ORIGINAL ARTICLE

Histological study of costal cartilage after transplantation and reasons for avoidance of postoperative resorption and retention of cartilage structure in rats

Yukiko Rikimaru-Nishi[a,b], Hideaki Rikimaru[a], Shinichiro Hashiguchi[a], Tomonoshin Kanazawa[b], Keisuke Ohta[b], Kei-ichiro Nakamura[b] and Kensuke Kiyokawa[a]

[a]Department of Plastic and Reconstructive Surgery and Maxillofacial Surgery, Kurume, Japan; [b]Division of Microscopic and Developmental Anatomy, Kurume University School of Medicine, Kurume, Japan

Corresponding author:

Hideaki Rikimaru, Ph.D., M.D.

Department of Plastic and Reconstructive Surgery and Maxillofacial Surgery,

Kurume University School of Medicine

67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan

Tel: +81-942-31-7569, Fax: +81-942-34-0834

E-mail: Hi_Rikimaru@yahoo.co.jp

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ABSTRACT

Background: Limited information is available on the biological status of transplanted cartilage from which the perichondrium has been removed. This paper describes the histological and three-dimensional structural picture of cartilage, using green fluorescent protein (GFP) transgenic rats and normal wild rats.

Methods: Three sections of costal cartilage were harvested from 10-week-old wild rats. One section was used as a specimen while two were subcutaneously collected from the dorsal region of 10-week-old GFP rats at 4 weeks and 8 weeks post-transplant. The experiment was performed in two randomized groups. The perichondrium was removed from transplanted cartilage in the first group and perichondrium of transplanted cartilage remained intact in the second group. Histology and focused ion beam/scanning electron microscope (FIB/SEM) tomography were used to evaluate the transplanted cartilage.

Results: All 40 transplanted sections were harvested and no infections, exposure or qualitative change of cartilage matrix were seen following transplant. Histological analyses showed that the surface layer of the GFP-negative transplanted cartilage was replaced with GFP-positive chondrocytes 8 weeks post-transplant in the first group. A three-dimensional layer of perichondrium-like tissue reconstructed around the cartilage at 8 weeks was confirmed, resembling normal perichondrium. However, the GFP-positive chondrocytes were not replaced in the second group.

Conclusions: The cell renewal of chondrocytes is necessary for subcutaneously transplanted cartilage to maintain its tissue composition over a long period of time. The histological and ultrastructural analyses revealed that cells from recipient tissue generated new chondrocytes even when cartilage was implanted after removing the perichondrium.

Introduction

Cartilage is a useful autologous transplant material in plastic surgery. Cartilage does not contain blood vessels; instead, nutrients are supplied through the spreading of interstitial fluid from the perichondrium [1]. Fibroblast-like cells present in the perichondrium are known to be progenitor cells of chondroblasts [1]. Chondrocytes are shaped from chondroblasts inside the perichondrium; however, during cartilage grafting, implantation is often carried out by removing the perichondrium [2]. Even when cartilage is transplanted in the subcutaneous tissue, it is rarely resorbed after surgery.

Green fluorescent protein (GFP) transgenic mice [3] and rats [4] are often used for organ transplantation research. GFP is a fluorescent protein isolated from *Aequorea Victoria* jellyfish. The GFP gene construct is designed for ubiquitous expression in GFP animals, and GFP protein emits green fluorescence under irradiation with blue light [3]. Since rejection rarely occurs when tissues from GFP rats are implanted into inbred wild-type rats, these animals are used as transplantation models [4]. Rejection is also rare when tissues from inbred wild-type rats are transplanted into GFP rats [5]. GFP fluorescent tissues/cells can therefore be used to observe the details of dynamic changes in tissues and organs [6,7].

Focused ion beam/scanning electron microscope tomography (FIB/SEM tomography) is a new three-dimensional (3D) analytical scanning electron microscopic method [8,9] that facilitates 3D structural analysis of biological tissues with a wider range and higher resolution than previous methods, thereby allowing details of the cellular and tissue ultrastructure to be evaluated. In the present study, FIB/SEM tomography was used to analyze the cellular structure of the normal perichondrium and transplanted cartilage.

Our hypothesis was that cartilage lacking the perichondrium might maintain the cartilage structure that receives the supply of cells from the wound bed. The aim of the

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present report was to clarify why cartilage is not resorbed after surgery, and to understand the

dynamics of transplanted cartilage-derived cells and wound bed-derived cells using GFP-

labeled rats. The study employed procedures that allowed histological and ultrastructural

analysis of transplanted cartilage to be performed.

Materials and methods

Ethical treatment of animals

All experiments were performed in accordance with the National Institutes of Health

guidelines for animal research. This study received the approval of the ethics review board of

Kurume University Animal Care Center (approval number: 2014-065).

Animals

Ten-week-old wild-type Sprague-Dawley rats (male, 310–330 g) in which cytoplasm does

not naturally express fluorescence, and ten-week-old GFP transgenic rats (male, 310–330 g)

['green rat CZ-004' SD TgN (act-EGFP) OsbCZ-004], which showed green fluorescence

protein under control of the actin promoter, were purchased from Japan SLC (Hamamatsu,

Japan).

Subcutaneous implantation and harvesting of cartilage grafts

Twenty animals (10 pairs of wild-type and GFP+ rats) were assigned to each group. The

perichondrium was removed from the cartilage in the first group (cartilage without

perichondrium [CWOP] group, while the perichondrium remained intact in the second group

(cartilage with perichondrium [CWP] group). After intraperitoneal anesthesia with

pentobarbital (0.05 mg/kg), two pieces of cartilage harvested from the wild-type rat of each

pair were implanted into the dorsal region of the GFP+ rat of the pair, followed by harvesting of one piece from each GFP+ rat after 4 weeks and harvesting of the other piece after 8 weeks. Thus, 10 cartilage pieces were harvested from the GFP+ rats in each group per time point, since one piece removed at 4 weeks and one piece at 8 weeks, with all animals remaining alive throughout the experiment (Figure 1). In both groups, the transplanted cartilages were harvested along with the surrounding soft tissues of the GFP+ rats. These time points were selected on the basis of reports that noted that no structural changes of the cartilage layer could be seen up to 4 weeks after transplantation [10]. Because the cells involved in tissue repair underwent adhesion, proliferation, migration and differentiation, a relatively long period was needed for the repaired cells to migrate into the extracellular matrix and synthesize or to reconstruct new tissue [10,11]. These reports noted that changes in the cartilage tissue were evaluated at 4 weeks and 8 weeks after transplantation. Evaluation was therefore performed at the same points in time after transplantation in the present study.

Processing of light microscopic specimens

Forty cartilage specimens obtained from 20 GFP+ rats (10 in the CWP group and 10 in the CWOP group) were fixed for 6 h in 4% paraformaldehyde (PFA) (pH 7.4) in phosphate-buffered saline (PBS) (pH 7.4). After a brief rinse in PBS, the fixed tissues were immersed overnight in 30% sucrose in PBS and subsequently embedded and frozen in optimal cutting temperature compound (Sakura Tissue-Tek, Tokyo, Japan). The entire cartilage specimen was cut into thin slices in the long axis direction so that the perichondrium was visible on both sides of the cartilage substance. Longitudinal sections (5 μm thick) were cut with a HM560 microtome (Micron, Germany), and were mounted on glass slides coated with poly-L lysine. The cartilage

specimens were subjected to multiple staining processes, with 15 slides per staining method being created from each cartilage sample.

Light microscopic examination and Immunohistochemistry

Sections of 40 cartilage specimens harvested from the 20 GFP+ rats in the two groups were rinsed three times with PBS (pH 7.4) and stained with toluidine blue [12].

Type I collagen is distributed in the matrices of perichondrium and collagen type II is found uniformly throughout the hyaline cartilage extracellular matrix. Sections from the 40 cartilage specimens were incubated with blocking solution containing 3% normal goat serum and 0.5% Triton X-100 in PBS, followed by incubation overnight at 4°C with anti-rabbit type I collagen or type II collagen polyclonal antiserum diluted with blocking solution (1:2000, Abcam plc, Cambridge, UK) and rinsing 4 times with PBS. Then the sections were reacted with ABC complex solution (Nacalai Tesque, Kyoto, Japan) and with 3,3'-diaminobenzidine tetrahydrochloride (DAB) according to the manufacturer's instructions (DAB; Dako Japan, Kyoto, Japan). Finally, nuclei were counterstained with hematoxylin. For negative controls, appropriate solutions without the primary antibody were applied, instead of those with specific anti-collagen antibodies [13].

Immunohistochemical fluorescence microscopy

The sections were reacted with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (Wako Pure Chemical Industries, Osaka, Japan) for nuclear counterstaining.

