



Biological aspects of tissue-engineered cartilage

Kazuto Hoshi¹ · Yuko Fujihara¹ · Takanori Yamawaki¹ · Motohiro Harai¹ · Yukiyo Asawa¹ · Atsuhiko Hikita¹

Accepted: 14 February 2018 / Published online: 6 March 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Cartilage regenerative medicine has been progressed well, and it reaches the stage of clinical application. Among various techniques, tissue engineering, which incorporates elements of materials science, is investigated earnestly, driven by high clinical needs. The cartilage tissue engineering using a poly lactide scaffold has been exploratorily used in the treatment of cleft lip-nose patients, disclosing good clinical results during 3-year observation. However, to increase the reliability of this treatment, not only accumulation of clinical evidence on safety and usefulness of the tissue-engineered products, but also establishment of scientific background on biological mechanisms, are regarded essential. In this paper, we reviewed recent trends of cartilage tissue engineering in clinical practice, summarized experimental findings on cellular and matrix changes during the cartilage regeneration, and discussed the importance of further studies on biological aspects of tissue-engineered cartilage, especially by the histological and the morphological methods.

Keywords Cartilage · Regenerative medicine · Chondrocyte · Tissue engineering · Scaffold

Introduction

Regenerative medicine is expected as an innovative therapy for patients who are hardly cured by conventional reconstructive surgery. In this procedure, a small volume of tissue is taken from a patient or a donor, and the cells isolated from those tissues are cultured *in vitro* for proliferation or differentiation. The cultured cells or the constructs consisting of those cells are then transplanted into the patient. The regenerative medicine has attracted further global attention, since the possibility of tissue engineering was reported in the journal *Science* by Langer and Vacanti from Harvard University (Langer and Vacanti 1993). This type of regenerative medicine is incorporating elements of materials science. It uses cells, stimulating factors (growth factors), and scaffold materials as the three major factors, and proposed the possibility of creating various tissues and organs with their shapes and functions by changing the combinations of those three factors.

According to high expectation for those possibilities, various tissues and organs have become the targets of active researches for the regenerative medicine. Among them, cartilage regenerative medicine has been progressed well, and it reaches the stage of clinical application. Probably, due to poor tissue repair ability in cartilage, the cartilage regenerative medicine has been investigated earnestly, driven by high clinical needs.

Principally, the cartilage widely exists in various parts of body, including nose, ear, trachea, joint, and intervertebral disc. The cartilage plays an important role in maintaining the body shape, daily movement, and exercise. From a histological view, the cartilage is composed of round or oval-shaped chondrocytes, surrounded by large amounts of extracellular matrices (Amizuka et al. 2012). The extracellular matrices in cartilage, which are termed cartilaginous matrices, are rich in type II collagen and proteoglycans. These organic materials form a dense meshwork in the physiological cartilage, containing a large volume of water, yielding sufficient mechanical strength (Graff et al. 2003). Therefore, the regeneration of cartilage in clinical practice nearly equals to regrowing healthy chondrocytes and, to speak of extremes, reforming those cartilaginous matrices.

✉ Kazuto Hoshi
pochi-tyk@umin.net

¹ Department of Sensory and Motor System Medicine, Division of Tissue Engineering, Graduate School of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8655, Japan

Recent trends of cartilage regeneration in clinical practice

As a typical example of cartilage regenerative medicine, autologous chondrocyte implantation (ACI) has been done all over the world. The original ACI method was designed to treat focal defects of articular cartilage caused by trauma or osteochondritis dissecans (Brittberg et al. 1994). A small volume of articular cartilage was harvested from edge of joint, and was digested by collagenase for isolation of chondrocytes. After the cells were cultured for proliferation, those cells in suspension were placed into the cartilage defect, which was then covered by a periosteal patch to prevent leakage (Brittberg et al. 1994). This ACI method was developed commercially by Genzyme in the USA, and the products have been sold as Cartice1™ worldwide. However, this method may have the room for improvement. Some papers reported adverse events of this method, including hypertrophy of the periosteal patches, while systematic review of the original ACI method indicated that there was no significant clinical advantage compared to just drilling on sclerotic bottom of cartilage defects or transplantation of some small cylinders consisting of autologous cartilage and bone, termed mosaic plasty (Ruano-Ravina and Jato Diaz 2006; Wood et al. 2006).

