium to form single cell-derived colonies. The cells were then cultured in calcification medium for an additional 21 days, and stained with Alizarin Red. Representative dish is shown.

was observed, although the pellet size did not increase. Interestingly, in the presence of TGFβ and DEX, the addition of BMP2 dramatically enhanced the growth of pellet and produced abundant cartilage extracellular matrix (Fig. 2B). The diameter of the pellet was also significantly increased by addition of BMP2 (Fig. 2C). A transmission electron micro-
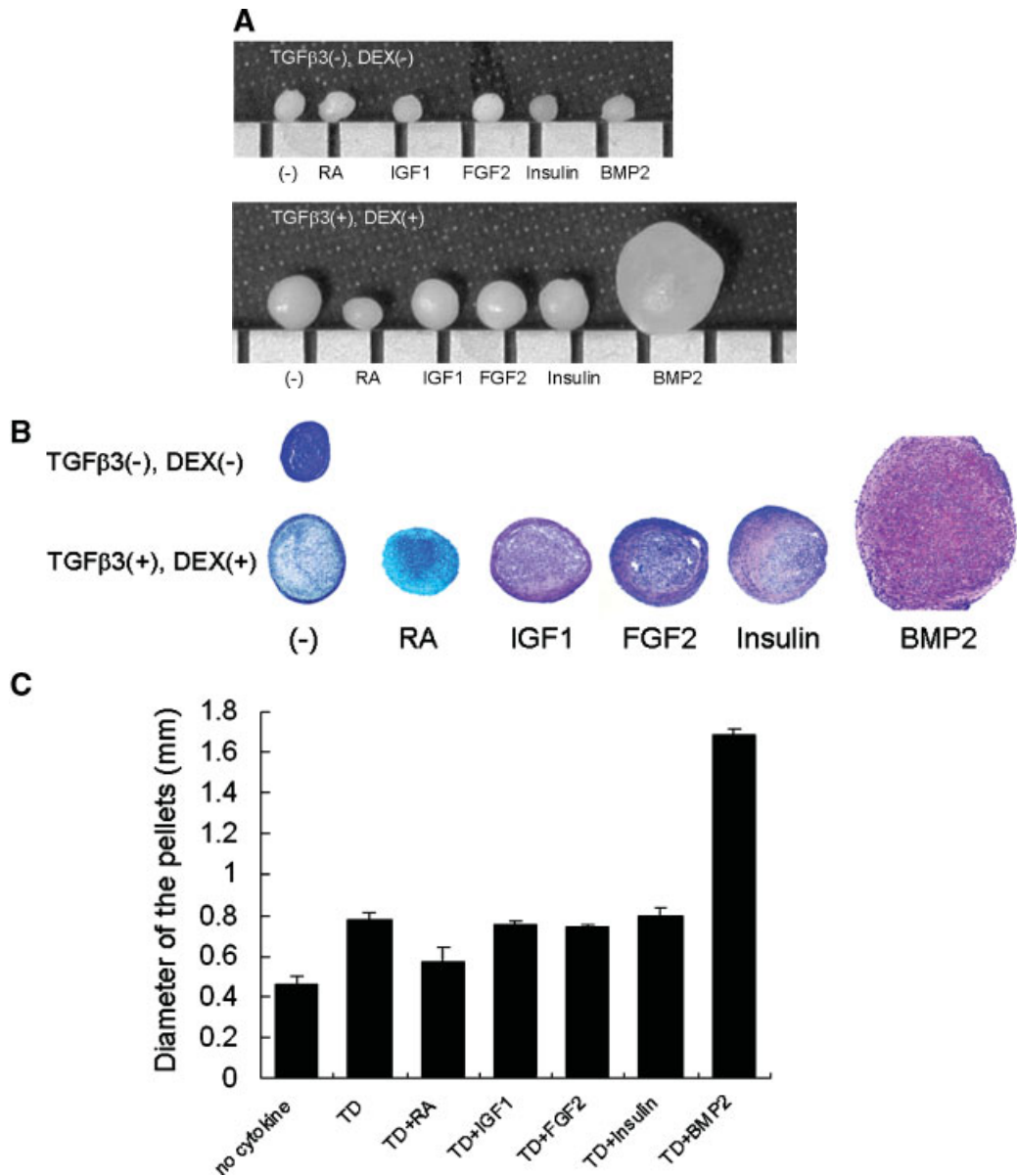
Fig. 2. The effect of cytokines on in vitro chondrogenesis of synovium-derived MSCs. Two hundred thousand synovium-derived MSCs were pelleted and cultured for 21 days in the defined medium in the presence or absence of transforming growth factor β-3 (TGFβ3) and dexamethasone (DEX) with the following cytokines: retinoic acid (RA), insulin like growth factor-1 (IGF1), fibroblast growth factor-2 (FGF2), insulin, and bone morphogenetic protein-2 (BMP2). **A**: Macro pictures of cell pellets with 1 mm scale are shown. **B**: Histological sections stained with Toluidine Blue. **C**: The diameters of the pellets cultured with various cytokines are indicated. The error bars are indicated standard deviation (n = 3). TD, TGFβ3, and DEX. Significant differences ($P < 0.01$) between TD + BMP2 and others were demonstrated by one-way ANOVA. **D**: Electron microscopic picture of a representative cell of the pellet. **E**: Immunohistochemical analysis for type II collagen. C–E: Synovium-derived MSCs were pelleted and cultured for 21 days in the defined medium containing TGFβ3, DEX, and BMP2. Macro picture of cartilage pellets with 1 mm scale is shown.

