

K. Sekine, T. Ogawa, Y. Nagashima, U. Yokoyama, and T. Hirose for critical reading of the manuscript. This work was supported by Grant 20592101 from the Grant-in-Aid for Developmental Scientific Research, the Ministry of

Education, Science and Culture. This work was also supported by Grant 19-15 from the Research and Development Project of Yokohama City University, Yokohama, Japan.

1. Chang SC, Tobias G, Roy AK, Vacanti CA, Bonassar LJ (2003) Tissue engineering of autologous cartilage for craniofacial reconstruction by injection molding. *Plast Reconstr Surg* 112(3):793-799; discussion 800-801.
2. Matton G, Anseeuw A, De Keyser F (1985) The history of injectable biomaterials and the biology of collagen. *Aesthetic Plast Surg* 9:133-140.
3. Nagata S (1994) Modification of the stages in total reconstruction of the auricle: Part I. Grafting the three-dimensional costal cartilage framework for lobule-type microtia. *Plast Reconstr Surg* 93(2):221-230; discussion 267-268.
4. Maas CS, Monhian N, Shah SB (1997) Implants in rhinoplasty. *Facial Plast Surg* 13: 279-290.
5. Matarasso A, Elias AC, Elias RL (1996) Labial incompetence: A marker for progressive bone resorption in silastic chin augmentation. *Plast Reconstr Surg* 98:1007-1014, discussion 1015.
6. Zeng Y, Wu W, Yu H, Yang J, Chen G (2002) Silicone implants in augmentation rhinoplasty. *Aesthetic Plast Surg* 26:85-88.
7. Laurie SW, Kaban LB, Mulliken JB, Murray JE (1984) Donor-site morbidity after harvesting rib and iliac bone. *Plast Reconstr Surg* 73:933-938.
8. Skouteris CA, Sotereanos GC (1989) Donor site morbidity following harvesting of autogenous rib grafts. *J Oral Maxillofac Surg* 47:808-812.
9. Kline RM, Jr., Wolfe SA (1995) Complications associated with the harvesting of cranial bone grafts. *Plast Reconstr Surg* 95:5-13, discussion 14-20.
10. Whitaker LA, et al. (1979) Combined report of problems and complications in 793 craniofacial operations. *Plast Reconstr Surg* 64:198-203.
11. Berry L, Grant ME, McClure J, Rooney P (1992) Bone-marrow-derived chondrogenesis in vitro. *J Cell Sci* 101:333-342.
12. Ma HL, Hung SC, Lin SY, Chen YL, Lo WH (2003) Chondrogenesis of human mesenchymal stem cells encapsulated in alginate beads. *J Biomed Mater Res A* 64:273-281.
13. Terada S, Fuchs JR, Yoshimoto H, Fauza DO, Vacanti JP (2005) In vitro cartilage regeneration from proliferated adult elastic chondrocytes. *Ann Plast Surg* 55:196-201.
14. Yanaga H, et al. (2006) Clinical application of cultured autologous human auricular chondrocytes with autologous serum for craniofacial or nasal augmentation and repair. *Plast Reconstr Surg* 117(6):2019-2030; discussion 2031-2032.
15. Bianco P, Robey PG (2001) Stem cells in tissue engineering. *Nature* 414:118-121.
16. Shieh SJ, Terada S, Vacanti JP (2004) Tissue engineering auricular reconstruction: In vitro and in vivo studies. *Biomaterials* 25:1545-1557.
17. Dickhut A, et al. (2009) Calcification or dedifferentiation: Requirement to lock mesenchymal stem cells in a desired differentiation stage. *J Cell Physiol* 219:219-226.
18. Afizah H, Yang Z, Hui JH, Ouyang HW, Lee EH (2007) A comparison between the chondrogenic potential of human bone marrow stem cells (BMSCs) and adipose-derived stem cells (ADSCs) taken from the same donors. *Tissue Eng* 13:659-666.
19. Koga H, et al. (2008) Comparison of mesenchymal tissues-derived stem cells for in vivo chondrogenesis: Suitable conditions for cell therapy of cartilage defects in rabbit. *Cell Tissue Res* 333:207-215.
20. Sakaguchi Y, Sekiya I, Yagishita K, Muneta T (2005) Comparison of human stem cells derived from various mesenchymal tissues: Superiority of synovium as a cell source. *Arthritis Rheum* 52:2521-2529.
21. Togo T, et al. (2006) Identification of cartilage progenitor cells in the adult ear perichondrium: Utilization for cartilage reconstruction. *Lab Invest* 86:445-457.
22. Duynstee ML, Verwoerd-Verhoef HL, Verwoerd CD, Van Osch GJ (2002) The dual role of perichondrium in cartilage wound healing. *Plast Reconstr Surg* 110:1073-1079.
23. Haberal Can I, Atilla P, Cakar AN, Onerci M (2008) An animal study on cartilage healing using auricular cartilage as a model. *Eur Arch Otorhinolaryngol* 265:307-311.
24. Quirici N, et al. (2002) Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp Hematol* 30:783-791.
25. Boiret N, et al. (2005) Characterization of nonexpanded mesenchymal progenitor cells from normal adult human bone marrow. *Exp Hematol* 33:219-225.
26. Aslan H, et al. (2006) Osteogenic differentiation of noncultured immunisolated bone marrow-derived CD105+ cells. *Stem Cells* 24:1728-1737.
27. Nimura A, et al. (2008) Increased proliferation of human synovial mesenchymal stem cells with autologous human serum: Comparisons with bone marrow mesenchymal stem cells and with fetal bovine serum. *Arthritis Rheum* 58:501-510.
28. Covas DT, et al. (2008) Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. *Exp Hematol* 36:642-654.
29. Wagner W, Ho AD, Zenke M (2010) Different facets of aging in human mesenchymal stem cells. *Tissue Eng Part B Rev* 16:445-453.
30. Vignon E, Arlot M, Patricot LM, Vignon G (1976) The cell density of human femoral head cartilage. *Clin Orthop Relat Res* (121):303-308.
31. Kode JA, Mukherjee S, Joglekar MV, Hardikar AA (2009) Mesenchymal stem cells: Immunobiology and role in immunomodulation and tissue regeneration. *Cytotherapy* 11:377-391.
32. Sarugaser R, Hanoun L, Keating A, Stanford WL, Davies JE (2009) Human mesenchymal stem cells self-renew and differentiate according to a deterministic hierarchy. *PLoS ONE* 4:e6498.
33. Gojo S, et al. (2003) In vivo cardiovascularogenesis by direct injection of isolated adult mesenchymal stem cells. *Exp Cell Res* 288:51-59.
34. Tondreau T, et al. (2005) Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: Proliferation, Oct4 expression, and plasticity. *Stem Cells* 23:1105-1112.
35. Gleghorn JP, Jones AR, Flannery CR, Bonassar LJ (2007) Boundary mode frictional properties of engineered cartilaginous tissues. *Eur Cell Mater* 14:20-28, discussion 28-29.
36. Roy R, et al. (2004) Analysis of bending behavior of native and engineered auricular and costal cartilage. *J Biomed Mater Res A* 68:597-602.
37. Kikuchi K, et al. (2009) Transcripts of unknown function in multiple-signaling pathways involved in human stem cell differentiation. *Nucleic Acids Res* 37:4987-5000.
38. Stock UA, et al. (2001) Dynamics of extracellular matrix production and turnover in tissue engineered cardiovascular structures. *J Cell Biochem* 81:220-228.

Supporting Information

Kobayashi et al. 10.1073/pnas.1109767108

SI Methods

Long-Term Cell Growth Assay. To test long-term growth, cells were seeded in 35-mm easy-grip cell culture dishes at a density of 1,200 cells/cm². Starting 24 h after the seeding, the medium was exchanged every 3 d. When cells reached confluence after ~14 d of culture, they were detached using 0.2% type II collagenase in Hank's balanced solution, and the cells were counted using a hemocytometer. Subsequently, the cells were seeded in 35-mm easy-grip cell culture dishes at a density of 1,200 cells/cm². These operations were performed repeatedly.

In Vitro Colony Assay. To assess colony formation, we cultured nonsorted or sorted cells in our standard culture medium at a density of 52 cells/cm² under an atmosphere of 5% CO₂ at 37 °C. The culture medium was replaced every 7 d. The colonies were stained with Giemsa and counted 14 or 21 d after seeding.

RT-PCR. Total RNA was isolated from confluent perichondrocytes and chondrocytes. RNA extraction was performed with RNeasy (Qiagen) according to the kit's protocol. cDNA was obtained using RNA PCR kit (Takara). The primers were designed with Primer 3 software and listed below. For chondrogenic differentiation-related markers: type I collagen (COL1A1), forward 5'

cgacagaggcataaagggtca3' and reverse 5'tacacgcaggtctcaccagtctc3'; type II collagen (COL2A1), forward 5'ctggctcccaactgccaacgtc3' and reverse 5'tccttgggttgcaacggattgt3'; type X collagen (COL10A1), forward 5'cccactaccaacaccaagac3' and reverse 5'tttctgtccattcataccagg3'; aggrecan (ACAN), forward 5'gtatgtgaggaggctggaaca3' and reverse 5'cgcttctgtagtctgcgtttgta3'; and elastin (ELN), forward 5'tatggactgcctacaccag3' and reverse 5'agcactgggacaactggaat3'. For adipogenic differentiation-related markers: lipoprotein lipase (Lpl/LPL), forward 5'tggacgtaacaggaatgtatgag3' and reverse 5'ccctctggtgaatgtgtgaaga3'; aP2 (Fabp4), forward 5'ggtagctgaaactgtctccag3' and reverse 5'catgacgcattccaccaccag3'; and PPAR γ (PPARGC1A), forward 5'gtgtgctctctggttggaagac3' and reverse 5'gttgctggtgcccagtaagacttc3'. For osteogenic differentiation-related markers: runt-related transcription factor 2 (Runx2), forward 5'gagttcaccttgaccataaccgtcttcac3' and reverse 5'gtggtagatggatggacggg3'; and alkaline phosphatase (ALPL), forward 5'tcccgtgcaacaccaccag3' and reverse 5'caacgaggtccaggccgtcc3'. PCR condition was 94 °C, 1 min; 58 °C, 32 s; 72 °C, 30 s, followed by 70 °C, 6 min. The cycle of PCR reactions was 35. The products of RT-PCR were separated by electrophoresis on agarose gels, stained with ethidium bromide, and photographed.

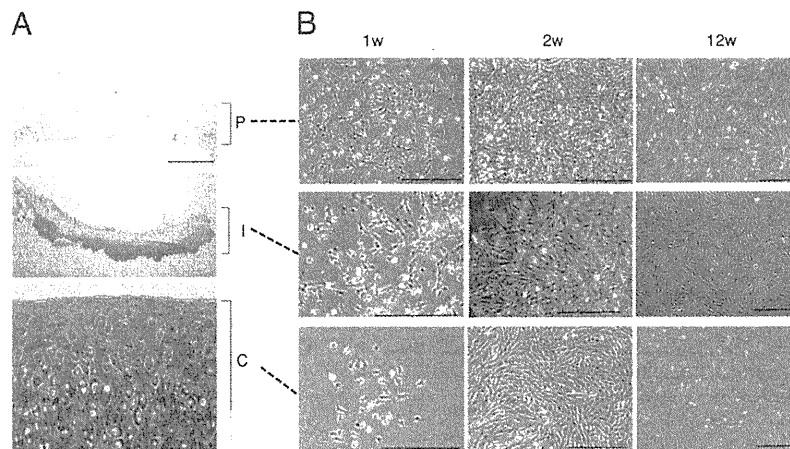


Fig. S1. Manual separation of perichondrium layer. (A) Alcian blue staining of each separated layer. Perichondrium layer never contains mature cartilage-derived cells. (B) Morphological observation of cells derived from each layer after 1, 2, and 12 wk of culture. (Scale bars, 500 μ m.)

