Pharmaceutical efficacy of human epiphyseal chondrocytes with differential replication numbers for cellular therapy products

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Running head: Efficacy of human chondrocytes in vivo

Keywords: passage expansion, angiogenesis, endochondral ossification, in vitro

Total number of figures and table: 9

1 **Abstract**

2 The cell-based therapy for cartilage or bone requires a large number of cells serial
3 passages of chondrocytes are, therefore, needed. However, fates of expanded
4 chondrocytes from extra fingers remains unclarified. The chondrocytes from human
5 epiphyses morphologically changed from small polygonal cells, to bipolar elongated
6 spindle cells and to large polygonal cells with degeneration at early passages. Gene of
7 type II collagen was expressed in the cells only at a primary culture (Passage 0) and
8 Passage 1 (P1) cells. The nodules by implantation of P0 to P8 cells were composed of
9 cartilage and perichondrium. The cartilage consisted of chondrocytes with round nuclei
10 and type II collagen-positive matrix, and the perichondrium consisted of spindle cells
11 with type I collage-positive matrix. The cartilage and perichondrium developed to bone
12 with marrow cavity through enchondral ossification. Chondrogenesis and osteogenesis
13 by epiphyseal chondrocytes depended on replication number in culture. It is noteworthy
14 to take population doubling level in correlation with pharmaceutical efficacy into
15 consideration when we use chondrocytes for cell-based therapies.

16
17

1 **Introduction**

2 Epiphyseal cartilage of long bone is destined to develop to bone with marrow cavity and
3 articular cartilage. The epiphyseal cartilage continues to proliferate and undergoes
4 enchondral ossification until apparent growth ceases at adolescence, and eventually is
5 replaced with bone except for articular surface which remains cartilaginous throughout
6 life [1]. Chondrocytes in culture have an ability to generate cartilage in vivo, and can be
7 a tool to simulate developmental process. Our previous study demonstrated that human
8 infant epiphyseal chondrocytes can also simulate developmental process of epiphyseal
9 cartilage as well as other cell types [2, 3]. Chondrocytes in culture regenerate the
10 cartilaginous tissue by a short-term implantation in vivo and exhibited enchondral
11 ossification after a longer period. The cell-based therapeutic strategies in cartilage and
12 bone require serial cell passage of expanded chondrocytes for acquisition of a large
13 number of cells. In this study, we extensively characterized chondrocytes derived from
14 human epiphyseal cartilage in correlation with regenerative medicine.

16 **Materials and Methods**

17 **Human epiphyseal chondrocytes (HECs)**

18 HECs were obtained from epiphyseal cartilage of the phalanx and metacarpal bones of
19 amputated supernumerary digitus with polydactyly of finger or toe of 5 patients
20 (patient's age 7-14 months). Signed informed consents were obtained from the parents
21 of the donors. Isolation of HECs was performed as described previously [2]. Briefly,
22 after confirmation of no development of secondary ossification center in the epiphyseal
23 ends of bone, the epiphyseal cartilage was dissected out from bones and cut into piece
24 of 1 mm³. The diced fragments were placed in 0.2% trypsin/0.02% EDTA solution

1 (Immuno-Biological Laboratories Co, Ltd. Gunma, Japan) diluted with PBS at 37°C for
2 30 min and incubated in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich
3 Japan K.K. Tokyo) with 0.1% collagenase (Wako Pure Chemical Industries, Ltd. Osaka,
4 Japan) at 37°C for 7 to 10 h. After centrifuge of the isolated cells, the cell-pellets were
5 resuspended in DMEM. Isolated chondrocytes were grown in MF basal medium with
6 1% fetal calf serum (Toyobo Life science, Fukui, Japan) at a density of $0.5-1 \times 10^5$ cells
7 / 100-mm dish and 10-mm chamber slide by a monolayer culture in 37°C and 5% CO₂
8 incubator.

9

10 **Cytology**

11 Culture cells on the chamber slides were stained by Papanicolaou's method after
12 fixation in 100% ethanol and by senescence-associated β -galactosidase [4].

