Supporting Information

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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/cm^2. 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/cm^2. 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/cm^2 under an atmosphere of 5% CO_2 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′ctggctcccaacactgccaacgtc3′ and reverse 5′tcctttgggtttgcaacggattgt3′; type X collagen (COL10A1), forward 5′cccactacccaacaccaagac3′ and reverse 5′tttctgtccattcataccaggg3′; aggrecan (ACAN), forward 5′ gtatgtgaggagggctggaaca3′ and reverse 5′cgcttctgtagtctgcgtttgta3′; and elastin (ELN), forward 5′tatggactgccctacaccacag3′ and reverse 5′agcacctgggacaactggaat3′. For adipogenic differentiation-related markers: lipoprotein lipase (Lpl/LPL), forward 5′ tggacggtaacaggaatgtatgag3′ and reverse 5′ccctctggtgaatgtgtgtaaga3′; aP2 (Fabp4), forward 5′ggtacctggaaacttgtctccag3′ and reverse 5′catgacgcattccaccaccag3′; and PPARγ (PPARGC1A), forward 5′gtgtgctgctctggttggtgaagac3′ and reverse 5′gttggctggtgccagtaagagcttc3′. For osteogenic differentiation-related markers: runt-related transcription factor 2 (Runx2), forward 5′gagtttcaccttgaccataaccgtcttcac3′ and reverse 5′gtggtagagtggatggacgggg3′; and alkaline phosphatase (ALPL), forward 5′tcccggtgcaacaccacccag3′ 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.

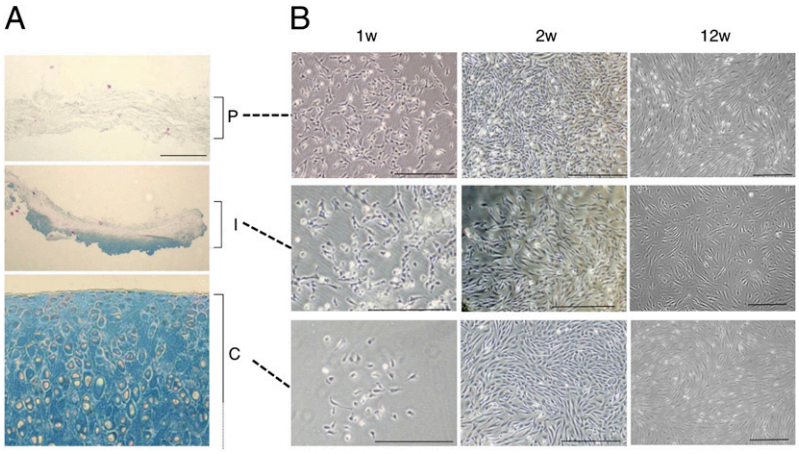


Fig. S1. Manual separation of perichondrium layer. (*A*) Alcian blue staining of each separated layer. Perichondrium layer never contains mature cartilage-derived cells. (*B*) Morphological observation of cells derived from each layer after 1, 2, and 12 wk of culture. (Scale bars, 500 μm.)

