Recent progress of animal transplantation studies in treating articular cartilage damage using pluripotent stem cells

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Abstract

Focal articular cartilage damage can eventually lead to the onset of osteoarthritis with degradation around healthy articular cartilage. Currently, there are no drugs available that effectively repair articular cartilage damage. Several surgical techniques exist and are expected to prevent progression to osteoarthritis, but they do not offer a long-term clinical solution. Recently, regenerative medicine approaches using human pluripotent stem cells (PSCs) have gained attention as new cell sources for therapeutic products. To translate PSCs to clinical application, appropriate cultures that produce large amounts of chondrocytes and hyaline cartilage. So too are assays for the safety and efficacy of the cellular materials in preclinical studies including animal transplantation models.

To confirm safety and efficacy, transplantation into the subcutaneous space and articular cartilage defects have been observed in animal models. All but one study we reviewed that transplanted PSC-derived cellular products into articular cartilage defects found safe and effective recovery. However, for most of those studies, the quality of the PSCs was not verified, and the evaluations were done with small animals over short observation periods. Large animals and longer observations times are preferred.

Cartilage is highly organized connective tissue consisting of chondrocytes and a cartilaginous extracellular matrix (ECM). Chondrocytes produce the components of the ECM, including collagen, proteoglycan and hyaluronic acid, and also maintain the ECM. On the other hand, the ECM helps sustain the chondrocytic property of chondrocytes. Thus, a mutually dependent relationship maintains cartilage function and homeostasis, and damage to either chondrocytes or the ECM affects the other.

There are two types of cartilage, growth plate cartilage and articular cartilage. Growth plate cartilage is where bones elongate in infants, juveniles and adolescents. Articular cartilage is permanent cartilage. It is located at the end of bones and composes the joints, providing smooth motion between bones through its lubricated articular surface.

The formation of the skeleton including cartilage and bone is a series of highly coordinated events through chondrogenesis. Progenitor cells accumulate at the presumptive site of the skeleton and generate mesenchyme condensations, initiating the differentiation into early chondrocytes (Olsen et al. 2000). Chondrocytes then extensively proliferate and produce the ECM, resulting in the formation of the cartilage primordia for future bones.

Cartilage primordia has a shape similar with future bone. At the center of the cartilage primordia, chondrocytes undergo hypertrophic differentiation. Vascular invasion then occurs, resulting in the formation of the primary ossification center at the center and epiphyseal cartilage at both ends of each skeletal element. This process is called endochondral ossification. The elongation of long bones is the result of continuously replacing cartilage with bone at the junction between epiphyseal cartilage and the primary ossification center (Long & Ornitz. 2013; Lefebvre & Bhattaram. 2010). In parallel, at the stage of primordial cartilage formation, articular cartilage formation begins at the interzones. These regions of undifferentiated progenitors separate developing skeletal elements and are where the joint cavity is formed.

Then, another endochondral bone formation event occurs at the center of the epiphyseal cartilage, producing the secondary ossification center. As the secondary ossification center grows, two types of cartilage are left: growth plate cartilage between the secondary ossification center and the primary ossification center, and articular cartilage at the end of skeletal elements. Articular cartilage permanently remains between the joint cavity and the secondary ossification center (Lefebvre & Bhattaram. 2010; Salva

& Merrill 2017).

Focal articular cartilage damage caused by a traumatic injury can eventually lead to the onset of osteoarthritis with degradation around healthy articular cartilage, resulting in activity-related pain. Currently, there are no drugs available that effectively repair articular cartilage damage. Arthroplasty using joint prosthesis is employed to treat end-stage osteoarthritis. However, arthroplasty is highly invasive and its failure rates increase after one or two decades of after implantation especially in young and middle-aged patients who are relatively active (Keeney et al., 2011). Therefore, surgical interventions that can treat focal damage of articular cartilage to prevent it from progression to osteoarthritis have been expected. Several surgical techniques, such as microfracture, mosaicplasty and autologous chondrocyte implantation, have been performed (Lee et al 2013, Andrade et al 2016, Brittberg et al 1994). Accordingly, these techniques have been proposed to restore normal joint function. However, some patients showed impaired cartilage repair as a long-term outcome, because the regenerated cartilage is fibrocartilage or fibrous tissue, whose ECM have inferior mechanical properties compared with the ECM of hyaline cartilage (Bentley et al 2012, Solheim et al 2018). Thus, there is a clinical need to develop regenerative medicine approaches for articular cartilage damage.

The generation of chondrocytes that can produce high-quality hyaline cartilage ECM reproducibly would remarkably advance the treatment of articular cartilage damage. Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are being considered, because they have the potential to proliferate indefinitely in an undifferentiated state and to differentiate into all cells and tissues of the body including chondrocytes by mimicking embryogenesis. Because ESCs are associated with ethical concerns due to their generation depending on the destruction of embryos, iPSCs are often preferred (Takahashi & Yamanaka 2006; Takahashi et al 2007). Originally, the chondrogenic differentiation of PSCs was done in 2D culture (Oldershaw 2010). However, in order for the PSC-derived chondrocytes to secrete ECM proteins and thus form cartilage tissues, scaffolds, 3D cultures and/or several growth factors (BMP, TGFβ, GDF5) have been adopted (Ko et al. 2014; Yamashita et al. 2015; Umeda et al. 2015; Nguyen et al. 2017; Mahboudi et al. 2018; Ferguson et al. 2018; Rim et al. 2018; Rim et al. 2020). In our case, PSCs were initially differentiated into mesoderm by activating Wnt and Activin signaling, and then chondrocytes were induced with BMP2, TGFβ and GDF5 and subjected to 3D culture. BMPs and GDF5 (also termed CDMP1) expand cartilage and induce chondrocyte

hypertrophy (Tsumaki et al. 1999; Tsumaki et al. 2002; Tsumaki & Yoshikawa. 2005; Horiki et al. 2004; Craft et al. 2015). On the other hand, TGFβ has been largely reported to inhibit the hypertrophy of chondrocytes (Ballock et al. 1993; Yang et al. 2001; Zhang et al. 2004; Mueller & Tuan 2008; Iwai et al. 2008; Craft et al. 2015).