scope showed that the cell had a territorial matrix, an abundant rough ER, Golgi-complex, and a very large round shaped nucleus consistent with a chondrocyte (Fig. 2D). The accumulation of type II collagen was confirmed by immunohistochemical analysis (Fig. 2E).

Optimal Cell Number for Chondrogenic Pellet Culture

Human synovium-derived MSCs at 200, 400, 800, 1,600, and $2,000 \times 10^3$ were pelleted and cultured for 21 days in the defined medium
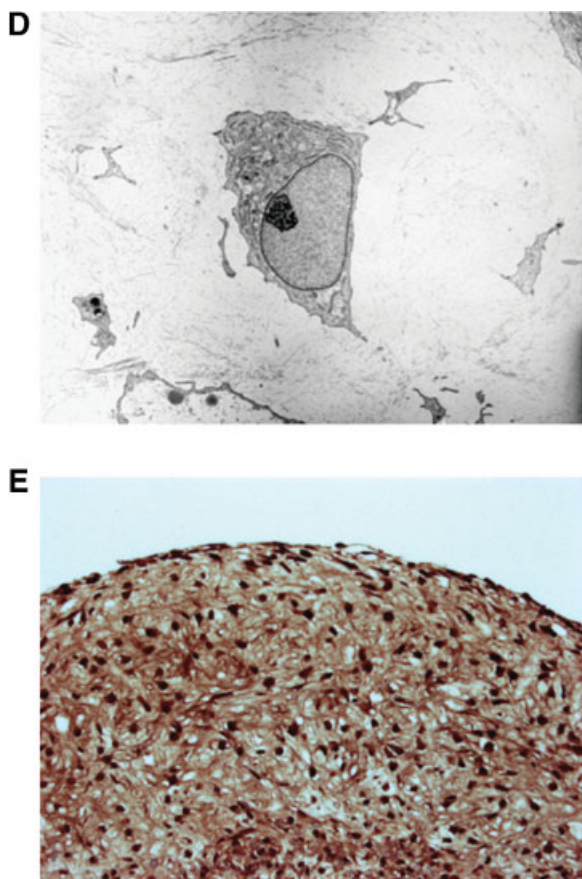
Fig. 2. (*Continued*)

containing TGFβ3, DEX, and BMP2 to examine the optimal cell number required to produce f the largest cartilage pellet. As shown in Figure 3, cartilage pellet size increased with the initial cell number up to 800×10^3. When $1,600 \times 10^3$ or more cells were used, the cells did not condense to a single pellet but dispersed into smaller pieces. In this assay, 800×10^3 cells per pellet were optimal for the formation of the largest cartilage pellet.
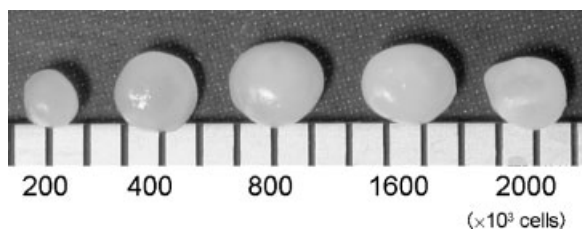

Fig. 3. The effect of initial cell number on in vitro chondrogenesis of synovium-derived MSCs. Human synovium-derived MSCs at 200, 400, 800, 1,600, and $2,000 \times 10^3$ were pelleted and cultured for 21 days in the defined medium containing TGFβ3, DEX, and BMP2. Macro pictures of cell pellets with 1 mm scale are shown.