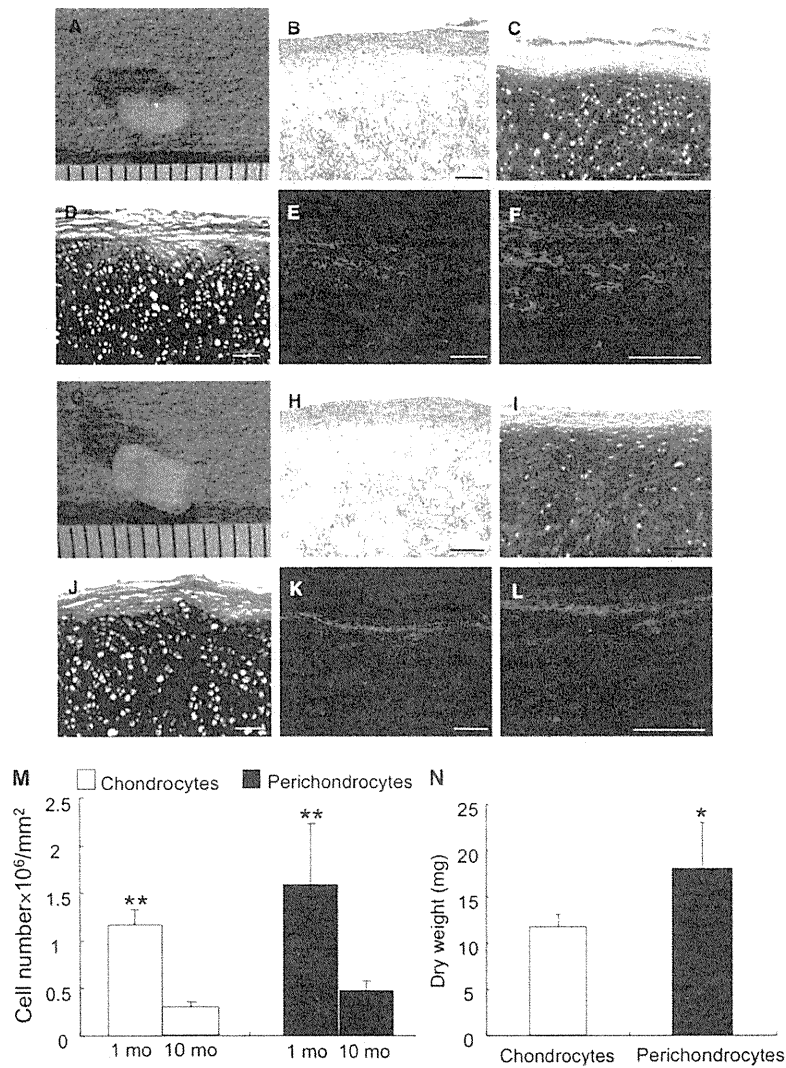


Fig. S2. Tissue restoration of regenerated elastic cartilage 6 (A–F) and 10 (G–L) mo after s.c. injection of human perichondrocytes. (A and G) Gross morphology, (B and H) Hematoxylin/eosin staining, (C and I) Alcian blue staining, (D and J) Elastica Van Gieson staining, (E, F, K, and L) immunohistochemistry for type I collagen (red) and type II collagen (green). (Scale bars, 100 μm .) (M) Decrease in cellularity between 1- and 10-mo construct. Data are shown as the mean \pm SD of repeated measures at four equally spaced areas, each with a cross-sectional area of $200 \times 200 \mu\text{m}^2$. ($n = 5$). ** $P < 0.01$. (N) Comparison of dry weight of 10-mo construct from chondrocytes and perichondrocytes. Data are shown as the mean \pm SD ($n = 4$). * $P < 0.05$.

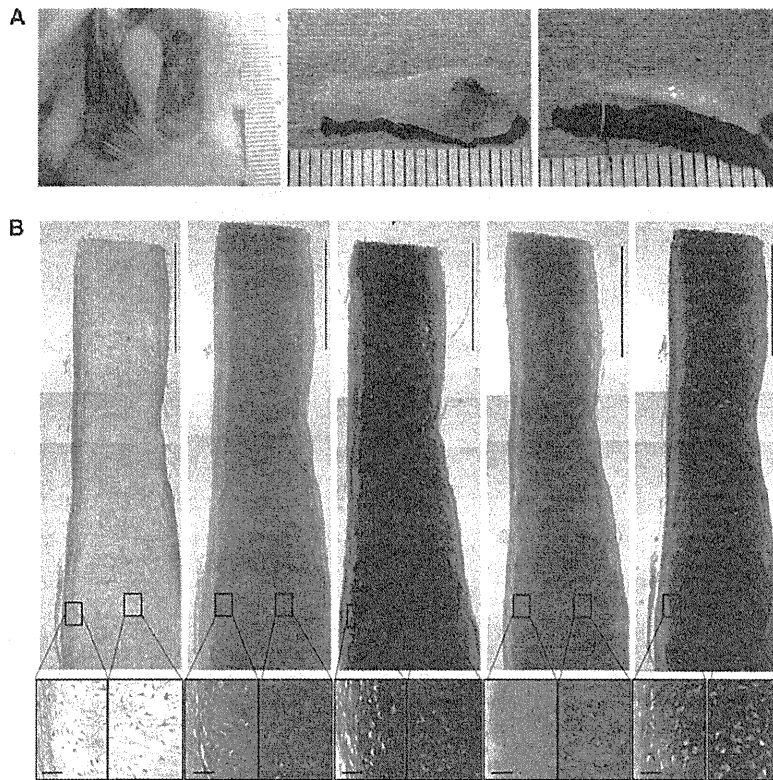


Fig. S3. Larger elastic cartilage reconstruction after direct cell injection. (A) Macroscopic observation of reconstructed cartilage 2 mo after high-volume cell injection. (Scale bars, 1 mm.) (B) Histological examinations show homogenous distribution of mature chondrocytes, which produce proteoglycan and elastic fibers, in every part of the construct. (Scale bars, 1 mm and 20 μ m.) (The figure is a composite of multiple panels.)

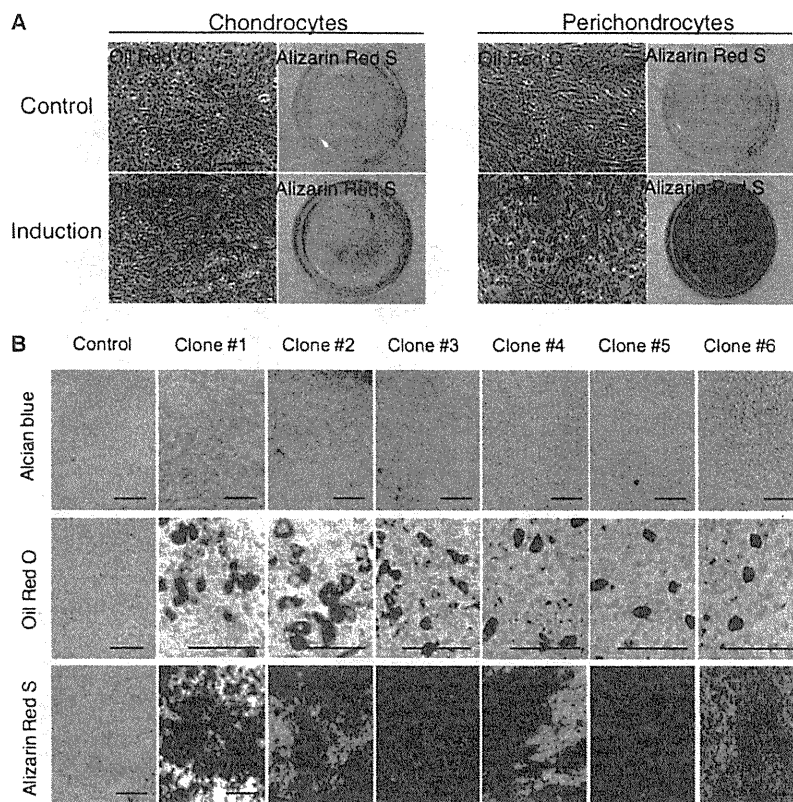


Fig. 54. Multilineage differentiation potential of clonally propagated CD44⁺ CD90⁺ cells. (A) Cytochemistry staining of chondrocytes (*Left*) and perichondrocytes (*Right*) after adipogenic and osteogenic induction. Control cells were cultured using our standard culture medium. Oil Red O and Alizarin Red S staining showed that the perichondrocytes differentiated into adipocytes and osteocytes, respectively. (Scale bar, 100 μ m.) (B) Multipotency of clone-sorted CD44⁺ CD90⁺ cells. All six different clonogenic progenies possess trilineage potential. (Scale bars, 200 μ m.)

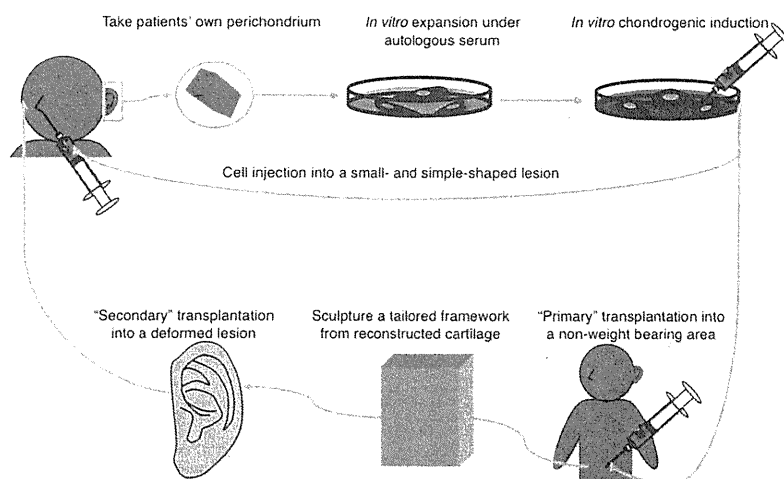
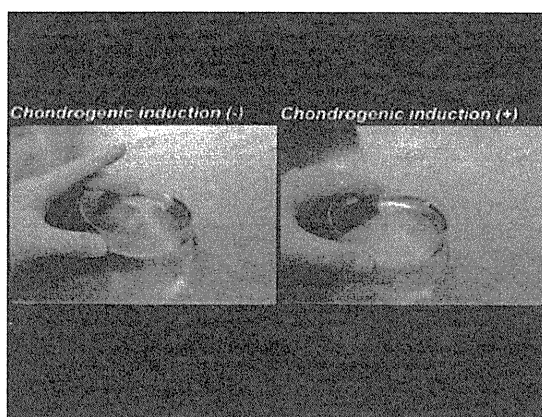


Fig. 55. Schematic representations of two different strategies toward clinical application: (i) direct cell injection and (ii) two-stage transplantation without scaffold.

