Establishment of a new technique for the fabrication of regenerative cartilage with a microslicer device to prepare three dimensional diced cartilage

Erika Aoki¹, Yukiyo Asawa², Atsuhiko Hikita², and Kazuto Hoshi¹, ², ³
¹ Department of Sensory and Motor System Medicine, Graduate School of Medicine, The University of Tokyo, Japan; ² Division of Tissue Engineering, The University of Tokyo Hospital, Tokyo, Japan; and ³ Department of Oral-maxillofacial Surgery, Dentistry and Orthodontics, The University of Tokyo Hospital, Tokyo, Japan

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ABSTRACT
Chondrocytes are utilized to cartilage regeneration by being harvested through enzymatic digestion and expanded by monolayer culture. However, these procedures will cause deterioration and dedifferentiation of the chondrocytes. In addition, scaffolds are often needed to provide the cartilage with mechanical strength and three-dimensional structures. We tried to use diced cartilage prepared using a micro-slicer without digestion, monolayer culture or scaffolds. In this study, an appropriate culture condition to induce the fusion of diced cartilage in vitro and cartilage regeneration in vitro and in vivo was determined to realize a scaffold-free cartilage regeneration. As a result, diced cartilages aggregated when they were cultured more than 5 weeks in the media containing 10% fetal bovine serum (FBS). Diced cartilage cultured for 7 weeks with the media containing 10%, followed by the culture with the media containing insulin-like growth factor-1 for 5 weeks in the ultralow attachment plate showed most prominent cartilage formation both in vitro and in vivo. The volume of regenerated cartilage was 2.14 times larger than that of the original cartilage. These results indicated that large regenerative cartilage from a small amount of cartilage was achieved without deterioration or dedifferentiation.

Cartilage is a semi-rigid but elastic avascular connective tissue found at various sites in the body. In the external ear, and the tip and septum of the nose, cartilage maintains the outline of the face and makes facial movement flexible. Aesthetic and functional disorders caused by congenital diseases, such as microtia and nasal deformity in cleft lip and palate, trauma, or malignant tumors, sometimes need reconstructive surgery using implants possessing three-dimensional (3D) structures and mechanical strengths. For example, prostheses made of Gore-Tex (5) or silicon (12) are sometimes used in the dorsal augmentation of the nose. These non-absorbable artificial materials may cause a foreign body reaction, calcification of surrounding tissues, positional anomalies, perforation and exposure. To avoid these issues, the use of autologous cartilage is desirable.

Auricular cartilage, nasal septal cartilage and costal cartilage are possible sources of implants. Costal cartilage is used for rhinoplasty or the treatment for microtia, but it has problems such as deformation over time, and invasiveness which causes thoracic deformity and pain. Auricular and nasal septal cartilages have a limitation in available amount. Even if a substantial amount of tissue is harvested, there is a risk of deformity of the auricle or the nose.

Regenerative medicine is a technique by which tissues or organs are regenerated by using cells, scaffolds, growth factors, or the combinations. Because cartilage has a poor ability to regenerate by itself, the regenerative medicine for cartilage has been applied to clinical practices. Our group has estab-
lished an implant-type regenerative cartilage for nasal deformity caused by a cleft lip and palate (8). To fabricate the implant, chondrocytes are isolated by enzymatic treatment of the autologous auricular cartilage, and administered to a scaffold made of poly-L-lactic acid which adds a 3D structure and mechanical strength. Although the safety and long-term maintenance of the shape were confirmed by clinical research (7), there are several points to be improved. First, chondrocytes dedifferentiate in a monolayer expanding culture and redifferentiate after transplantation, which makes the result of treatment uncertain (22). Secondly, enzymatic digestion of the cartilage may cause damage to the chondrocytes (19). Lastly, a biodegradable scaffold causes an immunoreaction which influences the cartilage formation (26). Although scaffold-free regenerative tissues have been developed (6), those with mechanical properties to withstand the load at the time of transplantation have not yet been realized.

