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Identification of cartilage progenitor cells in the adult ear perichondrium: utilization for cartilage reconstruction

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For cartilage reconstruction, it is still difficult to obtain a sufficient volume of cartilage and to maintain its functional phenotype for a long period. Utilizing tissue stem cells is one approach to overcome such difficulties. We show here the presence of cartilage progenitor cells in the ear perichondrium of adult rabbits by 5-bromo-2′-deoxyuridine labeling, clonogenicity, and differentiation analyses. Long-term label-retaining cells were demonstrated in the perichondrium. Cells from the perichondrium, that is, perichondrocytes were mechanically isolated using a raspatory and maintained in D-MEM/F-12 medium with 10% FCS. They proliferated more vigorously than chondrocytes from the cartilage. Perichondrocytes could differentiate into adipocytes as well as osteocytes in differentiation induction medium. For cartilage reconstruction *in vivo*, perichondrocytes were seeded on collagen sponge scaffolds and implanted in nude mice. After 4 weeks, the composites with perichondrocytes generated the same weight of cartilaginous tissue as those with chondrocytes. They produced glycosaminoglycan and type II collagen as shown by RT-PCR and immunohistochemical examination. On the contrary, rabbit bone marrow mesenchymal stem cells used as control could regenerate significantly smaller cartilage than perichondrocytes in the implant study. Based on these findings, we propose that the perichondrium containing tissue progenitor cells is one of the potential candidates for use in reconstructing cartilage and new therapeutic modalities.
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Although many researchers have attempted to regenerate articular cartilage, it is still difficult to maintain the quality for a long time because of environmental factors such as weight-bearing and friction. Meanwhile, cartilage taken from the nasal septum, the external ear or the rib has been used for supporting framework for microtia or augmentation of soft tissue to treat deformities of the face. However, the amount of cartilage that can be excised from the donor sites is limited. Tissue engineering uses organ-specific cells for seeding a scaffold *ex vivo*. This is thought to be a reasonable approach for preparing large quantities of tissues starting from a small tissue sample, because the tissue samples are of autologous origin and they can be obtained without causing substantial deformity or dysfunction.

Continuously self-renewing tissues such as skin, colon, and intestine are successively restored by stem cells. Stem cells have thus far been used for tissue engineering of several tissues, for example, mesenchymal stem cells from bone marrow for blood vessels,[1] bone,[2] dentin,[3] heart muscle,[4] liver,[5] and skeletal muscle,[6] epidermal stem cells from epidermis for skin,[7] pancreatic ductal stem cells from pancreatic tissue for pancreas,[8] adipo-stromal cells from fat tissue for bone,[9] and limbal epithelial stem cells from cornea for cornea,[10] and embryonic stem cells for nervous tissue.[11]

For cartilage reconstruction, it had been thought that mesenchymal stem cells[12,13] would be a possible cell source, but the rate of yield of chondrocytes and extracellular matrix was not sufficient to support three-dimensional auricular scaffolds.[14]

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Furthermore, cartilage reconstruction with ear cartilage has difficulties for practical use. Therefore, we focused on the perichondrium of the ear as a source of cells for auricular cartilage reconstruction. In addition, aiming at future clinical use, we chose the adult ear as a source of cells.

By 5-bromo-2′-deoxyuridine (BrdU) labeling, clonogenicity assays, and differentiation studies of the perichondrium-derived cells, the presence of tissue progenitor cells in the adult ear perichondrium was first demonstrated in the present study. The perichondrium was mechanically separated from the ear cartilage, and the cells were obtained from the perichondrium and cartilage. The perichondrocytes, expressing cartilage marker genes, were implanted into a subcutaneous space in nude mice with a collagen sponge. We also performed a head-to-head comparison of perichondrocytes with mesenchymal stem cells from rabbit bone marrow about the potency of cartilage reconstruction *in vivo*. Based on detailed evaluations of the implants, we demonstrated that cultured perichondrocytes could be used for cartilage reconstruction *in vivo*.

Materials and methods

Animals and Operations

Animals were maintained from the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. The number of animals used in this study was kept to a minimum, and all possible efforts to reduce their suffering were made in compliance with protocols established by the Animal Research Committee of Kyoto University.

BrdU Labeling and Detection

BrdU pulse-chase experiments were performed as described.[15] Two 4-week-old rabbits were injected daily subcutaneously with 50 mg/kg/day BrdU (Sigma-Aldrich, St Louis, MO, USA) for 5 days. The rabbits were killed after 4 weeks. Tissue sections were immersed in 3% hydrogen peroxide (Wako Pure Chemical Industries, Ltd, Osaka, Japan) for 10 min, digested with 0.2% trypsin (Kamiya Biomedical Company, Seattle, WA, USA) for 10 min, and denatured for 30 min with denaturing solution (Kamiya Biomedical Company, Seattle, WA, USA). After blocking in blocking solution (Kamiya Biomedical Company, Seattle, WA, USA) for 10 min, sections were incubated for 1 h at room temperature with anti-BrdU antibody (Kamiya Biomedical Company, Seattle, WA, USA). Color was developed by incubation with streptavidin-HRP conjugate (Kamiya Biomedical Company, Seattle, WA, USA) for 10 min and DAB (Kamiya Biomedical Company, Seattle, WA, USA) for 10 min. Hematoxylin was used for counterstaining.

Cell Culture

The perichondrium and the cartilage were harvested from proximal regions in both ears of two 4-week-old male Japanese white rabbits. The perichondrium and the cartilage were collected separately using a dental raspatory and weighed.

Tissues were washed in phosphate-buffered saline without magnesium and calcium ions, pH 7.4 (PBS (−), Takara Bio Inc., Otsu, Japan) containing 0.10% ethylenediamine tetraacetic acid (EDTA) and finely minced with scissors in 10% fetal calf serum (FCS) (Gibco, Grand Island, NY, USA) in D-MEM/F-12 medium (Gibco, Grand Island, NY, USA). Then they were digested with 0.25% collagenase-type I (Gibco, Grand Island, NY, USA)/D-MEM/F-12 medium with shaking at 37°C for 2 h. After passage through a 100 μm nylon mesh (Cell Strainer, BD Falcon, Bedford, MA, USA), cells were washed twice with D-MEM/F-12 medium with 10% FCS. The cell numbers were counted with a hemocytometer and viability was determined by the trypan blue exclusion test.

Clonogenicity assay

To analyze the clonogenicity of cells from the perichondrium and the cartilage, cells were seeded at a density of 60 cells/plate (10 cm diameter).[16] They were cultured in D-MEM/F-12 medium with 10% FCS, 10 000 U/ml of penicillin G sodium, 10 000 μg/ml of streptomycin sulfate, 25 μg/ml amphotericin B (Antibiotic-Antimycotic, Gibco, Grand Island, NY, USA) under an atmosphere of 5% CO_2 at 37°C. The culture medium was replaced every 7 days. The size and number of colonies were quantified after 4 weeks by staining with Giemsa.