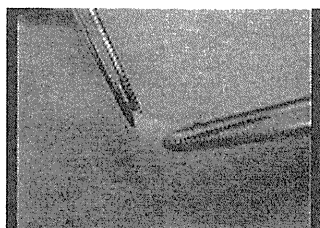
Table S1. The antihuman monoclonal antibodies used in this study

Specificity	Clone	Host	Isotype	Conjugate	Supplier
CD24	ML5	Mouse	IgG2a, κ	FITC	BD Pharmingen
CD34	AC136	Mouse	IgG2a	FITC	Miltenyi Biotec
CD44	G44-26	Mouse	IgG2b, κ	PE	BD Pharmingen
CD49e	NKI-SAM-1	Mouse	IgG2b, κ	FITC	BioLegend
CD73	AD2	Mouse	IgG1, κ	PE	BD Pharmingen
CD90	5E10	Mouse	IgG1, κ	APC	BD Pharmingen
CD105	43A3	Mouse	IgG1, κ	Alexa Fluor 488	BioLegend
CD117	A3C6E2	Mouse	IgG1	APC	Miltenyi Biotec
CD133/1	AC133	Mouse	IgG1	APC	Miltenyi Biotec
CD140a	αR1	Mouse	IgG2a, κ	PE	BD Pharmingen
CD146	P1H12	Mouse	IgG1, κ	PE	BD Pharmingen
CD271	ME20.4-1.H4	Mouse	IgG1, κ	FITC	Miltenyi Biotec



Movie S1. Secreted several mucopolysaccharides from the perichondrocytes after layered induction made the media viscous and matrix-like (*Right*), compared with standard culture media (*Left*). This viscosity let cells stay firmly aggregated under the skin when cells were directly injected.

Movie S1



Movie S2. Reconstructed cartilage possessed highly elastic property the same as normal auricular cartilage.

Movie S2

Presence of Cartilage Stem/Progenitor Cells in Adult Mice Auricular Perichondrium

Shinji Kobayashi^{1,4}*, Takanori Takebe¹*, Yun-Wen Zheng¹, Mitsuru Mizuno¹, Yuichiro Yabuki², Jiro Maegawa², Hideki Taniguchi^{1,3*}

1 Department of Regenerative Medicine, Yokohama City University Graduate School of Medicine, Kanazawa-ku, Yokohama, Kanagawa, Japan, **2** Department of Plastic and Reconstructive Surgery, Yokohama City University Graduate School of Medicine, Kanazawa-ku, Yokohama, Kanagawa, Japan, **3** Advanced Medical Research Center, Yokohama City University, Yokohama, Kanagawa, Japan, **4** Department of Plastic and Reconstructive Surgery, Kanagawa Children's Medical Center, Minami-ku, Yokohama, Japan

Abstract

Background: Based on evidence from several other tissues, cartilage stem/progenitor cells in the auricular cartilage presumably contribute to tissue development or homeostasis of the auricle. However, no definitive studies have identified or characterized a stem/progenitor population in mice auricle.

Methodology/Principal Findings: The 5-bromo-2'-deoxyuridine (BrdU) label-retaining technique was used to label dividing cells in fetal mice. Observations one year following the labeling revealed that label-retaining cells (LRCs) were present specifically in auricular perichondrium at a rate of $0.08 \pm 0.06\%$, but LRCs were not present in chondrium. Furthermore, LRCs were successfully isolated and cultivated from auricular cartilage. Immunocytochemical analyses showed that LRCs express CD44 and integrin- α_5 . These LRCs, putative stem/progenitor cells, possess clonogenicity and chondrogenic capability *in vitro*.

Conclusions/Significance: We have identified a population of putative cartilage stem/progenitor cells in the auricular perichondrium of mice. Further characterization and utilization of the cell population should improve our understanding of basic cartilage biology and lead to advances in cartilage tissue engineering and novel therapeutic strategies for patients with craniofacial defects, including long-term tissue restoration.

Citation: Kobayashi S, Takebe T, Zheng Y-W, Mizuno M, Yabuki Y, et al. (2011) Presence of Cartilage Stem/Progenitor Cells in Adult Mice Auricular Perichondrium. PLoS ONE 6(10): e26393. doi:10.1371/journal.pone.0026393

Editor: Samuel J. Lin, Harvard Medical School, United States of America

Received: July 4, 2011; **Accepted:** September 26, 2011; **Published:** October 19, 2011

Copyright: © 2011 Kobayashi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by grant from Grant-in-aid for Developmental Scientific Research, The Ministry of Education, Science and Culture (No. 20592101) and Health and Labour Sciences Research Grants Research on intractable disease (No. 2011-164). This work was also supported by the grant for Research and Development Project (No. 19-15) of Yokohama City University, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: zoukisasi@yokohama-cu.ac.jp

† These authors contributed equally to this work.

Introduction

Evidence from several tissues, including epidermis, cornea, adipose and skeletal muscle indicate that stem cells support tissue maintenance by balancing self-renewal and differentiation [1,2,3,4]. Auricular cartilage, known as elastic cartilage, also is considered that stem/progenitor cells support tissue maintenance after birth. In regard to the other type of cartilage maintenance, costal cartilage known as hyaline cartilage is clinically regenerated from surrounding perichondrium, suggesting the presence of stem/progenitor cells in perichondrium [5,6,7]. Although several *in vitro* studies indicate that the auricular perichondrium can support cartilage regeneration and that the auricular perichondrium, like that surrounding the costal cartilage, harbors multipotent progenitor cells, no definitive studies have demonstrated the existence of stem/progenitor cells in the auricle cartilage or surrounding perichondrium [8].

Identification of cartilage stem/progenitor cells in the auricle is useful for understanding the development of the auricle system

and potentially for developing a source of cells for cartilage tissue engineering. Conventional treatments for patients with craniofacial defects, which involve transplantation of auto-costal cartilage, have several critical problems, including donor limitation, donor involvement, and absorption over time [7,9,10]. To overcome these problems, alternative treatments that are based on elastic cartilage engineering and that use terminally differentiated chondrocytes derived from the auricle have been developed [11]. However, using differentiated chondrocytes puts a significant burden on donor sites; moreover, these grafts, which do not contain self-renewing stem cells, are absorbed over time not maintained. These clinical limitations may be overcome by the use of stem cells, as self-renewing stem cells can lead to permanent restoration of tissues characterized by high and continuous self-renewal [12].

The aim of our study was to determine whether stem/progenitor cells were present in auricular cartilage of adult mice. Initially, we performed a label-retention assay, which has been used to identify putative stem cells in several tissue types, in the

developing auricle of mice [13,14,15]. Furthermore, we successfully isolated and cultivated a population of cells that retained the label, designated, long-term label retaining cells (LRCs), and characterized these LRCs based on expression of cell-surface markers.

Results

Changes in the surface area of the external ear and in the thickness of the auricular cartilage

The mean surface area of the outer ear was $9.4 \pm 0.7 \text{ mm}^2$, $15.9 \pm 0.3 \text{ mm}^2$, $63.0 \pm 5.3 \text{ mm}^2$, $175.8 \pm 10.0 \text{ mm}^2$, $191.6 \pm 3.3 \text{ mm}^2$, and $235.9 \pm 11.1 \text{ mm}^2$ (N=6) 3 days, 1 week, 2 weeks, 4 weeks, 24 weeks, and 48 weeks, respectively, following birth (Figure 1A and B). Growth of the auricle was rapid during the 4 weeks following birth, but growth slowed after that point. Mean thickness, which was measured at the identical time points, was $32.0 \pm 2.6 \text{ mm}$, $37.0 \pm 2.0 \text{ mm}$, $38.7 \pm 1.5 \text{ mm}$, $29.7 \pm 1.4 \text{ mm}$, $24.7 \pm 1.5 \text{ mm}$, and $23.7 \pm 1.3 \text{ mm}$, respectively (N=6) (Figure 1B). The surface area of the auricular cartilage increased throughout the first 4 postnatal weeks, while the thickness of the auricular cartilage decreased over the first 2 weeks. These results indicate that cells in the auricular cartilage, which consists of chondrium and perichondrium, were proliferating rapidly within the first 4 weeks following birth. Consequently, putative stem cells, if exist, will transition to a dormant state 4 weeks post-birth. These observations let us examine the label-retaining approach to clarify the presence of stem cells in the auricle, which was well established technique to identify the dormant or slowly cycling cells.

Auricular perichondrium contains long-term DNA label-retaining cells (LRCs)

To identify putative stem cells in elastic cartilage of the auricle, we performed a label-retention assay that has been used to identify putative stem cells in many tissues. We performed the 5-bromo-2'-deoxyuridine (BrdU)-labeling assay on the auricle of adult mice (4

weeks old). BrdU was injected intraperitoneally, and the tissue was analyzed the following day. However, no cells in either the perichondrium or the chondrium were labeled within 24 h of the last BrdU injection (Figure S1).

Then, we treated pregnant mice with BrdU on day 17 to 19 of gestation to label proliferating cells in the fetuses during auricular development. The auricles of the offspring were analyzed at multiple time points (on day 0 and day 3 and at 1, 2, 4, 24, and 48 weeks) (Figure 2A–G). To assess the efficiency of BrdU incorporation, the auricles of neonate mice were immunohistochemically labeled with monoclonal antibodies against BrdU; the majority of cells ($83.6 \pm 5.4\%$) were labeled in neonates (N=6) (Figure 3A). Within two weeks, the number of BrdU-positive cells, i.e., label-retaining cells (LRCs), rapidly decreased to $2.5 \pm 0.2\%$ (N=6). Concomitantly, localization of LRCs is restricted to the perichondrium layer (50.0%, 53.8%, 80.3% and 69.7% of BrdU positive cells, respectively) (Figure 3B). After 4 weeks, no LRCs were observed in the chondrium. However, LRCs were observed in the perichondrium even after 4 weeks ($0.3 \pm 0.2\%$ of cells) (N=3). After 24 and 48 weeks, long-term LRCs were detected only in the perichondrium ($0.1 \pm 0.05\%$ and $0.08 \pm 0.06\%$ of all 4,6-diamidino-2-phenylindole (DAPI)-stained cells, respectively) (N=3, each timepoint) (Figure S2A, B, and Figure 3B). These LRCs, observed only in the perichondrium layer, seemed dormant, which is characteristic of putative stem cells.