Application of diced costal cartilage was first proposed by Peer in 1943. Peer reported an article on the use of diced cartilage for the treatment of microtia and calvarial depressions (16). The cartilage used had been cleanly diced by cutting. The cartilage was not absorbed but rather fused together as a cohesive mass, with connective tissue filling the interstices between the pieces of cartilage. Lu et al. reported that minced human articular cartilage fragments loaded on a polymer-based scaffold successfully repaired defects in the articular cartilage of SCID mouse (14). They also showed proliferation and outgrowth of the chondrocytes from the cartilage fragments when cultured with or without scaffolds in vitro, while the effectiveness of the cultured fragments for the cartilage regeneration was not examined. Nishiwaki et al. reported the usefulness of cubic microcartilages for cartilage regeneration. Microcartilages prepared using a micro-slicer were applied to Polyglycolic Acid Mesh with basic-FGF-loaded gelatin microsphers (15). These reports suggest that diced cartilage could be a cell source for cartilage regeneration. Although these methods do not need enzymatic digestion or expansion culture that causes dedifferentiation of the chondrocytes, scaffolds are still required for shape retention, which may result in an immunoreaction eventually leading to transplant deformity.

To avoid these shortcomings, we considered that we should attempt to use diced cartilage in order to substitute the cells isolated from enzyme digestion, to induce the fusion of those fragments without any support of scaffolds, and to make cartilage transplants. The purpose of this study was to establish a novel 3D cartilage culture method to induce the fusion of diced cartilage under in vitro conditions, and to make an aggregation that can serve as a transplant to treat tissue defects or deformities. For that, diced cartilage prepared by using a micro-slicer was cultured in various conditions, and we determined culture environment, medium, and period. We also examined the mechanisms of fragment fusion, focusing on the properties of cells that are emigrated from the diced cartilage.

**MATERIALS AND METHODS**

**Preparation of the diced cartilage using a micro-slicer device.** All procedures were approved by the ethics committee of the University of Tokyo Hospital (ethics permission #2573-(2)), and conducted according to Ethical Guidelines for Medical and Health Research Involving Human Subjects (Ministry of Health, Labour and Welfare, Japan). Auricular cartilage tissue was harvested from microtia patients who underwent the operation at Nagata Microtia and Reconstructive Plastic Surgery Clinic. Informed consent was obtained from all subjects. All procedures were approved by the ethics committee of the University of Tokyo Hospital (ethics permission #622). Auricular cartilage tissue was harvested from microtia patients who underwent the operation at Nagata Microtia and Reconstructive Plastic Surgery Clinic, Saitama, Japan. The tissues were processed at the University of Tokyo Hospital. A perichondrium was removed from the cartilage tissue, and the cartilage was stack on to the stage of the micro-slicer (Shibata System Service Co.) by DERMABOND® (Johnson & Johnson) (Fig. 1 a, b). Using the micro-slicer device, the tissues were cut into small cubes of 200 μm (diced cartilage, Fig. 1 c).

**Culture of diced cartilage.** The diced cartilage was cultured in a 6-well ultralow attachment surface plate (CORNING). One thousand pieces of the diced cartilage per well were cultured in 5 mL of culture medium. One of the following culture media was used; Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 Ham (DMEM/F12, Sigma-Aldrich) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (SIGMA A5955) (basal medium: DMEM), basal medium supplemented with 10% fetal bovine serum (FBS, GIBCO#10270-16) (10% FBS medium), or basal medium supplemented with 1 μg/mL insulin-like growth factor-1 (IGF-1, Somazon® 10 mg for Injection Orphan Pacific) (IGF-1 medium), at
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Experiment Committee of the University of Tokyo (#P14-104, #P15-019), and conducted according to the Guidelines for Animal Experiments at the Faculty of Medicine, the University of Tokyo, the Act on Welfare and Management of Animals, Standards Relating to the Care and Keeping and Reducing Pain of Laboratory Animals (Notice of the Ministry of the Environment), and the ARRIVE Guidelines. Aggregates of the diced cartilage cultured according to the conditions described above (8w/−, 5w/3w, 12w/−, 7w/5w), and 1,000 pieces of diced cartilage without culture were subcutaneously implanted into the back of a male nude mouse (BALB/cAJcl-nu/nu, 6 weeks of age, n = 3). The mice were anesthetized with 2% isoflurane (AbbVie) and the skins of the mice were sterilized with 70% ethanol. An incision was made with a scalpel, while the subcutaneous tissues were exfoliated with abrasion scissors. An aggregate of the diced cartilage, or 1,000 fragments of diced cartilage without culture were transplanted using a spatula. The incision was sewed together with two stitches using 5-0 nylon. The transplants were harvested eight weeks after the operation.

