Journal of Clinical Orthopaedics and Trauma 7 (2016) 157–163

Contents lists available at ScienceDirect

Journal of Clinical Orthopaedics and Trauma

journal homepage: www.elsevier.com/locate/jcot

Review article

Scaffold-free, stem cell-based cartilage repair

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ARTICLE INFO

Article history:
Received 27 May 2016
Accepted 3 June 2016
Available online 28 June 2016

Keywords:
Stem cell-based therapy
Scaffold free
Cartilage repair
Mesenchymal stem cell

ABSTRACT

Various approaches to treat articular cartilage have been widely investigated due to its poor intrinsic healing capacity. Stem cell-based therapy could be a promising approach as an alternative to chondrocyte-based therapy and some of these therapies have been already applied in clinical condition. This review discusses the current development of stem cell-based therapies in cartilage repair, specifically focusing on scaffold-free approaches.

1. Introduction

It is widely accepted that chondral injuries usually do not heal spontaneously. Therefore, a variety of approaches have been tested to improve cartilage healing.

Bone marrow stimulations, such as microfracture and subchondral drilling, are commonly applied first-line treatments for symptomatic small articular cartilage defects.[1–3] These procedures establish a communication of the cartilage defect with the bone marrow, by focal perforation of the subchondral plate allowing bone marrow cells to migrate into the chondral lesion and to stimulate formation of fibrocartilaginous tissue.[4] Repair tissues generated by bone marrow stimulation are not hyaline cartilage but fibrocartilaginous tissue, which is biochemically and biomechanically inferior to native hyaline cartilage.[5,6] Therefore, decrease in long-term clinical outcomes have been reported.[6–8]

Osteochondral autograft transfer (OAT) (or mosaicplasty) is another long-standing surgery. In this procedure, one or more cylindrical osteochondral autografts from a non-weight-bearing area of articular cartilage are transferred to the chondral lesions. It had demonstrated positive clinical benefits for young patients with an active lifestyle.[9] Lynch et al. reported in a systemic review that compared to microfracture, OAT/mosaicplasty offers patients better clinical outcomes, with a higher rate of return to sport and maintenance of their sports activity. When compared with autologous chondrocyte implantation (ACI), improvement of clinical outcomes was not conclusive; however, at 10-year follow-up, a greater failure rate was found to be present in the OAT/mosaicplasty group. They also suggested that OAT/mosaicplasty procedures might be more appropriate for lesions that are smaller than 2 cm^2 with the known risk of failure between 2 and 4 years.[10] Pareek et al. concluded in a systemic review that OAT showed successful outcomes in 72% of patients at mean follow-up of 10.2 years and concomitant surgical procedures negatively correlated with failure rate.[11]

Autologous chondrocyte implantation (ACI) was firstly demonstrated by Brittberg et al.[12] During ACI procedure, chondrocytes are isolated from the cartilage specimen harvested from non-weight-bearing area in the knee joint, and then, chondrocytes were culture expanded in vitro for subsequent implantation to the chondral lesions. Cultured chondrocytes were covered with autologous periosteal patch in the first-generation ACI and collagen type I/III membrane in the second-generation ACI. First-generation ACI has been shown to be associated with symptomatic chondral hypertrophy that requires subsequent shaving at a greater rate than second-generation ACI.[13] Both, first- and second-generation ACI are technically demanding because these procedure require suturing of the patch to the adjacent cartilage. Third-generation

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http://dx.doi.org/10.1016/j.jcot.2016.06.002
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ACI (matrix-associated autologous chondrocyte implantation (MACI)) is transplantation of cultured chondrocytes to the lesion with biomaterials made of either synthetic or natural polymers as the scaffolds.[14,15] MACI is technically less challenging because it does not require suturing technique and therefore is easy for surgeons to handle. Recently, Oussedik et al. reported in a systematic review that MACI has been shown to be more effective than microfracture[16] and Goyal et al. reported in another systematic review that either the second- or third-generation ACI procedure demonstrated better clinical outcomes than did the first generation, but with weak evidence.[17] Within each ACI procedure, the best technique has not been proven due to the great variety of techniques, absence of long-term follow-up, and heterogeneity of outcome measures.[16]

Both of the OAT and ACI has limitation regarding the sacrifice of the undamaged cartilage and the donor site morbidity.[9,18] In addition, dedifferentiation of chondrocytes during in vitro culture is a major concern about the ACI. Culture expansion of chondrocytes in a 2D environment is thought to lead to alterations in cellular phenotype, thereby compromising repair efficacy.[19,20] Tissue engineering approaches using chondrocytes also have the same limitations.[21]

After all, "gold standard" for the cartilage repair is still lacking, and therefore, stem cell therapy for cartilage repair has caught researcher's and clinician's attention as a next-generation therapy over the past decade.