Cell growth in vitro

To study the proliferation rate of perichondrocytes and chondrocytes, cells were seeded at a density of 1200 cells/cm^2 in 75 cm^2 culture flasks (Sumitomo Bakelite Co., Ltd, Tokyo, Japan) and subsequently passaged in D-MEM/F-12 medium with 10% FCS and antibiotic and antimicotic reagents. The culture medium was replaced every 3 days. Cells were trypsinized with 0.25% (w/v) trypsin for 5 min at subconfluency, and they were counted and seeded at the same density as above (approximately a 1:50 dilution).

Adipogenic and Osteogenic Differentiation

For adipogenic and osteogenic differentiation of perichondrocytes and chondrocytes, cells were seeded at a density of 2400 cells/cm^2 in a 12-well culture plate with cover glasses or in a six-well culture plate, and cultured in D-MEM/F-12 medium with 10% FCS. The culture medium was replaced every 3 days until confluency.[17,18]

For adipogenic differentiation, confluent cells were stimulated for 2 weeks in adipogenic induction medium containing h-insulin, L-glutamine, MCGS, dexamethasone, indomethacin, and 3-isobutyl-methyl-xanthine (hMSC Adipogenic Induction SingleQuots®, Cambrex, Walkersville, MD, USA). Cells in a 12-well plate were fixed with 4% phosphate buffered paraformaldehyde for 10 min, stained with oil red O solution (0.3% w/v) for 10 min at room temperature, rinsed with distilled water, and photographed with a digital camera (DXM 1200, Nikon, Tokyo, Japan).

For osteogenic differentiation, confluent cells were stimulated for 3 weeks in osteogenic induction medium containing dexamethasone, L-glutamine, ascorbate, MCGS, and β-glycerophosphate (hMSC Osteogenic SingleQuots®, Cambrex, Walkersville, MD, USA). Cells in a 12-well plate were detected about alkaline phosphatase expression using an alkaline phosphatase staining kit (Muto Pure Chemicals, Co., Ltd, Tokyo, Japan). Cells in a six-well plate were used for RT-PCR.

Isolation and Culture of Mesenchymal Stem Cells from Bone Marrow

MSCs were isolated according to the methods described previously[19,20] with minor modifications. Tibias were dissected from two 4-week-old male Japanese white rabbits. A hole of 2 mm in diameter was created with a drill and bone marrow was aspirated with 5 ml of D-MEM (Gibco, Grand Island, NY, USA) by an 18-gauge needle and syringe. Collected bone marrow tissues were dissociated by triturating with a pipette, and then filtered through a 100 μm nylon mesh. The suspension was centrifuged at 800 rpm for 5 min. The pellet of marrow cells were suspended with 10 ml of D-MEM and cultured in D-MEM with 15% FCS and antibiotic and antimicotic reagents. After 4 days, the non-adherent cells were removed by replacing the medium. Cells were trypsinized with 0.25% (w/v) trypsin for 5 min at subconfluency, and they were seeded at a density of 6000/cm^2 and allowed to grow.

Differentiation Studies of MSCs *In Vitro*

For chondrogenic differentiation, pellet cultures were performed.[17] MSCs (2.0×10^5) were spun down at 500 rpm in 15-ml polypropylene centrifuge tubes (Corning Incorporated, Corning, NY, USA), and cultured with chondrogenic induction medium containing dexamethasone, ascorbate, ITS + supplement, penicillin/streptomycin, sodium pyruvate, proline, and L-glutamine (hMSC Chondrogenic SingleQuots®, Cambrex, Walkersville, MD, USA) with 10 ng/ml transforming growth factor-β3 (TGF-β3) (Sigma-Aldrich Inc., Saint Louis, MO, USA). The culture medium was replaced every 3 days, and bottom of each tube was flicked to ensure that the pellets were free-floating after replacing the medium. After 3 weeks of culture, pellets were fixed with 10% phosphate-buffered formalin for 24 h at 4°C. Fixed tissue was embedded in paraffin, sliced into 7.0 μm sections, and stained with toluidine blue (pH 2.5) or Elastica van Gieson. Adipogenic and osteogenic differentiation studies of MSCs were performed as described in perichondrocytes and chondrocytes differentiation studies.

Preparation of Scaffolds and Assembly of the Cell-Scaffold Composites

Collagen sponge was synthesized as previously reported.[21] The thickness of the sheet was adjusted to 2 mm, and the average pore size was 90 μm. Collagen sponge was cut into round disks with 12 mm diameter.

For assembly of the MSC-scaffold composites (MSCSCs),[22–24] 2.3×10^6 cells were seeded on collagen scaffolds (1.0×10^7 cells/cm^3) and they were stimulated in chondrogenic induction medium replaced every day for 2 weeks before implantation. In reconstruction study using the convex perichondrocyte-scaffold composites (PCSCs (conv.)), the concave perichondrocyte-scaffold composites (PCSCs (conc.)), and the chondrocyte-scaffold composites (CSCs), they were made by seeding with 6.0×10^6 cells on collagen scaffolds, and followed by incubation for 24 h. The PCSCs with 2.3×10^6 cells were also made for comparison study with the MSCSCs.

They were then implanted into a subcutaneous pocket, made by blunt dissection on both sides of the midline incision at the dorsum of nude mice. The wound was closed in layers with 5-0 nylon-interrupted sutures.

Histochemical and Immunohistochemical Analyses

The MSCSCs, the PCSCs, and the CSCs were excised for histological analysis 4 weeks after implantation. All specimens were rinsed in PBS (−), and fixed with 10% phosphate-buffered formalin for 24 h at 4°C. Fixed tissue was embedded in paraffin, sliced into 7.0 μm sections, and stained with toluidine blue (pH 2.5) or Elastica van Gieson.

Immunohistochemical staining: Frozen sections were washed three times with PBS (−). After treatment with SaGlyPBS (0.005% saponin, 50 mM glycine in PBS) containing 20% BlockAce (Dainippon, Tokyo, Japan) for 30 min, sections were incubated with anti-hCL (II) antibody (Daiichi Fine Chemicals, Takaoka, Japan) diluted 1:1000 in 5% BlockAce for 30 min at 4°C. This antibody can react with human, rat, and rabbit type II collagen. After washing three times with SaGlyPBS, the sections were incubated for 12 h at 4°C with Alexa Fluor® 546 fragment-conjugated goat anti-mouse antibody

(Molecular Probes, Eugene, OR, USA) diluted 1:1000 in SaGlyPBS containing 5% BlockAce. TO-PRO3 (Molecular Probes, Eugene, OR, USA) was used for nuclear staining by adding it to the secondary antibody solutions. After washing, sections were observed by confocal microscopy (MRC 1024, Bio-Rad, Hercules, CA, USA).

Alizarin Red S (Junsei Chemical Co., Ltd, Tokyo, Japan) staining was used to examine calcification after fixing specimens with 10% phosphate-buffered formalin for 24 h at 4°C and soaking in 3% KOH solution for 1 week.