Histological evaluation. Each sample was fixed in 4% paraformaldehyde at 4°C overnight and embedded in paraffin. The fixed samples were cut into 4 μm sections by a microtome (Leica, RM2265) and stained with toluidine blue (TB) and hematoxylin and eosin (HE). The area of the cartilage regenerated in vivo was evaluated in the HE sections by ImageJ (downloaded from https://imagej.nih.gov/ij/index.html). Red area is newly-formed cartilage matrix, black area is original diced cartilage and background. Gray area indicates non-cartilaginous tissues. The end-point was a ratio of the newly-formed cartilage matrix or the enlargement ratio. The ratio of

37°C and 5% of CO₂. Two and a half milliliters of the culture media were changed twice a week. To determine the culture period for the aggregate formation, the diced cartilages were cultured for 8 weeks in any of the previously described media. To optimize the culture condition for the cartilage matrix formation, the diced cartilage was cultured in 10% FBS medium for 8 weeks (8w/−) or 12 weeks (12w/−), in 10% FBS medium for 5 weeks followed by a culture in IGF-1 medium for 3 or 7 weeks (5w/3w and 5w/7w, respectively), or in 10% FBS medium for 7 weeks followed by a culture in IGF-1 medium for 5 weeks (7w/5w).

Evaluation of the size for the aggregates of the diced cartilage. The size for the aggregates of the diced cartilage particles was measured using a non-contact optics-type three-dimensional scanner, ATOSIII Triple Scan (Marubeni Information Systems, Tokyo, Japan). Samples (n = 3) were put on the glass and impermeabilized with a titanium oxide spray. Composited 3D images were constructed using the observation data.

Time-lapse microscopy. Time-lapse observations were performed of the diced cartilage cultured for 3 weeks in 10% FBS medium using a BZ-H4XT/Time-lapse Module and BZ-X800 Microscope (KEYENCE, Osaka, Japan) for three days. The microscope was set in the phase difference mode with a ×10 objective lens, and images were taken every 15 min. Aggregates of the diced cartilage were incubated in an environmental control chamber at 37°C and 5% CO₂.

Evaluation of the cartilage regeneration in vivo. All animal experiments were approved by the Animal Experiment Committee of the University of Tokyo (#P14-104, #P15-019), and conducted according to the Guidelines for Animal Experiments at the Faculty of Medicine, the University of Tokyo, the Act on Welfare and Management of Animals, Standards Relating to the Care and Keeping and Reducing Pain of Laboratory Animals (Notice of the Ministry of the Environment), and the ARRIVE Guidelines. Aggregates of the diced cartilage cultured according to the conditions described above (8w/−, 5w/3w, 12w/−, 7w/5w), and 1,000 pieces of diced cartilage without culture were subcutaneously implanted into the back of a male nude mouse (BALB/cAJcl-nu/nu, 6 weeks of age, n = 3). The mice were anesthetized with 2% isoflurane (AbbVie) and the skins of the mice were sterilized with 70% ethanol. An incision was made with a scalpel, while the subcutaneous tissues were exfoliated with abrasion scissors. An aggregate of the diced cartilage, or 1,000 fragments of diced cartilage without culture were transplanted using a spatula. The incision was sewed together with two stitches using 5-0 nylon. The transplants were harvested eight weeks after the operation.
the newly-formed cartilage matrix was a percentage of the cartilaginous matrix that was distinguished from the remnants of original diced cartilage area to the whole regenerated cartilage. The enlargement ratio was a percentage of the whole regenerated cartilage to original diced cartilage (number of the diced cartilage included in the sections × 40,000 μm²).

Periostin, proliferating cell nuclear antigen (PCNA), matrix metalloproteinase 13 (MMP13), TRA1-60 and stage-specific embryonic antigen 3 (SSEA3) were analyzed by immunostaining. Antibodies were used and their concentrations were as follows. The primary antibodies were periostin (ab14041, 1 : 750), PCNA (ab92552, 1 : 500), SSEA3 (bs-3575R, 1 : 100), TRA1-60 (ab16288, 1 : 500) and normal rabbit IgG (ab172730, 1 : 200) for the negative control. The secondary antibodies were goat anti-mouse IgG H&L(HRP) (ab205719) for TRA1-60, and biotinylated anti-rabbit IgG (code 426011) for all the other primary antibodies.

**Biomechanical evaluation.** The dynamics intensity was measured five times per 1 sample using Venus-tron Alpha version 5.4J. (Axiom, Inc., Fukushima, Japan). According to the report by Aoyagi and Yoshida (21), the resonance frequency increases in the case when the body in contact is sufficiently hard, whereas it decreases in the case when the body is soft. Young’s modulus was calculated using this frequency equation. The resonance frequency of the sensor was set to 30 Hz, and the maximum pressure in depth was 0.3 mm.