However, to reach the numbers required to repair cartilage defects, appropriate cultures that produce large amounts of chondrocytes and hyaline cartilage are needed. Additionally, it is important to confirm the safety and efficacy of the cellular materials in preclinical tests including animal transplantation models (Fig 1). In this paper, we review these animal transplantation studies for the clinical application of PSC technology in the repair of articular cartilage defects.

Protocols for deriving chondrocytes from human PSCs

The chondrogenic differentiation of PSCs has been classified into four protocol strategies: 1) co-culture with primary chondrocytes, 2) passing the PSCs through an intermediate state of embryoid bodies (EB), 3) passing the PSCs through an intermediate state of mesenchymal stem cell (MSC)-like cells, and 4) differentiating the PSCs through intermediate steps composed of mesendoderm, mesoderm and chondrocyte stages (Oldershaw 2012; Park & Im. 2014). For the fourth strategy, new approaches have been developed for the differentiation. To investigate the conditions that differentiate PSCs toward cells that bear more chondrocytic properties, PSCs in which chondrocyte-specific reporter constructs were integrated have been investigated. The promoter/enhancer sequences of type XI collagen α2 chain gene (*COL11a2*) (Yamashita et al., 2015) or *COL2A1*（Adkar et al. 2018, Dicks et al. 2020) have been employed to direct the reporter expression in a chondrocyte-specific manner. To increase the efficiency of the chondrocytic differentiation, the transfection of non-viral vectors containing TGFβ3 and BMP2 to promote the chondrogenic differentiation of PSCs (Rim et al. 2020) and lithium-containing biomaterial to reduce hypertrophy (Hu et al. 2020) have also been applied.

Online search for animal transplantation models of PSC-derived chondrocytes and cartilage

Castro-Viñuelas et al. (2018) reviewed papers that investigated animal transplantation models of PSC-derived chondrocytes and cartilage and published by 2017. Therefore, in this review, we searched for publications from January 2017 to June 2020 on the PubMed database. As keywords, we searched ((chondrocyte) OR (cartilage)) and (pluripotent). We had 215 hits. We checked all titles and abstracts and found 64 reports were related to

PSCs including original research articles and reviews. Among these, 16 reports were studies of animal transplantation models using human (h)PSCs that were published after the review by Castro-Viñuelas et al. In addition, regarding the transplantation of undifferentiated PSCs into articular cartilage defects, we added 4 reports that used animal PSCs. Overall, we broke the collected reports down into three categories: 1) the subcutaneous transplantation of hPSC-derived chondrocytes or cartilage (Table 1), 2) the transplantation of undifferentiated PSCs into articular cartilage defects (Table 2), and 3) the transplantation of hPSC-derived chondrocytes or cartilage into articular cartilage defects (Table 3).

The purpose of animal transplantation models

Before the clinical application of PSC-derived chondrocytes/cartilage, the efficacy and safety of the cellular products must be assessed preclinically. Efficacy is assessed by evaluating how effectively the articular cartilage damage was healed. One experiment done for this purpose is to create a focal defect in the articular cartilage of a live animal in which the PSC-derived chondrocytes/cartilage is implanted. After a certain period, the animals are sacrificed, and the implanted sites are analyzed histologically. Safety is assessed in terms of toxicity and tumorigenicity. Regarding tumorigenicity, the PSC-derived chondrocytes/cartilage can give rise to two types of tumors: teratomas and malignant tumors. PSCs can form teratomas including the three germ layers when they are transplanted into immunodeficient animals. Thus, teratoma can appear after the implantation if the PSC-derived chondrocytes/cartilage are contaminated with remnants of undifferentiated PSCs. Malignant tumors are caused by cells transforming in two ways. One is during the reprogramming process. The other is during the culture period used to expand the PSCs or differentiate them toward chondrocytes/cartilage.

Subcutaneous transplantation of human PSC-derived chondrocytes or cartilage

We found 11 studies on the subcutaneous transplantation of PSC-derived chondrocytes/cartilage, none of which mentioned teratoma or other types of tumors including malignant tumor formation after the transplantation (Table 1).

Along with safety, transplantation into subcutaneous space provides new information about chondrogenesis. Articular cartilage is permanent, but the growth plate is eventually replaced by bone. Generally, PSC-derived cartilage is replaced by bone through endochondral ossification due to the invasion of blood vessels into the transplanted cartilage from the host subcutaneous space (Yamashita et al. 2015; Umeda et al. 2015).

Craft et al. (2015) observed the function of TGFβ and BMP signaling during chondrogenesis and skeletal formation. They found that cartilage generated by BMP was replaced by bone, but cartilage generated by TGFβ was maintained without calcification after the transplantation. Lee et al. (2018) demonstrated that Forskolin treatment, which increases cAMP levels in cultures, maintains cartilage without calcification after the transplantation. Additionally, we and another group used the character of endochondral ossification after transplantation to confirm drug efficacy in disease-specific iPSC models (Kimura et al. 2018; Hino et al. 2017). Thus, transplantation into subcutaneous space can be used to study skeletal development and drug efficacy.

Transplantation of undifferentiated PSCs into articular cartilage defects

Undifferentiated PSCs have been transplanted into articular cartilage defects in various animal transplantation models including mouse, rat, rabbit, pig and sheep. Wakitani et al. (2003) found 8 teratomas out of 25 transplants formed by 8 weeks when mouse ESCs were directly injected into mouse knee joints. However, when they transplanted mouse ESCs into osteochondral defects 2 mm diameter wide and 2 mm deep in rat, no teratoma formed, and the defects were repaired (Wakitani et al. 2004). Undifferentiated hPSCs embedded in atelocollagen gel were also reported to repair articular cartilage defects without tumors in rat and minipig (Zhu et al. 2016; Uto et al. 2018). Allogenic transplantation of sheep and porcine PSCs too could repair defects without tumors（Pilichi et al 2018; Uto et al. 2018) (Table 2). These results indicated that undifferentiated PSCs could differentiate into chondrocytes with abundant cartilaginous ECM and fill the defects. However, other reports using similar methodologies observed teratoma. Nakajima et al. (2008) reported that rats with knee fixation suffered from teratoma formation after mouse ESC transplantation into osteochondral defects, but those without the fixation were repaired without teratoma. Nakajima et al. discussed in the paper that the difference between two was under cyclic loading condition or not. Cyclic loading may promote the chondrogenic differentiation of ESCs. But, in spite of under the cyclic loading condition, two other groups demonstrated teratomas formation. Uto et al. (2013) showed no tumor formation in mouse osteochondral defects when 10^6 mouse iPSCs were transplanted, but teratoma formation did occur if the number was increased to at least 10^7. Kotaka et al. (2017) showed repaired osteochondral defects in rat after transplanting magnetically-labeled hiPSCs with an external magnetic field, but teratoma formation occurred without the field (Table 2). These results indicated that undifferentiated PSCs can repair osteochondral defects but can also form teratomas if they leak outside the defect and/or are transplanted at large numbers.