DNA Content During In Vitro Chondrogenesis

In chondrogenesis of synovium-derived MSCs, the amount of DNA per pellet decreased by 70% in 7 days and continued to be reduced thereafter (Fig. 4). These data indicate that the pellet increased its size due to accumulation of newly synthesized extracellular matrix, not due to proliferation of the cells.

Sequential Gene Expressions During In Vitro Chondrogenesis

Semi-quantitative reverse transcriptase-PCR showed the expression of COL2A1 was first detected at 7 days and increased thereafter (Fig. 5A). The expression of the link protein and COL10A1 was detected at 14 days and increased thereafter.

SOX4 increased markedly at 1 day and then decreased progressively. Three other SOX genes had previously been implicated in cartilage differentiation [Akiyama et al., 2002] and were detected in these cultures. SOX5 was detected at 7 and 14 days, SOX6 expression was detected in untreated synovium-derived MSCs, increased at 1 day and decreased at 21 days, and SOX9 expression increased from 1 day through 14 days.

The three BMPs involving to skeletal formation [Iwata et al., 1993; Hughes et al., 1995; Semba et al., 2000] were analyzed during the differentiation of synovium-derived MSCs into cartilage. Endogenous BMP2 expression increased at 1 day and decreased at 14 days. BMP4 expression decreased at 1 day, and BMP6 was not detected throughout the pellet culture.

Three BMP receptors were also analyzed. BMPR1A was not detected, BMPR1B increased
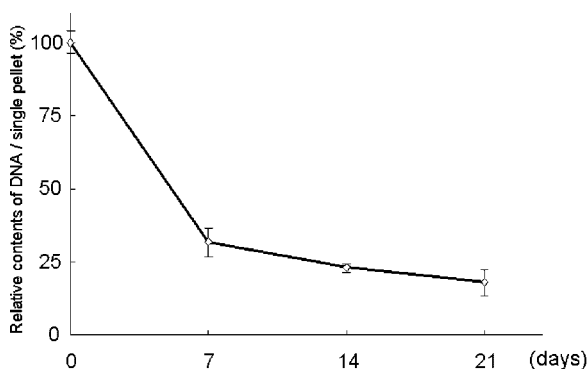

Fig. 4. Relative yield of DNA content per pellet during in vitro chondrogenesis of synovium-derived MSCs ($n = 3$ at day 0, $n = 4$ at day 7, $n = 6$ at day 14 and 21. Error bars indicated standard deviations).
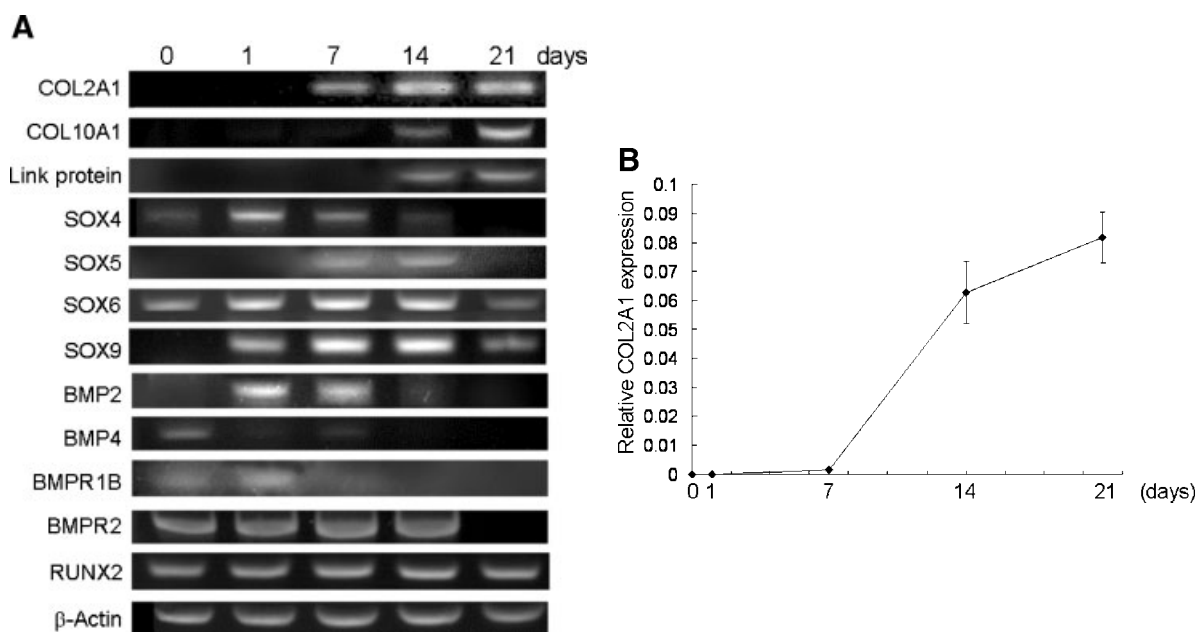
Fig. 5. **A**: Sequential expressions of chondrogenic-related genes assayed by reverse transcriptase-PCR. BMPR1B, bone morphogenetic protein receptor type 1B; BMPR2, bone morphogenetic protein receptor type 2. **B**: Quantitative real-time PCR analysis for COL2A1 gene expression. β-Actin was used as an internal control. The data are expressed as mean ± SD (n = 3).