**Statistical evaluation.** We determined the statistically significant difference using the Student t test between 2 groups. A comparison among three groups was performed by the one-way layout analysis of variance (ANOVA) and Tukey-Kramer test using Mac statistical analysis Ver.3.0 (Esumi).

### RESULTS

**Requirements for aggregation of diced cartilage**

Diced cartilage prepared using the micro-slicer device was cultured to determine the culture conditions for aggregation. The diced cartilage three-dimensionally aggregated in 5–6 weeks in the ultralow attachment plate with the 10% FBS medium (Fig. 2a indicated by arrows). A capsule (indicated by red arrow in Fig. 3) was formed around the particle of diced cartilage to assemble multiple particles into an aggregate. These phenomena did not occur in those with DMEM and the IGF-1 medium.

![Fusion of the diced cartilage in vitro](image_url)

Fig. 2  Fusion of the diced cartilage *in vitro*. Diced cartilages were cultured onto ultralow attachment surface plate in 10% FBS, DMEM or IGF-1. a. Aggregation up to 8 weeks of culture was recorded by a digital camera. When diced cartilages were cultured with 10% FBS medium, most of the samples aggregated within 6 weeks (indicated by arrows). b. Diced cartilages were cultured in 10% FBS medium (n = 30, each), DMEM (n = 4, each), and IGF-1 medium (n = 4, each). The graph shows the aggregation process. When the number of diced cartilages became less than 30 per well, it was counted as a well with aggregate. The photograph shows a representative example, and no change was found eight weeks later.
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Culture period for aggregation

Diced cartilages were cultured in ultralow attachment plate with 10% FBS medium, DMEM, and IGF-1 medium to determine the timing of the aggregation. When cultured with 10% FBS medium, most of the samples aggregated within 6 weeks. Only few samples did not aggregate during the observation period. Any aggregation did not occur in the DMEM or IGF-1 medium (Fig. 2b).

Culture conditions for cartilage matrix formation

To induce cartilage matrix formation, 10% FBS medium was changed to IGF-1 medium during the culture period. When comparing the group cultured for 5 weeks with 10% FBS medium and for another 3 weeks with IGF-1 (Fig. 4a, 5w/3w) to the group cultured for 8 weeks with 10% FBS medium (Fig. 4a, 8w/−), there was no apparent difference in the HE and TB staining. The matrix area of the diced cartilage was metachromatic in the TB staining, although the interstitial area among original diced cartilage was almost negatively stained for TB in both groups. On the other hand, metachromatic regions in the TB staining were observed in the interstitial areas of the 7w/5w groups, while not in the 12w/− groups (Fig. 4a).

The volumes of the aggregates of 5w/7w and 12w/− groups were measured with the ATOS capsules. 3D reconstructed data indicated the continuity of diced cartilage (Fig. 4b). The volumes of the 5w/7w samples were 18.53 mm$^3$ ± 4.064 mm$^3$, while those of the 12w/− samples were 15.69 mm$^3$ ±...
Fig. 4  Induction of matrix production by diced cartilage. a. The diced cartilage was cultured in 10% FBS medium for 8 weeks (8w/−) or 12 weeks (12w/−), in 10% FBS medium for 5 weeks followed by a culture in IGF-1 medium for 3 weeks (5w/3w), or in 10% FBS medium for 7 weeks followed by a culture in IGF-1 medium for 5 weeks (7w/5w). Metachromatic regions in the TB staining were observed in the interstitial areas of the 7w/5w groups, while not in other groups (Fig. 4a). All figures were adjusted to facilitated visibility.
Cell movement during aggregation

Time-lapse imaging was performed for the sample cultured with the 10% FBS medium for 3 weeks. Cells floating in the medium accumulated between the diced cartilages (Supplementary Fig. 1, the area surrounded by red line). These cells seemed to emerge from the diced cartilage, adhered to each other, and formed a stroma.

In vivo cartilage regeneration of the aggregates

When diced cartilages were transplanted immediately after the preparation, there were few areas of metachromasia.

Partial chondrogenesis was observed in the 8w/− group. Change of the medium from 10% FBS medium to IGF-1 medium at 5 weeks (5w/3w) did not affect chondrogenesis apparently.