2. Stem cell therapy for cartilage repair

Transplantation of autologous mesenchymal stem cells (MSC) is an attractive strategy to repair articular cartilage compared with the transplantation of articular chondrocytes.[22] MSCs have a potent differentiation capacity to the mesodermal lineage (chondrocytes, osteoblasts, and adipocytes). MSC can be isolated from various tissues, such as bone marrow, synovium, adipose tissue, and skeletal muscle.[23–27] The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy had defined the standard criteria for uniform characterization of MSCs: they must be plastic-adherent cells when maintained in standard culture conditions; they must express CD105, CD73, and CD90; they must lack surface expression of CD45, CD34, CD14 (CD11b), CD79α (CD19), and HLA-DR; and must be capable of differentiating to cells of the mesodermal lineage.[28–30]

As an alternative options for a cell source, allogeneic MSCs[31] or induced pluripotent stem (iPS) cells[32,33] may also be considered. However, there have not been much evidence about clinical safety of these cells, and thus further studies are needed to apply these cells in clinical condition. Regarding the safety of MSC, Wakitani et al. who transplanted autologous bone marrow MSC to repair articular cartilage in their clinical trial firstly in the world,[34] reported long-term safety of MSC followed for up to 11 years and 5 months.[35]

3. Type of MSC

In 1966, Friedenstein proved that bone marrow includes progenitor cells that can generate connective tissue-forming cells.[23] In the 1980–1990s, many researchers extended these observations and demonstrated that the cells identified by Frirdenstein had multipotency to differentiate into osteoblasts, chondrocytes, and adipocytes.[36–39] Caplan et al. named these cells as mesenchymal stem cells.[24] It is notable that bone marrow MSCs are most widely studied as a cell source of stem cell therapy and applied clinically.[30,40,41] However, aspiration of bone marrow is an invasive and painful procedure, often requiring anesthesia and often with attendant morbidity.[42]

In 2001, Zuk et al. identified adipose tissue-derived MSCs from lipoaspirates, which have multilineage potential.[26] However, several investigators demonstrated that the chondrogenic capacity of adipose tissue-derived MSCs is not as extensive as that of bone marrow MSCs.[43–46]

In 2001, De Bari et al. identified synovial MSCs from human synovium.[25] There have been several reports demonstrating that synovial MSCs have the greatest chondrogenic potential compared with MSCs from the other tissues.[47–49] In addition, multipotency of synovial MSCs is not influenced by donor age or cell passages, and synovial MSCs have less senescence and great proliferative capacity.[25,47] Koizumi et al. reported that synovial MSCs from patients with osteoarthritis or rheumatoid arthritis are no less appropriate for repairing cartilage than those from trauma patients.[50] To harvest synovial membrane, arthroscopic surgery is needed, but the procedure is less invasive than bone marrow aspiration because arthroscopic surgeons usually remove and discard synovial membrane to get clear vision during arthroscopy. Thus, synovial MSCs could be an attractive cell source for tissue engineering in cartilage repair.[46]

4. With or without exogenous 3-dimensional scaffold

In 1998, Wakitani et al. transplanted autologous bone marrow MSC to repair articular cartilage, which was the first clinical trial ever reported in the world.[34] They transplanted MSC suspension in collagen gel and covered it with autologous periosteum. Thereafter, other researchers have reported good clinical outcomes using MSC suspension.[51–53] Nejadnik reported direct comparison of first-generation ACI with transplantation of bone marrow MSC suspension in the same surgical procedure and concluded that using MSCs in cartilage repair is as effective as chondrocytes to repair articular cartilage.[54] However, in these techniques, the transplanted MSCs do not contain extracellular matrix (ECM) and therefore it must be difficult to maximize the cellular function of transplanted cells because appropriate 3-dimensional (3D) environment is an essential factor to optimize cell proliferation and chondrogenic differentiation.[55]

In this regard, 3D scaffolds have been investigated to enhance repair of the chondral lesions. Additional advantage of using 3D scaffolds is better handling to deliver MSC to the chondral lesion and possible barrier effect against fibroblast invasion of the graft that may otherwise induce fibrous repair.[56] As an alternative to transplantation of MSC in cell suspension, various 3D scaffolds, such as synthetic polymers,[57,58] natural polymers extracted from different species,[59,60] collagen,[61] fibrin,[62] and hyaluronan[63] have been investigated to transplant MSCs.