Transplantation of PSC-derived chondrocytes or cartilage into articular cartilage defects

The above studies suggest the *in vivo* environment may play a crucial role in the chondrogenesis of undifferentiated PSCs by providing paracrine factors and/or mechanical stimulation. At the same time, they indicate undifferentiated PSCs are tumorigenic if they escape the defect.

In general, the implantation of differentiated PSCs is recommended to treat lesions, because they have a lower risk of teratoma formation and higher chance of recovery. Accordingly, in the case of treating articular cartilage damage, the PSCs are differentiated to chondrocytes and cartilage prior to the implantation. Table 3 lists 16 reports for such experiments.

In order to evaluate the efficacy of the transplantation therapy as a pre-clinical study, long-term observations for the repair of large defects in large animals is desirable. However, for the studies listed in Table 3, most treatments were observed over short periods (4-16 weeks) in small animals such as mouse or rat. Kawata et al. (2019) made observations for 24 weeks, but of immunodeficient mice. Immunodeficient animals are only available for mice and rats and not for large animals. Although small animals are cheap and can be observed for a long time, evaluating the efficacy of the transplant is difficult, because the area of the articular cartilage defect is much smaller than that of humans (Lo Monaco, et al. 2018). We transplanted human PSC-derived cartilage into defects 6 mm diameter wide and 2 mm deep in minipigs but observed them only for 4 weeks (Yamashita et al, 2015), because immune suppressors were required, making longer observation periods difficult. Although cartilage does not elicit an immune rejection in allogeneic implantations, immune rejection is a concern for xenogeneic implantations. Considering this situation, it is difficult to observe long-term the repair of large cartilage defects in large animals currently. The development of more sophisticated immunosuppressive protocols for large animals or of immunodeficient large animals will make longer observation periods possible in the future (Itoh et al, 2019; Hara et al, 2018).

As for safety, observations must confirm no signs of teratoma or tumor formation after the transplantation. Among hiPSC studies, the transplanted chondrocytes or cartilage resided in and repaired the defects without tumor formation in all but one of the studies in Table 3 (Saito et al. 2015). There are likely two reasons for the exception, as

explained below.

Quality control is necessary to assess the maximum number of abnormal cells that can safely contaminate a transplant. To measure this number, we used HeLa cells as model transformed cells and transplanted them into nude rat osteochondral defects 1 mm diameter wide and 0.5 mm deep. Tumors were not observed when 10^4 or fewer HeLa cells were transplanted, but they did appear if 10^5 HeLa cells were transplanted (Takei et al. 2020). These results indicate that the implantation of up to 10^4 transformed cells in the knee joint does not risk tumor formation if assuming the transformed cells are equivalent to HeLa cells and rat knee is equivalent to human knee.

Another factor that could lead to tumorigenicity is the differentiation potential of the iPSC line. Yasuda et al. (2018) examined the *in vivo* tumorigenicity of 10 hiPSC lines established from different primary cells and reprogramming methods. When transplanted into NOG mice, the iPSCs showed remarkable variation in tumor incidence, formation latency, and volume. These variations could be explained by chromosomal abnormalities in the lines, the inclusion of c-Myc as one of the reprogramming factors, and the use of a retrovirus for the transfection. The primary cell type reprogrammed did not appear to be a factor. In the Saito paper that reported tumors, the iPSC lines were reprogrammed with retrovirus vectors that included c-Myc (Saito et al. 2015). The reactivation of the retrovirus or c-Myc might have been the cause of the tumor formation.

Another concern regarding the safety of iPSCs is genetic instabilities including gene mutations that can occur during the reprogramming process or the long culture period for maintenance or differentiation. To assess these possibilities, we subjected chondrocytes isolated from cartilage and generated from a clinical-grade iPSC line to expansion culture for a prolonged period. Notably, we found that the chondrocytes reached cell senescence, suggesting the cartilage was not contaminated with transformed cells (Takei et al. 2020).

However, the observation period (4-16 weeks) in the animal transplantation model was insufficient for evaluating safety. As a preclinical study to confirm safety for Parkinson's disease, Doi et al. (2020) injected hiPSC-derived dopaminergic progenitor cells into the striatum, which is the same target used in the clinical trial, of NOG mice and observed the cells for as long as possible, i.e., the life-long period. The researchers grafted the cells into 80 (male: 40, female: 40) and 50 mice (male: 25, female 25) as the cell product-transplanted group and control group, respectively. They then observed the

animals until the number of surviving mice was 30 of either gender in the transplant group or 20 of either gender in the control. At 52 weeks after the transplantation, the number of male mice in the control group reached the end-point. To evaluate safety pre-clinically, it is important to observe the life-long period following the chondrocyte/cartilage transplantation.

Conclusion and future perspective

In this review, we report the recent progress of chondrocytic differentiation from PSCs and related animal transplantation studies. To confirm the safety and efficacy of the differentiated PSCs, transplantation into the subcutaneous space and articular cartilage defects were observed in animal models. Although transplantation into subcutaneous space has been used to confirm safety, the field is moving away from this assay. The reason is that transplantation into the articular cartilage defect is more suitable for the evaluating safety and efficacy and is the same as the clinical transplantation site. Transplantation into subcutaneous space is still appreciated for the study of endochondral ossification or drug testing, however.