On the other hand, a substantial amount of the cartilage matrix was produced in the 7w/5w groups in vivo. Furthermore, in the 5w/3w group the boundaries between the original diced cartilage and the newly-formed cartilage matrix were visible (Fig. 5 surrounded by dotted lines). On the other hand, in the 7w/5w group, the boundary was hardly seen, suggesting good integration of both tissues, and a lump of cartilage was regenerated. No new cartilage matrix was formed between the diced cartilages at 12w/− (Fig. 5).

To evaluate the chondrogenesis quantitatively, histological sections were analyzed using ImageJ. In the analysis, the tissue was divided to 3 areas: the newly-formed cartilage matrix (indicated as red area), original diced cartilage (indicated as black area), and non-cartilaginous tissue (indicated as gray area) (Fig. 6a). As a result of quantitative analysis of each area, there was a significant difference in the ratio of the newly-formed cartilage matrix between 7w/5w and 12w/− (P = 0.000), and between 7w/5w and the direct transplant (P = 0.000) (Fig. 6a, b). There was no significant difference between 12w/− and the direct transplant (P = 0.296). Again, there was a significant difference in the ratio of the enlargement between 7w/5w and 12w/− (P = 0.024), and between 7w/5w and the direct transplant (P = 0.010). There was no significant difference between 12w/− and the direct transplant (P = 0.738) (Fig. 6b).

The dynamics intensity was significantly different among all the groups; 7w/5w and 12w/− (P = 0.000), 7w/5w and the direct transplant (P = 0.000), and 12w/− and the direct transplant (P = 0.000) (Fig. 6c).

Characteristics of interstitial cells

Expressions of periostin, PCNA, and MMP-13 in
Fig. 5 Histological finding in vivo. Transplants were harvested eight weeks after the operation, and were stained with TB and HE. A substantial amount of the cartilage matrix was produced in the 7w/5w. No new cartilage matrix was observed between the diced cartilages at 12w/−. In the 5w/3w group the boundaries between the original diced cartilage and the newly-formed cartilage matrix were visible (surrounded by dotted lines), while it was hardly seen in the 7w/5w group. All figures were adjusted to facilitate visibility. Scale bar: 500 μm (×4), 100 μm (×20).
Fig. 6  Quantification of regenerative tissue in vivo. a. Area of cartilage newly formed in vivo (red part) was evaluated in HE sections with Image J. Red area is newly-formed cartilage matrix, black area is original diced cartilage and background. Gray area indicates non-cartilaginous tissues. b. The ratio of newly-formed cartilage matrix: percentage of newly-formed cartilage matrix (red part) area to the whole regenerated cartilage. The enlargement ratio: percentage of the whole regenerated cartilage to original diced cartilage (number of the diced cartilage included in the sections × 40,000 μm²). c. The dynamics intensity was measured five times per 1 sample using Venustron Alpha version 5.4J. (n = 3 measured 5 times).*: P < 0.05, **: P < 0.01
the cultured diced cartilage were evaluated by immunostaining. In the 7w/5w group, both periostin and PCNA were positive in the cells present in the interstitial areas among diced cartilage. In the 7w/− group, periostin was negative and there were only few positive cells in the interstitial areas, while MMP-13 was positive in the cells near the edge of the diced cartilage (Fig. 7 MMP13, arrows). All were negative in the diced cartilage immediately after preparation (data not shown). To evaluate whether the interstitial cells have characteristics of stemness, immunostaining by TRA1-60 and SSEA3 was performed. The iPS cells, 7w/5w group and 12w/− group were positive for both markers (Fig. 8). On the other hand, both chondrocytes derived from the native cartilage and the diced cartilage explant cultures on adhesive dishes were negative for both TRA1-60 and SSEA3 (data not shown).

DISCUSSION

When cartilage is organ-cultured, cells migrate from the cartilage lacuna (3, 18). In this study, the optimal culture conditions for aggregation of the diced cartilage were determined. As a result, only diced cartilages cultured with 10% FBS medium and the ultralow attachment plate aggregated. This result agreed with that of a previous report which indicated that large aggregates could be obtained by culturing chondrocytes with 10% FBS (4). On the other hand, no aggregation occurred with the DMEM or IGF-1 medium. FBS contains various kind of factors, and some of these factors may have promoted aggregation. Factors which induce aggregation of the diced cartilages are to be determined.