Isolation of Total RNAs and RT-PCR

Total RNAs were isolated from cultured perichondrocytes, chondrocytes, and dermal fibroblasts using an RNeasy® Mini Kit (QIAGEN, Hilden, Germany). Total RNAs were isolated from the perichondrium and the cartilage excised from 4-week-old rabbits, and from PCSCs and CSCs using Trizol® Reagent (Invitrogen, CA, USA) and was purified with an RNeasy® Mini Kit. Using an Advantage RT-for-PCR Kit (BD Biosciences, Palo Alto, CA, USA), cDNA was synthesized with 1.0 μg of total RNAs in 100 μl of solution. Aliquots (one-tenth) of the cDNA products were used for PCR with Taq DNA polymerase (Toyobo Co., Ltd, Osaka, Japan) in a PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Primers for rabbit type I collagen, type II collagen, type X collagen, aggrecan, osteopontin and GAPDH were as follows: type I collagen,[25] sense primer (5′-GAGGAATTTCCGTGCC TGGC-3′) and antisense primer (5′-AGCTGTTCCGG GCAATCCTC-3′); type II collagen (GenBank™ accession no. S83370) (323 bp), sense primer (5′-GCACC CATGGACATTGGAGG-3′) and antisense primer (5′-AGCCCCGCACGGTCTTGCTT-3′); type X collagen (GenBank™ accession no. AF247705) (313 bp), sense primer (5′-AGCCAGGGTTGCCAGGACC-3′) and antisense primer (5′-CCAGGAGCACCATATCCTGT-3′); aggrecan (GenBank™ accession no. L38480) (313 bp), sense primer (5′-GAGGAGATGGAGGGTG AGGTCTTT-3′) and antisense primer (5′-CTTCGCC TGTGTAGCAGATG-3′); osteopontin (GenBank™ accession no. D16544) (249 bp), sense primer (5′-GCTCAGCACCTGAATGTACC-3′) and antisense primer (5′-CTTCGGCTCGATGGCTAGC-3′); GAPDH (GenBank™ accession no. L23961) (293 bp), sense primer (5′-TCACCATCTTCCAGGAGCGA-3′) and antisense primer (5′-CACAATGCCGAAGTGGTCGT-3′). PCR condition was 94°C, 30 s; 58°C, 30 s; 72°C, 30 s, followed by 72°C, 5 min. The cycle of PCR reactions was 35 for type I collagen, 30 for type II collagen, type X collagen, and aggrecan, 25 for osteopontin, and 22 for GAPDH. Products of RT-PCR were separated by electrophoresis on agarose gels, stained with ethidium bromide and photographed.

Statistical Analysis

Statistical analysis was performed using Student's *t*-test to examine differences between two groups. Values of $P<0.01$ were considered to be statistically significant. All values are presented as mean $\pm$ s.e.

Results

Identification of Long-Term Label-Retaining Cells (LRCs)

To identify tissue progenitor cells in the adult ear, we first tried to demonstrate the presence of LRCs, which are known to include stem cells.[15] To label the slow-cycling stem cells, BrdU was injected subcutaneously into 4-week-old rabbits for 5 consecutive days. After a 4-week chase period, BrdU was immunohistochemically probed with monoclonal antibody (Figure 1). LRCs were found in the nuclei of the cells at the junction between the cambium and fibrous layer of the perichondrium, but no label remained in the nuclei within the cartilage. Approximate 0.4% of the cells within the cambium layer of the concave perichondrium were positive for BrdU in the rabbits.

Separation of the Perichondrium from the Cartilage

The proximal region of the rabbit ear was examined histologically (Figure 2a and b). The perichondrium consists of two layers, the outer fibrous layer and the inner cambium layer (Figure 2b). After skin, fat, and muscle were removed (Figure 2c), the perichondrium was mechanically separated from the cartilage with a small dental raspatory (Figure 2d and e). The whole perichondrium was used as a source of

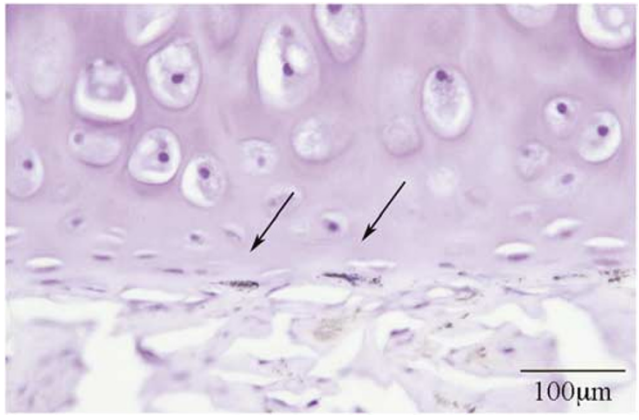


Figure 1 Detection of LRCs. Two 4-week-old rabbits were injected subcutaneously with BrdU for 5 days, and the ears were excised after a 4-week chase period. Paraffin-embedded sections were probed with anti-BrdU antibody, and then with HRP-conjugated anti-mouse antibody, and visualized with DAB. Hematoxylin was used for counterstaining. There were positive signals in the nuclei located at the junction between the cambium and fibrous layer of the perichondrium (arrows).