Recently, some biomaterials have been introduced to decrease the invasiveness during harvesting of periosteum that was used as the patch in the original ACI method. Collagen film (Chondro-Gide™) was used instead of periosteal patches (Marlovits et al. 2006). In addition, all-in-one style of regenerative cartilage has been developed, which did not use periosteal patches, but instead made use of a porous material comprised of collagen (Maix™) or hyaluronic acid (Hyaff-11™ or Hyalograft C™) (Marlovits et al. 2006). The clinical research and application of such a matrix-based ACI has been actively done. Future reports on clinical results are expected to provide valuable information to this field.

In addition, in the oral and maxillofacial region, the ACI has been clinically used for filling subcutaneous pockets in nasal dorsum of the patients who had transplanted silicone implants for the cosmetic rhinoplasty, but could not avoid removing them due to infection or their exposure to the skin (Yanaga et al. 2006). Auricular cartilage was biopsied from those patients under local anesthesia, and then, the chondrocytes were isolated and cultured to prepare sufficient numbers of the cells for injection. Cultured autologous auricular chondrocytes were injected into a subcutaneous pocket, where the silicone implants had been inserted, and then were removed due to the local infection or the exposure to skin. This group also performed another treatment, in which this type of regenerative cartilage was

“incubated” in the subcutaneous tissue of the patient’s abdomen, extracted after the incubation, fabricated to the desired shape *ex vivo*, like frame of auricles, and finally re-implanted the fabricated regenerative tissue (Yanaga et al. 2009). Although this method allowed implantation of shaped regenerative tissues to the treatment site, repeated surgeries were required, such as biopsy of donor cartilage, implantation of chondrocytes into the abdominal skin, and reimplantation of regenerative cartilage. In addition, the volume and the shape of regenerative cartilage harvested from the incubation site cannot be strictly managed, and it was, therefore, difficult to determine the final shape of the regenerative cartilage before surgery.

Tissue-engineered cartilage

Thus, it is necessary to create a regenerative cartilage with appropriate strength and three-dimensional shape, both of which have already been possessed before transplantation of the regenerative cartilage. To do so, usage of scaffold materials to reinforce the mechanical strength of regenerative cartilage is regarded essential.

To provide the regenerative tissue with mechanical strength, we examined the use of biodegradable polymer (Tanaka et al. 2010). The biodegradable polymers are defined as organic compounds that slowly undergo hydrolysis *in vivo* and that are ultimately broken down into water and carbon dioxide. Well-known biodegradable polymers include poly-L-lactic acid (PLLA), which has shown good clinical results as a material of plates and screws to fix bones, and poly-lactic acid-co-glycolic acid (PLGA), which are materials used to make absorbable surgical sutures. The PLGA undergoes rapid breakdown after transplantation, and interferes with the maturation of regenerative cartilage, because it induces severe foreign body reaction at the early stage of transplantation. This implied that period was important for the establishment of cartilage regeneration (Asawa et al. 2012). Based on those findings, we chose the PLLA, which undergoes relatively slow biodegradation, to reduce foreign body reaction in the early stage after transplantation (Asawa et al. 2012). Finally, we have established tissue-engineered cartilage with mechanical strength equivalent to that of physiological cartilage tissue, and term this regenerative cartilage “implant type”, because it is not injected, but can be surgically implanted into the body (Hoshi et al. 2013).

Now, we have used this type of tissue-engineered cartilage for secondary correction of cleft lip-nose (Hoshi et al. 2017a, b). Although numerous approaches have been proposed for the treatment of the cleft lip-nose, suitable graft materials cannot be obtained from any part of body or the artificial biomaterials. We primarily assessed the safety of the autologous tissue-engineered cartilage when used in the cleft lip-nose patients as an exploratory first-in-human trial,

and also explored the usefulness of the cartilage. After the acquisition of institutional and governmental permission, we used this implant-type tissue-engineered cartilage for three patients. As results, after 3 years of transplantation, we did not experience any serious adverse events that were related to the tissue-engineered cartilage. Nose shapes improved in all the patients, and more than 2 mm of nose augmentation maintained for 3 year post-surgery, as measured in cephalogram. In one patient, we did a secondary correction of the lip and nasal ala as the patient requested, 1 year and 6 month post-surgery. At that time, a little sample of the transplanted tissue-engineered cartilage could be biopsied in the vicinity of the nasal apex, and was histologically examined. In the HE staining, the cells were surrounded by abundant matrices and formed lacunae, while deep metachromasia in toluidine blue staining showed a substantial accumulation of proteoglycan in the extracellular matrices, indicating the evidence of cartilage regeneration (Fig. 1) (Hoshi et al. 2017a).