However, there are still several issues regarding the long-term safety and feasibility of these materials. Synthetic polymers, such as polyglycolic acid[57] and poly(lactic-co-glycolic acid),[58] may have potential problems associated with residual and degradation in situ[64,65] that can be the risk factor to cause subsequent inflammation. Biological materials have possibility to transmit infectious agents like bacteria, virus, and prion, which initiate immunological reactions.[66,67] For the above reasons, such materials should ideally be avoided to minimize unknown risk throughout the treatment procedure, and in this regard, cell delivery system without use of exogenous scaffold would be an excellent alternative.

To address these problems, we have developed a 3D tissue-engineered construct (TEC) without the use of exogenous scaffolds. TEC is composed of synovial MSCs and ECMs synthesized by the cells. Plasticity and adhesiveness of the TEC enable scaffold-free transplantation (Fig. 1). Such a new, exogenous scaffold-free MSC-based therapy could be considered as the next-generation construct for cartilage regeneration. In this review, we discuss

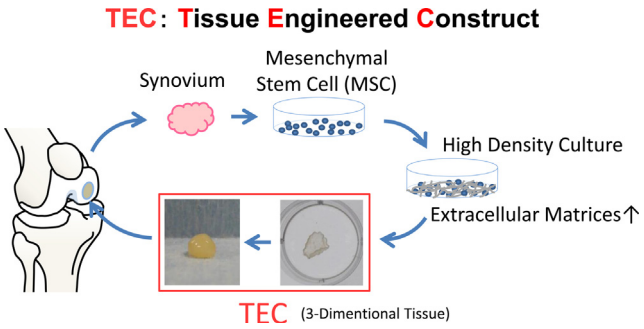

Fig. 1. Schematic representation of the TEC-mediated cartilage repair.

the feasibility and effectiveness of the TEC methodology repair and the advancement and problem in stem cell therapy for cartilage repair.

5. Development of the basic TEC

When MSCs were cultured to confluence in the basic growth medium (DMEM with 10% FBS), they did not deposit abundant collagenous matrices, because ascorbic acid is an essential cofactor to the formation of triple helix structure of collagen. In contrast, when Asc-2P was added to the medium, collagen synthesis significantly promoted. Subsequently, the monolayer cell–matrix complex cultured in growth medium added with Asc-2P became a stiff sheet-like structure, which could be easily detached from the substratum by applying gentle shear stress using pipette. After detachment, the monolayer cell–matrix complex immediately started active contraction and evolved into a thick 3D tissue. Such tissue contraction was partially, but significantly, inhibited by addition of dihydrocytochalasin B, an actin polymerization inhibitor, or Y-27632, a Rho kinase inhibitor, in a dose-dependent manner. These observations indicate that active contraction of the TEC is associated with cytoskeletal contraction.

Immunohistochemical evaluation showed that the TEC was rich in type I and III collagen and lacked expression of type II collagen. Besides that, fibronectin and vitronectin were also abundant in the TEC (Fig. 2a). It is notable that all the molecules detected within the TEC were diffusely distributed without obvious polarity to the matrix organization. As cell–matrix complex folded and contracted, TEC could change its morphology into one spherical body with several millimeters thickness or any other shape, because of its plasticity (Figs. 1 and 2b). This contracted cell–matrix complex was termed a tissue-engineered construct (TEC) derived from MSCs.[68,69]

6. Adhesive property of the TEC

To evaluate the adhesive property of TEC, basic porcine TECs were transplanted on the partial thickness defect created on the thawed fresh-frozen porcine chondral fragments. Just five minutes after transplantation, the TEC had adhered to the chondral fragments. During seven days of ex vivo culture of the TEC–chondral complexes, TEC adhered stably for the entire time. Histology at day 7 showed integration of the TEC to the bottom of the chondral defect on the fragments (Fig. 2c). Immunohistochemical finding revealed expression of fibronectin at the boundary surface between the TEC and the bottom of the chondral defect on the fragments (Fig. 2c).[69]