In addition, a section of cartilage harvested from each of the 10 GFP+ rats in the CWOP group at 8 weeks after transplantation was incubated overnight at 4°C with anti-rabbit type II collagen polyclonal antiserum diluted with blocking solution (1:2000, Abcam plc,

Cambridge, UK), followed by rinsing 4 times with PBS. Then the sections were incubated for

4 h at room temperature with Alexa 568-conjugated goat anti-rabbit IgG antibody (1:400;

Molecular Probes, Eugene, OR) as the secondary antibody [14], followed by reaction with

DAPI solution for nuclear counterstaining. After rinsing with PBS, the sections were

mounted using PermaFluor mounting medium (Thermo Shandon, PA).

Images were captured by a confocal laser-scanning microscope (FV-1000; Olympus,

Tokyo, Japan) at a magnification of 100× or 400×, with excitation at 473 nm and 559 nm.

Quantitative analysis of captured images of fluorescence microscopy

In cartilage specimens resected from 20 GFP+ rats in the two groups, GFP-positive cells were

detected on fluorescent images and the thickness of the cartilage containing GFP-positive

cells in the specimens was calculated by using ImageJ software (National Institutes of Health,

Bethesda, MD). The full thickness of the cartilage substance at the site of maximum

thickness of the GFP-positive cartilage was also measured simultaneously.

FIB/SEM tomography

Specimens were cut into small cubes and fixed with half Karnovsky fixative (2.5%

glutaraldehyde and 2% paraformaldehyde solution) in 0.1 M PB (pH 7.4) followed by post-

fixation with ferrocyanate and 1% OsO4. The specimens were then treated with 1%

thiocarbohydrazide and immersed in a 1% OsO4 solution, followed by further staining with

Walton's lead aspartate solution. They were then dehydrated in a graded ethanol series,

infiltrated with epoxy resin (EPON812, TAAB, Berkshire, UK) and polymerized at 60°C for

72 h [8,15]. The resin blocks were trimmed down to a 2 × 4-mm area. The surface of each

embedded specimen was exposed by trimming the resin block with a diamond knife and the

specimen was mounted in a special holder and set on the stage of the FIB/SEM apparatus. Serial images of the block face were acquired by repeated cycles of sample surface milling using a focused gallium ion beam (milling step: 100 nm, 1000 cycles) and by acquisition of compositional contrast SEM images from back scattered electrons (landing energy, 2.5 kV). Images were reconstructed that covered the area from the normal perichondrium to the internal region of the cartilage for control specimens in the CWP group, and the area from the perichondrium-like tissues to the internal region of the cartilage for 8-week specimens in the CWOP group. A total of 3600 block face images were obtained per specimen.

3D reconstruction and segmentation

In each region, cell morphology was evaluated after 3D reconstruction using Avizo 8.0 software (VSG Inc., Bordeaux, France). The images were briefly normalized using a histogram-based image filter. A median filter was then used to prepare threshold segmentation, the cellular regions were selected using the threshold method and irregular parts of the selected regions were manually removed [16].

Results

Macroscopic observations at the time of extraction

Forty sections each of transplanted cartilage from both groups were grafted without infections or exposure following transplant. No clear evidence of the resorption or destruction of cartilage was detected.

Light microscopic examination

Toluidine blue-stained sections from 20 cartilage specimens showed metachromatic staining

of glycosaminoglycans (GAGs) in the hyaline cartilage matrix before and after transplantation in the CWP group (Figure 2a, c, e). The type 1 collagen-stained sections from 20 cartilage specimens showed type I collagen fibers in the perichondrium as brown structures (Figure 3a). The type II collagen-stained sections from 20 cartilage specimens showed type II collagen fibers in the extracellular matrix as brown structures (Figure 3b). Toluidine blue-stained sections from 20 cartilage specimens obtained from the 10 GFP+ rats also showed GAGs in the hyaline cartilage matrix before and after transplantation in the CWOP group (Figure 4a, c, e). In the CWOP group, the perichondrium was completely removed before transplant (Figure 3c). The perichondrium-like fibrous structures began forming around the cartilage at 4 weeks and 8 weeks post-transplant (Figure 4c, e). This fibrous structure showed a brown-colored positive reaction upon collagen type I staining and perichondrium-like tissues similar to normal perichondrium had formed (Figure 3e, g). At 4 weeks and 8 weeks post-transplant, the type II collagen-stained sections from 20 cartilage specimens obtained from the 10 GFP+ rats showed type II collagen fibers in the extracellular matrix (Figure 3f, h). Degeneration of the extracellular matrix did not occur even when cartilage was transplanted without perichondrium, and it maintained its hyaline cartilage properties.

Immunohistochemical fluorescence microscopic analysis of cell morphology

In the CWP group, GFP-positive cells were detected in the normal perichondrium in seven out of ten cartilage specimens obtained from 10 GFP+ rats at 4 weeks post-transplant and in nine out of ten cartilage specimens obtained from 10 GFP+ rats at 8 weeks post-transplant. No GFP-positive cells were noted in the cartilage matrix (Table 1; Figure 2d, f).

DAPI and GFP images of cartilage specimens obtained from 10 GFP+ rats in the CWOP group at 4 weeks post-transplant showed that GFP rat-derived GFP-positive cells were aggregated into fibrous tissue lamina surrounding the wild-type rat-derived GFP-negative transplanted cartilage, forming perichondrium-like tissue (Figure 4d). At 8 weeks post-transplant, the surface layer of the transplanted GFP-negative cartilage matrix was replaced with GFP-positive cells in seven out of ten cartilage specimens obtained from 10 GFP+ rats (Table 1; Figure 4f).

The GFP-positive cells in the GFP-negative cartilage areas in these specimens were identified as chondrocytes by collagen type II immunoreactive (Figure 5). The thickness of transplanted cartilage replaced by GFP-positive cartilage ranged from 0 μm to 146 μm from the cartilage surface (mean: 54.7 μm) in longitudinal sections of seven cartilage specimens from ten GFP+ rats. The maximum thickness ratio of GFP-positive cartilage was calculated by dividing its thickness by the full cartilage thickness at the same site, revealing that the mean replacement rate by GFP-positive cartilage was 6.5% at 8 weeks post-transplant in seven cartilage specimens from the CWOP group (Table 2).

FIB/SEM tomography analysis of cell morphology

We used FIB/SEM tomography to examine the transitional zone between normal perichondrium and cartilage in the CWP group immediately after collection of specimens, as well as the transitional zone between perichondrium-like tissues and transplanted cartilage at 8 weeks after transplantation in the CWOP group. Areas for 3D reconstruction of the transitional zone between cartilage and perichondrium were randomly selected from the whole resin-embedded specimen (Figure 6a, colored area), and 500 to 700 serial images were acquired. The final reconstructed image was approximately $70 \times 70 \times 60$ μm^3 (Figure 6b).

Each block face image had sufficient resolution to characterize the pattern of cell distribution in the perichondrium (Figure 6c). Segmentation showed that the transitional tissues contained two different types of cells at each reconstructed site (Figure 6d).

FIB/SEM of the transitional zone between normal perichondrium and cartilage in the CWP group immediately following collection showed oval-shaped juvenile chondrocytes and revealed that elongated cells with protrusions similar to fibroblast cells were present in the perichondrium (Figure 7). In the transitional zone between the perichondrium-like tissues and transplanted cartilage in the CWOP group at 8 weeks after transplantation, the reconstructed cell layers were observed to have the same morphology as in the transitional zone of normal cartilage (Figure 8). These findings indicate that cartilage transplanted without the perichondrium eventually developed perichondrium-like tissue with substantially the same cellular and tissue architecture as the surrounding normal perichondrium.

Discussion

Growth of cartilage is thought to occur via two mechanisms, which are interstitial growth by mitosis of existing chondrocytes and appositional growth by differentiation of cells from the perichondrium. However, interstitial growth is not considered to be very important because it is only observed in the early stages of chondrogenesis, such as during fetal life, when tissue volumes increase very rapidly [1]. Therefore, we concentrated on appositional growth, which involves differentiation of cells derived from the perichondrium.

In 1978, Tanzer [17] reported on the long-term outcome of 44 microtia cases and observed that a reconstructed auricle frame grew even after reconstruction. Similar observations were noted by DellaCroce et al. in 2001 [18]. The authors reported that the reconstructed auricle frame grew to height and width. No histological evaluation was

conducted. However, it was not clear whether the post-transplant cartilage actually grew due to addition of new cells or whether only the connective tissue surrounding the transplanted cartilage was became thicker.

During surgery, the perichondrium is usually removed before cartilage is transplanted. Thus, in the transplanted cartilage from which the perichondrium is removed, new cells capable of differentiating into chondrocytes must be supplied from the surrounding tissues in order to maintain tissue structure. However, few reports focus on the changes that occur after the transplant of cartilage that has had the perichondrium removed.