Those evidences suggested that the implant-type tissue-engineered cartilage could surely reconstruct the nasal dorsum and apex of cleft lip–noses, although extent of cartilage regeneration was unknown except the case in which the histological samples could be obtained. Given that maturation of cartilage could not be evaluated in many cases, it is important to increase the possibility of successful regeneration of cartilage. Host reactions are induced by transplantation of the construct containing cultured cells and biomaterials such as PLLA scaffold, affecting the cartilage regeneration either positively or negatively. Appropriate regulation of such reactions is indispensable for the success of cartilage

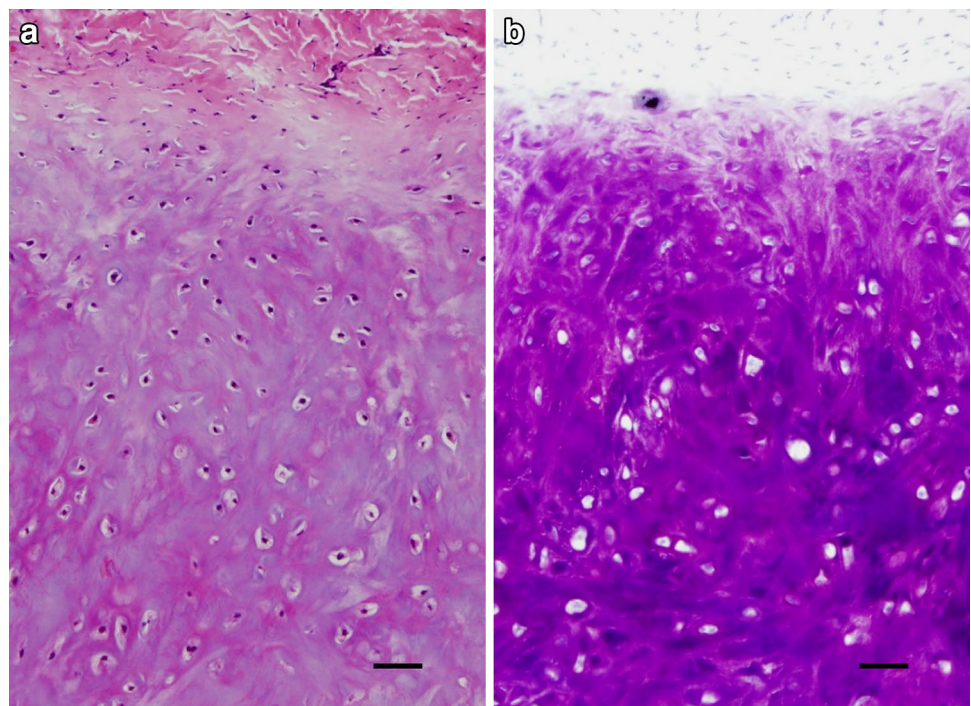
regeneration. To understand the detail of cellular and matrix changes during the cartilage regeneration approaches in the *in vivo* conditions would provide valuable improvement on techniques of cartilage tissue engineering.

Cellular and matrix changes during cartilage regeneration

Several experiments have been reported, regarding the cellular and the matrix changes during the transplantation of tissue-engineered cartilage. Electron microscopy is a tool with which cell morphology and minute structures in and around the cells can be analyzed (Jeong and Hollister 2010; Kwon et al. 2015; Schlegel et al. 2008).

By transmission electron microscopy, experimentally-transplanted chondrocytes *in vivo* were observed (Yamawaki et al. 2018). In this study, human auricular chondrocytes cultured on coverslips were transplanted into the peritoneal cavities of nude mice. On the first week of transplantation, several types of cells were detected, including chondrocytes with some fibroblast-like characteristics such as elongated cell processes, and macrophage-like cells (Fig. 2). Cell-to-cell contact was also found between chondrocytes and macrophage-like cells (Fig. 3). At the second week, the development of rough endoplasmic reticula and the Golgi apparatus were clearly confirmed in chondrocytes, suggesting active production of extracellular matrices (Fig. 3). Macrophage-like cells functioning to phagocytose cells were also noticed (Fig. 3). By the third week, the number of cells other than

Fig. 1 Histological findings of tissue-engineered cartilage in a patient. Hematoxylin and eosin stain (a) and toluidine blue stain (b). Bar = 100 μ m. The images were modified from the Ref (Hoshi et al. 2017a)