7. Chondrogenic differentiation potential of the TEC

Human basic TECs were replated on the bottom of the culture dishes and then subsequent chondrogenic differentiation was performed in a chondrogenic medium containing BMP2. Such chondrogenic-differentiated TEC showed increased GAG synthesis and deposition as evidenced by intense alcian blue staining (Fig. 3a). Semiquantitative RT-PCR of cartilage-specific markers,

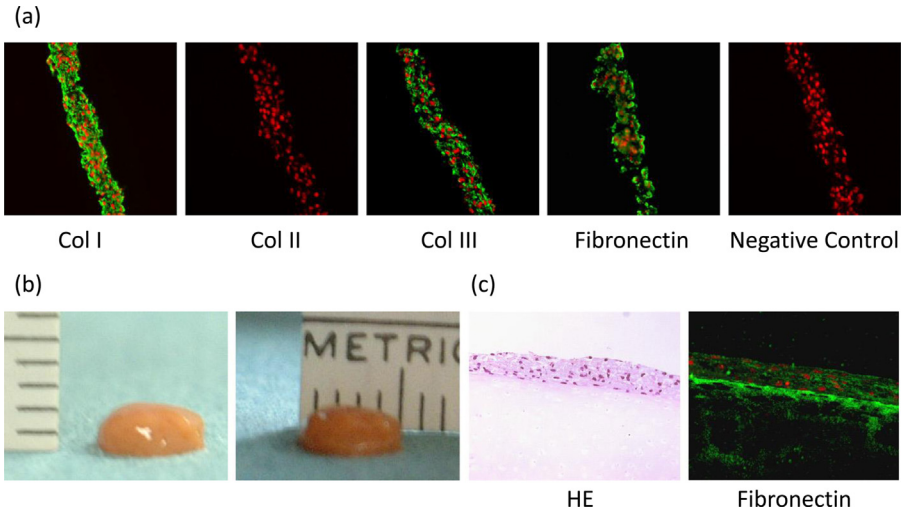

Fig. 2. Development of the tissue-engineered construct. (a) Immunohistochemical analysis of the basic TEC stained with type I collagen (Col I), type II collagen (Col II), type III collagen (Col III), fibronectin, and negative IgG (control). Bar = 100 mm. (b) Macroscopic view of the TEC, which was integrated to one spherical body. The diameter of this TEC was 5 mm and the thickness was 2 mm. (c) Microscopic view of HE staining (left side) and fibronectin staining (right side) of the cultured porcine chondral fragment for 7 days after the implantation of the basic TEC on the injured surface. Bar = 100 mm.
Cited from Ref. 69.

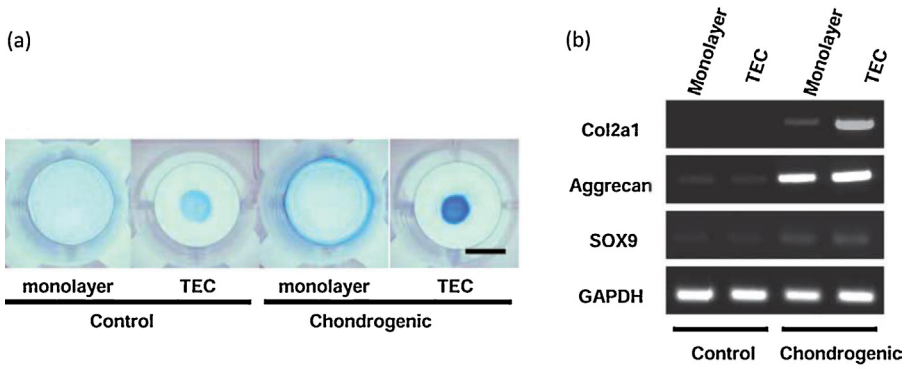

Fig. 3. Chondrogenic differentiation potential of the TEC. (a) Alcian blue staining of monolayer cultured MSCs or a basic TEC in control medium or in the chondrogenic medium containing 500 ng/mL BMP2 for 14 days. Bar = 1 cm. (b) RT-PCR analysis of monolayer cultures or TEC for chondrogenic marker genes, type II collagen (COL2A1), aggrecan (ACAN), SOX9, and GAPDH.
Cited from Ref. 68.