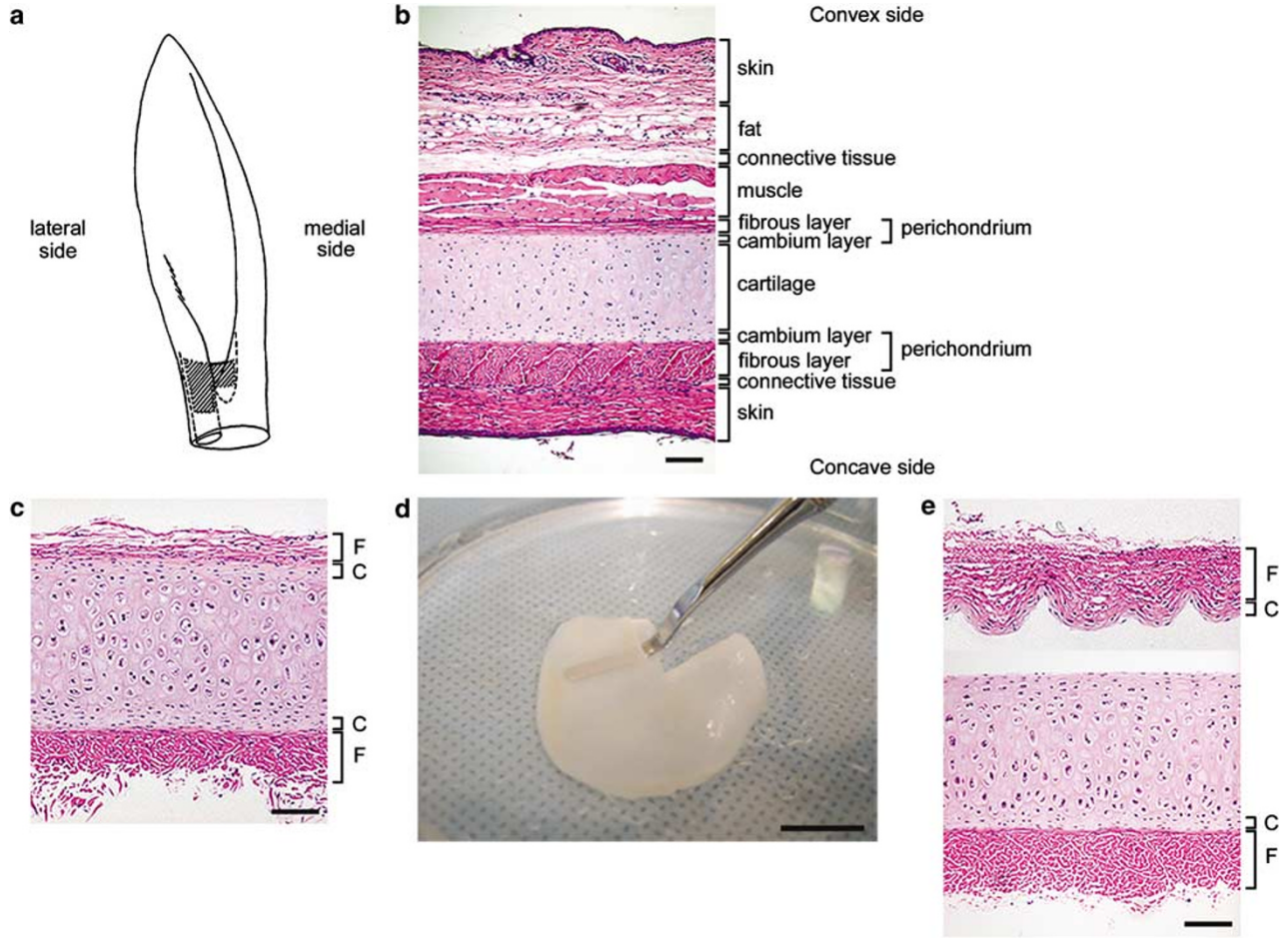


Figure 2 Separation of the perichondrium from the cartilage. (**a**) The hatched area of the rabbit ear was used. (**b**) Histology of the excised rabbit ear. (Hematoxylin and eosin stain. Scale bar: 100 μm.) (**c**) Histology of the rabbit ear after removing skin, fat, and muscle. Scale bar: 100 μm. (**d**) Separation of the perichondrium from the cartilage was performed with a dental raspatory. Scale bar: 10 mm. (**e**) Separation of the convex perichondrium from cartilage. Scale bar: 100 μm. (F) fibrous layer of the perichondrium; (C) cambium layer of the perichondrium.

perichondrocytes.[26] The perichondrium and the cartilage were dissected into small pieces and digested with 0.25% collagenase. In a representative experiment using two rabbits, we were able to prepare 3.4×10^4 cells from 0.19 g of the convex perichondrium, 5.0×10^3 cells from 0.21 g of the concave perichondrium, and 1.4×10^5 cells from 0.51 g of the cartilage. It was difficult to prepare a large amount of the perichondrium from the cartilage on the concave side, because the perichondrium is connected to the cartilage with a tightly packed, thicker fibrous layer on this side (Figure 2b).

Clonogenicity Assay

We analyzed the clonogenicity using freshly isolated cells from the perichondrium and the cartilage to assess the proliferation potency of single cells.[16] Cells were maintained in monolayer cultures in D-MEM/F-12 medium with 10% FCS. At 4 weeks after seeding 60 cells on a plastic dish, colonies formed by cells from the perichondrium and the cartilage were stained and counted (Figure 3a and b). Cells (35%) from the convex perichondrium and 20% of cells from the concave perichondrium generated colonies with 14–21 mm diameter. In contrast, only 8.5% of cells from the cartilage made colonies with 14–21 mm diameter. A certain population of cells from the convex perichondrium (4.8%) and from the concave perichondrium (1.6%) formed colonies larger than 21 mm in diameter, but cells from the cartilage did not. These findings suggest that cells in the perichondrium proliferate more vigorously than those in the cartilage.

Differentiation of Cells into Adipocytes and Osteocytes

To examine the pluripotency of cells from the perichondrium, differentiation studies were performed. To induce adipogenic differentiation, mono-

layers of confluent perichondrocytes and chondrocytes were treated with adipogenic induction medium (Figure 3c). A change in shape from polygonal to oval was detected 3 days after stimulation. The stratification of cells advanced and lipid-rich vacuoles within cells increased during the following 11 days. Lipid-rich vacuoles within both type of cells were stained with oil red O (Figure 3c).

To induce osteogenic differentiation, confluent perichondrocytes and chondrocytes were treated with osteogenic induction medium (Figure 3d). Cells formed aggregates or nodules that were strongly positive for alkaline phosphatase 3 weeks after stimulation, but cells under unstimulated conditions were only weakly stained (Figure 3d). Total RNAs were collected from cells 3 weeks after stimulation. RT-PCR was performed for osteopontin, type I collagen and type X collagen (Figure 3d). Type X collagen is specific for hypertrophic chondrocytes and is not expressed in osteogenic regions. With osteogenic differentiation, type I collagen and osteopontin were upregulated, but type X collagen were drastically downregulated. It is concluded that increase of alkaline phosphatase after induction is not a result of hypertrophic chondrocytes differentiation of these cells. These results indicated that cells from the perichondrium and the cartilage had pluripotency to differentiate into adipocytes as well as into osteocytes. Existence of LRCs in the perichondrium, strong clonogenicity, and pluripotency of perichondrocytes suggest that there are tissue progenitor cells in the perichondrium of the rabbit ear.

Characterization of Cultured Perichondrocytes and Chondrocytes

Cells isolated from the perichondrium and the cartilage, that is, perichondrocytes and chondrocytes, respectively, were maintained in D-MEM/F-12 medium with 10% FCS. Under these conditions, the convex and concave perichondrocytes were similar in cell shape, but they were different from chondrocytes with polygonal shape (Figure 4a). The rate of proliferation of perichondrocytes and chondro-