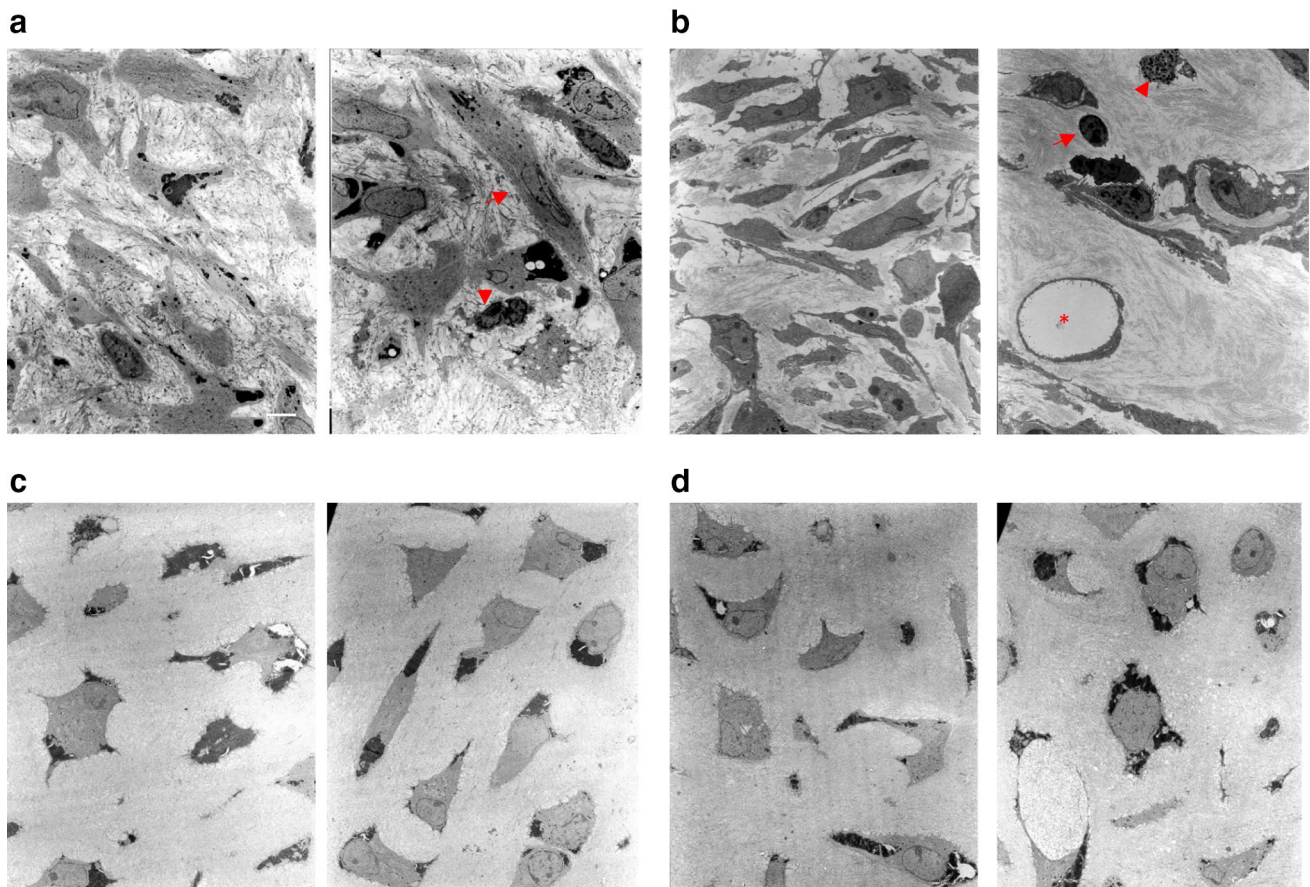


Fig. 2 Transmission electron microscopic images of chondrocytes on coverslip incubated in peritoneal space of nude mice after 1–4 weeks. **a** First week, **b** second weeks, **c** third weeks, **d** fourth weeks. In **a**, the arrowhead and the arrow indicate a macrophage-like cell and a

chondrocyte, respectively. In **b**, the arrowhead, the arrow, and the asterisk indicate a macrophage-like cell, lymphocyte-like cell, and a capillary, respectively. Bar = 5 μ m. The images were modified from Ref (Yamawaki et al. 2018)

chondrocytes had decreased, while the volume of the cytoplasm of the chondrocytes decreased (Fig. 2).

The cell-to-cell contact may suggest the possibility of intercellular signaling. After 3 weeks, such findings were diminished, characterized by extended intercellular distances and no cell-to-cell contact. Macrophage-like cells appeared relatively early in the maturation process of cartilage, suggesting some effects on maturation of cartilage. Recent studies have classified macrophages mainly into two subsets; classically, activated macrophages (M1-type) that basically stimulate immune response, and alternatively activated macrophages (M2-type) that are anti-inflammatory and involved in tissue repair (Mantovani et al. 2002; Mills et al. 2000). Macrophages that initially infiltrated into tissue-engineered cartilage were speculated to be predominantly M1-type, which may suppress the maturation of cartilage. Therefore, for effective cartilage regeneration, it would be desirable to suppress macrophages when M1-type is dominant. Contrarily, it may be hypothesized that macrophages present in and around tissue-engineered cartilage form immune privilege

to assist the maturation of the transplanted chondrocytes (Fujihara et al. 2014).