All but one study we reviewed that conducted transplantation into articular cartilage defects found the PSC-derived chondrocytes or cartilage resided in the defect and were safe and effective. However, for most of those studies, the quality of the PSCs was unknown, and the evaluation was done with small animals over short observation periods. Recently, hiPSCs derived from human leukocyte antigen (HLA)-homozygous healthy volunteers or hiPSCs that retained HLA-C after HLA-class II knockout by CRISPR-Cas9 (Xu et al. 2019) were established. Further, these cells were made under quality control for clinical application, potentially minimizing cell and tissue transplantation immune rejection. Also, two new options could become available for the long-term observation of animal transplantation studies to treat cartilage defect. One is a humanized animal that carries functioning human genes, cells and/or organs (Wunderlich et al. 2015; Deuse et al. 2019). This animal allows for allogenic hPSC grafts. The other is allotransplantation using PSCs derived from large animals (Morizane et al. 2017, Sugita et al. 2017, Kashiyama et al. 2019), as seen with the transplantation of monkey iPSC-derived cells into monkey heart (Shiba et al. 2016). To realize the clinical application of hPSC-derived chondrocytes or cartilage, quality-control PSCs and animal transplantation models are recommended.

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Reference

Adkar, S.S., Wu, C.L., Willard, V.P., Dicks, A., Ettyreddy, A., Steward, N., … Guilak F. (2019). Step-Wise Chondrogenesis of Human Induced Pluripotent Stem Cells and Purification Via a Reporter Allele Generated by CRISPR-Cas9 Genome Editing. Stem Cells, 37, 65-76.

Andrade, R., Vasta, S., Pereira, R. Pereira, H., Papalia, R., Karahan, M., … Espregueira-Mendes, J. (2016). Knee donor-site morbidity after mosaicplasty-a systematic review. J. Exp. Orthop, 3, 31.

Ballock, R.T., Heydemann, A., Wakefield, L.M., Flanders, K.C., Roberts, A.B. & Sporn, M.B. (1993). TGF-β1 prevents hypertrophy of epiphyseal chondrocytes: Regulation of gene expression for cartilage matrix proteins and metalloproteases. Dev. Biol, 158, 414 - 429.

Bentley, G., Biant, L.C., Vijayan, S., Macmull, S., Skinner, J.A. & Carrington, R.W. (2012). Minimum ten-year results of a prospective randomised study of autologous chondrocyte implantation versus mosaicplasty for symptomatic articular cartilage lesions of the knee. J Bone Joint Surg Br, 94, 504-509.

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.

Castro-Viñuelas, R., Sanjurjo-Rodríguez, C., Piñeiro-Ramil, M., Hermida-Gómez, T., Fuentes-Boquete, I.M., de Toro-Santos, F.J, … Díaz-Prado SM. (2018). Induced pluripotent stem cells for cartilage repair: current status and future perspectives. Eur Cell Mater, 36, 96-109.

Cheng, A., Kapacee, Z., Peng, J., Lu, S., Lucas, R.J., Hardingham, T.E. & Kimber, S.J. (2014). Cartilage repair using human embryonic stem cell-derived chondroprogenitors. Stem Cells Transl Med, 3, 1287-1294.

Chijimatsu, R., Ikeya, M., Yasui, Y., Ikeda, Y., Ebina, K., Moriguchi, Y., … Norimasa N. (2017). Characterization of Mesenchymal Stem Cell-Like Cells Derived From Human iPSCs via Neural Crest Development and Their Application for Osteochondral Repair. Stem Cells Int, 2017, 1960965.

Craft, A.M., Rockel, J.S., Nartiss, Y., Kandel, R.A., Alman, B.A. & Keller, G.M. (2015). Generation of articular chondrocytes from human pluripotent stem cells. Nat Biotechnol, 33, 638-45.

Deuse, T., Hu, X., Gravina, A., Wang, D., Tediashvili, G., De, C., … Schrepfer, S. (2019). Hypoimmunogenic derivatives of induced pluripotent stem cells evade immune rejection in fully immunocompetent allogeneic recipients. Nat Biotechnol, 37, 252-

258.

Dicks, A., Wu, C.L., Steward, N., Adkar, S.S., Gersbach, C.A. & Guilak, F. (2020). Prospective isolation of chondroprogenitors from human iPSCs based on cell surface markers identified using a CRISPR-Cas9-generated reporter. Stem Cell Res Ther, 11, 66.

Doi, D., Magotani, H., Kikuchi, T., Ikeda, M., Hiramatsu, S., Yoshida, K., … Takahashi, J. (2020). Pre-clinical study of induced pluripotent stem cell-derived dopaminergic progenitor cells for Parkinson's disease. Nat. Commun, 11, 3369.

Ferguson, G.B., Van Handel, B., Bay. M., Fiziev, P., Org, T., Lee, S., … Evseenko, D. (2018). Mapping molecular landmarks of human skeletal ontogeny and pluripotent stem cell-derived articular chondrocytes. Nat. Commun. 9, 3634.

Gardner, O.F., Juneja, S.C., Whetstone, H., Nartiss, Y., Sieker, J.T., Veillette, C., … Craft AM. (2019). Effective repair of articular cartilage using human pluripotent stem cell-derived tissue. Eur Cell Mater, 38, 215-227.

Hara, H., Shibata, H., Nakano, K., Abe, T., Uosaki, H., … Hanazono, Y. (2018). Production and rearing of germ-free X-SCID pigs. Exp Anim, 67, 139-146.

Hino, K., Horigome, K., Nishio, M., Komura, S., Nagata, S., Zhao, C., … Ikeya, M. (2017). Activin-A enhances mTOR signaling to promote aberrant chondrogenesis in fibrodysplasia ossificans progressiva. J Clin Invest, 127, 3339-3352.

Horiki, M., Imamura, T., Okamoto, M., Hayashi, M., Murai, J., Myoui, A., … Tsumaki, N. (2004). Smad6/Smurf1 overexpression in cartilage delays chondrocyte hypertrophy and causes dwarfism with osteopenia. J. Cell Biol, 165, 433-445.

Hu, Y., Chen, L., Gao, Y., Cheng, P., Yang, L., Wu, C. & Jie, Q. (2020). A lithium-containing biomaterial promotes chondrogenic differentiation of induced pluripotent stem cells with reducing hypertrophy. Stem Cell Res Ther, 11, 77.