Transition to a dormant state after experiencing transient amplification

To determine the ratio of proliferating cells during auricular development, Ki-67 immunocytochemistry was performed. The nuclear antigen Ki-67 is a marker of proliferation and is expressed only in cycling cells. In the chondrium, Ki-67 staining was seen in $3.4 \pm 1.7\%$, $34 \pm 5.9\%$, $8.4 \pm 2.0\%$, and $0.4 \pm 0.8\%$ of all DAPI-stained cells at postnatal day 0, day 3, week 1, and week 2,

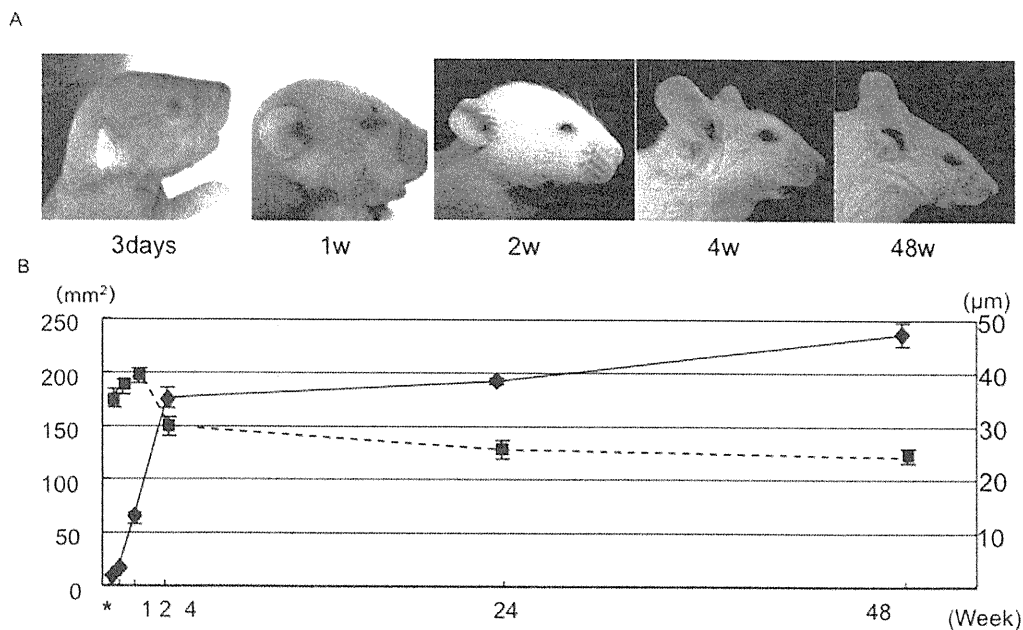


Figure 1. Development of murine external ears. (A) The external ear at several developmental stages. From the left, ears were photographed 3 days, 1 week, 4 weeks, and 48 weeks after birth. (B) Mean surface area of the external ear and mean thickness of auricular cartilages changed during postnatal development. * ; 3 days, ; mean surface area of the external ear (mm²), ; mean thickness of auricular cartilage in the middle area of the external ear (µm) (N=6).

doi:10.1371/journal.pone.0026393.g001

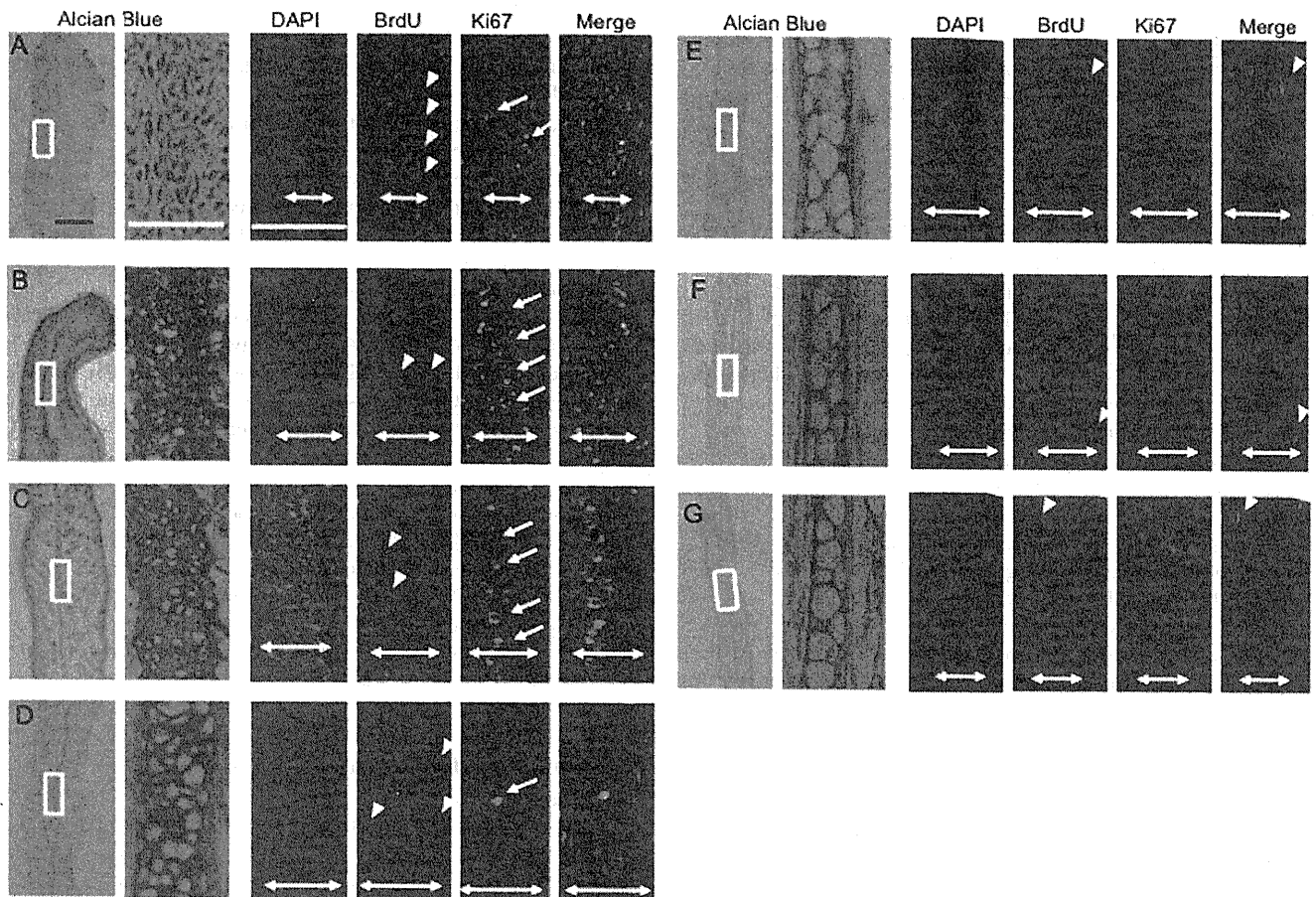


Figure 2. 48-week chase analysis of BrdU-labeled cells and Ki67-positive cells. BrdU-labeled cells gradually decreased in auricular cartilage (White arrowheads). A few BrdU-labeled cells were present in the perichondrium of 48-week-old, BrdU-labeled offspring. No LRCs were observed in chondrocytes of the chondrium 4 weeks after BrdU labeling. No Ki67-positive cells were seen in perichondrium 1 week following birth (White arrows). No Ki67-positive cells were observed in the chondrium 2 weeks following birth. (A) 0 day, (B) 3 day, (C) 1 week, (D) 2 weeks, (E) 4 weeks, (F) 24 weeks and (G) 48 weeks. From the left, Alcian blue staining, DAPI, BrdU, Ki67, and a merged image. Two-headed arrows: the cartilage width including perichondrium. Black scale bar = 200 μm , White scale bar = 50 μm . doi:10.1371/journal.pone.0026393.g002

respectively ($N = 3$, each timepoint) (Figure 2A–G and 3C). After 4 weeks, no Ki-67-positive cells were observed in the chondrium. In the perichondrium, Ki-67 staining was seen in $1.5 \pm 0.8\%$ and $8.5 \pm 3.5\%$ of all DAPI-stained cells at postnatal day 0 and day 3 respectively, and no Ki-67-positive cells were observed at week 1 or week 2. These observations may indicate that cells in the perichondrium transitioned to the dormant state of stem cells after a transient period of amplification sometime within the first week following birth.

LRCs localize to the opening of the external acoustic meatus

To determine the distribution of LRCs in the auricular cartilage, the auricles of 24-week-old BrdU-labeled mice were analyzed (Figure 4A). Interestingly, the proximal part, i.e., the opening of the external acoustic meatus, contained a higher percentage of LRCs ($23.87 \pm 0.07\%$) than the distal ($0.02 \pm 0.03\%$) or middle ($0.01 \pm 0.00\%$) parts of the auricle ($N = 3$) (Figure 4B).

Specific expressions of CD44 and integrin α_5 in auricular perichondrium, but not chondrium

To further characterize the LRCs (putative stem cells), the auricles of 24-week-old mice were stained with antibodies against

well-characterized cell-surface molecules. Integrin- α_1 , α_2 , α_L , and α_V were expressed by all cells in the chondrium (Figure S3). Cells in the perichondrium and the chondrium expressed integrin- β_1 , but not integrin- α_6 , α_M , α_X , β_2 , β_3 , syndecan-1, 3, 4, PECAM, VCAM-1, or Flk-1. Interestingly, expression of CD44 and integrin- α_5 was specific to the perichondrium, indicating that these molecules may be markers for the putative auricular stem/progenitor cell population (Table 1).

Characterization of long-term LRCs in vitro

Based on the immunohistochemistry analyses, we next examined whether auricular LRCs expressed CD44 and integrin- α_5 . Long-term BrdU labeled mice auricles were harvested at 24 weeks post birth. After enzymatic digestion of harvested auricle, we successfully isolated and cultivated the LRCs *in vitro*. Primary cultures of auricle cells isolated from 24-week-old BrdU-labeled mice were stained with antibodies against CD44 and integrin- α_5 . The long-term LRCs expressed both CD44 and integrin- α_5 , indicating both are potential stem cell markers (Figure 5A).

To examine the clonogenic capability of LRCs in BrdU-labeled 24-week-old mice auricle, cells were plated onto a cell culture dish under clonal culture condition ($1,000$ cells/ cm^2). Immunocytochemistry showed that long-term LRC possessed a capacity to