The immune privilege exists physiologically in certain tissues such as the eye, brain, ovary, testis, and pregnant uterus, where antigen ordinarily does not initiate an immune reaction. The molecular mechanisms maintaining immune privilege in these tissues are considered to be a lack of lymphatic drainage, the presence of a physical barrier and the production of immunosuppressive cytokines/neuropeptides (Green and Ferguson 2001). Classical immune privilege maintains physiological protection of tissues from overly activated reactions of T cells that could be destructive to tissues. On the other hand, immune privilege in tissue-engineered cartilage could be induced by stimulation of the surrounding microenvironment, and mainly acts to inhibit the accumulation of macrophages. The tissue-engineered cartilage constructs containing FasL-dysfunctional chondrocytes (gld) showed more intense infiltration of macrophages than those containing wild-type chondrocytes, suggesting that FasL on

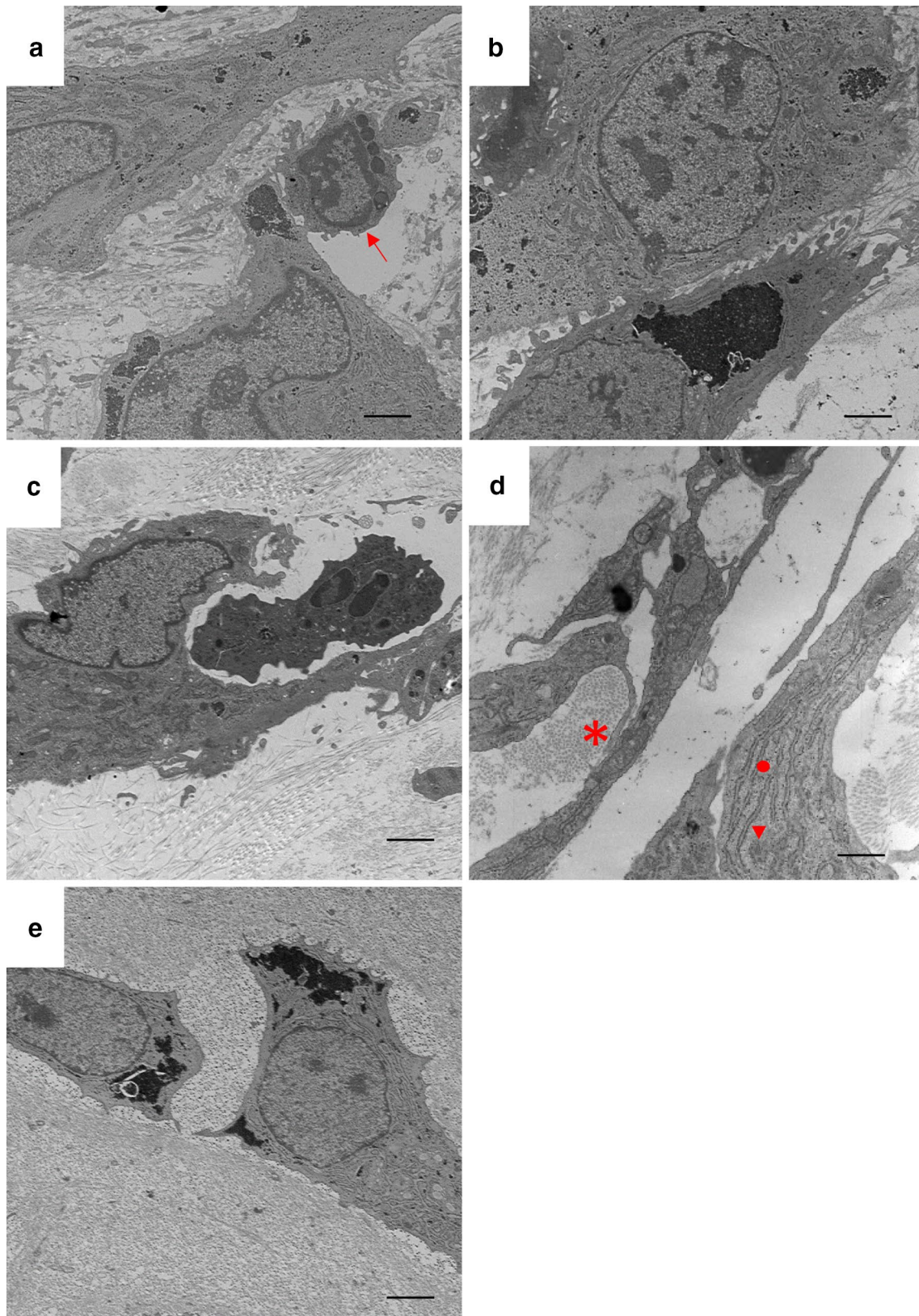
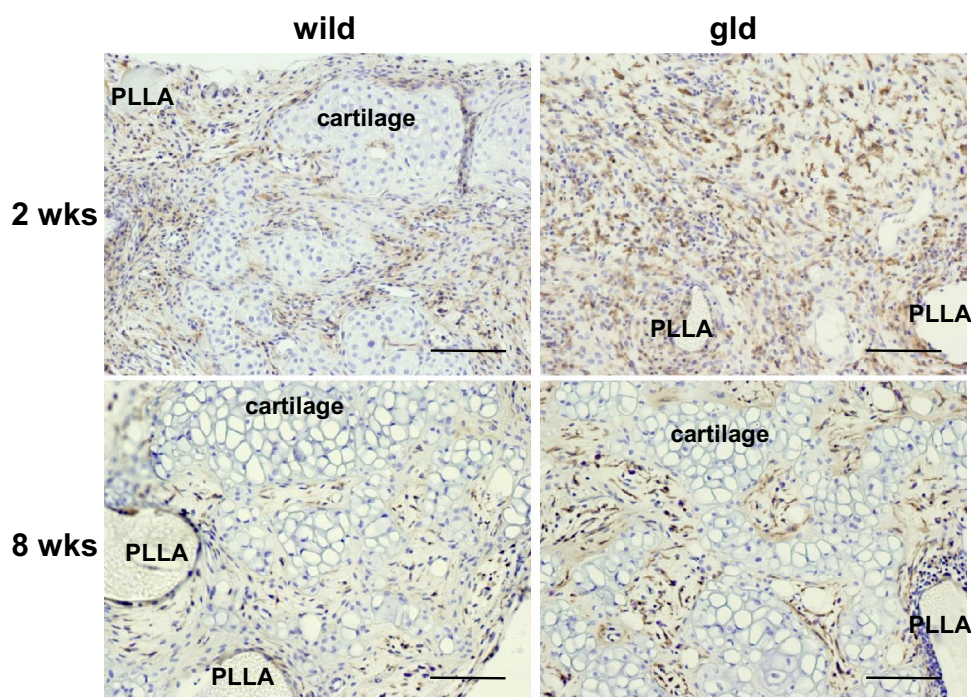