In a 2002 report by Duynstee et al. [19] a study using the cartilage of laboratory rabbits was conducted to explore the role of the perichondrium in the healing process of cartilage wounds. The authors noted that the presence of the inner layer of perichondrium played a major role in new cartilage formation. The report did not clarify whether cell renewal of chondrocytes actually occurs in transplanted cartilage, however.

In the orthopedic surgery field, attempts have been made to create hyaline cartilage with normal mechanical properties by using bone marrow-derived mesenchymal stem cells (BM-MSC) or other methods. However, the wound bed for implanted cells tends to be a highly inflammatory or osteogenic microenvironment, which affects the viability of the implanted cells. It has been proven that the microenvironment regulates the mechanisms of cell integration. Accordingly, it is important to recognize that the tissue microenvironment may be involved in regulating the articular cartilage repair response [20]. Therefore, accumulation of more knowledge about the subcutaneous tissue microenvironment surrounding hyaline cartilage and cellular dynamics in that tissue may be necessary to improve the outcome of cartilage transplantation for plastic surgery.

We hypothesized that cartilage lacking the perichondrium might maintain the cartilage

structure that receives the supply of cells from recipient tissue.

The present experiment used two different transplant models, a CWP group of transplanted cartilage with intact perichondrium and a CWOP group of transplanted cartilage with perichondrium eliminated, to observe temporal changes with histological analysis using a technique for detection of GFP fluorescent cells.

In the CWP group, hyaline cartilage was maintained, but GFP-positive cartilage was not formed in all cases. In this group, the original perichondrium was intact and it was considered possible for cells to differentiate into chondrocytes in the future; hence, it was considered that there was no need for ingrowth of cells from outside the perichondrium.

In the CWOP group, four observations were noted. First, when the perichondrium was removed and the cartilage was transplanted, a perichondrium-like tissue was formed around the transplanted cartilage in all cases and the morphology of cells contained in this membrane-like tissue was similar to the original perichondrium. Second, hyaline cartilage always persisted, even in transplanted cartilage that had the perichondrium removed. Third, the perichondrium-like tissue-derived cells originated from the recipient and differentiated into new chondrocytes in 70% of the transplanted cartilage specimens. Therefore, we confirmed that the transplanted cartilage showed growth of cells derived from the perichondrium-like tissue and cell renewal. Since this was observed in 7 out of 10 rats, it was a highly reproducible finding. Fourth, the interaction between transplanted cartilage lacking perichondrium and the subcutaneous microenvironment led to development of new chondrocytes in the cartilage implants, which is a novel finding of this study.

These results suggest that maintaining healthy subcutaneous tissue around transplanted cartilage may be as important as the state of the graft itself (thickness and shape, etc.) when cartilage is transplanted and may affect the long-term outcome.

The present study had several limitations. First, the origin of GFP-positive chondrocytes in the transplanted cartilage is unclear. These stem cells have two possible sources, which are mesenchymal stem cells originally present in the surrounding soft tissues, such as adipose tissue, and bone marrow stem cells mobilized to the wound bed. Although fibroblasts are abundant inside subcutaneous tissues, many reports have indicated that among of these are cells that function as mesenchymal stem cells [21]. Further research is warranted to clarify the origin of cells differentiating into new chondrocytes by placing luminescent surface-marker labeled cells that are different from GFP labeled cells derived from different origins around the transplant cartilage.

Second, the cell cycle may not be the same in rat cartilage and human cartilage, and it is not clear that the same results would be obtained with human tissues. However, the present model is potentially important because specific tissues or cells were implanted in the subcutaneous microenvironment, after which it could be determined whether tissue changes were driven by donor cells or recipient cells by using two different rat strains. Therefore, this model may be useful for providing information about interactions between transplanted and recipient tissues.

Third, only the effects of a relatively normal microenvironment surrounding the implanted cartilage were assessed in this study. However, surgery for microtia may involve multiple operations at the same site that could lead to local inflammation after cartilage transplantation. Further research will be required to assess the influence of such factors.

The present study revealed that recipient -derived perichondrium-like tissues formed around transplanted cartilage that had been stripped of the perichondrium, and the cartilage grafts contained new chondrocytes which developed from recipient cells. These findings suggest that the local tissue microenvironment plays a crucial role in regulating the

development and function of cartilage grafts without perichondrium. Accordingly, it is important to recognize that the wound bed microenvironment may determine whether subcutaneously transplanted cartilage undergoes successful engraftment.

Acknowledgments

Disclosure statements

References

1. Junqueira LC, Carneiro J. Basic Histology Text and Atlas. 11th ed. New York: McGraw-Hill, 2005.

2. Tanzer RC. Total reconstruction of the external ear. Plast Reconstr Surg 1959;23:1–15.

3. Okabe M, Ikawa M, Kominami K, et al. 'Green mice' as a source of ubiquitous green cells. FEBS Lett 1997;407:313–9.

4. Hakamata Y, Tahara K, Uchida H, et.al. Green fluorescent protein-transgenic rat: a tool for organ transplantation research. Biochem Biophys Res Com 2001;286:779–85.

5. Takei I, Mura N, Momonoi Y, et al. Shoulder muscle power and postoperative results of fascia lata patch grafting for a massive rotator cuff tear. Katakansetsu (The Shoulder Joint) 2007;31:341–4. (in Japanese)

6. Lu B, Kwan T, Kurimoto Y, et al. Transplantation of EGF-responsive neurospheres from GFP transgenic mice into the eyes of rd mice. Brain Research 2002;943:292–300.

7. Hayakawa J, Migita M, Ueda T, et al. Generation of a chimeric mouse reconstituted with green fluorescent protein-positive bone marrow cells: a useful model for studying the behavior of bone marrow cells in regeneration in vivo. Int J Hematol 2003;77:456–62.

8. Ohta K, Sadayama S, Togo A, et al. Beam deceleration for block-face scanning electron microscopy of embedded biological tissue. Micron 2012;43:612–20.

9. Knott G, Marchman H, Wall D, et al. Serial section scanning electron microscopy of adult brain tissue using focused ion beam milling. J Neurosci 2008;28:2959–64.

10. Hotokebuchi T, Arai K, Takagishi K, et al. Limb allografts in rats immunosuppressed with cyclosporine: as a whole-joint allograft. Plast Reconstr Surg 1989;83:1027–36.

11. Shalabi MA, Elshakaa NM, Kalleny NK, et al. Bone marrow stem cells as a potential treatment of experimentally induced cartilage defects in the knee joint of adult male

albino rats. Egypt J Histol 2012;35:159–75.

12. Drury RAB, Wallington EA. Carleton's histological technique. 5th ed. Oxford: Oxford University Press, 1980.

13. Verwoerd CD, Verwoerd-Verhoef HL, Meeuwis CA, et al. Wound healing of autologous implants in the nasal septal cartilage. ORL J Otorhinolaryngol Relat Spec 1991;53:310–4.

14. Katsuda S, Okada Y, Minamoto T, et al. Collagens in human atherosclerosis. Immunohistochemical analysis using collagen type-specific antibodies. Arterioscler Thromb 1992;12;494–502.

15. Villinger C, Gregorius H, Kranz C, et al. FIB/SEM tomography with TEM-like resolution for 3D imaging of high-pressure frozen cells. Histochem Cell Biol 2012;138:549–56.

16. Kanazawa T, Gotoh M, Ohta K, et al. Novel characteristics of normal supraspinatus insertion in rats: an ultrastructural analysis using three-dimensional reconstruction using focused ion beam/scanning electron microscope tomography. Muscles Ligaments Tendons J 2014;14:182–7.

17. Tanzer RC. Microtia—a long-term follow-up of 44 reconstructed auricles. Plast Reconstr Surg 1978;61:161–6.

18. DellaCroce FJ, Green S, Aguilar EF 3rd. Framework growth after reconstruction for microtia: is it real and what are the implications? Plast Reconstr Surg 2001;108:1479–86.

19. Duynstee ML, Verwoerd-Verhoef HL, Verwoerd CD, et al. The dual role of perichondrium in cartilage wound healing. Plast Reconstr Surg 2002;110:1073–9.

20. Jayasuriya CT, Chen Y, Liu W, et al. The influence of tissue microenvironment on stem cell-based cartilage repair. Ann N Y Acad Sci 2016;1383:21–33.

21. Haniffa MA, Wang XN, Holtick U, et al. Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. J Immunol 2007;179:1595–604.