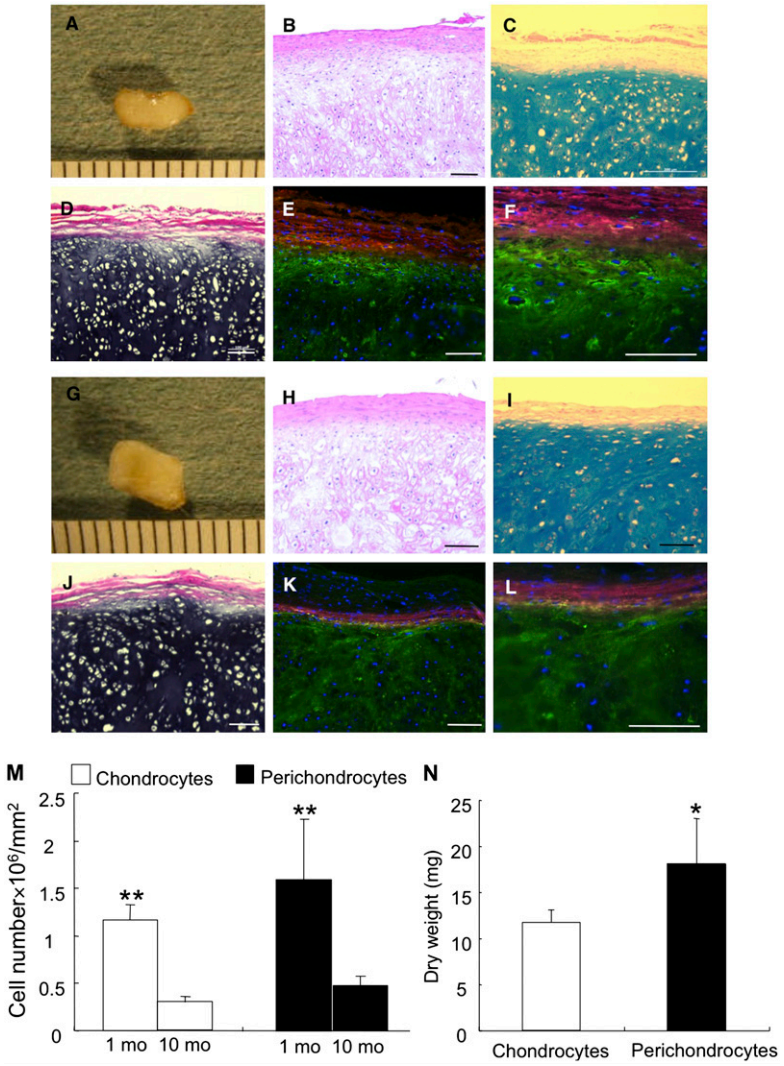


Fig. S2. Tissue restoration of regenerated elastic cartilage 6 (*A*–*F*) and 10 (*G*–*L*) mo after s.c. injection of human perichondrocytes. (*A* and *G*) Gross morphology, (*B* and *H*) Hematoxylin/eosin staining, (*C* and *I*) Alcian blue staining, (*D* and *J*) Elastica Van Gieson staining, (*E*, *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 ± SD of repeated measures at four equally spaced areas, each with a cross-sectional area of 200 × 200 μm^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 ± SD ($n = 4$). $^{*}P < 0.05$.

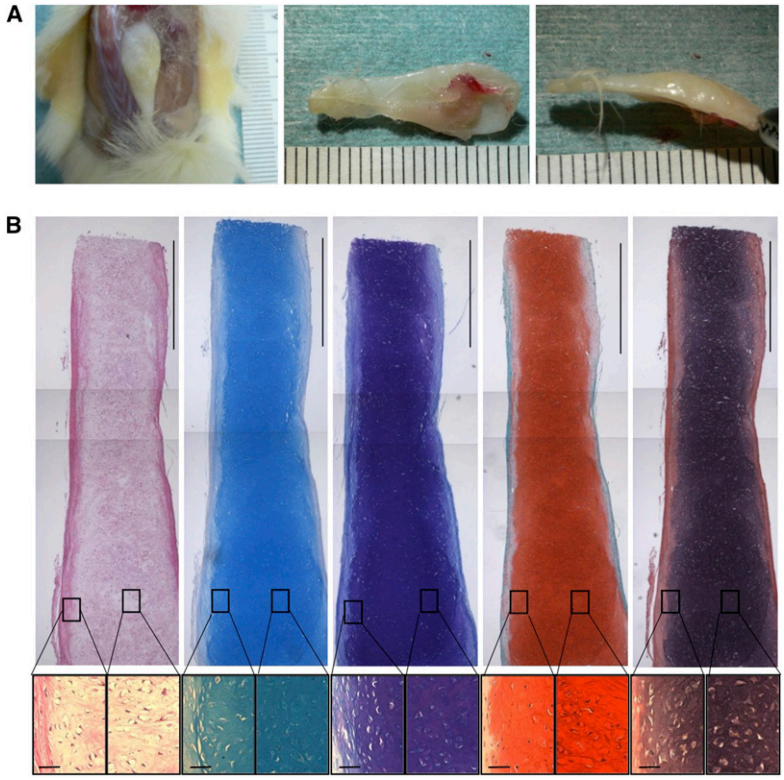


Fig. S3. Larger elastic cartilage reconstruction after direct cell injection. (*A*) Macroscopic observation of reconstructed cartilage 2 mo after high-volume cell injection. (Scale bars, 1 mm.) (*B*) Histological examinations show homogenous distribution of mature chondrocytes, which produce proteoglycan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