13

14 **Reverse transcription polymerase chain reaction (RT-PCR)**

15 Total RNA was isolated from HECs, using the RNeasy kit (Qiagen, Valencia, Ca, USA).
16 Total RNA (2 μ g) was reverse-transcribed to cDNA at 50°C for 50 min in a volume of
17 20 μ l containing the following reagents: 0.5 mM dNTP mix, 0.5 μ M oligo-dT₁₂₋₁₈
18 primer, first strand buffer, RNase inhibitor and 200 unit Superscript III (RNase H-free
19 reverse transcriptase). All the reagents were purchased from Invitrogen (Carlsbad, CA,
20 USA). After terminating the reaction at 70°C for 15 min, 1 U RNase H (Invitrogen) was
21 added to the reaction mixture, followed by incubation at 37°C for 10 min to remove
22 RNA. PCR was performed in a total volume of 50 μ l, using the primers (Table 1) and
23 the Ex Taq Hot Start version (Takara Bio Inc. Shiga, Japan). The PCR products were
24 electrophoresed, and visualized on ethidium bromide-stained 1.5% agarose gels.

1

2 **Cell implantation**

3 To determine the ability of HECs in a monolayer culture, P0 to P8 cells of $1.5-3 \times 10^7$ in
4 number were subcutaneously inoculated into the back skin of 6 to 8 week old,
5 NOD/Shi-*scid*, IL-2R γ^{null} (NOG) mice (Central Institute for Experimental Animals,
6 Kanagawa, Japan). The subcutaneous nodules of the mice were removed, and
7 photographed. Their weight and size were then measured.

8

9 **Microscopic analysis with immunohistochemistry**

10 Subcutaneous nodules in mouse backs were fixed in 4% paraformaldehyde and
11 embedded in paraffin, after decalcification with 5% EDTA (pH 6.8) solution if
12 necessary. Cutting sections were deparaffinized, dehydrated, and stained with
13 hematoxyline and eosion (HE), and with toluidine blue (pH 7.0) and alcian blue (pH
14 2.5) for cartilaginous matrix.

15

16 For immunohistochemical analysis, the following primary antibodies were used: mouse
17 monoclonal anti-human collagen type II (clone: II-4c11) and type I (clone: I-8H5)
18 antibodies (Daiichi Fine Chemical Co. Ltd, Toyama, Japan). Additionally, anti-human
19 antibodies (Dako, Glostrand, Denmark) of vimentin (clone: V9), CD31 (clone: JC70A)
20 and CD34 (clone: QBEnd 10) were used. These antibodies do not react with mouse
21 antigens according to applications for use, and furthermore these things have been
22 reconfirmed in our previous experiment [2]. Cut paraffin sections were deparaffinized,
23 dehydrated and treated with 2% proteinase K (Dako) in Tris-HCl buffer pH 7.5 solution
24 for 5 min at room temperature, or heated in ChemMate Target Retried Solution (Dako)

1 for 5-20 min in a high-pressure steam sterilizer for epitope unmasking. After blocking
2 of endogenous peroxidase by 1% hydrogen peroxide/methanol for 15 min, the sections
3 were incubated at room temperature for 60 min in primary antibodies diluted with
4 antibody diluent (Dako). The sections were washed with 0.01 M Tris buffered saline
5 (TBS) solution (pH 7.4) and incubated with goat anti-mouse and anti-rabbit
6 immunoglobulin labeled with dextran molecules-horseradish peroxidase (EnVision,
7 Dako) for 30 min at room temperature. After washing with TBS, the sections were
8 incubated in 3,3'-diaminobenzidin in substrate-chromogen solution (Dako) for 5-10 min.
9 Negative controls were performed by omitting the primary antibody. The sections were
10 counterstained with hematoxylin.