at 1 day and decreased thereafter, and BMPR2 expression was observed from 0 days through 14 days and then decreased.

The expression of RUNX2, a transcription factor for early osteoblast differentiation [Komori et al., 1997], was detected at 0 days, and kept the same expression level during the culture period.

Real time quantitative PCR analysis for COL2A1 gene expression was performed to verify the reverse transcriptase-PCR. As shown in Figure 5B, COL2A1 gene expression was increased with time in agreement with the reverse transcriptase-PCR data.

Long-Term Pellet Culture

800×10^3 synovium-derived MSCs were cultured as a pellet in the defined medium containing TGFβ3, DEX, and BMP2. It should be noted that the size of the cell pellet and its weight increased continuously up to 42 days and its diameter reached 3 mm (Fig. 6A,B).

Human synovium-derived MSCs have a differentiation potential for calcification (Fig. 1C-ii) and BMP2 has a variety of functions including bone induction [Iwata et al., 1993; Iwasaki et al., 1994; Hanada et al., 2001; Dragoo et al., 2003a]. Therefore, we examined osteogenesis-

related gene expression during in vitro chondrogenesis of synovium-derived MSCs (Fig. 6C).

The expression of RUNX2 was still observed at 42 days. The expression of Osterix, a transcription factor for late osteoblast differentiation [Nakashima et al., 2002], was detected at 21 and 42 days. The expression of bone sialoprotein (BSP), an early bone specific matrix marker, was detected slightly at 42 days. The expression of Osteocalcin, a bone specific matrix protein, was not detected at all (Fig. 6C). Though some of osteogenesis-related gene expressions were observed during in vitro chondrogenesis of synovium-derived MSCs, the extracellular matrix was filled with GAGs, and neither hypertrophic change nor bone formation was observed at all histologically (Fig. 6D).