Fig. 3 Transmission electron microscopic images of chondrocytes on coverslip with high magnification. **a, b** First week, **c, d** second weeks, **e** third weeks. In **a**, the arrow indicates a macrophage-like cell. In **d**, the closed circle, the arrowhead, and the asterisk indicate

rER, Golgi apparatus, and dense collagen fibrils, respectively. **a, b, c, e** Bar = 2 μm ; **d** bar = 1 μm . The images were modified from the Ref (Yamawaki et al. 2018)

Fig. 4 Immunohistochemical staining for F4/80 in tissue-engineered cartilage constructs containing wild-type chondrocytes (wild) and FasL-dysfunctional chondrocytes (gld) at 2 and 4 weeks after transplantation. Bar = 100 μ m. The images were modified from the Ref (Fujihara et al. 2014)



chondrocytes could create an immunologically privileged microenvironment against macrophages (Fujihara et al. 2014) (Fig. 4). The wild-type constructs showed increased accumulation of cartilaginous matrix, so immune privilege induced in tissue-engineered cartilage is advantageous to promote cartilage maturation by suppressing the localization of macrophages. Macrophages produce various enzymes, complement factors, and other inflammatory cytokines (Beekman et al. 1998; Loeser 2006), which potentially decrease the accumulation of cartilage matrix, hampering the regeneration of engineered tissues.

Electron microscopic imaging also revealed detailed morphological changes of extracellular matrices (Fig. 3). By the second week, interstitial fibrous matter appeared rough. Dense collagen fibrils were observed in the vicinity of cells, suggesting the production of extracellular matrix formation. On the fourth week, the fibers became fine without directionality, and filled with fine granules. Extracellular matrix formation increased until the fourth week. The role of extracellular matrix in the modulation of immunoreaction has not been studied intensively. It is suggested that cartilage matrix contributes to immune privilege because of its physical characteristics (Fujihara et al. 2018).