type II collagen (COL2A1), aggrecan (ACAN), and SOX9 revealed the cartilage phenotype of the chondrogenic-differentiated TEC. In contrast, undifferentiated basic TEC, as well as monolayer cell cultures, showed no expression of the cartilage-specific marker, type II collagen (Fig. 3b). These observations indicate that TEC provided appropriate 3D microenvironment to the MSC to differentiate into chondrogenic lineage.[68]

8. Implantation of in vitro generated basic TEC into porcine chondral defects in vivo

Porcine basic TECs were prepared as an allograft. The medial femoral condyles of the 4-month-old pigs were exposed, and chondral defects of 8.5 mm diameter and 2.0 mm depth were made. Then, the basic TECs were transplanted without suture or glue. At 6 months post-transplantation, the mean macroscopic score for the TEC-treated group was significantly lower than that for the untreated group (Fig. 4b), where a higher score is suggestive of a failure. Histological evaluation revealed that the chondral lesions in the nontreatment control group showed apparent osteoarthritic changes (Fig. 4c) while patients in the TEC-treated group were repaired with hyaline cartilaginous tissue exhibiting good integration to the adjacent native cartilage (Fig. 4d and e). Regarding the histological score, all assessment category for the TEC-treated group was significantly higher than those for the untreated group (Fig. 4f). These data indicated that the TEC