Figure legends

Figure 1. Transplantation model of costal cartilages. A total of three cartilages were harvested from a wild rat, with one immediately fixed as a specimen while the remaining two were each subcutaneously removed from the dorsal region of a GFP rat. The costal cartilages were harvested one at a time at 4 weeks and 8 weeks following transplant. Confocal laser microscope image of the costal cartilage collected from a wild rat. Only the nucleus produces blue luminescence by DAPI staining. Confocal laser microscope image of the costal cartilage collected from a GFP rat. The nucleus produces blue luminescence while the cytoplasm produces green luminescence under the same conditions. Scale bar = 50 μm.

Figure 2. Toluidine blue staining of transplanted cartilage of the CWP group (left row) and a confocal laser microscope image (right row; DAPI and GFP merged images).

(a): Cartilage with perichondrium attached before transplantation showed metachromatic staining of GAGs in the hyaline cartilage matrix.

(b): Cells derived from wild rats did not show GFP fluorescence in the cartilage with perichondrium attached before transplantation.

(c): Four weeks following transplant. No apparent resorption of cartilage was observed.

(d): Wound bed-derived GFP-positive cells are observed in the perichondrium in 4 weeks following transplant. White arrowheads indicate the GFP-positive cells.

(e): Eight weeks following transplant. No apparent resorption of cartilage was observed.

(f): Recipient-derived GFP-positive cells are observed in the perichondrium but not in the cartilage in 8 weeks following transplant. White arrowheads indicate the GFP-positive cells. p, perichondrium. (a-f) Scale bar = 50 μm.

Figure 3. Immunohistochemical stained image (collagen type I staining (left row), and collagen type II stained image (right row) of the sections of the CWP group at pre-transplant and CWOP group at 8 weeks post-transplant.

a: Cartilage of the CWP group before transplant

    Perichondrium (arrow) is stained brown by collagen type I staining.

b: Cartilage of the CWP group before transplant

    Cartilage extracellular matrix is stained brown by collagen type II staining.

c: Cartilage of the CWOP group before transplant.

    Perichondrium is completely removed (black arrowheads).

d: Cartilage of the CWOP group before transplant.

    Cartilage extracellular matrix is stained brown by collagen type II staining.

e: Removed cartilage of the CWOP group at 4 weeks following transplant

    Fibrous structures (arrow) stained is stained brown by collagen type I staining in the same

    manner as the normal perichondrium (a), and is a perichondrium-like tissue.

 f: Collected cartilage of the CWOP group at 4 weeks following transplant.

    Cartilage extracellular matrix stained brown by collagen type II staining.

 g: Collected cartilage of the CWOP group at 8 weeks following transplant.

    Perichondrium-like tissue stained brown by collagen type I staining are formed, with a

    thicker layered structure compared to week 4.

 h: Collected cartilage of the CWOP group at 8 weeks following transplant.

    Cartilage extracellular matrix was stained brown by collagen type II staining even at

    8 weeks.   (a-h) Scale bar = 50 μm.

Figure 4. Toluidine blue staining of transplanted costal cartilage of the CWOP group (left

row) and a confocal laser microscope image (right row; DAPI and GFP merged images).

(a): Pre-transplant cartilage of the perichondrium removed showed metachromatic staining of GAGs in the hyaline cartilage matrix. Perichondrium is completely removed.

(b): Cells derived from wild rats did not show GFP fluorescence in pre-transplant cartilage of the perichondrium removed.

(c): Four weeks following transplant. No apparent resorption of cartilage was observed. Perichondrium-like fibrous structures were confirmed surrounding the transplanted cartilage.

(d): Recipient-derived GFP-positive cells are observed in the perichondrium-like tissue in 4 weeks following transplant. White arrowheads indicate the GFP-positive cells.

(e): Eight weeks following transplant. No apparent resorption of cartilage was observed.

(f): Cartilage was generated by recipient-derived GFP-positive cells. Yellow dotted line indicates the range of GFP-positive cells in the GFP-negative cartilage.

p', perichondrium-like tissue. (a-f) Scale bar = 50 μm.

Figure 5. Type II collagen fluorescent immunostaining of the sections of the CWOP group at 8 weeks post-transplant. The presence of GFP positive chondrocytes were confirmed with collagen type II immunoreactive. White arrowheads indicate GFP-positive chondrocytes. White asterisks indicate GFP-negative cartilage lacuna. Scale bar = 50 μm.

Figure 6. Three-dimensional FIB/SEM tomography reconstruction of the transitional zone between cartilage and perichondrium.

(a) Low magnification SEM micrograph showing the entire resin-embedded specimen. Small arrows indicate the reconstructed area.

(b) Stack of serial images.

(c) Higher magnification view of the area enclosed by a rectangle in (b).

(d) Three-dimensional rendered views of juvenile chondrocytes and fibroblast-like cells.

Scale bars, (a) 300 μm; (b-d) 20 μm.

Figure 7. FIB/SEM analysis of the transitional zone between the normal innermost layer of the perichondrium and cartilage in the CWP group immediately after collection.

Purple, juvenile chondrocytes; green, fibroblast cell-like cells.

Scale bar = 20 μm.

Figure 8. FIB/SEM analysis of the transitional zone between the innermost layer of perichondrium-like tissue and cartilage at 8 weeks after transplantation in the CWOP group. Substantially the same tissue construction as normal perichondrium was reconstructed. Purple, juvenile chondrocytes; green, perichondrium-like tissue cells. These cells were long and thin, similar to fibroblasts and in the same manner as normal perichondrium. Scale bar = 20 μm.
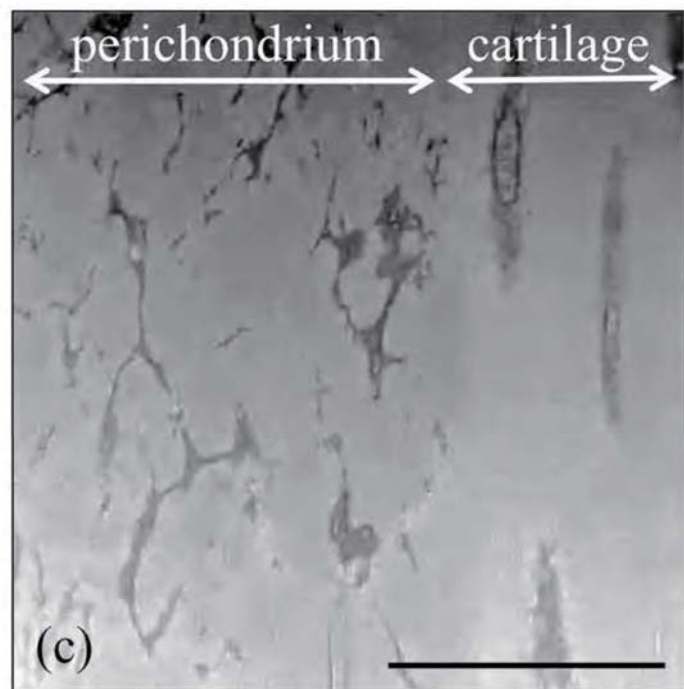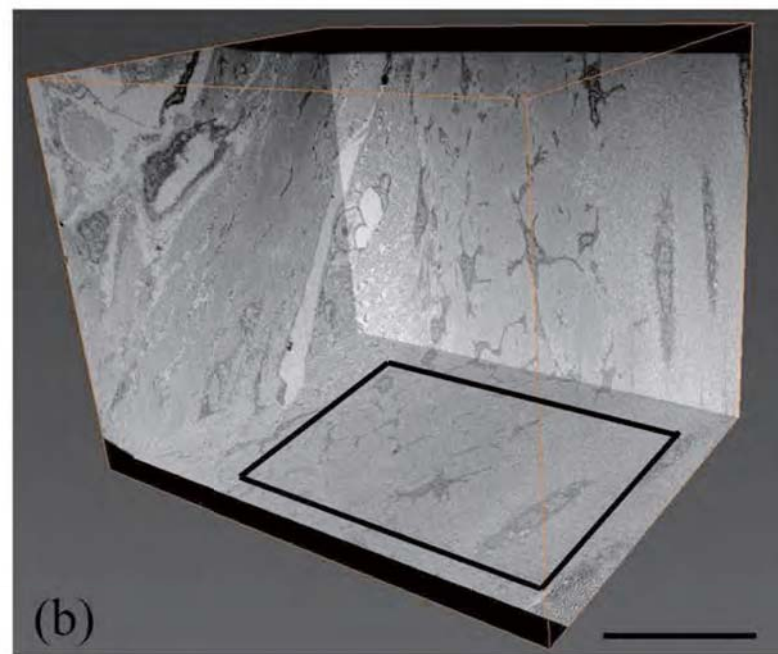
3 sections of cartilage