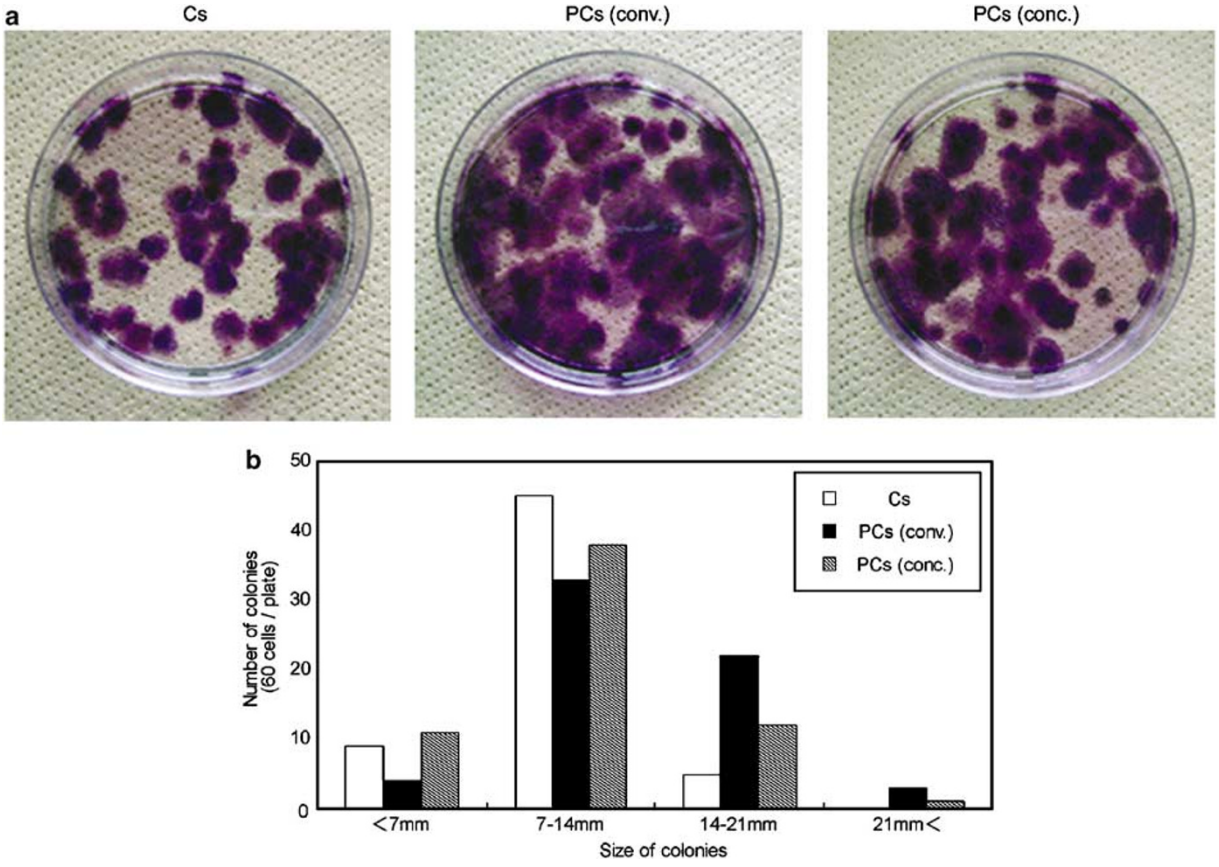
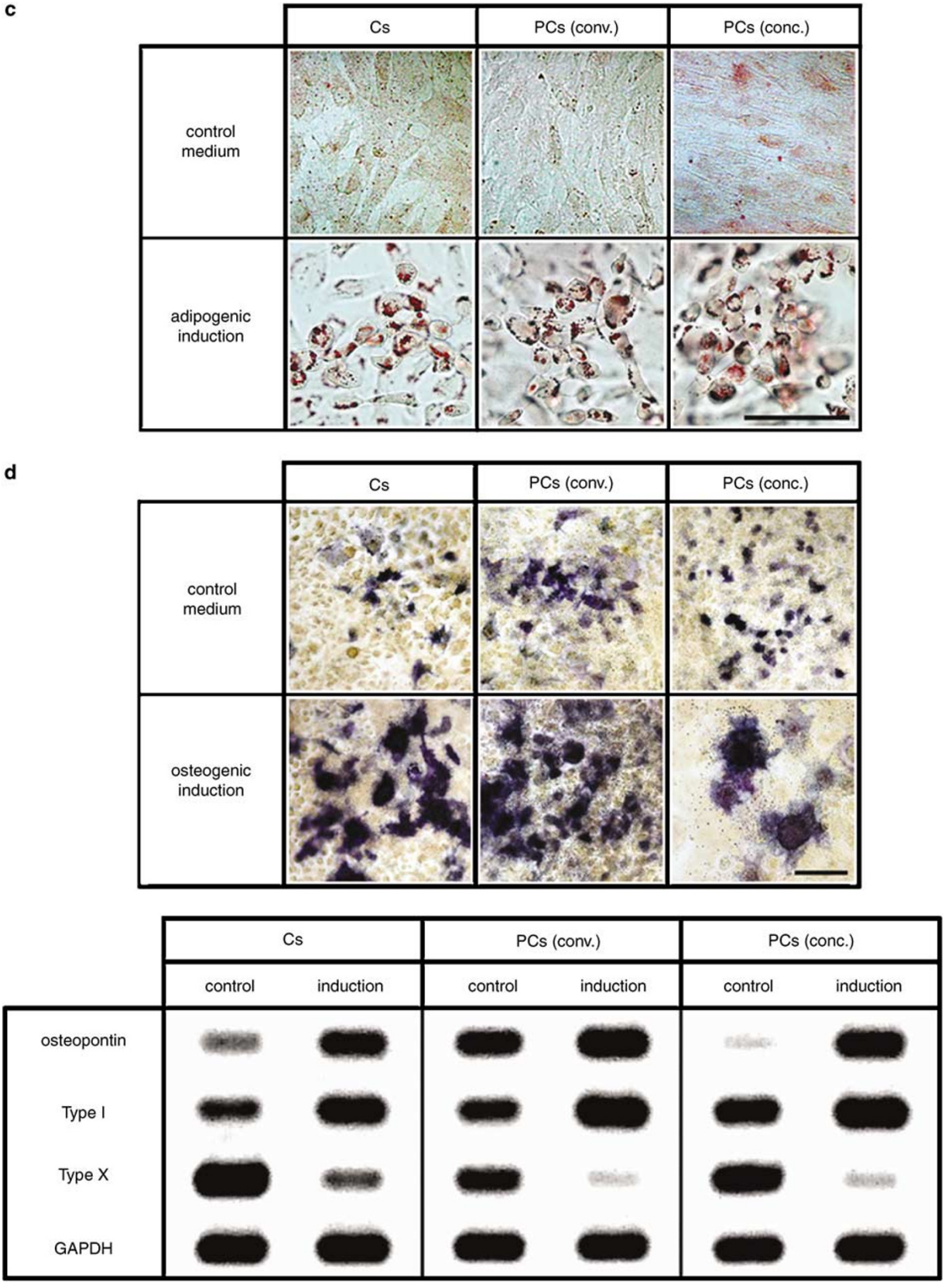


Figure 3 Clonogenicity assay and differentiation induction. (**a**) Cells were freshly isolated and seeded at a density of 60 cells/10 cm culture plate, and cultured for 4 weeks. (Giemsa stain) (**b**) Size and number of colonies from PCs (conv.), PCs (conc.) and Cs were quantified. (**c**) Convex perichondrocytes were stimulated in the adipogenic induction medium for 2 weeks. Adipogenesis was indicated by the accumulation of lipid vacuoles stained with oil red O. Scale bar: 100 μm. (**d**) upper; Convex perichondrocytes were stimulated in the osteogenic induction medium for 3 weeks. Alkaline phosphatase staining was used for the detection of osteocytes. Scale bar: 100 μm. PCs (conv.), convex perichondrocytes; PCs (conc.), concave perichondrocytes; Cs, chondrocytes. lower; RT-PCR study for osteopontin, type I collagen, and type X collagen gene expression using convex perichondrocytes stimulated in the osteogenic induction medium (induction) for 3 weeks.