11

12 **Results**

13 **HEC in culture**

14 HECs were fibroblast-like in morphology at a primary culture. HECs slowly
15 proliferated until 4th day after start of the cultivation, and then rapidly proliferated at
16 5-6th day (Figure 1A). The cells reached to senescence before PDL 60. PDLs of the
17 senesced cells were 52.4 in an average at Passage 8. We then examined HECs for
18 expression of cartilage-associated genes at each passage by RT-PCR analysis (Figure
19 1B). The type-II collagen gene was expressed at P0 and P1, but was not detected at P2
20 to P8. The genes for collagen type I, III and X remained to be expressed from P0 to P8.

21

22 HECs showed extensive morphological alternations during passage expansions (Figure
23 2): P0, small polygonal cells with many mitoses; P1 and P2, bipolar elongated spindle
24 cells; P3-P5, bipolar short spindle cells of with appearance of large cells; P6-P8, large,

1 fusiform or polygonal cells with long cytoplasmic processes, indistinct nuclear
2 chromatin structures and occasional vacuolations. P8 cells became enlarged and deeply
3 stained with senescence-associated β -galactosidase.

4

5 **Implantation of HECs for two weeks**

6 We subcutaneously implanted HECs at each passage to investigate ability of
7 chondrogenesis and osteogenesis in vivo. P0 and P1 cells generated the nodules that
8 hardly changed in size, weight and in degree of transparency with whitish color on
9 surface (Figure 2). In contrast, P2 cells foamed the nodules that decreased in weight,
10 size and degree of transparency. The nodule by P0 cells was clearly divided into two
11 different parts, cartilage composing of chondrocytes with lacunae and abundant
12 extracellular matrix in the center, and perichondrium with spindle cells-layers at the
13 periphery (Figure 3), as demonstrated in the previous study [2]. Extracellular matrix in
14 the cartilage generated by P0 and P1 cells demonstrated metachromasia with Toluidine
15 Blue stain. Vimentin was detected in both chondrocytes and perichondral spindle cells.
16 Type II collagen-positive matrix was diffusely distributed in the cartilage by P0 and P1
17 cells, and type I collagen-positive matrix was localized in the perichondrium.

18

19 Unlike P0, P1 and P2 cells, P3 to P8 cells generated small and flattened nodules without
20 clear vascular invasion on opaque, whitish to brownish surface color, and failed to
21 generate cartilage. Microscopically, the nodules showed fibrous tissues with lack of
22 chondrocytes with lacunae, and the increase of spindle and dedifferentiated
23 chondrocytes. The fibrous tissue included inflammatory cells, reactions of foreign-body
24 giant cells and vascular invasions.

1

2 **Long-term observation of cartilage and bone by P0 to P2 HEC implantation**

3 We then followed up the nodule of bone and cartilage following implantation of HEC at
4 P0 (PDL 7) until 20 and 40 weeks (Figure 4). Macroscopically, the nodules showed
5 smooth surface with white-yellow and reddish portion in color at 20 weeks and 40
6 weeks. Enchondral ossification progressed in the cartilage at 20 weeks and new bone
7 and marrow cavity were formed (Figure 4A-G). Bone marrow cavity was surrounded by
8 osteoid and new bone with osteoblasts, osteoclasts, immature adipocytes, endothelial
9 cells and fibroblastoid cells. The nodules with bone and cartilage increased in size and
10 weight, became reddish on surface and developed prominent vascular networks at 40
11 weeks (Figure 4H-Q). The nodules resembling articular cartilage and cortical bone with
12 periosteum demonstrated the thin trabecular and spicular networks of the lamellar bone
13 with diffuse accumulation of unilocular mature adipocytes and vascular development.

14

15 At 20 weeks, type II collagen was detected in the extracellular matrix of cartilage, but
16 gradually reduced (Figure 4E). In contrast, type I collagen became positive in matrix of
17 osteoid, new bone and periosteum (Figure 4F). The CD 31-positive endothelial cells
18 were present along with the vessels at the periphery of cartilage (Figure 4G). At 40
19 weeks, type II collagen remained positive in peripheral cartilage and occasionally in
20 core of enchondral ossification (Figure 4M, N). Type I collagen-positive matrix was
21 clearly distributed in trabecular bone (Figure 4O, P). The vasculogenesis with CD
22 31-positive endothelial cells became prominent (Figure 4Q).