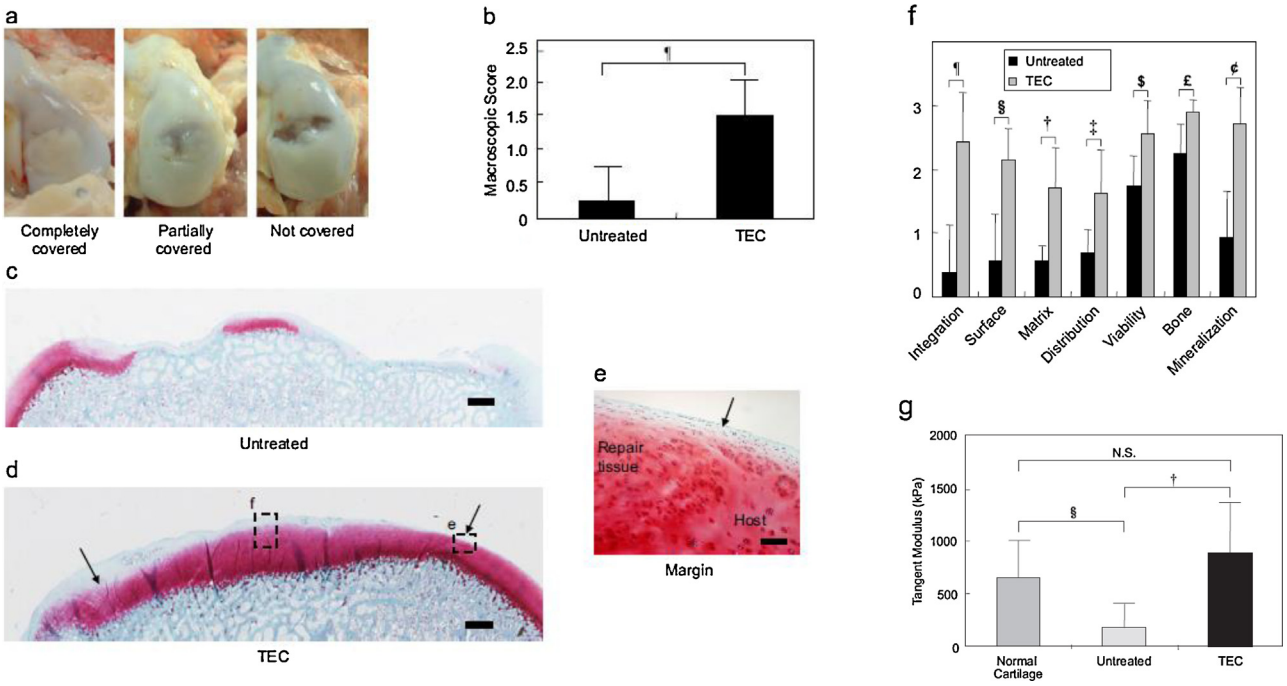

Fig. 4. Macroscopical and histological assessment of in vivo TEC implantation on chondral defect. (a) Macroscopic view of porcine chondral lesion treated with or without the TEC at 6 months after implantation. When treated with the TEC, 4 of 8 defects are completely covered with repair tissue (left side) and the others are partially covered (middle). Without the TEC, most of the chondral lesions have little tissue coverage (right side). (b) Macroscopic score of the chondral lesion treated with (TEC, $N = 8$) or without (untreated, $N = 4$) TEC at 6 months. ¶: $p = 0.017$. (c, d) Safranin O staining of chondral lesion treated with (c) or without (d) the TEC at 6 months after operation. Bar = 1 mm. (e) Light magnification view in the area enclosed by dotted rectangle in (d) at the margin area. Bar = 100 mm. Note that the defect treated with the TEC is completely filled with repair tissue with good tissue integration to the adjacent cartilage and with restoration of smooth surface (arrow). In contrast, the chondral defect in the control group (c) shows osteoarthritic change with loss of cartilage and destruction of subchondral bone. Modified ICRS Visual Histological Assessment Scale of repair tissue treated with (TEC; $N = 8$) and without (untreated; $N = 4$) TEC. ¶: $p = 0.009$, §: $p = 0.008$, †: $p = 0.010$, ‡: $p = 0.026$, $: $p = 0.037$, £: $p = 0.011$, ¢: $p = 0.006$.
Cited from Ref. 69.

maintains good tissue integration to the adjacent cartilage matrix, and the repair tissue exhibits chondrogenic differentiation without any evidence of central necrosis up to 6 months after sutureless transplantation.

Biomechanical analysis also revealed that the repaired tissue by the TEC transplantation exhibited modulus similar to the properties of native articular cartilage (Fig. 4g). To our knowledge, this study was the first demonstration of a successful MSC-based therapy for the repair of chondral lesions in a clinically relevant injury model without breaching the subchondral plate.[69]

9. Clinical trials to repair chondral defect using a TEC derived from human synovial MSCs

Based on the results of the preclinical studies discussed above, we have stepped forward to a clinical study at Osaka University Hospital, which has a current good manufacturing practice-grade cell processing center, and submitted the application of the "first-in-men" clinical trial to the Ministry of Health and Labour of Japan in 2011 (UMIN ID: UMIN000008266; Authorization number: HM1201). We got the approval in 2012, and the clinical trial was initiated in 2013 after the preparation of good clinical practice-based protocols. Patients who suffered from symptomatic chondral lesions of the knee, and who met the inclusion criteria (isolated chondral lesion $\leq$5 cm^2, 20–60 years of age, with normal alignment), have been registered. Approximately 1 g of synovium was obtained arthroscopically from the knee aseptically, and MSCs were isolated and expanded in the cell processing center. 3–5 weeks after harvest of synovium, the TECs were prepared for autologous implantation. Affected chondral lesion was exposed by mini-arthrotomy, and then chondral lesion was debrided so as to not breach the subchondral plate (Fig. 5a). Before transplantation, the TEC was trimmed and the shape of the TEC was adjusted to match that of the chondral lesion (Fig. 5b). Transplantation was completed within 5–10 min, without any suture or glue (Fig. 5c). After the transplantation of the TEC, joint capsule was closed temporary and knee joint was passively flexed and extended several times to confirm stable attachment of the TEC to the lesion.

As a postoperative treatment, immobilization of the knee joint was done in a brace for 2 weeks, and then, range-of-motion exercises and muscle exercises were initiated. Full weight bearing was allowed 6 to 8 weeks after transplantation surgery. Return to strenuous activity was allowed approximately 12 months following transplantation. The duration of follow-up was 1 year, and the primary end point of this study was to evaluate the adverse effects. The secondary end point was the assessment of effectiveness, including clinical scores (visual analog score [VAS], Knee Injury and Osteoarthritis Outcome Score [KOOS], Lysholm Knee Questionnaire, and Tegner Activity Scale), MRI (conventional and quantitative, such as T2-weighted mapping) at 3, 6, and 12 months, and histological assessment of a biopsy sample at 12 months. The preliminary results indicated that TEC transplantation restored normal joint function by completely covering the cartilage defect with cartilage-like repair tissue (Fig. 5d) with high, T2-weighted mapping profile (Fig. 5e).[70] This clinical study was completed in March 2015 and we will report the outcomes of this clinical trial in the near future.