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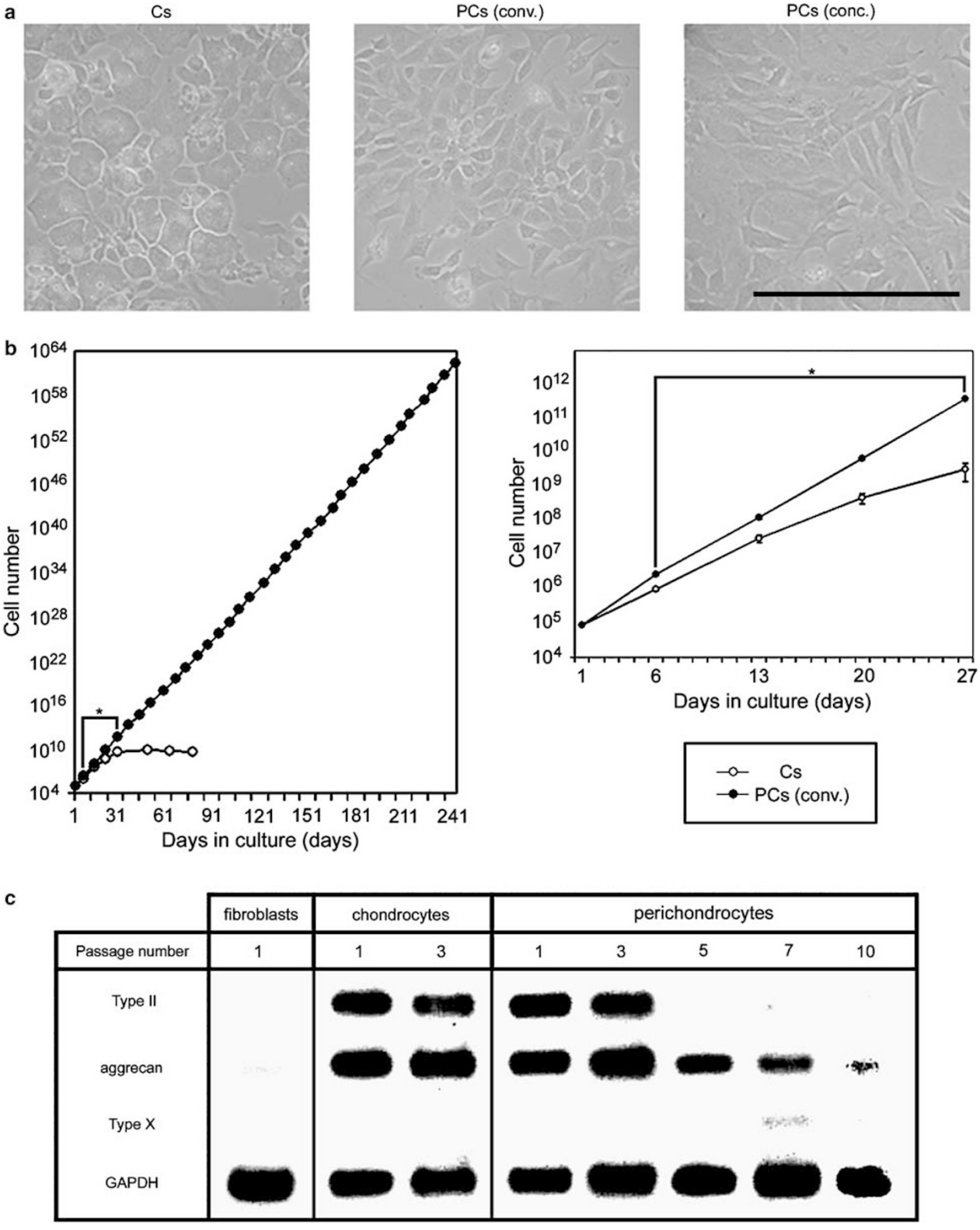


Figure 4 Characterization of cultured perichondrocytes and chondrocytes. (**a**) Cells at 5 days of the second passage maintained in D-MEM/F-12 medium with 10% FCS. Scale bar: 100 μm. (**b**) Growth curves of PCs (conv.) (-●-) and Cs (-○-) (left). The detailed data from the first 4 passages are shown on the right. Each point represents 1 passage. $^*P<0.01$, $n=3$. Data are shown as mean $\pm$ s.e. (**c**) RT-PCR study for type II collagen, aggrecan, and type X collagen gene expression using cultured convex perichondrocytes and chondrocytes at different passages as indicated. RT-PCR study with dermal fibroblasts at the first passage was also performed. PCs (conv.), convex perichondrocytes; PCs (conc.), concave perichondrocytes; Cs, chondrocytes.

cytes was analyzed. Perichondrocytes proliferated more rapidly than chondrocytes from the beginning of the primary culture (Figure 4b). The proliferation of chondrocytes gradually became slower and the cells stopped growing after 31 days (five passages). In contrast, perichondrocytes continued to grow at the same rate for more than 8 months (35 passages).

Total RNAs were collected from cells at different passages and RT-PCR was performed (Figure 4c). Cultured perichondrocytes and chondrocytes expressed the type II collagen and aggrecan genes until the third passage. Cultured dermal fibroblasts with a similar cell shape (data not shown) did not express either type II collagen or aggrecan, indicating that the cultured perichondrocytes established by the mechanical separation were different cell populations from dermal fibroblasts. After the fifth passage, only perichondrocytes were used for RT-PCR assays, because chondrocytes stopped growing by the fifth passage. In perichondrocytes, there was type II collagen gene expression until the third passage. Aggrecan gene expression was observed until the tenth passage but was drastically reduced after the fifth passage. At the seventh passage, perichondrocytes expressed a very low level of type X collagen, an early marker of calcification.