23

24 We then investigated if P1 cells are able to generate cartilage and bone like P0 cells

1 (Figure 5). To this end, we performed 10 implantations of P1 cells (PLD 10). P1 cells
2 under PDL 12 exhibited cartilage formation with enchondral ossification after
3 implantation. The nodules at 25 weeks were composed of the perichondrium and
4 cartilage with accumulation of extracellular matrix (Figure 5A, B). The cartilage was
5 consisted of small chondrocytes with round or occasional spindle nuclei and lacunae,
6 and covered by perichondrium with thin layer of elongated fibroblast-like spindle cells
7 (Figure 5C). At 41 weeks (Figure 5G-L), enchondral ossification was focally
8 demonstrated in the nodules with invasion and proliferation of peripheral
9 undifferentiated mesenchymal cells (Figure 5H, I). Marrow-like structure with
10 vasculogenesis, adipogenesis and osteoclastogenesis were confirmed, however, bone
11 marrow cavity with differentiation into trabecular bone or mature adipocytes was not
12 detected. Type I collagen-positive matrix diffusely distributed at the perichondrium at
13 25 weeks (Figure 5E) and at the osteoid and new bone in the enchondral ossification at
14 41 weeks (Figure 5K). Vasculogenesis with CD 34-positive endothelia (Figure 5F)
15 started to be seen during enchondral ossification at 41 weeks (Figure 5L).
16
17 P1 cells (PDL 14) generated cartilage that were macroscopically flattened or
18 club-shaped, translucent and whitish surface, exhibited type II collagen-positive matrix
19 in the cartilage at 25 and 42 weeks after the implantation, but failed to develop bone
20 marrow even after follow-up observation (Figure 6). The cartilage at 25 weeks level was
21 composed of small chondrocytes with round or spindle nuclei and lacunae, and
22 hypertrophic cells with large lacunae. The perichondrium showed the structure of thick
23 or thin layers with elongated fibroblast-like spindle cells (Figure 6B). The nodules at 42
24 weeks showed morphologies similar to those at 25 weeks, except decrease of

1 hypertrophic cells with large lacunae and slight increase of chondrocyte with spindle
2 nuclei and narrow lacunae in number, and increase in eosinophil of the perichondrial
3 matrix and in thickness of the perichondrium. Type-II collagen-positive matrix was
4 detected in the cartilage at 25 weeks (Figure 6D) and 42 weeks (Figure 6I). Type-I
5 collagen-positive matrix was distributed in the thick perichondrium at 25 weeks (Figure
6 6E), and 42 weeks (Figure 6J).

7
8 In contrast, P2 cells also generated cartilage that was maintained for up to 17 weeks
9 (Figure 7). Macroscopically, the cartilage showed translucent and smooth, surface that
10 were white, partly light brown or yellow in color (Figure 7A). The cartilages at 9 weeks
11 consisted of hypertrophic chondrocytes with round or spindle nuclei and large lacunae
12 (Figure 7B, C). The cartilages at 17 weeks showed increase of small chondrocyte with
13 spindle nuclei and eosinophilic extracellular matrix at the perichondrium (Figure 7G, H).
14 Type II collagen-positive matrix remained in the cartilage at 9 weeks (Figure 7D) and at
15 17 weeks (Figure 7I). Type-I collagen-positive matrix was distributed in the
16 perichondrium at 9 weeks (Figure 7E), and extended into the cartilage at 17 weeks, in
17 addition to the perichondrium (Figure 7J).

18

19 **Lack of chondrogenic activity in P3 cells**

20 P3 cells (PDL 23) generated fibrous tissue that was macroscopically translucent and
21 flattened, and tightly adhered to the subcutaneous tissue (Figure 8A). The tissue was
22 ill-defined in the boundaries between the perichondrium and cartilage (Figure 8B, C).
23 Type II collagen-positive matrix was irregularly distributed in the cartilage (Figure 8D).
24 Type I collagen-positive matrix was irregularly found in the cartilage and not

1 overlapped with type II collagen-positive matrix (Figure 8E).