Transient vascularization is also important for cartilage regeneration (Takebe et al. 2014). Actually, luminal structures indicating capillary formation were observed around an early stage of cartilaginous tissue in the *in vivo* experiments (Yamawaki et al. 2018) (Fig. 2). These results suggested that vascular endothelial cells may have some influences on early regeneration process and cartilage organization.

Perspectives

To constantly supply safe and stable treatment by cartilage tissue engineering, not only accumulation of clinical evidence on safety and usefulness of the tissue-engineered products, but also establishment of scientific background on biological mechanisms, are considered essential. Further detailed analyses are needed on cellular changes of transplants, and biological stimulations or signals that induce those changes. In addition, the temporal changes of host cells should be evaluated, using flow cytometry or other quantitative devices. It would help the clarification of the association and interaction between transplanted chondrocytes and host cells.

Moreover, we should have profound insights on properties and structures of cartilaginous matrices. Although it has been reported that chondrocytes help the functions of immune privilege, complicated and fine structures of the cartilaginous matrices may also play specific roles in the suppression of the immunoreaction. Even the similarity and the difference between the matrices of tissue-engineered cartilage and native one have remained an issue to be understood, up to date. Further studies on biological aspects of tissue-engineered cartilage are needed, especially by the histological and the morphological methods. By piling up the scientific evidences on the biological mechanisms, we would increase clinical reliability of tissue engineering, and realize to further expand the indication of regenerative medicine.

Acknowledgements This study was supported by Grants-in-Aid for Medical Research and Development Programs Focused on Technology Transfer (AMED A-STEP, D07-05), and Research Project for Practical Applications of Regenerative Medicine (AMED).