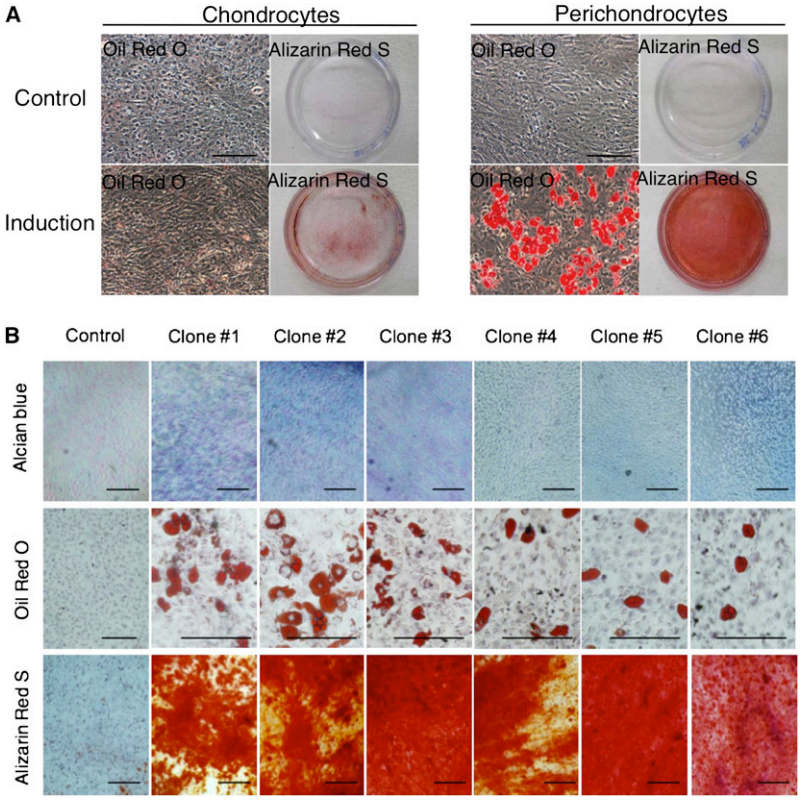


Fig. S4. Multilineage differentiation potential of clonally propagated CD44$^+$ CD90$^+$ cells. (*A*) Cytochemistry staining of chondrocytes (*Left*) and perichondrocytes (*Right*) after adipogenic and osteogenic induction. Control cells were cultured using our standard culture medium. Oil Red O and Alizarin Red S staining showed that the perichondrocytes differentiated into adipocytes and osteocytes, respectively. (Scale bar, 100 μm.) (*B*) Multipotency of clone-sorted CD44$^+$ CD90$^+$ cells. All six different clonogenic progenies possess trilineage potential. (Scale bars, 200 μm.)

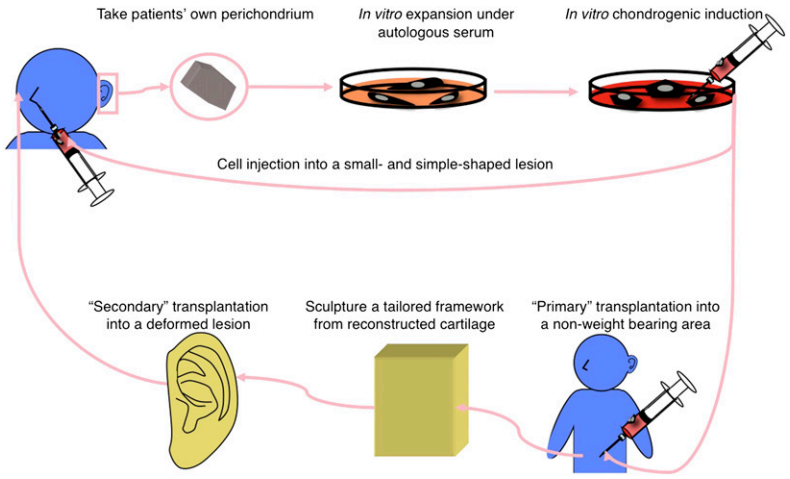
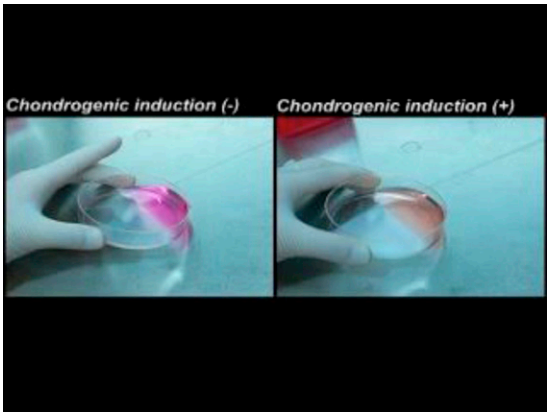


Fig. S5. Schematic representations of two different strategies toward clinical application: (*i*) direct cell injection and (*ii*) two-stage transplantation without scaffold.

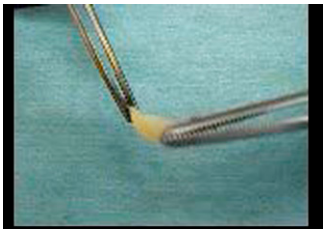
Table S1. The antihuman monoclonal antibodies used in this study

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