2

3 **Discussion**

4 For the cell-based therapeutic strategies of cartilage and bone formation, the expansion

5 of cultivation are required to gain sufficient number of cells, and to preserve for a long

6 period in recipient, therefore the detailed studies are essential with regards to the

7 characteristics of expanded and/or dedifferentiated chondrocytes in vitro and in vivo.

8 We have previously reported that chondrocytes from epiphyseal cartilage at a primary

9 culture have the ability to regenerate cartilage and to develop bone with marrow cavity

10 through enchondral ossification in vivo [2]. This present study demonstrated the

11 detailed alterations of the human infant epiphyseal chondrocytes during expansions

12 from P0 to P8 cells in monolayer culture and the ability of in vivo chondrogenesis and

13 osteogenesis by implantation, and long-term observation after implantation.

14

15 The fate of cartilage generated by chondrocytes depended on population doubling levels

16 of chondrocytes in culture. Primarily, implanted chondrocytes need to retain the abilities

17 to generate cartilage with production of type II collagen-positive cartilaginous matrix,

18 and to produce type I-positive perichondrium. In addition to chondrocytes, participation

19 of undifferentiated mesenchymal cells that differentiate to osteoblasts, adipocytes and

20 endothelia is required for bone development through enchondral ossification [1, 5, 6]. In

21 our previous study [2], we showed that perichondrial spindle cells participate in bone

22 development as undifferentiated mesenchymal cells after implantation of cultivated

23 chondrocytes, like perichondrial cells in a cartilage anlage of human embryonic bone.

24

1 Chondrogenic ability of cultivated chondrocytes is associated with surface markers,
2 gene expression, morphology, synthesis of the type of collagen and capacity of
3 redifferentiation, using human articular and other cartilages [7, 8] or animal [9] as cell
4 sources [10-12]. Surface markers include CD26 for the chondrogenic potential of
5 human articular chondrocytes [13] and ratio of CD54 to CD44 as an index of
6 differentiation status [7]. Morphology of chondrocytes changes from a differentiated
7 round to polygonal cell shape to dedifferentiated fibroblast-like phenotype [9, 14]. In
8 gene expression, type-II collagen is essential for chondrocytes capable of generating
9 cartilage [8] and inversely correlated with increasing PDLs [15]. The ratio of type-I and
10 type-II collagen, defined as an index of cell differentiation, is significantly higher at a
11 primary culture and become lower along with expansion [13]. in vivo chondrogenesis is
12 also associated with PDLs of cultivated chondrocytes in rabbit [16, 17], bovine [18],
13 and human fetal and adult articular chondrocytes [19]. Chondrocytes at a primary
14 culture from articular cartilage show mature and well-formed cartilage with
15 extracellular matrix of type-II collagen, however those with chondrocytes with a longer
16 cultivation do not generate cartilage with type-II collagen but form fibrous tissue [16]
17 and almost same results have been presented in chondrocytes from the costal cartilage
18 [17]. Chondrocytes can be not only isolated from cartilage, but also differentiated from
19 somatic stem cells including mesenchymal stem cells and marrow stromal cells [2,
20 20-23]. Chondrocytes have recently been generated from pluripotent stem cells like
21 other cell types [2, 24-27].
22
23 To reverse de-differentiation process during cultivation, several approaches have been
24 developed. Dedifferentiated chondrocytes after a longer cultivated period regain

1 chondrogenic activity by the high-density culture method [28]. To acquire sufficient cell
2 number for cartilage engineering, the co-culture system of in vitro-expanded
3 dedifferentiated chondrocytes with small numbers of primary chondrocyte is useful to
4 re-differentiate the de-differentiated cells and form thicker cartilage tissue with type-II
5 collagen [18]. Wnt/beta-catenin signaling may be involved in maintenance of
6 chondrogenic activity [29, 30]. Cultivation medium is shown to affect cell phenotypes
7 in vitro [31]. In addition to the re-differentiation strategies, the selection approach was
8 employed to isolate functionally active chondrogenic cells with high levels of type-II
9 collagen from dedifferentiated cells or hypertrophic cells with product of type-X
10 collagen for in vivo chondrogenesis [19].