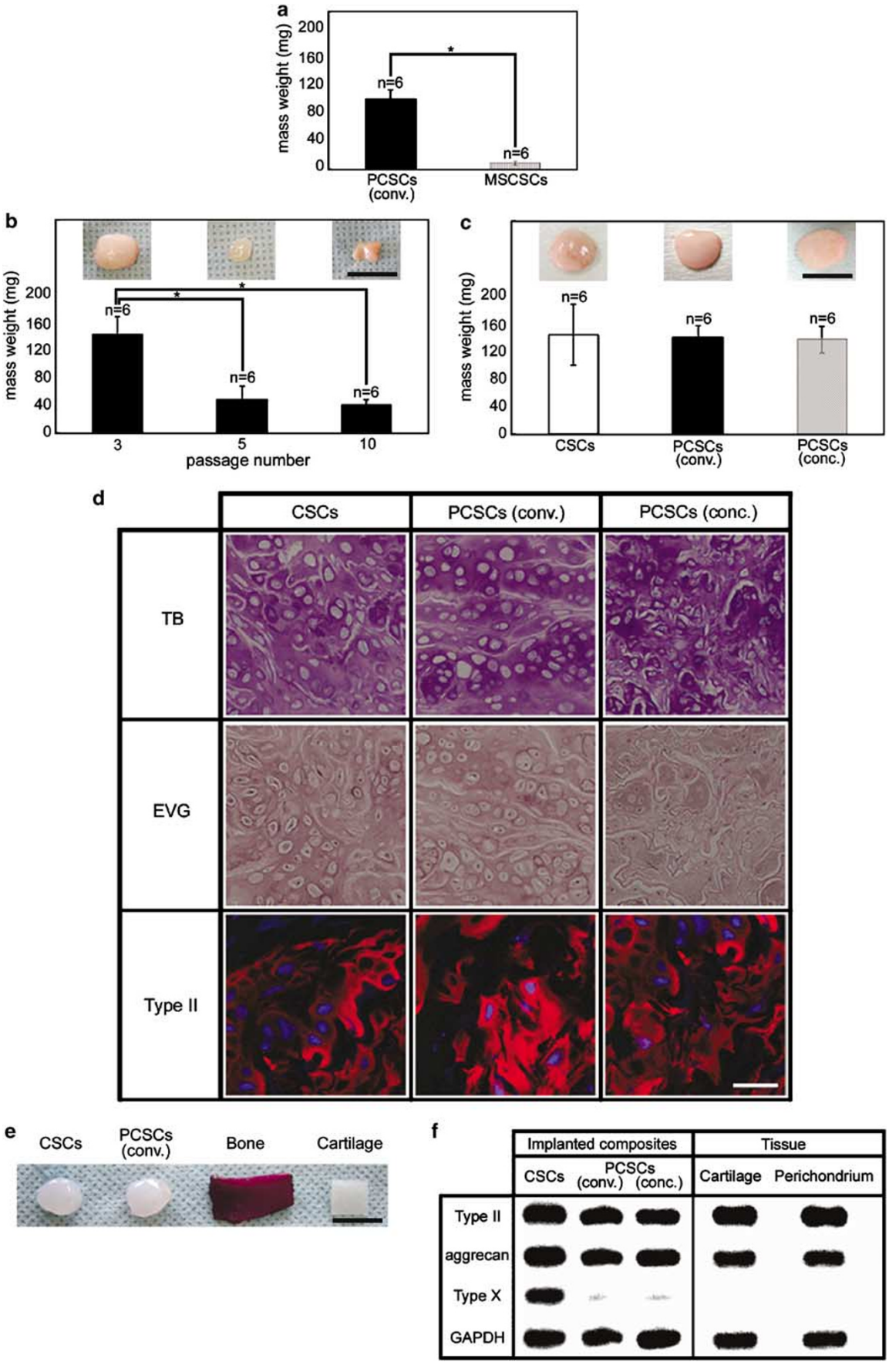
These findings showed that cultured perichondrocytes proliferated and had the capacity for production of cartilage-specific molecules even in D-MEM/F-12 medium with 10% FCS. However, type II collagen expression continued until only the third passage, suggesting that perichondrocytes should be used for cartilage reconstruction within the first few passages.

Cartilage Reconstruction with Collagen Sponge *In Vivo*

To study cartilage reconstruction *in vivo*, we utilized the collagen sponge scaffold system. For comparison study, we chose rabbit mesenchymal stem cells (MSCs) from bone marrow. MSCs were isolated from rabbit bone marrow.[19,20] First, pluripotency of these cells was confirmed by adipogenic, osteogenic, and chondrogenic induction study *in vitro* as described in materials and methods (data not shown). Then, we analyzed potency of cartilage reconstruction of perichondrocytes and MSCs from bone marrow. The MSC-scaffold composites (MSCSCs) seeded with 2.3×10^6 cells at second passage were stimulated in chondrogenic induction medium for 2 weeks *in vitro*, followed by implantation into a subcutaneous space in the dorsum of nude mice ($n = 6$). The convex perichondrocyte-scaffold composites (PCSCs (conv.)) seeded with 2.3×10^6 cells at second passage were incubated for 24 h before implantation ($n = 6$). 4 weeks after implantation, the MSCSCs were significantly smaller than the PCSCs (conv.) (Figure 5a). The weights of the PCSCs (conv.) and the MSCSCs were 98.5 ± 12.0 and 8.90 ± 2.90 mg, respectively. These results indicated that perichondrocytes are superior to MSCs for cartilage reconstruction *in vivo*. We examined the potency of cartilage reconstruction using convex perichondrocytes at different passage numbers. The PCSCs (conv.) seeded with 6.0×10^6 cells at the third ($n = 6$), fifth ($n = 6$), and tenth ($n = 6$) passage were implanted into a subcutaneous space in the dorsum of nude mice. At 4 weeks after implantation, the PCSCs (conv.) seeded with the fifth- and tenth-passage cells were significantly smaller than those seeded with third-passage cells (Figure 5b). The weights of the PCSCs (conv.) seeded with the third-, fifth-, and tenth-passage cells were 139 ± 24.1, 46.5 ± 20.2, and 39.1 ± 7.9 mg, respectively (Figure 5b). The PCSCs (conv.) seeded with the third-passage cells showed sulfated glycosaminoglycan deposits around the cells by toluidine blue staining (pH 2.5), but those with the fifth- and the tenth-passage cells did not show such signals (data not shown). Therefore, 6.0×10^6 convex and concave perichondrocytes and chondrocytes at the second passage were seeded on collagen sponge scaffolds. At 4 weeks after implantation, composites of these three groups showed indistinguishable appearance (Figure 5c). The weights of the PCSCs (conv.), the concave perichondrocyte-scaffold composites (PCSCs (conc.)) and the chondrocyte-scaffold composites (CSCs) were not different, 137 ± 15.4, 133 ± 18.3, and 140 ± 42.8 mg, respectively (Figure 5c). Histological and immunohistochemical examinations were performed (Figure 5d). Neither the PCSCs nor the CSCs had distinguishable signals of sulfated glycosaminoglycan around the cells by toluidine blue staining (pH 2.5). Elastica van Gieson staining gave rise to clear signals of collagenous components in all groups. Immunohistochemical staining showed that the level of type II collagen was similar in the PCSCs and the CSCs. Calcification did not occur in either the PCSCs or the CSCs, as shown by the lack of signal with Alizarin Red S staining (Figure 5e).

These results indicated that not only the CSCs but also the PCSCs could produce sulfated glycosaminoglycan and collagenous components and maintain a noncalcificated phenotype in the reconstructed cartilage.

To examine whether the PCSCs implanted in nude mice produce cartilage-specific molecules, RT-PCR was performed using the PCSCs and the CSCs at 4 weeks after implantation. The convex perichondrium and the cartilage were also excised from the rabbit ear and their RNAs were extracted. RT-PCR showed type II collagen and aggrecan gene expression in the PCSCs and the CSCs, which were detected in the tissue of the ear perichondrium and cartilage (Figure 5f). Type X collagen gene expression, which is specific for hypertrophic chondrocytes, was detected only in the CSCs, not in the PCSCs or in the tissue of the ear cartilage. The RT-PCR results suggested that the PCSCs would be


a
mass weight (mg)
200
160
120
80
40
0
n=6
n=6
*
PCSCs (conv.)
MSCSCs
b
mass weight (mg)
n=6
n=6
n=6
*
*
3
5
10
passage number
c
mass weight (mg)
n=6
n=6
n=6
CSCs
PCSCs (conv.)
PCSCs (conc.)
d
CSCs
PCSCs (conv.)
PCSCs (conc.)
TB
EVG
Type II
e
CSCs
PCSCs (conv.)
Bone
Cartilage
f
Implanted composites
Tissue
CSCs
PCSCs (conv.)
PCSCs (conc.)
Cartilage
Perichondrium
Type II
aggrecan
Type X
GAPDH

more useful for cartilage reconstruction than the CSCs, because the CSCs might induce undesirable hypertrophic chondrocytes. These results showed that the cultured second-passage perichondrocytes had the capacity for cartilage reconstruction *in vivo*.