Itoh, M., Mukae, Y., Kitsuka, T., Arai, K., Nakamura, A., Uchihashi, K., … Kobayashi E. (2019). Development of an immunodeficient pig model allowing long-term accommodation of artificial human vascular tubes. Nat Commun, 10, 2244.

Iwai, T., Murai, J., Yoshikawa, H. & Tsumaki, N. (2008). Smad7 Inhibits chondrocyte differentiation at multiple steps during endochondral bone formation and down-regulates p38 MAPK pathways. J. Biol. Chem, 283, 27154-27164.

Kashiyama, N., Miyagawa, S., Fukushima, S., Kawamura, T., Kawamura, A., Yoshida, S., … Sawa, Y. (2019). MHC-mismatched Allotransplantation of Induced Pluripotent Stem Cell-derived Cardiomyocyte Sheets to Improve Cardiac Function in a Primate Ischemic Cardiomyopathy Model. Transplantation. 103, 1582-1590.

Kawata, M., Mori, D., Kanke, K., Hojo, H., Ohba, S., Chung, U.I, … Saito T. (2019).

Simple and Robust Differentiation of Human Pluripotent Stem Cells toward Chondrocytes by Two Small-Molecule Compounds. Stem Cell Reports, 13, 530-544.

Keeney, J.A., Eunice, S., Pashos, G., Wright, R.W. & Clohisy, J.C. (2011). What is the evidence for total knee arthroplasty in young patients?: a systematic review of the literature. Clin Orthop Relat Res, 469, 574-583.

Kimura, T., Ozaki, T., Fujita, K., Yamashita, A., Morioka, M., Ozono, K. & Tsumaki, N. (2018). Proposal of patient-specific growth plate cartilage xenograft model for FGFR3 chondrodysplasia. Osteoarthritis Cartilage, 26, 1551-1561.

Ko, J.Y., Kim, K.I., Park, S. & Im, G.I. (2014). In vitro chondrogenesis and in vivo repair of osteochondral defect with human induced pluripotent stem cells. Biomaterials, 35, 3571-3581.

Kotaka, S., Wakitani, S., Shimamoto, A., Kamei, N., Sawa, M., Adachi, N. & Ochi, M. (2017). Magnetic Targeted Delivery of Induced Pluripotent Stem Cells Promotes Articular Cartilage Repair. Stem Cells Int, 2017, 9514719.

Lee, J. J., Lee, S. J., Lee, T. J., Yoon, T. H. & Choi, C. H. (2013). Results of microfracture in the osteoarthritic knee with focal full-thickness articular cartilage defects and concomitant medial meniscal tears. Knee Surgery & Related Research, 25, 71-76.

Lee, J., Taylor, S.E., Smeriglio, P., Lai, J., Maloney, W.J., Yang, F. & Bhutani, N. (2015). Early induction of a prechondrogenic population allows efficient generation of stable chondrocytes from human induced pluripotent stem cells. FASEB J, 29, 3399-3410.

Lee, J.Y., Matthias, N., Pothiawala, A., Ang, B.K., Lee, M., Li, J., … Nakayama, N. (2018). Pre-transplantational Control of the Post-transplantational Fate of Human Pluripotent Stem Cell-Derived Cartilage. Stem Cell Reports, 11, 440-453.

Lefebvre, V. & Bhattaram, P. (2010). Vertebrate skeletogenesis. Curr. Top Dev. Biol, 90, 291-317.

Lo Monaco, M., Merckx, G., Ratajczak, J., Gervois, P., Hilkens, P., Clegg, P., … Lambrichts I. (2018). Stem Cells for Cartilage Repair: Preclinical Studies and Insights in Translational Animal Models and Outcome Measures. Stem Cells Int, 2018, 9079538.

Long, F. & Ornitz, D. M. (2013). Development of the endochondral skeleton. Cold Spring Harb. Perspect. Biol, 5, a008334.

Mahboudi, H., Soleimani, M., Enderami, S.E., Kehtari, M., Hanaee-Ahvaz, H., Ghanbarian, H., … Kazemi, B. (2018). The effect of nanofibre-based polyethersulfone (PES) scaffold on the chondrogenesis of human

induced pluripotent stem cells. Artif Cells Nanomed Biotechnol, 46, 1948-1956.

Morizane, A., Kikuchi, T., Hayashi, T., Mizuma, H., Takara, S., Doi, H., … Takahashi J. (2017). MHC matching improves engraftment of iPSC-derived neurons in non-human primates. Nat Commun, 8, 385.

Mueller, M.B. & Tuan, R.S. (2008). Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. Arthritis Rheum, 58, 1377-1388.

Nakajima, M., Wakitani, S., Harada, Y., Tanigami, A. & Tomita, N. (2008). In vivo mechanical condition plays an important role for appearance of cartilage tissue in ES cell transplanted joint. J Orthop Res, 26, 10e7.

Nejadnik, H., Diecke, S., Lenkov, O.D., Chapelin, F., Doing, J., Tong, X., … Daldrup-Link, H.E. (2015). Improved approach for chondrogenic differentiation of human induced pluripotent stem cells. Stem Cell Rev Rep, 11, 242-253.

Nguyen, D., Hägg, D.A., Forsman, A., Ekholm, J., Nimkingratana, P., Brantsing, C., … Simonsson, S. (2017). Cartilage Tissue Engineering by the 3D Bioprinting of iPS Cells in a Nanocellulose/Alginate Bioink. Sci Rep, 7, 658.

Oldershaw, R. A., Baxter, M. A., Lowe, E. T. Bates, N., Grady, L.M., Soncin, F., … Kimber SJ. (2010). Directed differentiation of human embryonic stem cells toward chondrocytes. Nat. Biotechnol, 28, 1187-1194

Oldershaw, R.A. (2012). Cell sources for the regeneration of articular cartilage: the past, the horizon and the future. Int J Exp Pathol, 93, 389–400.

Olsen, B. R., Reginato, A. M. & Wang, W. (2000). Bone development. Annu. Rev. Cell Dev. Biol. 16, 191-220.

Park, S., & Im, G.I. (2014). Embryonic stem cells and induced pluripotent stem cells for skeletal regeneration. Tissue Eng Part B Rev, 20, 381-91.