11
12 With regard to epiphyseal cartilage, however, the study in concerning cytological
13 descriptions of the isolated culture cells have not been presented and there are a few
14 morphological studies in vivo on the new bone formation by the implantation of
15 chondrocytes isolated from cartilage of rat [32] and mouse [33]. In human epiphyseal
16 chondrocytes, nodular structures resembling mature articular cartilage are formed by
17 primary culture of human fetal epiphyseal chondrocytes for long-term of up to 180 days
18 on hydrogel substrate [34], although the experiment in passage-expansion of cultivated
19 cells was not performed. Our observations in the present studies of epiphyseal
20 chondrocytes showed the morphology in vivo on the regenerated cartilage and new bone
21 formation with marrow cavity by the implantation of expanded cells for short to long
22 period. Large cartilage without ossification needs to be formed by implantation of
23 cultivated cells with higher PDLs by usage of cytokines in future.

24

1 Finally, the present studies showed the changes in cytology and gene expression of the
2 infant epiphyseal chondrocytes in a monolayer culture by cell expansion from the P0 to
3 P8 cells. The experiment in vivo by implantation of expanded cells for a short to long
4 periods demonstrated the fate of the epiphyseal chondrocytes and elucidated that the
5 proceeded passage-cells can regenerate the tissue for a short period; however these
6 tissues can't be preserved for a longer period and absorbed, except those of the P0 and
7 P1 cells. The bone development through enchondral ossification occurred in cartilage by
8 the P0 cells and by the P1 cells with only lower PDLs. The P1 cells with rather higher
9 PDLs form the articular cartilage-like tissue without ossification. These findings would
10 be a help of chondrocyte-based therapy.

11

1 **Acknowledgement**

2 We would like to express our sincere thanks to T. Sugiki, H. Takahashi, and Y. Ito for
3 support throughout the work, H. Abe for excellent technical assistances, and E. Suzuki
4 and K. Saito for assistant in preparation of the manuscript.

5

6 **Conflict of interest**

7 The authors declare that there is no conflict of interest regarding the publication of this
8 manuscript.

9

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- 12

1 **Figure legends**

2 **Figure 1. Characteristics of the human epiphyseal chondrocytes (HECs)**

- 3 A. Proliferative capacity of HECS. The number of cells was counted at each passage.
 4 Total number of population doubling levels (PDLs) was calculated, using the formula of
 5 $\log_{10} (\text{total number of cells}/\text{starting number of cells})/\log_{10} 2$.
 6 B. RT-PCR analysis for cartilage-associated genes. P: Passage number.

7

8 **Figure 2. Morphological changes from P0 (PDL 8) to P8 (PDL 45) cells of human** 9 **epiphyseal chondrocytes during expansion.**

10 **Top panels:** Cytology of the culture cells with Papanicolaou's stain (Pap stain). Cell
 11 morphology changed from small polygonal cells with mitosis (P0) to short or long (P1),
 12 to long spindle cells (P2-P4) with occasional large spindle or polygonal cells (P4-P5)
 13 and to large, polygonal cells with long cytoplasmic processes and vacuolation, and
 14 nuclei with indistinct chromatin structures (P6-P8).

15 **Middle panels:** Macroscopic views of the tissues by implantation of P0 to P8 cells for
 16 two weeks. The nodules of P0 to P2 cells are the same in size, in weight and in degree
 17 of transparency with whitish color on surface. The nodule of P3 and P4 cells, however,
 18 decreased in size along with the increase of passages, and were accompanied central
 19 vascular invasion. The nodules of P5 to P8 cells became flattened and to be white to
 20 brown in color. Bars: 3 mm.