Comparison With Bone Marrow-Derived MSCs

For further understanding of the characteristics of synovium-derived MSCs, we compared them with bone marrow-derived MSCs, which are regarded as the most popular MSCs at present. To compare their proliferation potencies, both synovium- and bone marrow- derived MSCs from three independent donors were plated at 50 cells/cm^2 and harvested after 14 days. Though some donor variation exists,
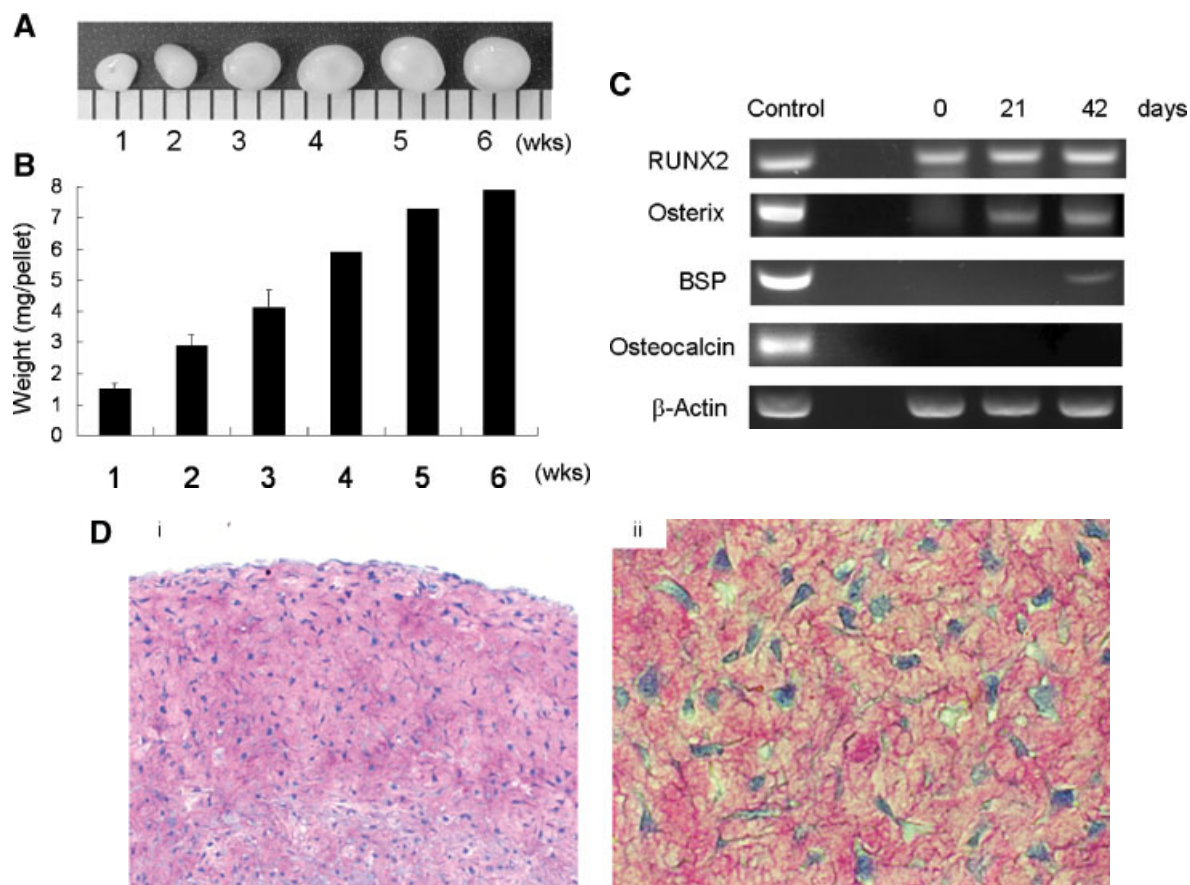
Fig. 6. In vitro chondrogenesis of synovium-derived MSCs up to 6 weeks. Eight hundred thousand cells were pelleted and cultured in the defined medium containing TGFβ3, DEX, and BMP2. **A:** Macro picture of cartilage pellets with 1 mm scale. **B:** The weight of the pellet cultured for 1 through 6 weeks. **C:** Reverse transcriptase-PCR for osteogenesis-related gene expressions. For the positive control, human bone marrow-derived MSCs were differentiated into osteoblast and the total RNA was used. RUNX2, Runt-related transcription factor 2; BSP, bone sialoprotein. **D:** Histological sections of cartilage pellet cultured for 6 weeks stained with Toluidine Blue. (i) Low (×40) and (ii) high (×200) magnification.

similar proliferation potencies were observed in cells obtained from the same donor (Fig. 7A).

With regard to surface epitopes, no significant differences were found between synovium- and bone marrow- derived MSCs. Both were positive for CD44, CD90, CD105, CD147, partially positive for CD10, CD54, CD106, CD166, STRO-1 and negative for CD34, CD45, CD117, CD31, Flk-1, NGFR (Table II).

In terms of the chondrogenesis potential of each cell type, cartilage pellets from synovium- and bone marrow- derived MSCs looked similar histologically (Fig. 7B); however, cartilage pellets from synovium-derived MSCs were larger and heavier than cartilage pellets from bone marrow-derived MSCs in patient-matched comparison (Fig. 7C). This indicates that the synovium-derived MSCs have a greater chondrogenesis potential than bone marrow-derived MSCs.