Pilichi, S., Rocca, S., Dattena, M., Pool, R.R., Mara, L., Sanna, D., … Passino, E.S. (2018). Sheep embryonic stem-like cells engrafted into sheep femoral condyle osteochondral defects: 4-year follow-up. BMC Vet Res, 14, 213.

Rim, Y.A., Nam, Y., Park, N., Jung, H., Lee, K., Lee, J. & Ju, J.H. (2020). Chondrogenic Differentiation from Induced Pluripotent Stem Cells Using Non-Viral Minicircle Vectors. Cells, 9, 582.

Rim, Y.A., Nam, Y., Park, N., Lee, J., Park, S.H., & Ju, J.H. (2018). Repair potential of nonsurgically delivered induced pluripotent stem cell-derived chondrocytes in a rat osteochondral defect model. J. Tissue Eng. Regen. Med, 12, 1843-1855.

Saito, T., Yano, F., Mori, D., Kawata, M., Hoshi, K., Takato, T., … Tanaka, S. (2015). Hyaline cartilage formation and tumorigenesis of implanted tissues derived from human induced pluripotent stem cells. Biomed Res, 36, 179-186.

Salva, J.E. & Merrill, A.E. (2017). Signaling networks in joint development. Dev Dyn, 246, 262–274.

Shiba, Y., Gomibuchi, T., Seto, T., Wada, Y., Ichimura, H., Tanaka, Y., … Ikeda, U. (2016). Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. Nature., 538, 388-391.

Solheim, E., Hegna, J., Strand, T., Harlem, T. & Inderhaug, E. (2018). Randomized Study of Long-term (15–17 Years) Outcome After Microfracture Versus Mosaicplasty in Knee Articular Cartilage Defects. Am J Sports Med, 46, 826–831.

Sugita, S., Makabe, K., Fujii, S., Iwasaki, Y., Kamao, H., Shiina, T., … Takahashi, M. (2017). Detection of Retinal Pigment Epithelium-Specific Antibody in iPSC-Derived Retinal Pigment Epithelium Transplantation Models. Stem Cell Reports, 9, 1501-1515.

Takahashi, K. & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell, 126, 663-676.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. & Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell, 131, 861-872.

Takei, Y., Morioka, M., Yamashita, A., Kobayashi, T., Shima, N. & Tsumaki, N. (2020). Quality assessment tests for tumorigenicity of human iPS cell-derived cartilage. Sci Rep, 10, 12794.

Tsumaki, N. & Yoshikawa, H. (2005). The role of bone morphogenetic proteins in endochondral bone formation. Cytokine Growth Factor Rev, 16, 279-285.

Tsumaki, N., Nakase, T., Miyaji, T., Kakiuchi, M., Kimura, T., Ochi, T. & Yoshikawa, H. (2002). Bone morphogenetic protein signals are required for cartilage formation and differently regulate joint development during skeletogenesis. J. Bone Miner. Res, 17, 898-906.

Tsumaki, N., Tanaka, K., Arikawa-Hirasawa, E., Nakase, T., Kimura, T., Thomas, J.T., … Yamada, Y. (1999). Role of CDMP-1 in skeletal morphogenesis: promotion of mesenchymal cell recruitment and chondrocyte differentiation. J. Cell Biol, 144, 161-173.

Umeda, K., Oda, H., Yan, Q., Matthias, N., Zhao, J., Davis, B.R. & Nakayama, N. (2015). Long-term expandable SOX9+ chondrogenic ectomesenchymal cells from human pluripotent stem cells. Stem Cell Reports, 4, 712-726.

Uto, S., Nishizawa, S., Hikita, A., Takato, T. & Hoshi, K. (2018). Application of induced pluripotent stem cells for cartilage regeneration in CLAWN miniature pig osteochondral replacement model. Regen Ther, 9, 58-70.

Uto, S., Nishizawa, S., Takasawa, Y., Asawa, Y., Fujihara, Y., Takato, T. & Hoshi, K. (2013). Bone and cartilage repair by transplantation of induced pluripotent stem cells in murine joint defect model. Biomed Res, 34, 281-288.

Wakitani, S., Aoki, H., Harada, Y., Sonobe, M., Morita, Y., Mu, Y., … Tanigami, A. (2004). Embryonic stem cells form articular cartilage, not teratomas, in osteochondral defects of rat joints. Cell Transplant, 13, 331e6.

Wakitani, S., Takaoka, K., Hattori, T., Miyazawa, N., Iwanaga, T., Takeda, S., …Tanigami, A. (2003). Embryonic stem cells injected into the mouse knee joint form teratomas and subsequently destroy the joint. Rheumatology (Oxford), 42, 162-165.

Wang, T., Nimkingratana, P., Smith, C.A, Cheng, A., Hardingham, T.E. & Kimber, S.J. (2019). Enhanced chondrogenesis from human embryonic stem cells. Stem Cell Res, 39, 101497.

Wei, Y., Zeng, W., Wan, R., Wang, J., Zhou, Q., Qiu, S. & Singh, S.R. (2012). Chondrogenic differentiation of induced pluripotent stem cells from osteoarthritic chondrocytes in alginate matrix. Eur Cell Mater, 23, 1-12.

Wunderlich, M., Chou, F.S., Link, K.A., Mizukawa, B., Perry, R.L., Carroll, M., & Mulloy, J.C. (2010). AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. Leukemia, 24, 1785–1788.

Xu, H., Wang, B., Ono, M., Kagita, A., Fujii, K., Sasakawa, N., … Hotta, A. (2019). Targeted Disruption of HLA Genes via CRISPR-Cas9 Generates iPSCs with Enhanced Immune Compatibility. Cell Stem Cell, 24, 566-578.

Xu, X., Shi, D., Liu, Y., Yao, Y., Dai, J., Xu, Z., Chen, D., … Jiang, Q. (2017). *In vivo* repair of full-thickness cartilage defect with human iPSC-derived mesenchymal progenitor cells in a rabbit model. Exp Ther Med, 14, 239-245.

Yamashita, A., Morioka, M., Yahara, Y., Okada, M., Kobayashi, T., Kuriyama, S., …Tsumaki N. (2015). Generation of scaffoldless hyaline cartilaginous tissue from human iPSCs. Stem Cell Reports, 4, 404-418.