21 **Bottom panels:** Microscopic views with hematoxylin and eosin stain. P0 and P1 cells
 22 generated two distinct portions, i.e. cartilage and perichondrium at the implanted sites.
 23 As the increase in passage number, the boundary of two portions became to be
 24 ill-defined. P4 to P8 cells exhibited spindle-shaped dedifferentiated chondrocytes,

1 accompanied with reactions of inflammatory cells, macrophages and foreign-body giant
2 cells.

3

4 **Figure 3. Histological and immunohistochemical analysis of the implanted sites.**

5 Hematoxylin and eosin (HE) stain, Toluidine blue (TB) stain, and immunohistochemical
6 analysis by using antibodies against vimentin, type II collagen (COL II), and type II
7 collagen (COL II) are shown from left to right. With TB stain, the extracellular matrix
8 of cartilage exhibited metachromasia diffusely in the nodules by P0 and P1 cells, and
9 focally in the nodules by P2 cells. The matrix of the nodules by P0 and P1 cells was
10 positive for COL II at the center and with type I collagen (COL I) at the periphery. The
11 nodule by P2 cells was focally COL II-positive and diffusely COL I-positive matrix.
12 The matrix in the nodules by P3 and P5 cells was not stained with COL II and showed
13 weakly positive reaction with COL I. The nodules by P7 cells were negative for COL II
14 and COL I. The microscopic views (the same as Figure 2C) with HE stain (HE) are also
15 shown for reference.

16

17 **Figure 4. Long-term observation at the P0 cell (PDL 7)-implanted sites.**

18 P0 cell-implanted sites were analyzed at 20 weeks (A-G) and 40 weeks (H-Q).
19 Macroscopic examination of the P0 cell-nodules (606 mg in weight) at 20 weeks
20 showed smooth and whitish surface with focal reddish portion and vascular networks
21 (A). Microscopic examination showed progress of enchondral ossification (B) with new
22 bone formation and poor marrow cavity (C). Marrow cavity consisted of osteoblasts,
23 osteoclasts, undifferentiated mesenchyme cells and a few of immature adipocytes (D).
24 Cartilage and core of the enchondral ossification were positive for COL II (E).

1 Perichondrium, bone and osteoid was positive for COL I (F). Endothelium in the early
2 marrow cavity was positive for CD31 (G). Macroscopic examination of the P0
3 cell-nodules (686 mg in weight) at 40 weeks showed increased reddish portion with
4 prominent vascular networks on the surface (H). Bone marrow cavity was surrounded
5 by the cartilage and bone (I), which morphologically resembled to articular cartilage (J)
6 and cortical bone (K), respectively. Trabecular bone was extended with the fatty marrow
7 (L). Peripheral cartilage (M) and core of the enchondral ossification (N) were positive
8 for COL II. Perichondrium, periosteum (O) and trabecular bones (P) were positive for
9 COL I. The vessels in the bone marrow cavity were positive for CD31 (Q).

10

11 **Figure 5. Long-term observation at the P1 cell (PDL 10)-implanted sites.**

12 P1 cell-implanted sites were analyzed at 25 weeks (A-F) and 41 weeks (G-L).
13 Macroscopic examination of the P1 (PDL 10) cell-nodules (86 mg in weight) at 25
14 weeks showed smooth and semi-translucent surface with irregular light reddish portion
15 (A). Microscopic examination showed cartilage with thin perichondrium (B). The
16 cartilage was composed of small chondrocytes with round or spindle nuclei and lacunae
17 in the extracellular matrix (C). Matrix of the cartilage (D) and perichondrium (E) were
18 positive for COL II and COL I, respectively. CD34-positive vessels were not detected
19 (F). Macroscopic examination of the P1 cell-nodules (69 mg in weight) at 41 weeks
20 showed smooth and whitish surface with focal yellowish portion (G). Microscopic
21 examination revealed focal enchondral ossification in the cartilage (H) and bone
22 formation with early marrow cavity (I). Cartilage with enchondral ossification was
23 positive for COL II (J), and perichondrium, osteoid and new bone (K) were positive for
24 COL I. CD34-positive vessels were expanded in the early bone marrow cavity (L).