control

4W

8W

wild rat

GFP rat
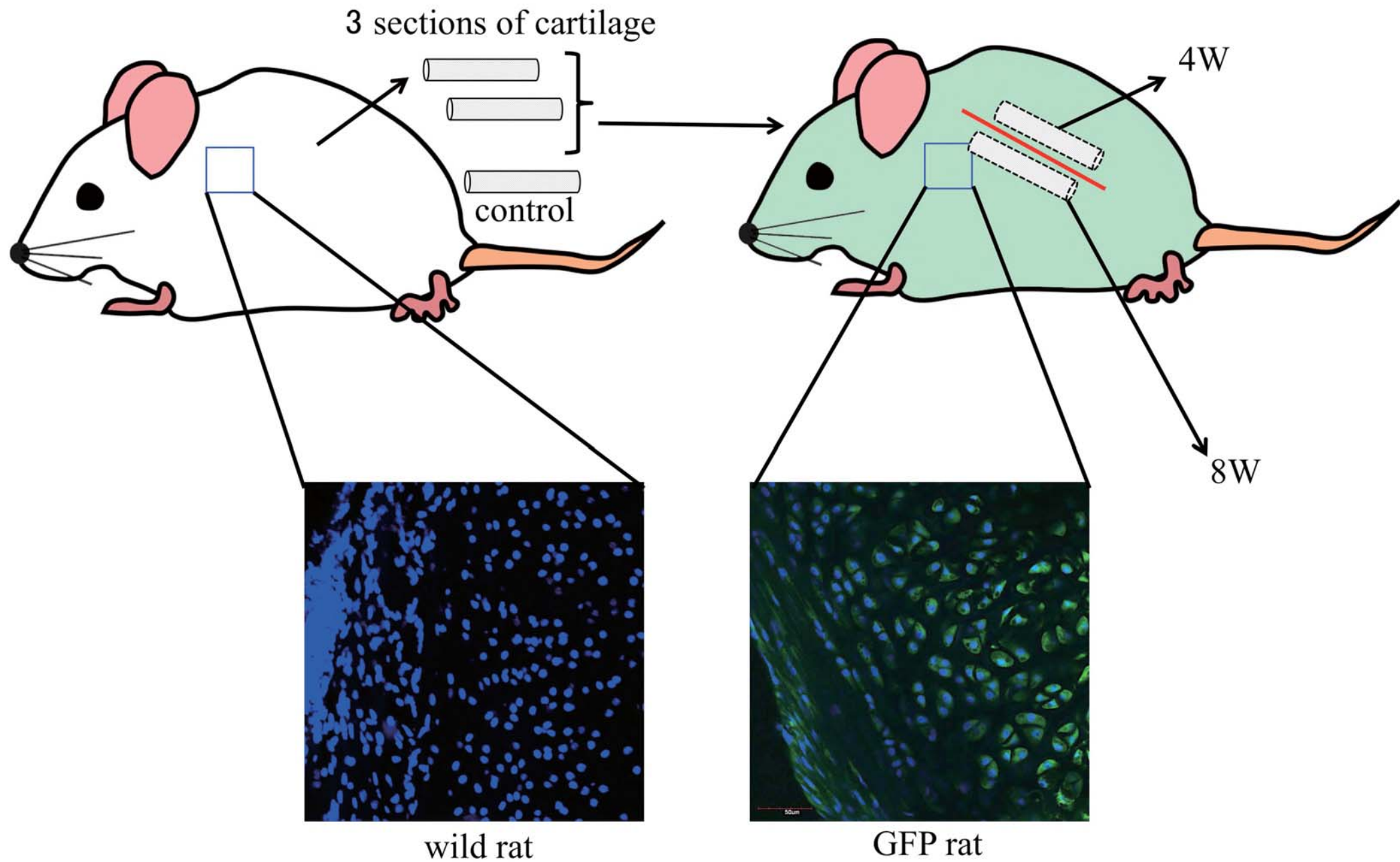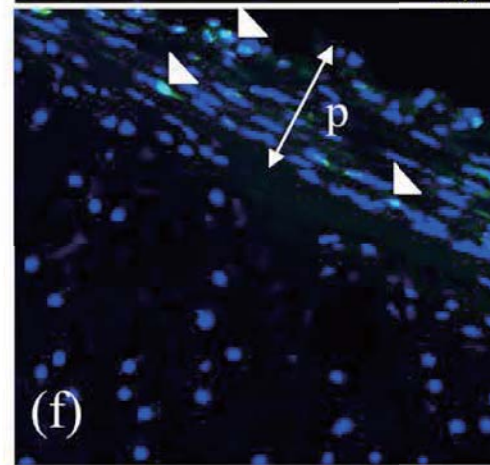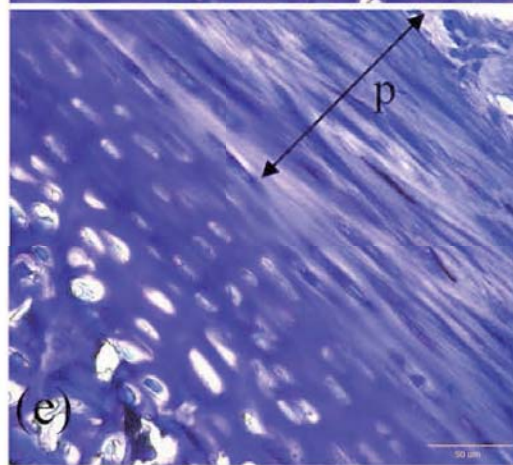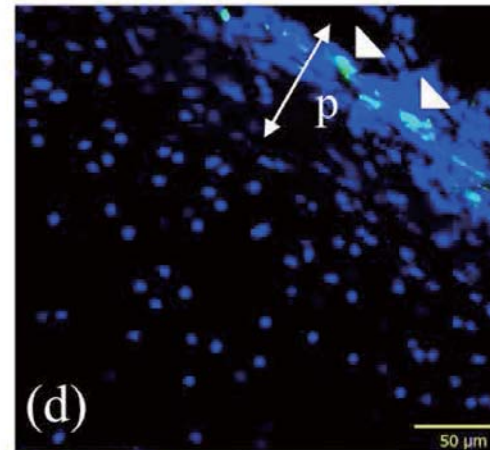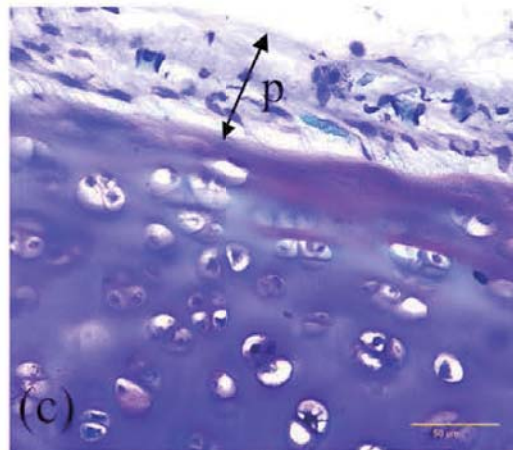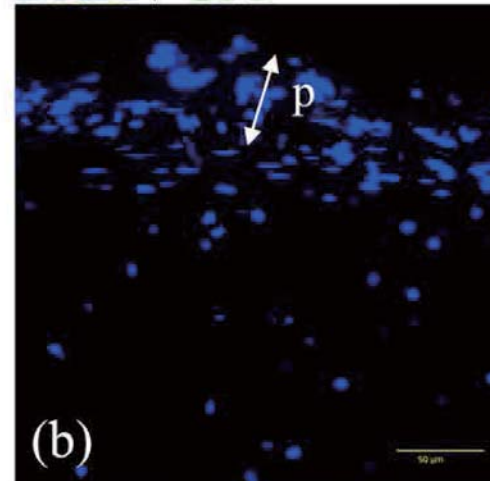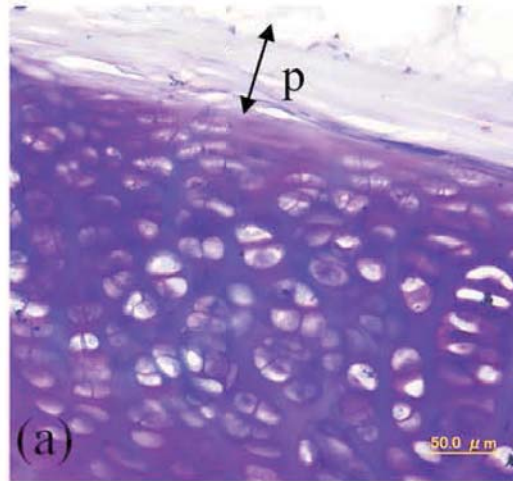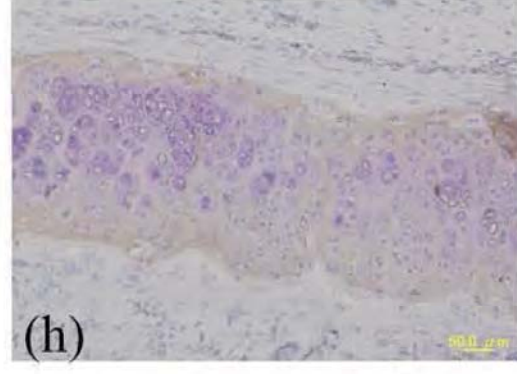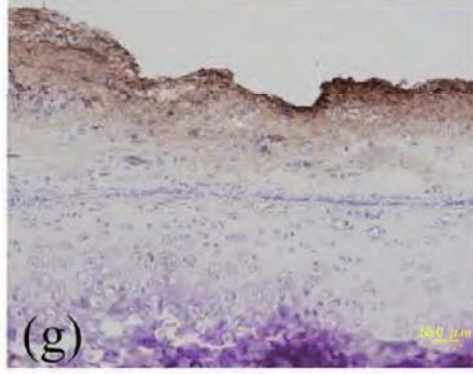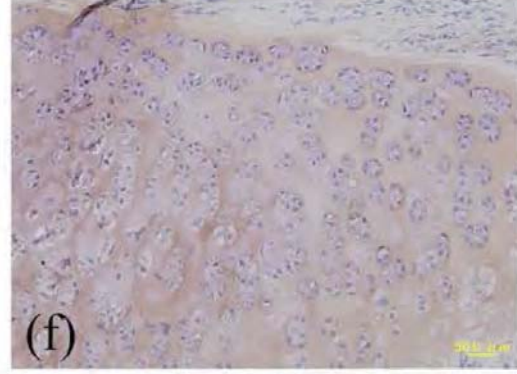