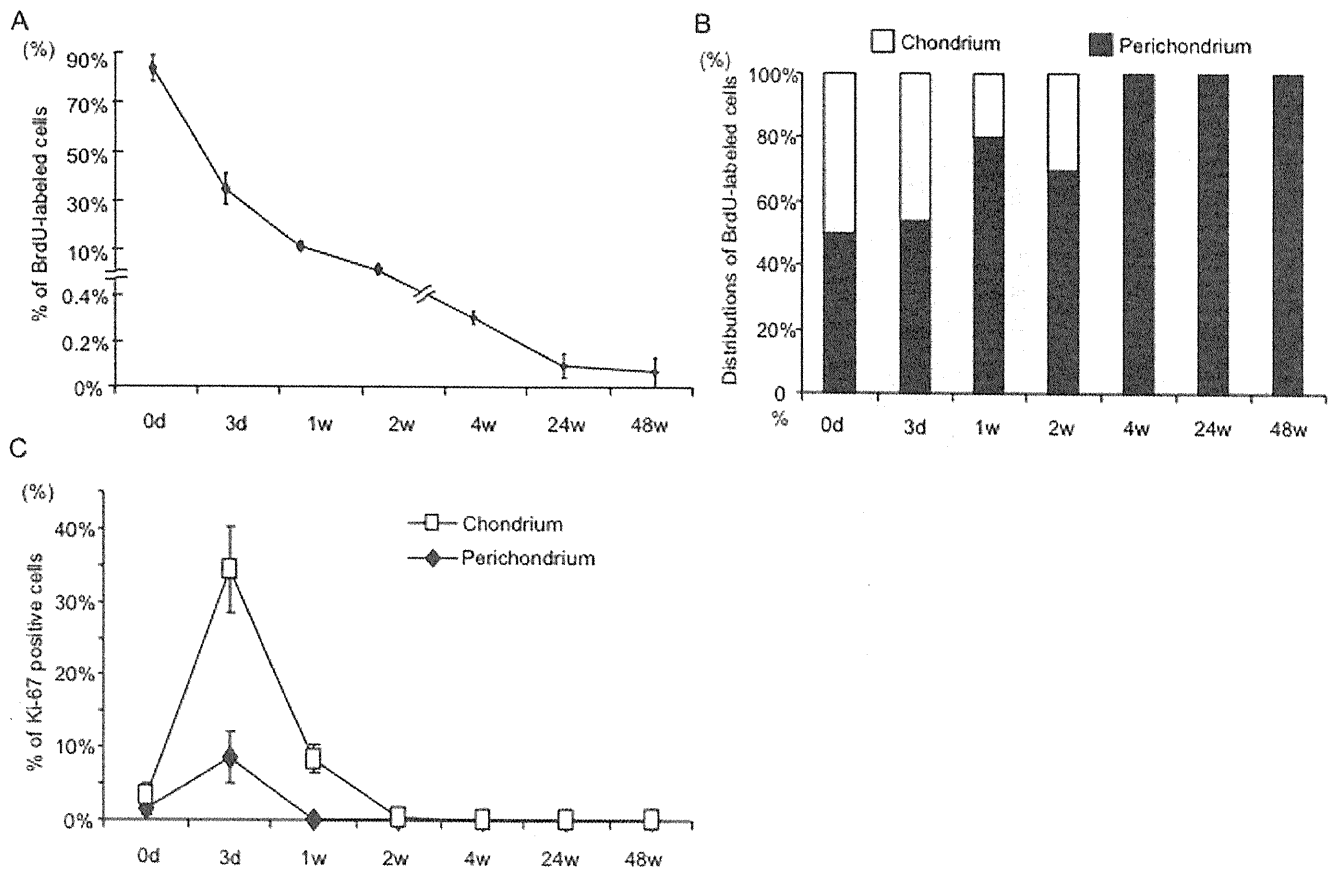


Figure 3. Presence of long-term label retaining cells in auricular perichondrium 48 weeks following birth. (A) Quantification of BrdU-label retaining cells from 0 day to 48 weeks in mice auricular cartilage (N=6). (B) The distributions of LRCs between chondrium and perichondrium layer. 4 weeks after birth, all LRCs were detected in perichondrium, but not chondrium layer. (C) Proliferation of cells in auricular cartilage. Cells in both layers extensively proliferate till 2 weeks post birth. Graph shows time course-dependent changes of Ki67-positive cell index (KI) in perichondrium and chondrium for 48 weeks after birth. ;KI of Perichondrium, ; KI of Chondrium. doi:10.1371/journal.pone.0026393.g003

form a colony following 14 days cultivation (Figure 5B). Interestingly, BrdU-label was confined to a part of cells inside the colonies. This phenomenon seemed to be a result of asymmetric DNA segregation, indicating an asymmetric cell division, that is, one major characteristic of stem cells [16].

Identification of stem/progenitor cells will facilitate future efforts towards auricular cartilage regeneration. To determine the chondrogenic potential of cells, semi-clonally expanded cells containing long-term LRCs were cultivated under high-density condition *in vitro*. Alcian blue staining showed the production of cartilage proteoglycans, indicating the chondrogenic potential (Figure 5C).

Discussion

We demonstrated the presence of putative stem/progenitor cells in auricular elastic cartilage using a BrdU-labeling assay, a well-established technique that identifies stem cells based on their quiescent nature [17]. Long-term LRCs were found in the perichondrium, surrounding the auricular chondrium. LRCs are characterized by having a longer cell cycle than actively proliferating cells. During cartilage growth and maturation, mature cells experience a transient period of active proliferation and finally execute a terminal differentiation program. As a result, BrdU labeling diminishes during several cycles of division in the active proliferation period. We successfully incorporated the BrdU

label into the majority of the cells of E17 fetuses that were to develop into the auricle. Observations the 48 weeks after the birth of BrdU-labeled offspring revealed that long-term LRCs remained at a rate of $0.08 \pm 0.06\%$ in perichondrium and that no cells in the chondrium retained BrdU label. Consistent with this, Ki67 immunocytochemistry showed that during the 4 weeks following birth, cells in chondrium were proliferating vigorously and contributed to the rapid enlargement of auricle. However, after 4 weeks, no proliferating cells were detected in the chondrium. In contrast, one week after birth, no proliferating cells were found in the perichondrium. Stem/progenitor cells go into a dormant state more rapidly than differentiated cells; therefore, the long-term LRCs in the perichondrium may be a putative stem/progenitor population in auricular cartilage. Furthermore, this population of rare slow-cycling cells may contribute to long-term maintenance of the auricle.

Several reports indicate that stem cells are present in hyaline cartilage. The surface layer, growth plate, and synovium of the articular cartilage contain multipotent stem/progenitor cells [18,19,20]. The fibrous layer surrounding costal cartilage contains cells with regenerative capacity, and the cartilage surrounding costal bone might have high growth activity [21]. However, there are no reports that describe the stem/progenitor cells in the elastic cartilage of mice. Using a long-term LRC assay, we demonstrated that a population of putative stem/progenitor cells was present in the auricular perichondrium of 48-week-old mice. Interestingly,

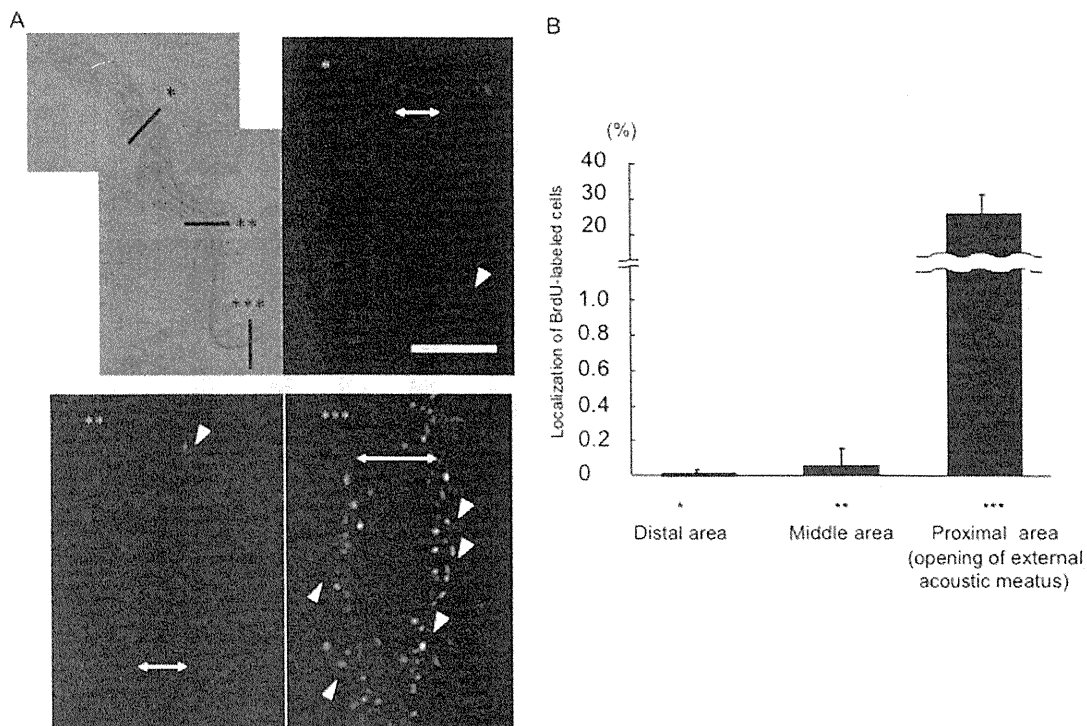


Figure 4. Localization of long-term LRCs in different parts of the external ear. (A) A few LRCs were present in the perichondrium in the distal and middle areas of the external ear (White arrowheads). Many LRCs were present in the opening of the external acoustic meatus (White arrowheads). Two-headed arrows: Chondrium. Scale bar = 50 μ m. Original magnification: $\times 40$ (Alcian blue staining), $\times 100$ (BrdU staining). (B) The LRCs of the distal and middle areas of the external ear and in the opening of the external acoustic meatus was much higher than that of the distal and middle areas (N = 6). doi:10.1371/journal.pone.0026393.g004

Table 1. Cell surface marker characterization of chondrium and perichondrium layer using 24-week-old mice auricles.

Marker	Perichondrium	Chondrium
Integrin alpha-1	-	++
Integrin alpha-2	-	++
Integrin alpha-5	++	-
Integrin alpha-6	-	-
Integrin alpha-V	-	++
Integrin alpha-L	-	++
Integrin alpha-M	-	-
Integrin alpha-X	-	-
Integrin beta-1	++	++
Integrin beta-2	-	-
Integrin beta-3	-	-
Syndecan 1	-	-
Syndecan 3	-	-
Syndecan 4	-	-
PECAM ^a	-	-
VCAM 1 ^b	-	-
Flk 1 ^c	-	-
CD44	+	-

NOTE. (-) = no immunostained cells; (+) = 0 < positive cells \leq 5%; (++) = over 5% cells positive.

^a; PECAM 1 = platelet/endothelial cell adhesion molecule.

^b; VCAM 1 = vascular cell adhesion molecule 1.

^c; Flk1 = Fetal Liver Kinase1.

doi:10.1371/journal.pone.0026393.t001

most LRCs were in the opening of the external acoustic meatus, but not at the distal part of the auricle. Generally, injured auricular cartilage has a limited capacity to heal, resulting in uncontrollable scar formation. Further characterization of stem/progenitor cells may help to develop a method to guide these cells to differentiate and treatments that can control scar formation. Finally, maximizing intrinsic regenerative capacity may lead to a novel strategy for auricular regeneration or repair.

Thus, characterization of LRCs, i.e., stem cells, will pave the way to for cartilage regenerative therapies. Based on the cell-surface markers expressed in articular chondrocytes, we analyzed expression of several cell-surface molecules to identify markers for the putative stem cell population in the auricular perichondrium. Of the molecules analyzed, CD44 and integrin- α_5 antigens were specifically expressed in cells of the auricular perichondrium. CD44 is a multifunctional adhesion molecule that binds to hyaluronan (HA), type I collagen, and fibronectin. Mesenchymal stem cells, neural crest-derived cells, and embryonic stem cells express CD44 antigens [22,23,24,25]. Integrin- α_5 , a type 1 collagen and fibronectin ligand, is a component of the auricular perichondrium [26,27,28,29,30,31]. Stem cells in the articular surface and growth plate also express integrin- α_5 , indicating that these are potential stem/progenitor cell markers [26,32,33]. Based on these observations, long-term LRCs were examined in vitro and shown to co-express CD44 and integrin- α_5 . Both molecules are thus promising markers for future enrichment of stem cells.