Interstitial cell proliferation and cartilage matrix formation were further analyzed with the following protocol. IGF-1 is reported to promote proliferation of the chondrocyte (2), cartilage formation in the scaffold-free three-dimensional culture (20) and cartilage differentiation of mesenchymal stem cells (13). On the other hand, culturing with 10% FBS after aggregation was not enough to induce stromal formation. In addition, a 2-week difference in timing of the change in culture media from 10% FBS medium to IGF-1 medium significantly affected the cartilage formation both in vitro and in vivo. One possible explanation for this difference is that cells after the 7w culture will be in a more appropriate state for induction of chondrogenesis by IGF-1, which should be confirmed by comparison between cells after 5 and 7 weeks of culture in the 10% FBS medium.

It was unclear how the capsule and interstitial areas observed in the tissue sections of the aggregates were formed in vitro (Fig. 4). We observed cell movement during aggregation by time-lapse imaging (Supplementary Fig. 1), suggesting that cells floating in the medium accumulated between the diced cartilage to form an interstitial area. It suggested that the cells that probably emerged from the diced cartilage formed a capsule and aggregated, as shown by the report by Lu et al. (16), although we could not catch the moment when the cells run out by the diced cartilage.

Chondrogenesis after transplantation to mice was also best in the samples cultured for 7 weeks in 10% FBS, followed by the culture in the IGF-1 medium for 5 weeks in vitro (7w/5w), reflecting the chondrogenesis in vitro (Figs. 5, 6). This result agreed with that of a previous study, in which the differentiation medium showed a better ability to induce chondrogenesis in vivo compared to the proliferation medium (13).

Matrix degradation is usually involved in the migration of cells from the matrix (23). Catabolic enzyme MMP-13 is known to be expressed in chondrocytes under certain circumstances, such as arthritis (1). In some reports, IGF-1 is shown to decrease the expression of MMP-13 (10, 27) and protect cartilage (25) by maintaining chondroitin sulfate-rich proteoglycan, whereas other reports indicated that it promotes the MMP13 expression (24). In accordance with some previous reports, positive cells increased after differentiation induction with IGF-1 (7w/5w), while MMP13 immunostaining was positive only in a few cells in 10% FBS 7w (7w/−). Because cell migration occurred during aggregate formation (up to 7w), other proteolytic enzymes would be involved in the substrate degradation.

Periostin is known to contribute not only to the maintenance of the morphology of regenerated tissues by promoting the 3D structure of collagen tissues, but also chondrogenesis (11). In vitro, 7w/5w stromal cells had many periostin-positive cells, and when this aggregate was transplanted into mice, good substrate production was observed, suggesting that periostin positively affected chondrogenesis (Fig. 7).

TRA1-60 and SSEA3 are known as stem cell markers (9, 17). In this study, we used iPSCs as the positive control of evaluation of stemness. TRA1-60 and SSEA3 were positive when suspended in the non-adherent plates (12w/−, 7w/5w) and iPSC cells (Fig. 8). In vivo, to examine that the interstitial cells are derived from diced cartilage or invaded
Fig. 7 Evaluation of interstitial cells. Periostin, proliferating cell nuclear antigen (PCNA), and matrix metalloproteinase 13 (MMP13), were analyzed by immunostaining. In the 7w/5w group, both periostin and PCNA were positive in the cells present in the interstitial areas. In the 7w/− group, MMP-13 was positive in the cells near the edge of the diced cartilage (indicated by arrows). Scale bar: 100 μm (×20).
Stemness of interstitial cells. To evaluate whether interstitial cells have stemness, immunostaining of TRA1-60 and SSEA3 was performed. 12w/− group, 7w/5w group and iPS cells were positive for both markers. Scale bar: 50 μm (×40).
from fibroblasts of host, we transplanted samples of 5w/− culture into the back of GFP mouse (our unpublished data) and evaluated by immunohistochemistry of GFP. This suggests that interstitial cells may have acquired properties resembling stem cells by the 3D culture of the aggregates, although this hypothesis needs much more verification.

The cartilage fragment produced a new cartilage matrix without collagenase treatment or monolayer culture, indicating that large regenerative cartilage from a small amount of cartilage was achieved without damaging cells or dedifferentiation. If this new culture technology is established, it will be useful to expand the application not only in the oral surgery field but also in the plastic and orthopedic fields, and a new regenerative medicine technology can be provided.

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Supplementary Fig. 1  Cell movement during aggregation. Using samples after the 10% FBS 3w culture, time lapse was photographed for 3 days. Images were taken of cells migrating around the cartilage fragments. The states immediately after 12 h, 24 h and 36 h later are shown.