DISCUSSION

MSCs derived from synovium were first reported by De Bari et al. [2001]. Their findings and ours seem similar in that MSCs can be obtained from human adult synovium; however, some differences in the methods and results exist. For expansion, De Bari et al. replated the cells at a 1:4 dilution of the confluence and cultured for 10–25 days for subsequent passages; using this method the cells will expand approximately 4-fold. We plated the cells at a low density of 50 cells/cm^2, and the cells expanded more than 100 fold in 14 days. Our method enables sufficient number cells to be obtained more quickly with fewer passages. Previous analyses for quantification demonstrated the differentiation potential for chondrogenesis [Sekiya et al., 2001] and adipogenesis [Digirolamo et al., 1999] decreased
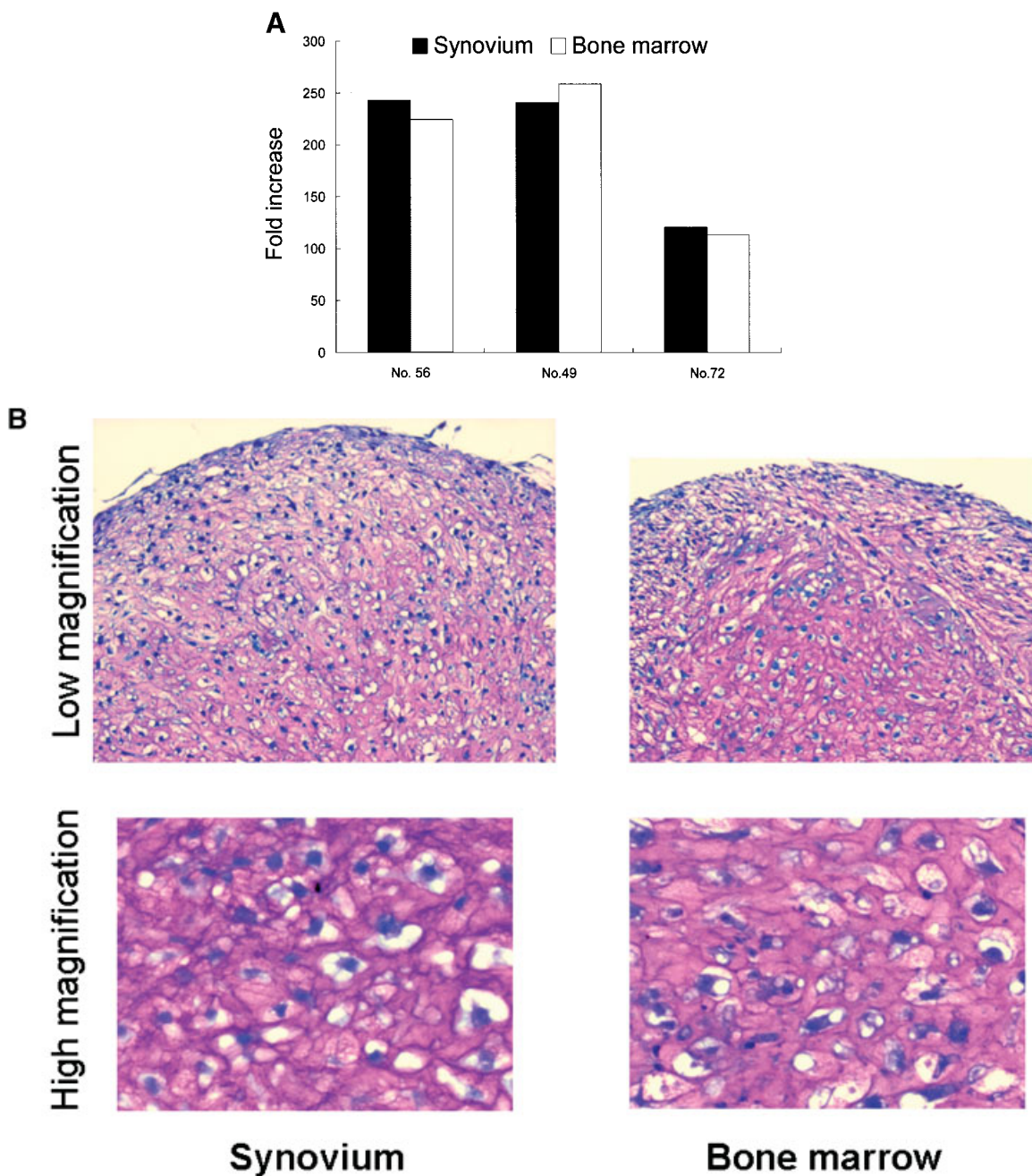
Fig. 7. Patient-matched comparison of synovium-derived MSCs with bone marrow-derived MSCs. **A**: Proliferation ability. The cells from three independent donors were plated at 50 cells/ cm^2 in 60 cm^2-culture dishes, cultured for 14 days, and counted. **B**, **C**: Chondrogenesis potential. Two hundred-fifty thousand cells at Passage 3 from three independent donors were pelleted and cultured in the chondrogenesis medium for 21 days. B: Representative histology of cartilage pellet from synovium and from bone marrow stained with Toluidine Blue with low ($\times 40$) and high ($\times 200$) magnification. C: Average weight of cartilage pellets with standard deviation (n = 3).

after many passages. These data indicate that expansion of multipotential synovial-derived MSCs through low density plating will be useful for clinical applications.