References

- Amizuka N, Hasegawa T, Oda K, Luiz de Freitas PH, Hoshi K, Li M, Ozawa H (2012) Histology of epiphyseal cartilage calcification and endochondral ossification *Front Biosci. (Elite Ed)* 4:2085–2100
- Asawa Y, Sakamoto T, Komura M, Watanabe M, Nishizawa S, Takazawa Y, Takato T, Hoshi K (2012) Early stage foreign body reaction against biodegradable polymer scaffolds affects tissue regeneration during the autologous transplantation of tissue-engineered cartilage in the canine model. *Cell Transplant* 21:1431–1442. <https://doi.org/10.3727/096368912X640574>
- Beekman B, Verzijl N, de Roos JA, TeKoppele JM (1998) Matrix degradation by chondrocytes cultured in alginate: IL-1 beta induces proteoglycan degradation and proMMP synthesis but does not result in collagen degradation. *Osteoarthritis Cartilage* 6:330–340. <https://doi.org/10.1053/joca.1998.0132>
- Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N. Engl J Med* 331:889–895. <https://doi.org/10.1056/NEJM199410063311401>
- Fujihara Y, Takato T, Hoshi K (2014) Macrophage-inducing FasL on chondrocytes forms immune privilege in cartilage tissue engineering enhancing in vivo regeneration. *Stem Cells* 32:1208–1219. <https://doi.org/10.1002/stem.1636>
- Fujihara Y, Hikita A, Takato T, Hoshi K (2018) Roles of macrophage migration inhibitory factor in cartilage tissue engineering. *J Cell Physiol* 233:1490–1499. <https://doi.org/10.1002/jcp.26036>
- Graff RD, Kelley SS, Lee GM (2003) Role of pericellular matrix in development of a mechanically functional neocartilage. *Biotechnol Bioeng* 82:457–464. <https://doi.org/10.1002/bit.10593>
- Green DR, Ferguson TA (2001) The role of Fas ligand in immune privilege. *Nat Rev Mol Cell Biol* 2:917–924. <https://doi.org/10.1038/35103104>
- Hoshi K, Fujihara Y, Asawa Y, Nishizawa S, Kanazawa S, Sakamoto T, Watanabe M, Ogasawara T, Saijo H, Mori Y, Takato T (2013) Recent trends of cartilage regenerative medicine and its application to the oral and maxillofacial surgery. *Oral Sci Int* 10:15–19. [https://doi.org/10.1016/S1348-8643\(12\)00049-3](https://doi.org/10.1016/S1348-8643(12)00049-3)
- Hoshi K, Fujihara Y, Saijo H, Asawa Y, Nishizawa S, Kanazawa S, Uto S, Inaki R, Matsuyama M, Sakamoto T, Watanabe M, Sugiyama M, Yonenaga K, Hikita A, Takato T (2017a) Implant-type tissue-engineered cartilage for secondary correction of cleft lip-nose patients: an exploratory first-in-human trial. *J Clin Trials* 7:1000315. <https://doi.org/10.4172/2167-0870.1000315>
- Hoshi K, Fujihara Y, Saijo H, Kurabayashi K, Suenaga H, Asawa Y, Nishizawa S, Kanazawa S, Uto S, Inaki R, Matsuyama M, Sakamoto T, Watanabe M, Sugiyama M, Yonenaga K, Hikita A, Takato T (2017b) Three-dimensional changes of noses after transplantation of implant-type tissue-engineered cartilage for secondary correction of cleft lip-nose patients. *Regen Ther* 7:72–79. <https://doi.org/10.1016/j.reth.2017.09.001>
- Jeong CG, Hollister SJ (2010) A comparison of the influence of material on in vitro cartilage tissue engineering with PCL, PGS, and POC 3D scaffold architecture seeded with chondrocytes. *Biomaterials* 31:4304–4312. <https://doi.org/10.1016/j.biomaterials.2010.01.145>
- Kwon H, Rainbow RS, Sun L, Hui CK, Cairns DM, Preda RC, Kaplan DL, Zeng L (2015) Scaffold structure and fabrication method affect proinflammatory milieu in three-dimensional-cultured chondrocytes. *J Biomed Mater Res A* 103:534–544. <https://doi.org/10.1002/jbm.a.35203>
- Langer R, Vacanti JP (1993) *Tissue Eng Sci* 260:920–926
- Loeser RF (2006) Molecular mechanisms of cartilage destruction: mechanics, inflammatory mediators, and aging collide. *Arthritis Rheum* 54:1357–1360. <https://doi.org/10.1002/art.21813>
- Mantovani A, Sozzani S, Locati M, Allavena P, Sica A (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 23:549–555
- Marlovits S, Zeller P, Singer P, Resinger C, Vecsei V (2006) Cartilage repair: generations of autologous chondrocyte transplantation. *Eur J Radiol* 57:24–31. <https://doi.org/10.1016/j.ejrad.2005.08.009>
- Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM (2000) M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* 164:6166–6173
- Ruano-Ravina A, Jato Diaz M (2006) Autologous chondrocyte implantation: a systematic review. *Osteoarthritis Cartilage* 14:47–51. <https://doi.org/10.1016/j.joca.2005.07.017>
- Schlegel W, Nurnberger S, Hombauer M, Albrecht C, Vecsei V, Marlovits S (2008) Scaffold-dependent differentiation of human articular chondrocytes. *Int J Mol Med* 22:691–699
- Takebe T, Kobayashi S, Suzuki H, Mizuno M, Chang YM, Yoshizawa E, Kimura M, Hori A, Asano J, Maegawa J, Taniguchi H (2014) Transient vascularization of transplanted human adult-derived progenitors promotes self-organizing cartilage. *J Clin Invest* 124:4325–4334. <https://doi.org/10.1172/JCI76443>
- Tanaka Y, Yamaoka H, Nishizawa S, Nagata S, Ogasawara T, Asawa Y, Fujihara Y, Takato T, Hoshi K (2010) The optimization of porous polymeric scaffolds for chondrocyte/atelocollagen based tissue-engineered cartilage. *Biomaterials* 31:4506–4516. <https://doi.org/10.1016/j.biomaterials.2010.02.028>
- Wood JJ, Malek MA, Frassica FJ, Polder JA, Mohan AK, Bloom ET, Braun MM, Cote TR (2006) Autologous cultured chondrocytes: adverse events reported to the United States Food and Drug Administration. *J Bone Joint Surg Am* 88:503–507. <https://doi.org/10.2106/JBJS.E.00103>
- Yamawaki T, Fujihara Y, Harata M, Takato T, Hikita A, Hoshi K (2018) Electron microscopic observation of human auricular chondrocytes transplanted into peritoneal cavity of nude mice for cartilage regeneration. *Regen Ther* 8:1–8. <https://doi.org/10.1016/j.reth.2017.11.002>
- Yanaga H, Yanaga K, Imai K, Koga M, Soejima C, Ohmori K (2006) Clinical application of cultured autologous human auricular chondrocytes with autologous serum for craniofacial or nasal augmentation and repair. *Plast Reconstr Surg* 117:2019–2030. <https://doi.org/10.1097/01.prs.0000210662.12267.de> (**discussion 2031–2012**)
- Yanaga H, Imai K, Fujimoto T, Yanaga K (2009) Generating ears from cultured autologous auricular chondrocytes by using two-stage implantation in treatment of microtia. *Plast Reconstr Surg* 124:817–825. <https://doi.org/10.1097/PRS.0b013e3181b17c0e>