In conclusion, we identified long-term label-retaining stem/progenitor cells in auricular perichondrium by analyzing BrdU-labeled auricles of mice. Utilization of these stem/progenitor cells from auricular cartilage will not only improve our understanding of basic cartilage biology, but it may also lead to novel therapeutic

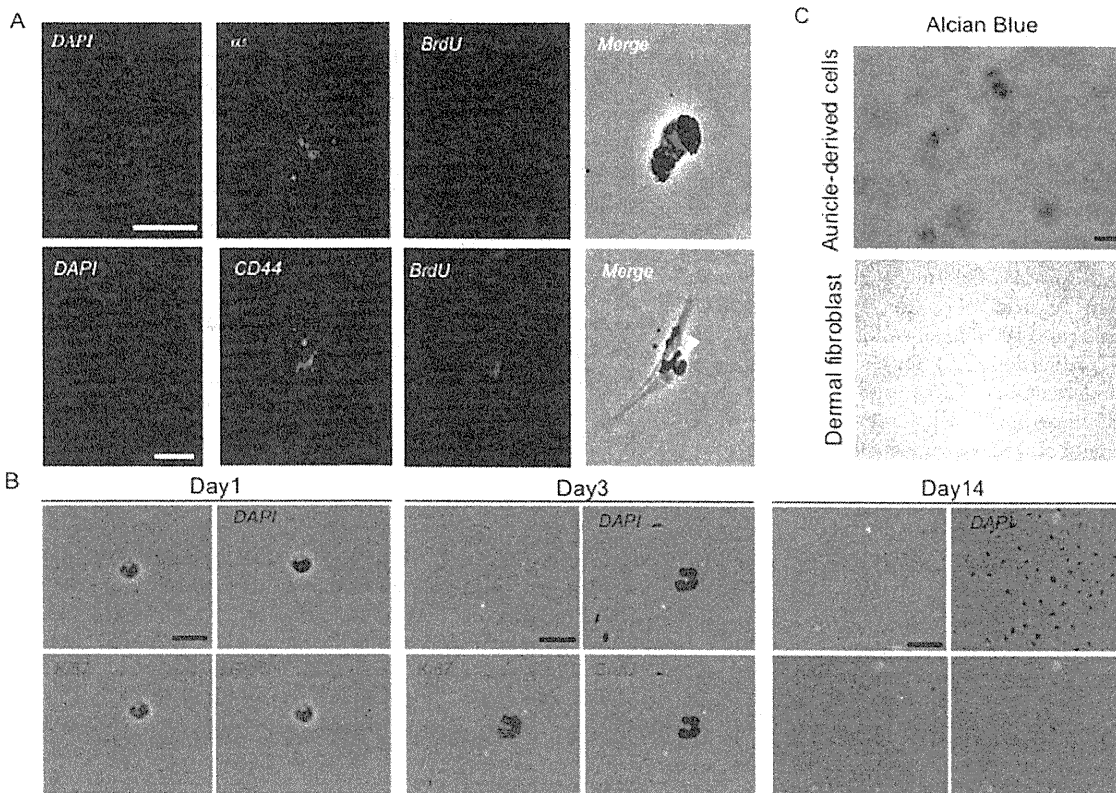


Figure 5. In vitro characterization of CD44+ integrin- α 5+ LRCs. (A) Immunocytochemistry of LRCs in vitro. Integrin- α 5 and CD44 were co-expressed in BrdU-labeled cells. CD44 staining: scale bar = 20 μ m; integrin- α 5 staining: scale bar = 40 μ m. Original magnification: \times 200. (B) Colony formation of LRCs. Cells from 24-week-old mice, which were injected with BrdU as E17 to E19 fetuses, were harvested from mice auricle following collagenase digestion. Colony assay was performed to examine the clonogenicity of LRCs. Cells were stained at 1, 3 and 14 day after plated. Clonal colonies were stained with antibodies against DAPI, Ki-67 and BrdU. Scale bars = 20 μ m or 100 μ m (C) Alcian blue staining of cells isolated from auricular cartilage or dermal fibroblasts. Scale bar = 200 μ m.
doi:10.1371/journal.pone.0026393.g005

strategies, including long-term tissue restoration, for patients with craniofacial defects.

Materials and Methods

Time-dependent changes in the surface area of the external ear and in the thickness of auricular cartilage

The surface area of the external ear was calculated using measurements from the bottom of the ear lobe to the distal end of the auricle. The thickness of the auricular cartilages was measured in Alcian blue-stained specimens using 4 equidistant points from the bottom of the ear lobe to the distal end of the auricle. The surface area and thickness were measured on days 1, 2, and 4 and weeks 24 and 48 after birth. Mean values were calculated from the measurements of 6 mice using the WinROOF software (MITANI, Fukui, Japan).

BrdU labeling

To label slow-cycling cells, ICR pregnant mice with embryonic day 17 mice (Japan SLC Co, Shizuoka, Japan) were injected with BrdU (50 μ g/g body weight [WTg]) (Sigma-Aldrich, St. Louis, MO) intraperitoneally on twice daily for 3 days. The offspring were sacrificed as newborns or on day 2, day 4, week 24, or week 48 following birth. All animal experimentation was conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan), and all experimental procedures were approved by institutional review board of Animal

Research Center, Yokohama City University School of Medicine (11–68).

Histochemical and immunohistochemical analysis

Tissue sections were stained with Hematoxylin/eosin or Alcian blue. For immunohistochemical analysis, the tissue sections were immunolabeled with primary antibodies: anti-BrdU antibody (sheep, 1:1200; Sigma-Aldrich, St. Louis, MO, USA) Ki67 (rabbit, 1:200; Abcam, Cambridge, MA, USA) α 1-integrin (hamster, 1:100; BD Biosciences, San Jose, CA USA), α 2-integrin (hamster, 1:100; BD Biosciences), α 5-integrin (rabbit, 1:100; Chemicon, Temecula, CA USA), α 6-integrin (rat, 1:100; Chemicon), α V-integrin (rabbit, 1:100; Chemicon), α L-integrin (rat, 1:100; eBioscience, San Diego, CA, USA), α M-integrin antibody (rat, 1:100; eBioscience), α X-integrin (hamster, 1:100; eBioscience), β 1-integrin (hamster, 1:100; BD Biosciences), β 2-integrin (rat, 1:100; eBioscience), β 3-integrin (hamster, 1:100; BD Biosciences), CD44 (rat, 1:100; Chemicon), Syndecan-1 (rat, 1:100; BD Biosciences), Syndecan-3 (rabbit, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Syndecan-4 (rat, 1:100; BD Biosciences), PECAM (rat, 1:100; BD Biosciences), VCAM-1 (rat, 1:100; Chemicon), or Flk-1 (rat, 1:100; BD Biosciences) at 4°C overnight.

After washing, the sections were incubated with secondary antibody, Cy3-conjugated Donkey anti-sheep IgG antibody (1:1600; Chemicon), Cy3-conjugated Donkey anti-Rabbit IgG antibody (1:1600; Jackson Immuno Research Laboratories, Inc. West Grove, PA, USA), and/or Alexa488-conjugated Donkey

anti-rabbit IgG antibody (1:1200; Molecular Probes, Junction City, OR, USA) depending on the primary antibody used. Tissue sections were incubated with secondary antibody for 1 h at room temperature. The samples were counterstained with DAPI and analyzed with a LSM510 laser-scanning microscope (Zeiss).

Calculation of BrdU-labeling Index (LI) and the Ki67-positive cell Index (KI)

The number of BrdU-positive cells was divided by the total number of DAPI-stained cells in a field to calculate the residual LRC percentage within a field of cells. The mean LRC percentages from five fields at $\times 100$ magnification were calculated to determine the BrdU-labeling Index (LI) for each time point. To compare the rate of cell division, the Ki67-positive cell Index (KI) was calculated from the mean of the Ki67 ratio in five fields at $\times 100$ magnification. The LI and KI were calculated separately for the auricular perichondrium and the chondrium at days 0, 2, and 4, and weeks 24 and 48 following birth.

Isolation and cultivation of mice auricular cartilage

Auricular cartilage was obtained from 24-week-old, BrdU-labeled mice under a SZ 60 Stereo Microscope (Olympus). Dissected tissues were cut into small pieces and digested in Hank's balanced solution (Sigma) with 0.2% collagenase type II (Worthington) during a 2-h incubation at 37°C with shaking.

After passing the tissue suspension through a 40- μ m nylon mesh (BD Falcon), the cells were washed three times with PBS. The isolated cell suspensions were cultured in Dulbecco's modified Eagle medium and Ham's F-12 medium (D-MEM/F-12; Nissui Pharmaceutical Japan) supplemented with 10% fetal bovine serum (FBS; Moregate, Bulimba city, Australia) and 1% antibiotic antimycotic solution (AMS; Sigma) in 5% CO₂ at 37°C. Cells were seeded in 10%FBS+DMEM at the density of 10²/cm².

References

- Jackson KA, Mi T, Goodell MA (1999) Hematopoietic potential of stem cells isolated from murine skeletal muscle. *Proc Natl Acad Sci U S A* 96: 14482–14486.
- Bickenbach JR, Chism E (1998) Selection and extended growth of murine epidermal stem cells in culture. *Exp Cell Res* 244: 184–195.
- Brandl C, Florian C, Driemel O, Weber BH, Morszeck C (2009) Identification of neural crest-derived stem cell-like cells from the corneal limbus of juvenile mice. *Exp Eye Res* 89: 209–217.
- Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, et al. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 7: 211–228.
- Bairati A, Comazzi M, Gioria M (1996) A comparative study of perichondrial tissue in mammalian cartilages. *Tissue Cell* 28: 455–468.
- Engkvist O, Wilander E (1979) Formation of cartilage from rib perichondrium grafted to an articular defect in the femur condyle of the rabbit. *Scand J Plast Reconstr Surg* 13: 371–376.
- Kawanabe Y, Nagata S (2007) A new method of costal cartilage harvest for total auricular reconstruction: part II. Evaluation and analysis of the regenerated costal cartilage. *Plast Reconstr Surg* 119: 308–315.
- Togo T, Utani A, Naitoh M, Ohta M, Tsuji Y, et al. (2006) Identification of cartilage progenitor cells in the adult ear perichondrium: utilization for cartilage reconstruction. *Lab Invest* 86: 445–457.
- Kawanabe Y, Nagata S (2006) A new method of costal cartilage harvest for total auricular reconstruction: part I. Avoidance and prevention of intraoperative and postoperative complications and problems. *Plast Reconstr Surg* 117: 2011–2018.
- van der Meulen JC, Hauben DJ, Vaandrager JM, Birgenhager-Frenkel DH (1984) The use of a temporal osteoperiosteal flap for the reconstruction of malar hypoplasia in Treacher Collins syndrome. *Plast Reconstr Surg* 74: 687–693.
- Yanaga H, Yanaga K, Imai K, Koga M, Soejima C, et al. (2006) Clinical application of cultured autologous human auricular chondrocytes with autologous serum for craniofacial or nasal augmentation and repair. *Plast Reconstr Surg* 117: 2019–2030; discussion 2031–2012.
- Bianco P, Robey PG (2001) Stem cells in tissue engineering. *Nature* 414: 118–121.
- Szotek PP, Chang HL, Brennand K, Fujino A, Pieretti-Vanmarcke R, et al. (2008) Normal ovarian surface epithelial label-retaining cells exhibit stem/progenitor cell characteristics. *Proc Natl Acad Sci U S A* 105: 12469–12473.
- Duvillie B, Attali M, Aiello V, Quemeneur E, Scharfmann R (2003) Label-retaining cells in the rat pancreas: location and differentiation potential in vitro. *Diabetes* 52: 2035–2042.
- Ito M, Kizawa K, Toyoda M, Morohashi M (2002) Label-retaining cells in the bulge region are directed to cell death after plucking, followed by healing from the surviving hair germ. *J Invest Dermatol* 119: 1310–1316.
- Wu LJ, Errington J (1994) Bacillus subtilis SpoIIIE protein required for DNA segregation during asymmetric cell division. *Science* 264: 572–575.
- Cotsarelis G, Sun TT, Lavker RM (1990) Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* 61: 1329–1337.
- Dowthwaite GP, Bishop JC, Redman SN, Khan IM, Rooney P, et al. (2004) The surface of articular cartilage contains a progenitor cell population. *J Cell Sci* 117: 889–897.
- Shirasawa S, Sekiya I, Sakaguchi Y, Yagishita K, Ichinose S, et al. (2006) In vitro chondrogenesis of human synovium-derived mesenchymal stem cells: optimal condition and comparison with bone marrow-derived cells. *J Cell Biochem* 97: 84–97.
- Nishimura K, Solchaga LA, Caplan AI, Yoo JU, Goldberg VM, et al. (1999) Chondroprogenitor cells of synovial tissue. *Arthritis Rheum* 42: 2631–2637.
- Peltomaki T, Hakkinen L (1992) Growth of the ribs at the costochondral junction in the rat. *J Anat* 181(Pt 2): 259–264.
- Arufo A, Stamenkovic I, Melnick M, Underhill CB, Seed B (1990) CD44 is the principal cell surface receptor for hyaluronate. *Cell* 61: 1303–1313.
- Chow G, Knudson CB, Homandberg G, Knudson W (1995) Increased expression of CD44 in bovine articular chondrocytes by catabolic cellular mediators. *J Biol Chem* 270: 27734–27741.