De Bari et al. described synovial-derived MSCs as appearing to be a relatively homogeneous population of fibroblast-like cells when viewed microscopically. We demonstrated that human synovium-derived MSCs were heterogeneous, in that they contain at least two distinct types of cells. In agreement with this finding, Colter et al. demonstrated that bone
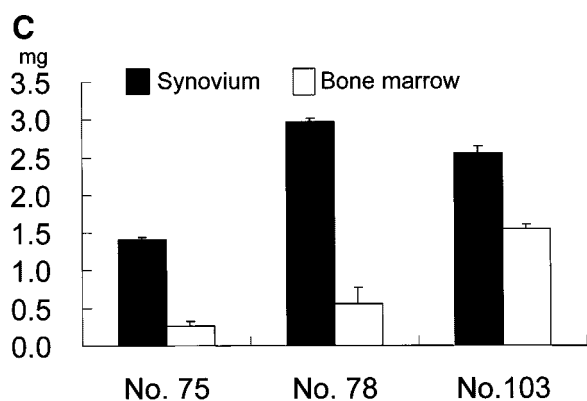
C

Fig. 7. (Continued)

marrow-derived MSCs contained two morphologically distinct cell types: spindle-shaped cells and large flat cells. The subtypes were also distinguished by a series of surface epitopes and expressed proteins [Colter et al., 2001]. Furthermore, the samples enriched for the small cells had a greater potential for expansion and multipotential differentiation than the samples enriched for the large cells [Colter et al., 2001; Sekiya et al., 2001]. In the synovium-derived MSCs, large colonies consisted of dense fibroblastic spindle shape cells, while small colonies contained sparse flat cells. This suggests that the fibroblastic, spindle shape cells have greater proliferation ability than the large, flat cells, as was observed in bone marrow-derived MSCs [Colter et al., 2000; Sekiya et al., 2001, 2002a].

For the chondrogenesis analysis, De Bari et al. obtained micromass by pipetting 20 μl droplets of cell suspension (4×10^5 cells) into individual wells and, after the cells were allowed to attach without medium for 3 h, they were incubated for 6 days in chondrogenesis medium containing 10 ng/ml TGFβ. Their resulting cartilage was thin with only several cell layers, while our cartilage was spherical with three-dimensional

structure. During in vitro chondrogenesis of MSCs using our method, the pellet increased its size and weight due to the production of cartilage matrix. This makes it possible to evaluate the chondrogenic potential of a population of MSCs using pellet wet weight. Therefore, any difference in chondrogenic ability can be shown in more detail in contrast to the micromass culture system.

We found that the combination of TGFβ3, dexamethasone and BMP2 had the strongest positive effect on in vitro chondrogenesis. This result is similar to our previous data using bone marrow-derived MSCs [Sekiya et al., 2001, 2002a,b, 2005]. Nishimura et al. reported that TGFβ and dexamethasone differentiated synovial cells into chondrocytes in a pellet culture system [Nishimura et al., 1999]; however, as shown in Figure 2, the addition of BMP2 to the media reported by Nishimura et al. dramatically enhanced the production of cartilage extracellular matrix, indicating our chondrogenic medium seems better than theirs.

RT-PCR showed progressive expression of the cartilage specific extracellular matrix molecules: COL2A1 and the link protein, and the chondrogenic transcription factors SOX -9, -5, -6 during the in vitro chondrogenesis of synovium-derived MSCs. It is remarkable that SOX4 expression increased at day 1, before the significant increase in the other SOX genes, and decreased thereafter. Similar results were obtained during both chondrogenesis and adipogenesis of bone marrow-derived MSCs [Sekiya et al., 2002b, 2004]. SOX4 may act to initiate differentiation into several lineages, including chondrogenesis and adipogenesis.

Of the three BMP receptors dedicated to a specific function in chondrogenesis [Zou et al., 1997], BMP receptor-1A was not expressed, and BMP receptor-1B and -2 were detected only up to 14 days of culture. Also, the expression of endogenous BMP2 and 4 could not be detected at 21 days. BMP6 was not expressed throughout the culture period (data not shown). These results may suggest that during the later phase of chondrogenesis in synovium-derived MSCs, the BMP signal may not be needed. However in this study, exogenous BMP2 was provided continuously and exogenous BMP2 possibly suppresses endogenous BMP expression and/or affects downstream signal cascades. Therefore, the importance of theses molecules can not be ignored.