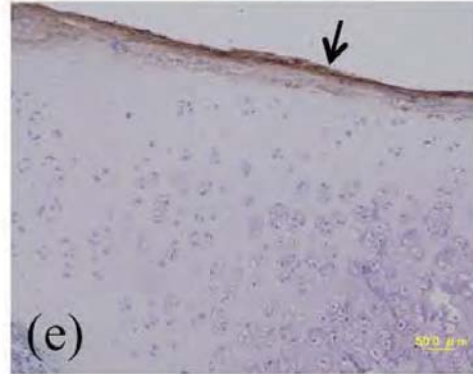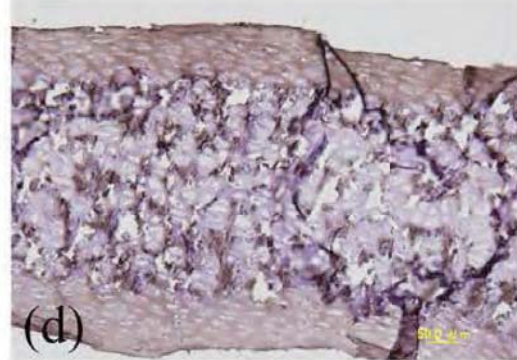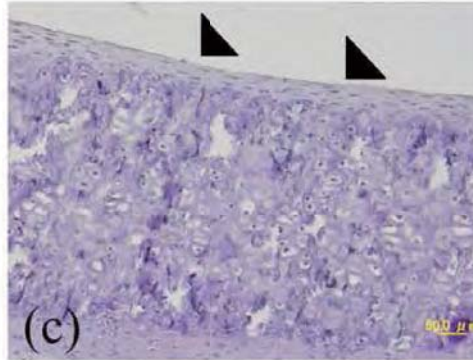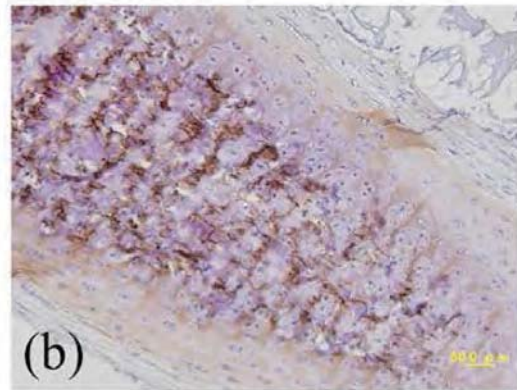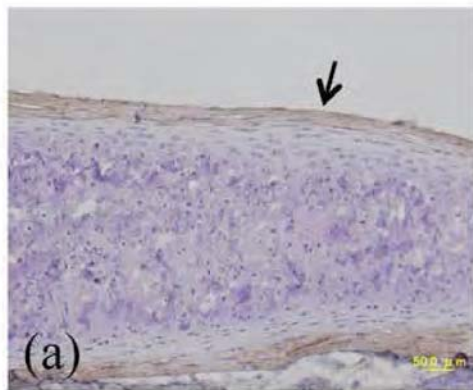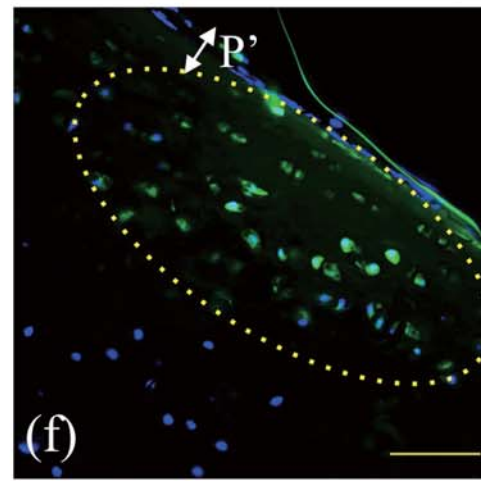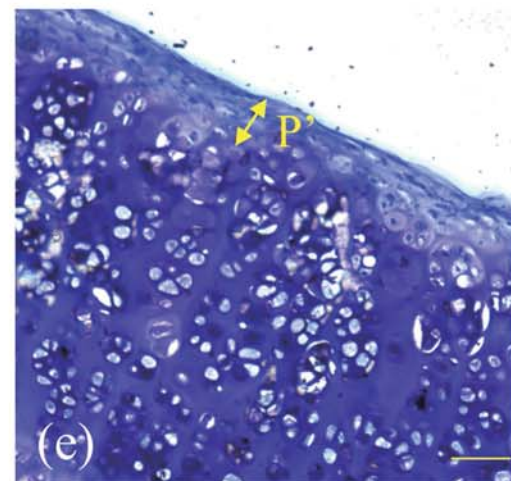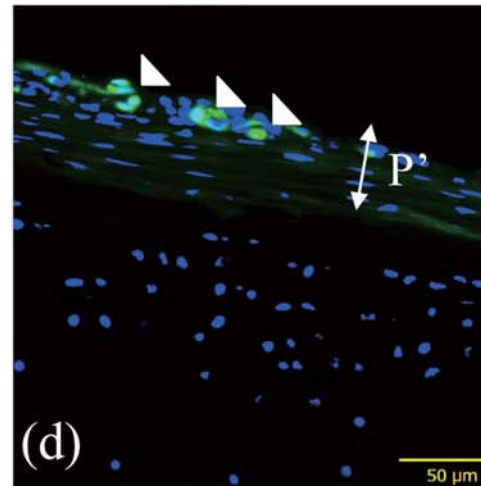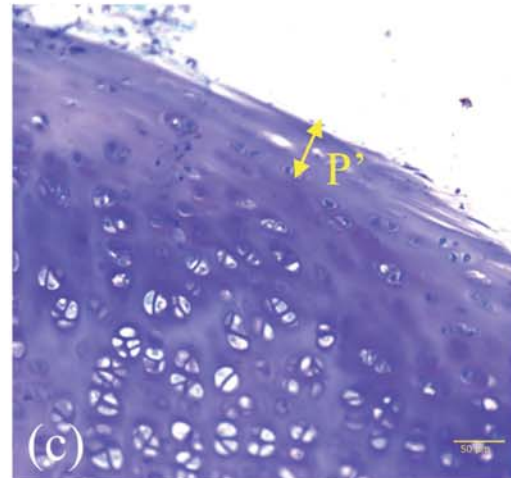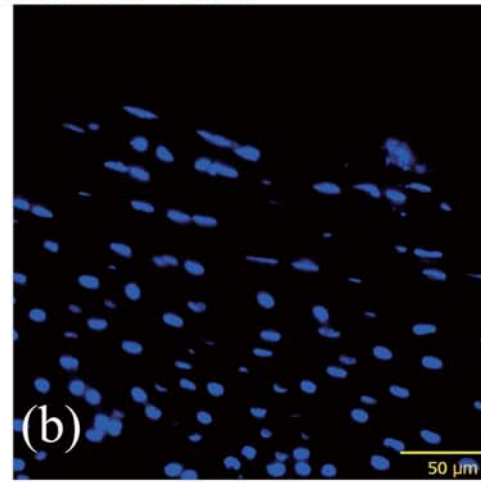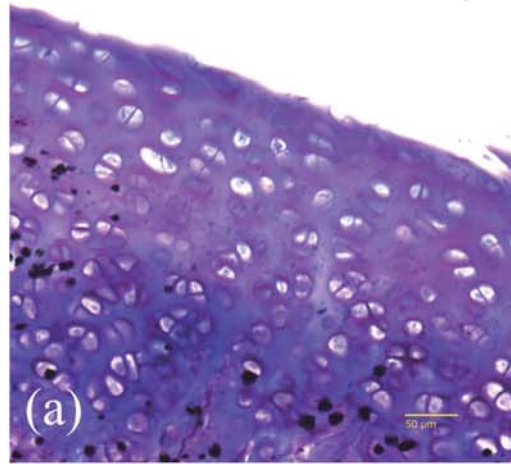
DAPI / GFP