Yang, X., Chen, L., Xu, X., Li, C., Huang, C. & Deng, C.X. (2001). TGFβ/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol, 153, 35-46.

Yasuda, S., Kusakawa, S., Kuroda, T., Miura, T., Tano, K., Takada, N., … Sato, Y. (2018). Tumorigenicity-associated characteristics of human iPS cell lines. PLoS One, 13, e0205022.

Zhang, X., Ziran, N., Goater, J.J, Schwarz, E.M., Puzas, J.E., Rosier, R.N., … O'Keefe, R.J. (2004). Primary murine limb bud mesenchymal cells in long-term culture

complete chondrocyte differentiation: TGF-β delays hypertrophy and PGE2 inhibits terminal differentiation. Bone, 34, 809-817.

Zhu, Y., Wang, Y., Zhao, B., Niu, X., Hu, B., Li, Q., … Wang, Y. (2017). Comparison of exosomes secreted by induced pluripotent stem cell-derived mesenchymal stem cells and synovial membrane-derived mesenchymal stem cells for the treatment of osteoarthritis. Stem Cell Res Ther, 8, 64.

Zhu, Y., Wu, X., Liang, Y., Gu, H., Song, K., Zou, X. & Zhou, G. (2016). Repair of cartilage defects in osteoarthritis rats with induced pluripotent stem cell derived chondrocytes. BMC Biotechnol, 16, 78.

Figure legend

Figure 1. Animal transplantation studies of undifferentiated PSCs or PSC-derived chondrocytes/cartilage.

To evaluate safety and efficacy, undifferentiated PSCs or PSC-derived chondrocytes/cartilage were transplanted into the subcutaneous space of mouse and rat or articular cartilage defects of mouse, rat, rabbit, minipig, and sheep. Red spots indicate transplantation sites.

Figure (Yamashita et al)

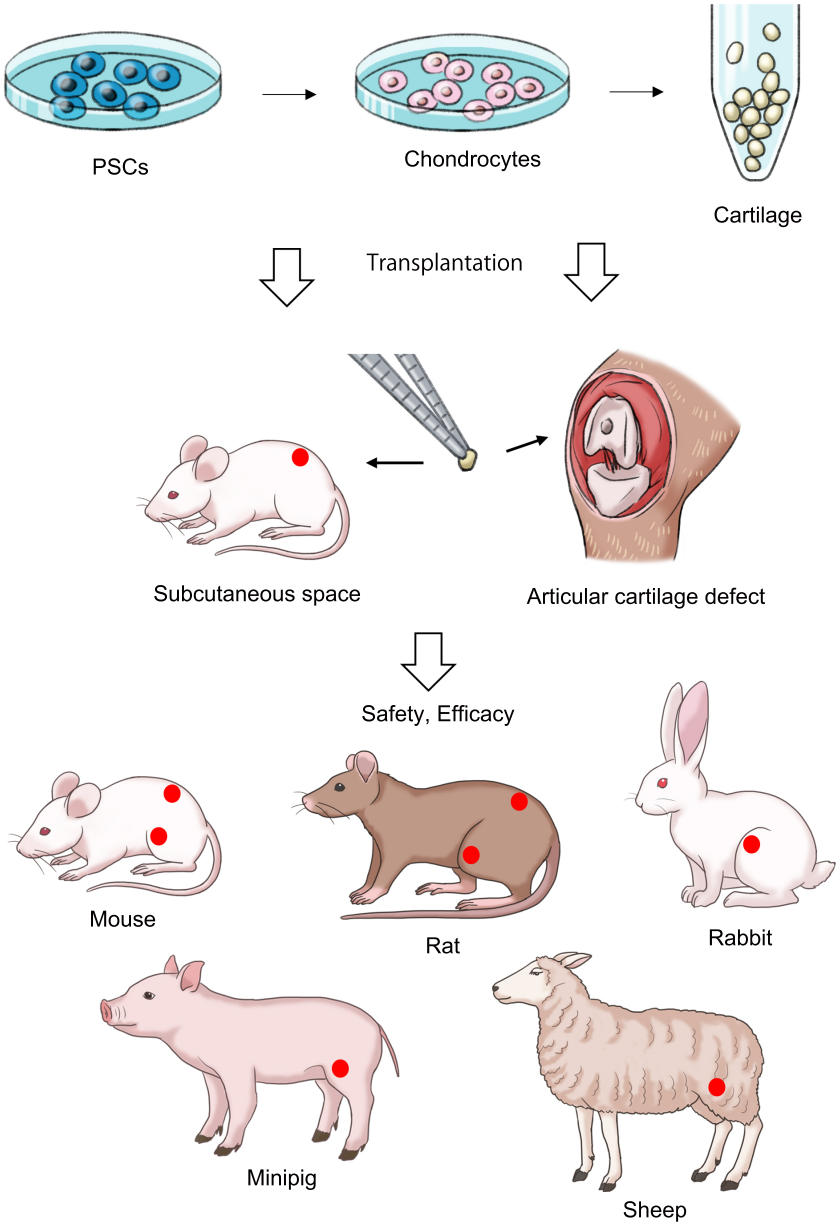

Table1 : The subcutaneous transplantation of hPSC-derived chondrocytes or cartilage