TABLE II. Surface Apitopes for Synovium- and Bone Marrow-Derived MSCs

+	±	−
CD44	CD10	CD34
CD90	CD54	CD45
CD105	CD106	CD117
CD147	CD166	CD31
	STRO-1	Flk-1
		NGFR

+, >95% positive; −, <10% positive.

The expression of COL10A1, hypertrophic chondrocyte marker, increased during chondrocyte differentiation of synovium-derived MSCs in culture. This is similar to the results observed during *in vitro* chondrogenesis of cells derived from bone marrow [Sekiya et al., 2001, 2002b] and adipose tissue [Zuk et al., 2002]. Also, the results obtained here are consistent with observations of synovium-derived MSCs by other groups without BMPs [Nishimura et al., 1999; De Bari et al., 2001]. This early COL10A1 expression seems to be characteristic of *in vitro* chondrogenesis of MSCs regardless of the original tissue. However, Dragoo et al. reported that IGF1, FGF2, and growth hormones promoted chondrogenesis of fat-derived cells in a three dimensional culture and COL10A1 mRNA was not detected by RT-PCR [Dragoo et al., 2003b].

The expression of RUNX2, known as an osteogenesis-related transcription factor, was stable through 21 days and stayed at the same level through 42 days. Runx2 is also reported to be expressed by chondroprogenitors [Lengner et al., 2002] and matured chondrocytes [Enomoto et al., 2000]. Recently Lengner et al. showed that Runx2 played an antichondrogenic role in C3H10T1/2 cells, pluripotent mesenchymal progenitor cell, prior to the onset of chondrogenesis, and that Runx2 suppression by Nkx3.2 was prerequisite for the initiation of chondrogenesis [Lengner et al., 2005]. In their results, Runx2 expression decreased at day 1 and increased at day 4, while in our experiment, Runx2 expression appeared similar between at day 1 and at day 7 and thereafter. One possible explanation of this variation is that the gene expression level was investigated at different time points. Lengner et al. also mentioned that the osteogenic phenotype cannot be induced in high-density micromass cultures despite the presence of exogenous Runx2 and BMP2, suggesting cell-cell contact in the culture system predisposes the cells to undergo chondrogenesis in response to BMP2 signaling. This finding is consistent with our data that despite continuous expression of Runx2, the progression into osteogenic change that is represented by the expression of Osterix or BSP did not start until the later phase of the pellet culture.

Osterix was expressed weakly at 3 weeks and was slightly increased at 6 weeks. Osterix is one of the master genes for bone formation [Nakashima et al., 2002]; however, its effect on chondrocyte differentiation still remains to be elucidated. Yagi et al. reported that the Osterix gene was expressed in the primary cultures of chondrocytes and its expression was upregulated by BMP2, which is consistent with our data [Yagi et al., 2003].

The BSP promoter has a number of functional Runx2 DNA binding sites [Javed et al., 2001] and RUNX2 regulates BSP expression directly [Barnes et al., 2003]; however, BSP expression was not observed until day 21, even though the cells expressed Runx2 continuously in our chondrogenic model. Upregulation of BSP by RUNX2 is controlled by multiple factors and its late upregulation in this model may be explained by osterix expression because BSP expression followed osterix expression.

A possible tendency of synovium-derived MSCs to differentiate into bone might limit their clinical usefulness for cartilage regeneration. After 6 weeks of pellet culture, BSP mRNA was only slightly detected; however, neither Osteocalcin mRNA nor calcification was detected.

Synovium-derived MSCs looked similar to bone marrow-derived MSCs according to our investigations into the expression of surface epitopes, their proliferation potential and histological findings. However, cartilage pellets from synovium-derived MSCs tended to be larger than those from bone marrow, indicating that synovium-derived MSCs are very suitable for cartilage regeneration.

We demonstrated the optimal conditions for in vitro chondrogenesis of MSCs derived from synovium. This system mimics cartilage formation during skeletogenesis, indicating the validity of this cartilage produced from the MSCs. Furthermore, synovium-derived MSCs had a greater chondrogenic ability than bone marrow-derived MSCs. We conclude that synovium-derived MSCs can be a potent cell source for tissue engineering of autologous cartilage in vitro with our method.

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