Discussion

A major problem of cartilage reconstruction is failure of the maintenance of the implant because of its invariable absorption. The long-term success of a skin graft depends on suitable supplementation of stem cells in the graft. Similarly, for successful cartilage reconstruction, it will likely be necessary to use a sufficient number of self-renewing stem cells.[27] Stem cells have the ability to self-renew as well as to generate more-differentiated progeny, and many recent studies have indicated that most adult tissues contain stem cells. These adult stem cells are usually involved in homeostatic processes but are rapidly recruited to repair injured tissues.

In this study, the existence of LRCs was demonstrated in the perichondrium of the adult ear cartilage for the first time. LRCs are subpopulations of cells characterized by having a longer cell cycle than transient amplifying cells. They have been identified in the bulge region of hair follicles as stem cells. Following several cycles of division, the transient amplifying cells withdraw from the cell cycle and execute a terminal differentiation program.[28] Adult stem/progenitor cells from various tissues, such as articular cartilage,[29] epidermal keratinocytes,[16] and mammary glands,[30] are capable of proliferating vigorously after stimulation, shown by colony forming capacity. In this study, it is shown that more cells from the perichondrium could form large colonies than cells from the cartilage. Furthermore, perichondrocytes could differentiate into adipocytes and osteocytes *in vitro* and also into cartilage *in vivo*. Taken these data together, cartilage progenitor cells have been identified in the perichondrium of the adult ear for the first time.

Mesenchymal stem cells in the perichondrium of the hyaline cartilage of fetal limbs have been shown to be pluripotent.[31] However, for practical cartilage reconstruction, it is important to obtain cells from autologous cartilaginous tissues, not from allogenic grafts such as fetal tissues.

It was reported that chondrocytes from the articular cartilage of shoulder joints were implanted in nude mice with poly (L-lactide-ε-caprolactone) scaffolds, in which the cartilage was successfully reconstructed and kept its shape for over 10 months.[32] The cell population used in that study may have contained cells corresponding to those from the cambium layer of the perichondrium at the auricular cartilage used in the present study, because it seems to be difficult to maintain the regenerated tissue for a long time without stem cells.

In the present study, we showed that the perichondrocytes from the ear perichondrium are useful for regenerating cartilage. Perichondrocytes have been demonstrated to be superior to mesenchymal stem cells from bone marrow in this type of application *in vivo*. The perichondrocytes can be prepared without removing the ear cartilage itself, resulting in preservation of the shape of the donor site. Furthermore, the ear is the easiest site from which to obtain the perichondrium in clinical cases.

Shieh *et al*[14] claimed that cartilage was preferable as donor tissue, because the auricular perichondrium might be influenced by various factors, such as donor age or culture conditions, so the reproducibility of cartilage reconstruction and rate of yield of the new cartilage were inconsistent.

In our study, cultured perichondrocytes suddenly stopped producing type II collagen after only the third passage (Figure 4c), and lost the ability to generate cartilage (Figure 5a). However, van Osch *et al*[33] reported that the young auricular perichondrium produced more sulfated glycosaminoglycan when cultured in serum-free medium in combination with insulin-like growth factor 1 (IGF-1) and TGF-β2. Therefore, they claimed perichondrium became a useful source of cells for tissue engineering of cartilage.

It is known that cultured chondrocytes, probably containing perichondrocytes, gradually lose the activity to produce cartilage specific molecules after several passages. Redifferentiation of dedifferentiated chondrocytes has been attempted by the addition of various cytokines, such as insulin,[34] triiodothyronine,[35] bone morphogenetic protein-2,[36]

Figure 5 Cartilage reconstruction *in vivo*. (**a**) The average weight of the MSCSCs and the PCSCs (conv.) at 4 weeks after implantation. Collagen composites were seeded with 2.3×10^6 cells. The MSCSCs were maintained for 2 weeks in cartilage induction medium before implantation. The PCSCs were incubated for 1 day in D-MEM/F-12 medium with 10% FCS before implantation. Data are shown as mean ± s.e. $^*P<0.01$, $n=6$. (**b**) The average weights and appearances at 4 weeks after implantation of the PCSCs (conv.) seeded with 6.0×10^6 cells of the third-, fifth-, and tenth-passage. Data are shown as mean ± s.e. $^*P<0.01$, $n=6$. Scale bar: 10 mm. (**c**) The average weights and appearances at 4 weeks after implantation. The PCSCs (conv.), the PCSCs (conc.) and the CSCs were seeded with 6.0×10^6 cells. Data are shown as mean ± s.e. $n=6$. Scale bar: 10 mm. (**d**) Toluidine blue (pH 2.5) staining (TB) and Elastica van Gieson staining (EVG) of the PCSCs and the CSCs. Type II collagen was probed with a specific antibody at 4 weeks after implantation. Scale bar: 100 μm. (**e**) Alizarin Red S staining of the PCSCs (conv.) and the CSCs at 4 weeks after implantation. The bone and the ear cartilage of 4-week-old rabbits were also stained as a positive and negative control, respectively. Scale bar: 10 mm. (**f**) RT-PCR study for type II collagen, aggrecan, and type X collagen from the PCSCs, the CSCs at 4 weeks after implantation, the cartilage and the convex perichondrium from 4-week-old rabbit ear. MSCSCs, mesenchymal stem cell-scaffold composites; PCSCs (conv.), convex perichondrocyte-scaffold composites; PCSCs (conc.), concave perichondrocyte-scaffold composites; CSCs, chondrocyte-scaffold composites.

IGF-1,[37] TGF-β1,[38] epidermal growth factor, platelet-derived growth factor-bb, and fibroblast growth factor-2. However, the effects of these cytokines have not been elucidated by *in vivo* study. Perichondrocytes at the 13th passage were cultured with the combination of 10 ng/ml TGF-β1 and 10 ng/ml IGF-1 or with that of 10 ng/ml TGF-β3[39] and 10 ng/ml IGF-1. However, only aggrecan mRNA was induced, but type II collagen was not (data not shown). Thus, the optimal combination of cytokines for redifferentiation of dedifferentiated perichondrocytes is now under investigation. In the future, we will need to determine the culture conditions to maintaining the phenotype of perichondrocytes during further passages in order to reconstruct larger cartilage.

As we examined the composites 1 month after implantation in this study, it could not be determined whether perichondrocytes are able to maintain the cartilaginous phenotype for a longer period, for example, for several years *in vivo*. As this is an indispensable requirement for clinical therapy, we are now preparing human perichondrocytes from a patient with microtia for cartilage reconstruction with at least 1-year incubation *in vivo*. Although the culture conditions, requirement for growth factors, and kinds of scaffold materials must still be optimized, perichondrocytes from the adult ear showed much promise for cartilage reconstruction.

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