Author	Year	Cell	Line name	Reprograming method	Origin	Approach	Main growth factor / Compound	Transplants	Animal	Period	Tumor	Purpsoe
Wei Y, et al.	2012	iPSC	-	retrovirus / 4 factor	OA patient fibroblast	1)	lentivirus-TGFβ1	iPSC-derived chondrocytes in alginate	mouse	6w	-	efficacy, safety
Yamashita A, et al.	2015	iPSC	409B2	episomal	fibroblast	4)	BMP2, TGFβ1, GDF5	scaffold-free iPSC-derived cartilage particles	mouse	1 year	-	efficacy, safety
Lee J, et al.	2015	iPSC	-	retrovirus / 4 factors	fibroblast	4)	BMP4, GDF5, FGF2, etc.	iPSC-derived chondrocytes	mouse	4w	-	efficacy, safety
Craft AM, et al.	2015	ESC / iPSC	HES2, H7, iBJ, MSC-IPS1/Y2-1	retrovirus / 4 factors	fibroblast	4)	TGFβ3 or BMP4	iPSC-derived cartilage	mouse	12w	-	development
Umeda K, et al.	2015	ESC	H9, HES3	-	-	4)	TGFβ3, FGF2	ESC-derived chondrocyte pellets	mouse	12w	-	efficacy, safety
Hino K, et al.	2017	iPSC	FOP, res FOP	retrovirus / 4 factors	fibroblast	3)	-	patient iPSC-derived MSCs	mouse	6w	-	drug efficacy
Rim YA, et al.	2018	iPSC	-	Sendai virus / 4 factors	cord blood	2)	BMP2, TGFβ3	iPSC-derived chondrocytes or pellets	mouse	12w	-	efficacy, safety
Kimura T, et al.	2018	iPSC	409B2, HCH, ACH, TD	episomal	fibroblast	4)	BMP2, TGFβ1, GDF5	patient iPSC-derived cartilage particles	mouse	6w	-	drug efficacy
Ferguson GB, et al.	2018	ESC	H1	-	-	4)	BMP4, TGFβ1, IGF2, FGF2, SHH, etc.	ESC-derived chondrocyte aggregates	rat	4w	-	efficacy, safety
Lee JY, et al.	2018	ESC	H9	-	-	4)	PDGF, TGFβ3, BMP4, Forskolin	ESC-derived chondrocyte pellets	mouse	8w	-	development
Kawata M, et al.	2019	iPSC	7F3955	retrovirus / 4 factors	fibroblast	4)	CHIR99021, TTNPB	iPSC-derived chondrocytes	mouse	24w	-	efficacy, safety

Table2 : The transplantation of undifferentiated PSCs into articular cartilage defects

Author	Year	Cell	Species	Reprograming method	Origin	Approach	Allogenic / Xenogenic	Animal	Period	Tumor
Wakitani S et al.	2003	ESC	mouse	-	-	-	Allogenic	mouse	8w	+
Wakitani S et al.	2004	ESC	mouse	-	-	-	Xenogenic	rat	8w	-
Nakajima M et al.	2008	ESC	mouse	-	-	-	Xenogenic	rat	8w	+
Uto S, et al	2012	iPSC	mouse	retrovirus / 4 factors	Fibroblast	-	Allogenic	mouse	8w	+
Zhu Y, et al	2016	iPSC	human	retrovirus / 4 factors	OA patient fibroblast	-	Xenogenic	SD rat with OA	15w	-
Kotaka S et al	2017	iPSC	human	retrovirus / 4 factors	Fibroblast	magnetic label	Xenogenic	rat	8w	+
Pilichi S, et al	2018	ES-like	sheep	-	-	-	Allogenic	sheep	4 years	-
Uto S, et al	2018	iPSC-like	porcine	retrovirus / 4 factors	Fibroblast	-	Allogenic	minipig	8w	-
Uto S, et al	2018	iPSC	human	retrovirus / 3 factors	Fibroblast	-	Xenogenic	minipig	8w	-

Table3 : The transplantation of hPSC-derived chondrocytes or cartilage into articular cartilage defects

Author	Year	Cell	Line name	Reprograming method	Origin	Approach	Main growth factor / Compound	Transplants	Animal	Number	Period	Tumor	Detection of transplanted cells	Score for efficacy
Ko JY, et al.	2014	iPSC	SC802A-1	cell peptide	fibroblast	2)	TGFβ3	chondrocytes pellets / in alginate	rat	6	12w	-	+	+
Cheng A, et al.	2014	ESC	HUES1	-	-	4)	BMP4, GDF5, FGF2, etc.	chondrocytes	rat	6	12w	-	+	+
Yamashita A, et al.	2015	iPSC	409B2	Episomal	fibroblast	4)	BMP2, TGFβ1, GDF5	cartilage particles	rat	3	12w	-	+	-
Yamashita A, et al.	2015	iPSC	409B2	Episomal	fibroblast	4)	BMP2, TGFβ1, GDF5	cartilage particles	minipig	3	4w	-	+	-
Saito T, et al.	2015	iPSC	7F3955	retrovirus / 4 factors	fibroblast	4)	BMP4, GDF5, FGF2, etc.	chondrocytes	mouse	3	16w	+	+	+
Nejadnik H, et al.	2015	iPSC	-	non-viral minicircle	adipose stem cell	3)	TGFβ3	MSCs / chondrocyte pellets	rat	3	6w	-	+	+
Zhu Y, et al.	2016	iPSC	-	retrovirus / 4 factors	OA patient's fibroblast	2)	TGFβ1	chondrocytes	SD rat with OA	5	15w	-	-	-
Chijimatsu R, et al.	2017	iPSC	414C2	Episomal	fibroblast	3)	-	MSCs	rat	7	8w	-	+	+
Xu X, et al.	2017	iPSC	0209-001	retrovirus / 6 factors	fibroblast	3)	-	MSCs	rabbit	6	6w	-	-	-
Zhu Y, et al.	2017	iPSC	C1P33	retrovirus / 4 factors	fibroblast	-	-	MSC exosomes	mouse with OA	10	4w	-	-	+
Rim YA, et al.	2018	iPSC	-	Sendai virus / 4 factors	cord blood	2)	BMP2, TGFβ3	chondrocytes / chondrocyte pellets	rat	5	8w	-	+	+
Ferguson GB, et al.	2018	ESC	H1	-	-	4)	BMP4, TGFβ1, IGF2, FGF2, SHH, etc.	chondrocyte aggregates	rat	3	4w	-	-	-
Kawata M, et al.	2019	iPSC	7F3955	retrovirus / 4 factors	fibroblast	4)	CHIR99021, TTNPB	chondrocyte particles	mouse	6	24w	-	+	+
Wang T, et al.	2019	ESC	HUES1	-	-	4)	BMP2 or 4, GDF5, FGF2, etc.	chondrocytes	rat	4	12w	-	-	+
Gardner OF, et al.	2019	ESC	HES2	-	-	4)	TGFβ3	chondrocytes	rat	6	12w	-	+	+
Rim YA, et al.	2020	iPSC	-	Sendai virus / 4 factors	cord blood	4)	BMP2, TGFβ3	chondrocyte pellets	rat	5	8w	-	-	-