DAPI / GFP

DAPI / GFP/ Anti-type II collagen
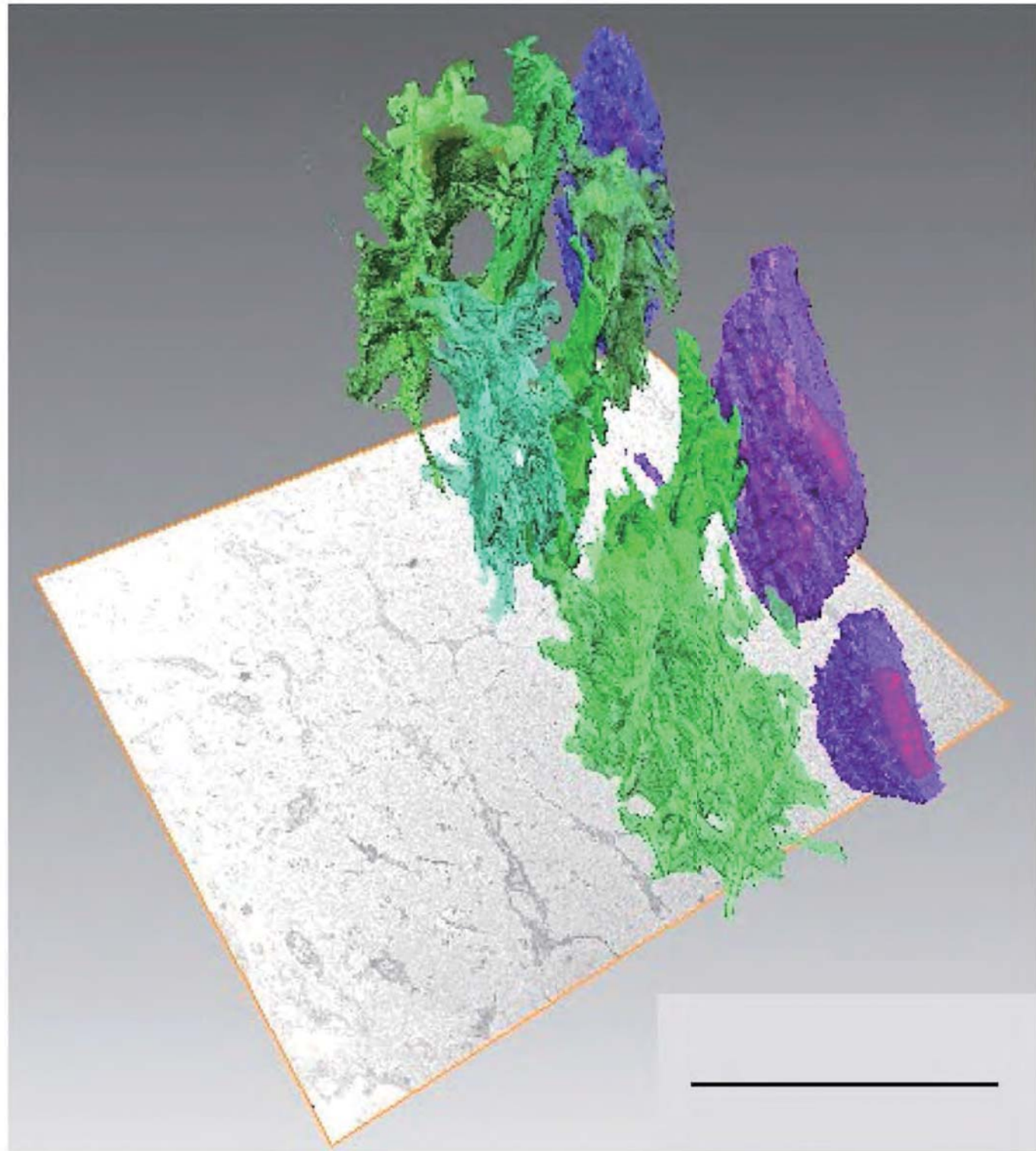
(a) perichondrium, cartilage, Reconstruction area

(b)