1

2 **Figure 6. Long-term observation at the P1 cell (PDL 14)-implanted sites.**

3 P1 cell-implanted sites were analyzed at 25 weeks (A-E) and 41 weeks (F-J).

4 Macroscopic examination of the P1 (PDL 14) cell-nodules (73 mg in weight) at 25 weeks

5 showed club-shaped, semi-translucent and whitish surface (A). Microscopic examination

6 showed cartilage and perichondrium (B). The cartilage was composed of small

7 chondrocytes with lacunae and of hypertrophic cells with large lacunae (C). The cartilage

8 and perichondrium were positive for COL II (D) and COL I (E), respectively.

9 Macroscopic examination of the P1 cell-nodules (70 mg in weight) at 42 weeks showed

10 the same appearance as that of 25 weeks (F). Microscopic examination revealed

11 decrease of hypertrophic chondrocytes in the cartilage and increase of matrix in the

12 perichondrium (G, H). Again, the cartilage was positive for COL II (I) and the

13 perichondrium was positive for COL I (J).

14

15 **Figure 7. Long-term observation at the P2 cell (PDL 18)-implanted sites.**

16 P2 cell-implanted sites were analyzed at 9 weeks (A-E) and 17 weeks (F-J).

17 Macroscopic examination of the P2 (PDL 18) cell-nodules (9 mg in weight) at 9 weeks

18 showed light-brown surface (A). Microscopic examination showed the cartilage with

19 small chondrocytes with round or spindle nuclei and hypertrophic cells with lacunae (B)

20 and thick perichondrium (C). Immunohistochemically, the cartilage was positive for

21 COL II (D) and the perichondrium was positive for COL I (E). Macroscopic

22 examination of the P2 (PDL 18) cell-implanted sites (32 mg in weight) at 17 weeks

23 showed whitish surface with partly yellowish area (F). Microscopic examination

24 showed the cartilage and irregularly thickened perichondrium (G) and the

1 perichondrium with decrease of hypertrophic chondrocytes and increase of eosinophilic
2 matrix (**H**), compared with nodules at 9 weeks. The cartilage was positive for COL II (**I**),
3 and partially positive for COL I (**J**). The perichondrium was positive for COL I.

4

5 **Figure 8. Implantation of P3 cell (PDL 23).**

6 P3 (PDL 23) cell-implanted sites were analyzed at 7 weeks. Macroscopic examination
7 showed the flattened and smaller nodules (11 mg in weight) with translucent whitish
8 surface (**A**). Microscopic examination showed the cartilaginous tissue with tight
9 adhesion to the mouse muscle tissue and reactions of foreign-body type giant cells (**B**).
10 The nodule was consisted of chondrocytes with spindle nuclei and narrow lacunae and a
11 few hypertrophic cells (**C**) in myxomatous matrix. The matrix was focally positive for
12 COL II (**D**) and diffusely positive for COL I in the cartilage (**E**).

13

1 **Table 1. Primer pairs and experimental conditions for RT-PCR**

Table 1. Primer pairs and experimental conditions for RT-PCR		
Gene product	Forward and reverse primers (5'–3')	Expected product size (bp)
Collagen I	CCCAAGGACAAGAGGCAT	159
	GCAGTGGTAGGTGATGTTCTG	
Collagen II	AGATGGCTGGAGGATTTGAT	301
	CTTGGATAACCTCTGTGACTT	
Collagen III	GGGTCCTACTGGTCCTATTGG	229
	TCGAAGCCGAATTCCTGGTCT	
Collagen X	AGCCAGGGTTGCCAGGACCA	387
	TTTTCCCACTCCAGGAGGGC	
β2-microglobulin	CTCGCGCTACTCTCTCTTTCTGG	335
	GCTTACATGTCTCGATCCCACTTAA	

2

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8