Supporting Information

Figure S1 BrdU labeling of auricular cartilage in a 4-week-old mouse. 4-week-old mice were injected with BrdU and were sacrificed the day. None of BrdU-labeled cells and Ki67-positive cells were observed in both the perichondrium and chondrium of an auricular cartilage. From the left, Alcian blue staining, DAPI, BrdU, Ki67, and a merged image. Two-headed arrows: the cartilage width including perichondrium. Scale bar = 100 μ m.

(TIFF)

Figure S2 Long-term LRCs specifically reside in auricular perichondrium. 24 (A) or 48 (B) weeks-old mice auricle were immunohistochemically examined. Although none of BrdU labeling cells was recognized in chondrium, rare long-term LRCs specifically existed in perichondrium layer. Two-headed arrows: perichondrium width, but not chondrium of auricular cartilage. Arrowheads: long-term LRCs. Scale bars = 200 μ m.

(TIFF)

Figure S3 Immunohistochemical analysis of cell surface marker proteins in a 24-week-old mouse. Perichondrocytes of 24-week-old mice expressed integrin- $\alpha 5$ and CD44 (Arrowheads). Chondrocytes expressed integrin- $\alpha 1,2,V,L$ and integrin- $\beta 1$. Two-headed arrows: the cartilage width including perichondrium. Scale bar = 50 μ m.

(TIFF)

Acknowledgments

We thank N. Sasaki for technical support. We are especially grateful to K. Sekine for critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: SK TT HT. Performed the experiments: SK TT. Analyzed the data: SK TT HT. Contributed reagents/materials/analysis tools: Y-WZ MM YY JM. Wrote the paper: SK TT.

24. Knudson W, Loeser RF (2002) CD44 and integrin matrix receptors participate in cartilage homeostasis. *Cell Mol Life Sci* 59: 36–44.
25. Nakamura H, Kato R, Hirata A, Inoue M, Yamamoto T (2005) Localization of CD44 (hyaluronan receptor) and hyaluronan in rat mandibular condyle. *J Histochem Cytochem* 53: 113–120.
26. Enomoto-Iwamoto M, Iwamoto M, Nakashima K, Mukudai Y, Boettiger D, et al. (1997) Involvement of alpha5beta1 integrin in matrix interactions and proliferation of chondrocytes. *J Bone Miner Res* 12: 1124–1132.
27. Holmvall K, Camper L, Johansson S, Kimura JH, Lundgren-Akerlund E (1995) Chondrocyte and chondrosarcoma cell integrins with affinity for collagen type II and their response to mechanical stress. *Exp Cell Res* 221: 496–503.
28. Homandberg GA, Costa V, Wen C (2002) Fibronectin fragments active in chondrocytic chondrolysis can be chemically cross-linked to the alpha5 integrin receptor subunit. *Osteoarthritis Cartilage* 10: 938–949.
29. Hynes RO (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69: 11–25.
30. Kim SJ, Kim EJ, Kim YH, Hahn SB, Lee JW (2003) The modulation of integrin expression by the extracellular matrix in articular chondrocytes. *Yonsei Med J* 44: 493–501.
31. Tavella S, Bellese G, Castagnola P, Martin I, Piccini D, et al. (1997) Regulated expression of fibronectin, laminin and related integrin receptors during the early chondrocyte differentiation. *J Cell Sci* 110(Pt 18): 2261–2270.
32. Salter DM, Godolphin JL, Gourlay MS (1995) Chondrocyte heterogeneity: immunohistologically defined variation of integrin expression at different sites in human fetal knees. *J Histochem Cytochem* 43: 447–457.
33. Hausler G, Helmreich M, Marlovits S, Egerbacher M (2002) Integrins and extracellular matrix proteins in the human childhood and adolescent growth plate. *Calcif Tissue Int* 71: 212–218.



Human Elastic Cartilage Engineering from Cartilage Progenitor Cells Using Rotating Wall Vessel Bioreactor

T. Takebe, S. Kobayashi, H. Kan, H. Suzuki, Y. Yabuki, M. Mizuno, T. Adegawa, T. Yoshioka, J. Tanaka, J. Maegawa and H. Taniguchi

ABSTRACT

Transplantation of bioengineered elastic cartilage is considered to be a promising approach for patients with craniofacial defects. We have previously shown that human ear perichondrium harbors a population of cartilage progenitor cells (CPCs). The aim of this study was to examine the use of a rotating wall vessel (RWV) bioreactor for CPCs to engineer 3-D elastic cartilage *in vitro*. Human CPCs isolated from ear perichondrium were expanded and differentiated into chondrocytes under 2-D culture conditions. Fully differentiated CPCs were seeded into recently developed pC-HAp/ChS (porous material consisted of collagen, hydroxyapatite, and chondroitinsulfate) scaffolds and 3-D cultivated utilizing a RWV bioreactor. 3-D engineered constructs appeared shiny with a yellowish, cartilage-like morphology. The shape of the molded scaffold was maintained after RWV cultivation. Hematoxylin and eosin staining showed engraftment of CPCs inside pC-HAp/ChS. Alcian blue and Elastica Van Gieson staining showed of proteoglycan and elastic fibers, which are unique extracellular matrices of elastic cartilage. Thus, human CPCs formed elastic cartilage-like tissue after 3-D cultivation in a RWV bioreactor. These techniques may assist future efforts to reconstruct complicate structures composed of elastic cartilage *in vitro*.

RECONSTRUCTION OF CRANIOFACIAL INJURIES or abnormalities requires reconstructive materials, such as auto-costal cartilage grafts. Collecting autologous tissues, however, puts a significant burden on donor sites and the absence of self-renewing stem/progenitor cells leads to invariable absorption of the transplanted grafts. An effective treatment for craniofacial defects is currently lacking despite the tremendous demands.¹ To overcome the limitations of current therapies, the use of engineered elastic cartilage using autologous stem/progenitor cells is expected to provide a

promising alternative. Recently, we showed that auricular (ear) perichondrium contains self-renewing cartilage progenitor cells (CPCs) in both mice and humans.^{2,3} Transplantation of human CPCs into immunodeficient mice resulted in elastic cartilage reconstruction with long-term morphological preservation. However, under conventional two-dimensional culture conditions, it was difficult to control the size or shape of the construct. The aim of this study was to engineer larger, molded elastic cartilage using our newly identified CPCs with a 3-D culture technique employing a rotating wall vessel (RWV) bioreactor.

From the Department of Regenerative Medicine (T.T., H.K., H.S., M.M., H.T.), Yokohama City University Graduate School of Medicine, Kanazawa-ku, Yokohama, Kanagawa, Japan; Department of Plastic and Reconstructive Surgery (S.K.), Kanagawa Children's Medical Center, Minami-ku, Yokohama, Japan; Department of Plastic and Reconstructive Surgery (Y.Y., J.M.), Yokohama City University Graduate School of Medicine, Yokohama, Kanagawa and Department of Metallurgy and Ceramics Science (T.A., T.Y., J.T.), Tokyo Institute of Technology, Tokyo, Japan.

This work was supported by a Grant-in-aid for Developmental Scientific Research, The Ministry of Education, Science and

Culture (No. 20592101) and Health and Labour Sciences Research Grants Research on intractable disease (No. 2011-164). This work was also supported by the grant for Research and Development Project (No. 19-15) of Yokohama City University, Japan.

Address reprint requests to Dr Hideki Taniguchi, Department of Regenerative Medicine, Yokohama City University Graduate School of Medicine, 3-9, Fukuoka, Kanazawa-ku, Yokohama, Kanagawa 236-004, Japan. E-mail: rtanigu@med.yokohama-cu.ac.jp

MATERIALS AND METHODS.

Isolation and Cultivation of Human CPCs

We obtained human elastic cartilage samples from microtia patients following the approved guidelines set by our Ethical Committee (approval #03-074). The samples were separated microscopically into cartilage and perichondrium layers. Minced dissected tissues were digested with shaking for 2 hours at 37°C in phosphate-buffered saline containing 0.2% collagenase type II (Worthington). After passing through a 100- μ m nylon mesh (BD Falcon), the cells were cultured in Dulbecco's modified Eagle's (D-MEM) and Ham's F-12 media (D-MEM/F-12; Nissui Pharmaceutical) supplemented with 10% fetal bovine serum (FBS; Morgante) and 1% antibiotic antimycotic solution (AMS; Sigma) in 5% CO₂ at 37°C.

Chondrogenic Differentiation of Human CPCs

Cells were cultured for 5 days using D-MEM/F-12 containing 10% FBS, 1% AMS, L-ascorbic acid 2-phosphate, dexamethasone, insulin-like growth factor-1, and fibroblast growth factor-2. After 7 days of induction, detached cells from the same donor-derived subculture were adjusted to a density of 2.5×10^4 cells/cm² for seeding on to monolayer cultures. These steps were repeated twice at an interval of 1 week.