(c) perichondrium, cartilage

(d)
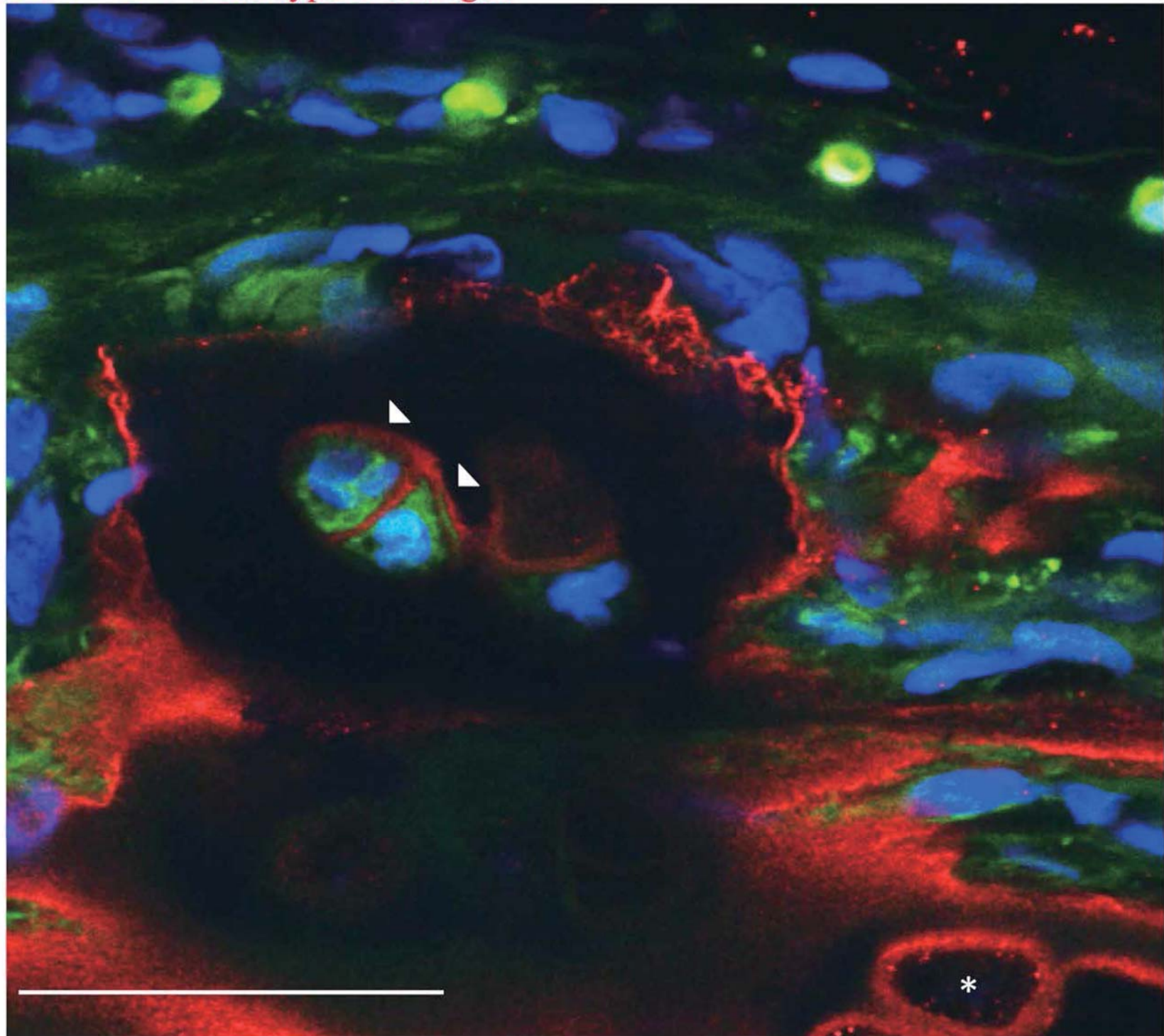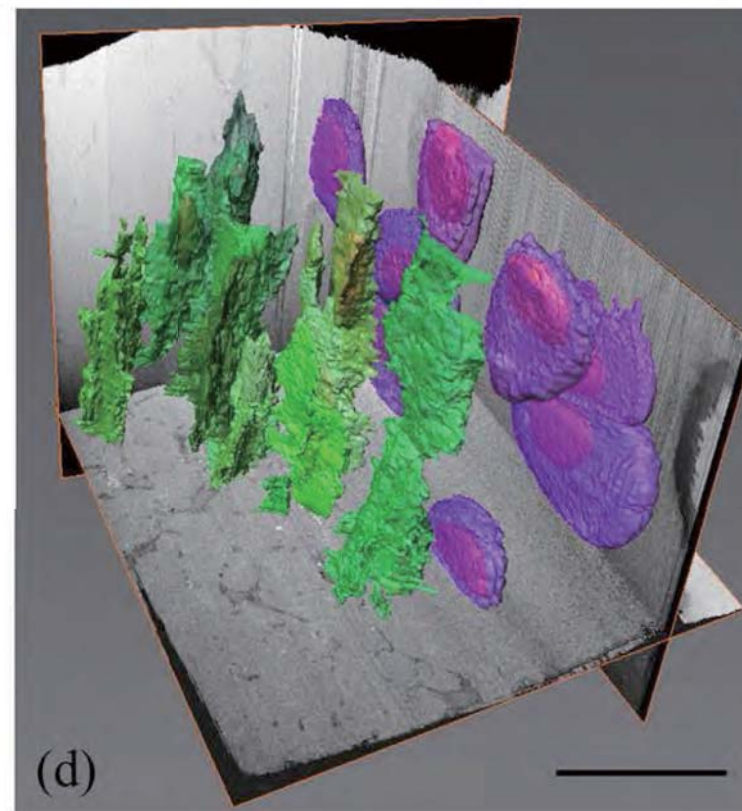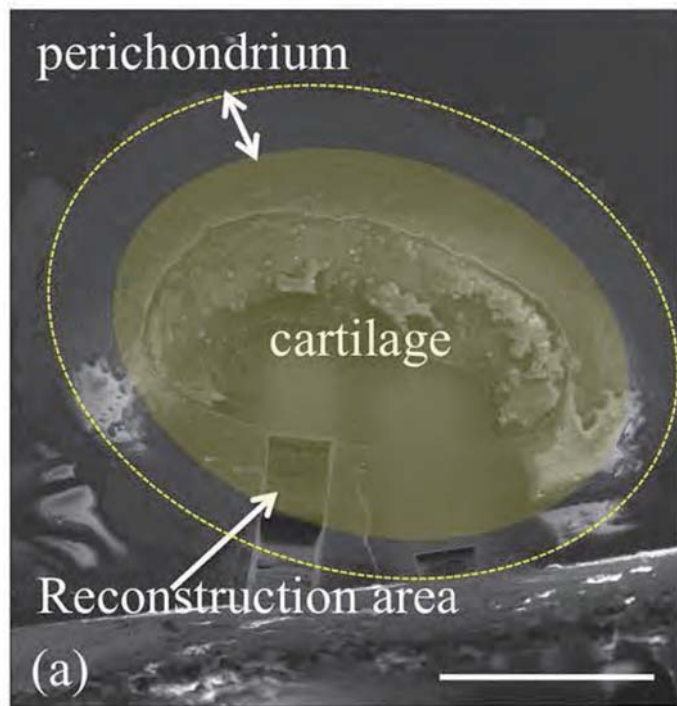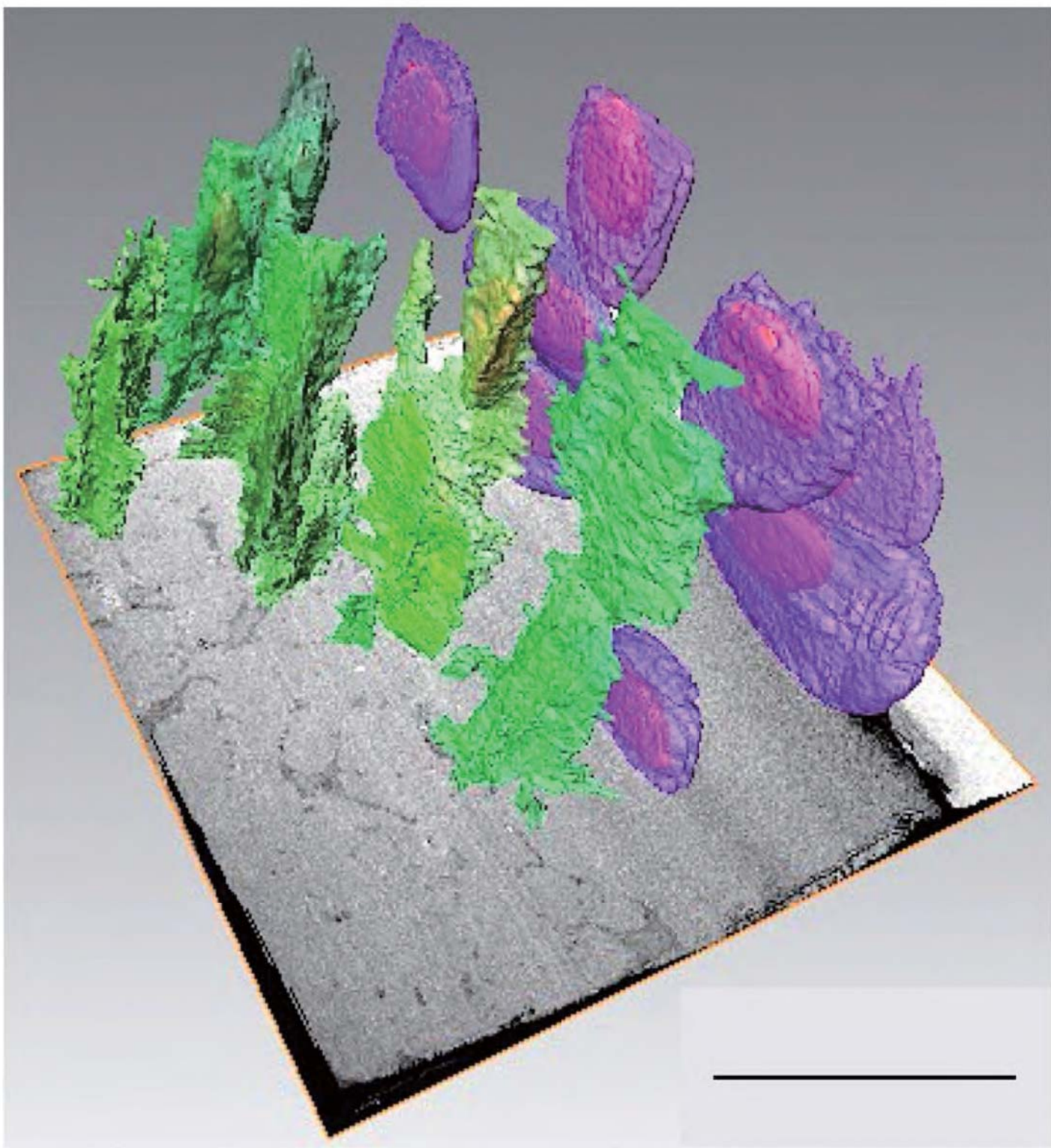
Table 1　Dynamic GFP-positive cells in transplanted cartilage.

Perichondrium and perichondrium-like tissues				
Period following transplant		0 weeks	4 weeks	8 weeks
CWP group (*n* = 10)	Replacement with GFP-positive cells of perichondrium	-	7/10	9/10
CWOP group (*n* = 10)	Replacement with GFP-positive cells of perichondrium-like tissue	-	7/10	10/10

Cartilage substance				
Period following transplant		4 weeks	8 weeks	Thickness of GFP-positive cartilage
CWP group (*n* = 10)	Replacement with GFP-positive cells of cartilage substance	0/10	0/10	0 µm
CWOP group (*n* = 10)		0/10	7/10	0–146 µm (average, 54.7µm)

CWP = cartilage with perichondrium; CWOP = cartilage without perichondrium.

Table 2 Thickness of GFP-positive cartilage from the surface of the long axis of the transplanted cartilage at 8 weeks post-transplantation (10 rats in the CWOP group).

Rat no.	Maximum thickness of GFP-positive cartilage (μm)	Full cartilage thickness at the site of maximum thickness of GFP-positive cartilage
1	0	-
2	57	814
3	146	730
4	0	-
5	53	1060
6	115	766
7	28	933
8	86	1075
9	0	-
10	62	885

CWOP = cartilage without perichondrium.