Fabrication of pC-HAp/ChS Scaffolds

The detailed method for the preparation of pC-HAp/ChS scaffolds was described previously.⁴

3-D Culture Utilizing RWV Bioreactor

The discoidal vessels of an RWV reactor (RCCS-4 system with 50-mL disposable vessels, Synthecon Incorporated, Houston, Tex, USA) were filled with 50 mL of differentiation media. CPCs that had been subjected to the chondrogenic differentiation

conditions using the layered culture system were scraped with a cell lifter. The scraped cells were collected in a 2.5-mL syringe (Terumo) equipped with a 23-gauge needle (Terumo). One milliliter of the culture was injected into pC-HAp/ChS, before introduction into the vessels and cultivation in a CO₂ incubator. The rotary culture was performed for 6 weeks at a speed that was manually adjusted to maintain cell aggregates freely suspended within the vessel.

Histochemical and Immunohistochemical Analysis

We stained the sections of culture constructs with hematoxylin/eosin (HE), Alcian blue, or Elastica Van Gieson (EVG; Muto Pure Chemicals).

RESULTS

Our overall study design to engineer larger and molded elastic cartilage is shown in Fig 1. Rotating wall vessel (RWV) bioreactor was employed to the three-dimensional culture system (Fig 2A).

Human CPCs were isolated from auricular cartilage remnant of microtia patients. After extensive expansion, cells differentiated into chondrocytes under defined conditions. Differentiated CPCs were seeded into a porous scaffold pC-HAp/ChS, consisting of collagen, hydroxyapatite, and polysaccharide with appropriate porosity, high elasticity, and biodegradability. Before seeding, we sculpted pC-HAp/ChS into auricle-like shapes (Fig 2B). CPCs seeded pC-HAp/ChS were introduced into a RWV bioreactor and 3-D cultivated for 6 weeks. Engineered construct appeared to be a yellowish with shiny cartilaginous tissue (Fig 2C). Histological analyses were performed to evaluate cell engraftment and matrix production. HE staining

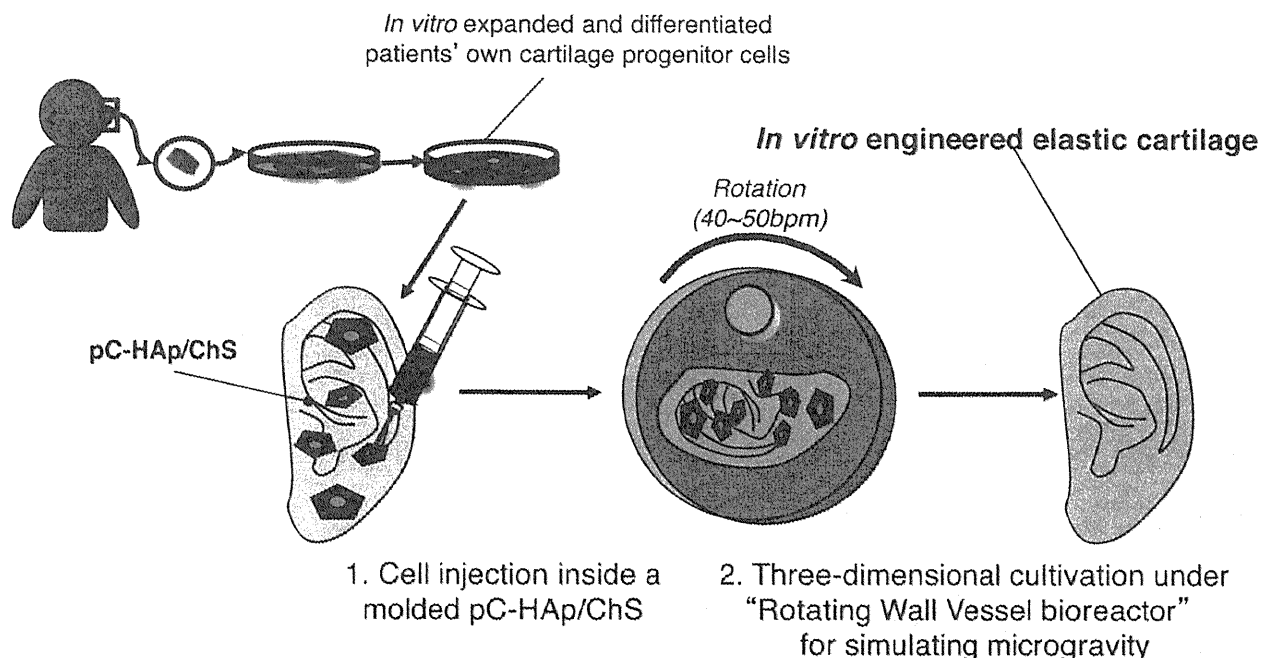


Fig 1. Schematic strategy of human elastic cartilage engineering in vitro by human cartilage progenitor cells.

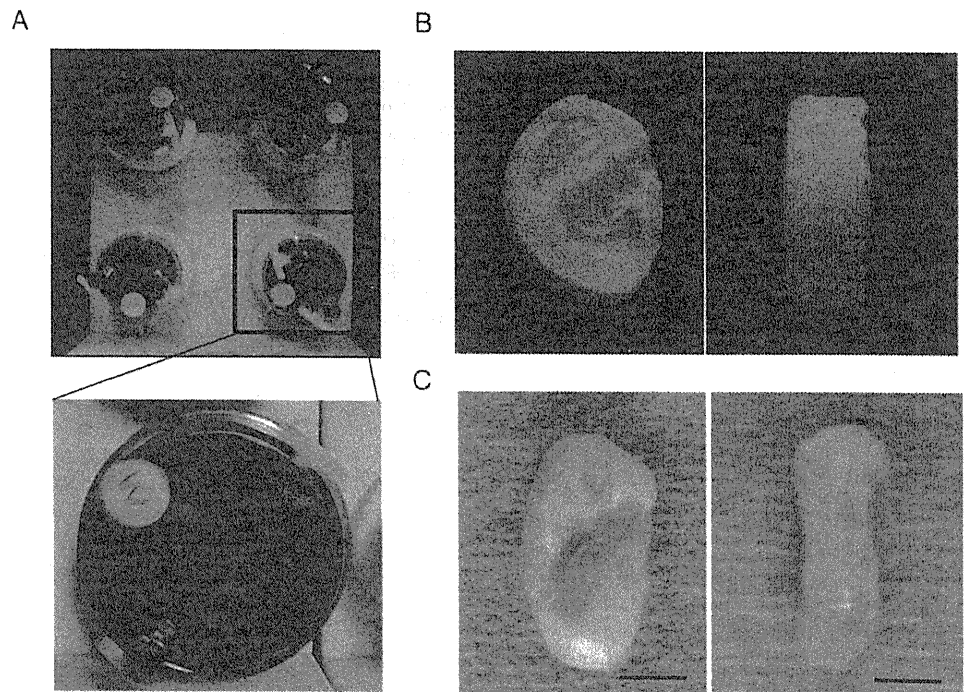


Fig 2. In vitro engineering of human elastic cartilage using a rotating wall vessel bioreactor. **(A)** Macroscopic view of the bioreactor and rotating wall vessels. **(B and C)** Gross morphology before **(B)** and after **(C)** 3-D cultivation. Bars, 3 mm.

showed engraftment of CPCs inside pC-HAp/ChS as compared with control samples (Fig 3). Alcian blue staining demonstrated that CPCs differentiated into chondrocytes to produce lots of proteoglycans. Presence of elastic fibers were confirmed by EVG staining.

DISCUSSION

Identification of CPCs from human auricular cartilage will contribute to provide novel therapeutic strategies, including long-term tissue restoration, in patients with craniofacial defects. However, conventional 2-D culture techniques limit the size or shape of the construct. The RWV bioreactor, which simulates a microgravity envi-

ronment, is becoming recognized as a useful tool for several kinds of tissue engineering such as hyaline cartilage, aiming at clinical application for articular cartilage defect.⁵ However, no other studies to date have evaluated the use of RWV toward elastic cartilage reconstruction.

Here, we evaluated the usefulness of RWV for 3-D elastic cartilage engineering using CPCs. Engineered construct seemed to be elastic cartilage-like tissue consisting of mature chondrocytes and unique extracellular matrices of elastic cartilage. One major goal of elastic cartilage reconstruction is to generate the complicated structures designed for each patients' deformities. Our

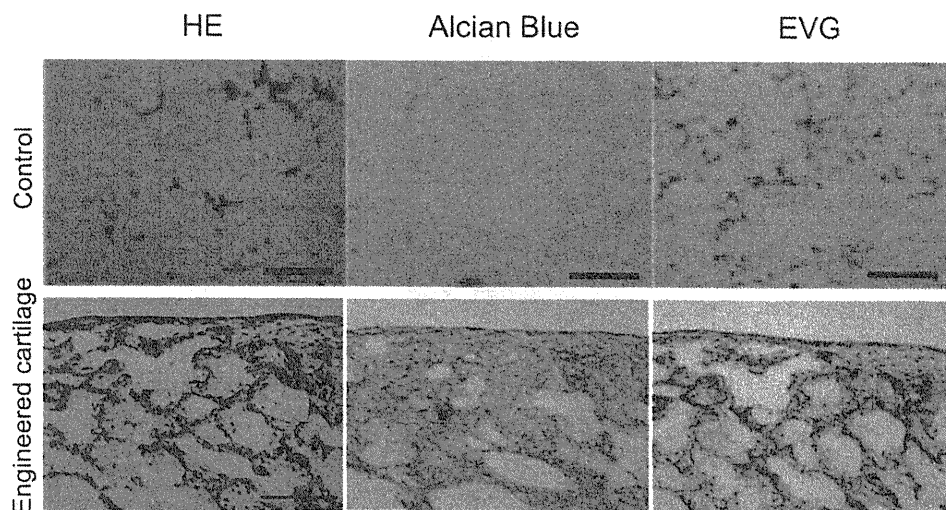


Fig 3. Histological analyses of engineered human elastic cartilage from cartilage progenitor cells. Engineered elastic cartilage (bottom) was compared with no-cell seeded construct (upper). HE, hematoxylin/eosin staining; EVG, Elastica Van Gieson staining. Bars, 200 μm.

preliminary results open a new possibility for achieving this goal using the RWV culture system. Further characterization of their mechanical character may allow these approaches to be employed to patients with larger, complicated craniofacial defects like microtia.

REFERENCES

1. Chang SC, Tobias G, Roy AK, et al: Tissue engineering of autologous cartilage for craniofacial reconstruction by injection molding. *Plast Reconstr Surg* 112:793, 2003
2. Kobayashi S, Takebe T, Inui M, et al: Reconstruction of human elastic cartilage by a CD44+ CD90+ stem cell in the ear perichondrium. *Proc Natl Acad Sci USA* 108:14479, 2011
3. Kobayashi S, Takebe T, Zheng Y-W, et al: Presence of cartilage stem/progenitor cells in adult mice auricular perichondrium. *PLoS ONE* 6:e26393, 2011
4. Adegawa T, Ohyabu Y, Uemura T, et al: Preparation and characterization of porous scaffolds consisted of hydroxyapatite, polysaccharides and collagen for cartilage tissue engineering. *Key Eng Mater* 396:707, 2009
5. Ohyabu Y, Kida N, Kojima H, et al: Cartilaginous tissue formation from bone marrow cells using rotating wall vessel (RWV) bioreactor. *Biotechnol Bioeng* 95:1003, 2006