10. Nonhomologous use of stem cell therapy

The present review has discussed the feasibility of exogenous scaffold-free TEC generated by synovial MSCs for effective stem cell therapy to repair articular cartilage. The strategy discussed above is nonhomologous use of stem cell therapy, that is, transplantation of cells or tissue that is different from target tissue. Therefore, transplanted cells have to differentiate into chondrocytes according to the host microenvironment and differentiated chondrocyte has to generate cartilage matrices to achieve cartilage repair.

Nonhomologous use of stem cell therapies for cartilage repair has been investigated and some of the therapies are already clinically applied. Orth et al. reviewed outcomes of nonhomologous use of stem cell therapies for cartilage repair in 2014. In this

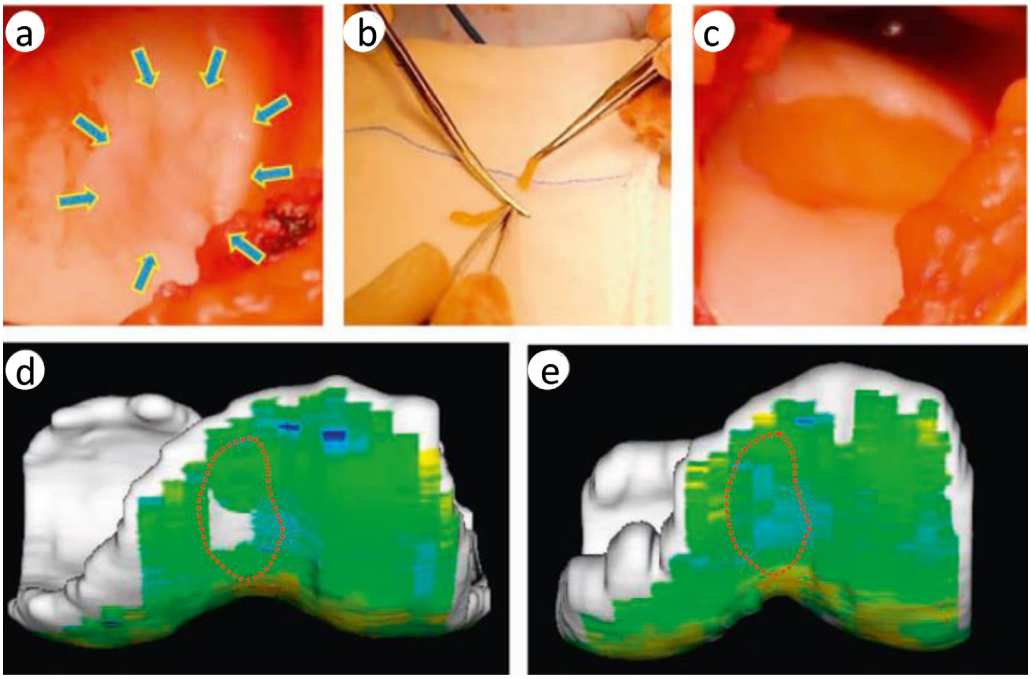

Fig. 5. Arthroscopic and magnetic resonance imaging (MRI) analyses of repair tissue following implantation of a tissue-engineered construct (TEC) to repair human chondral defects in clinical trial. (a) A ICRS grade III lesion in the medial femoral chondyle after debridement. (b) Adjustment of the size of the TEC to match the lesion size just before implantation. (c) Implanted TEC into the lesion. (d, e) T2 mapping of the lesion at the femoral groove. (d) Before implantation and (e) 6 months after implantation. Cited from Ref. 70.

review, although many studies reported clinical improvement, the repair tissue was hyaline-like tissue or fibrocartilage and none of these studies achieved pure hyaline cartilage restoration. Therefore, long-term outcome may be compromised due to the inferior mechanical property of fibrocartilage. We precisely evaluated the morphological and mechanical property of the repair cartilage generated by TEC in porcine cartilage defect model. Although macroscale compressive and lubrication properties were comparable to uninjured cartilage, microindentation evaluation showed that the surface stiffness of the repair tissue by TEC was significantly lower than that of native articular cartilage. Morphological observation showed that the superficial zone of the repair tissue by TEC was more fibrocartilaginous, in contrast to the middle or deep zones that were of more hyaline cartilaginous morphology. Then, histological scores were compared between superficial, middle, and deep zones of repair tissue by TEC and superficial zone was significantly compromised.

To overcome such limitation of nonhomologous use of stem cell therapy, various approaches have been investigated to achieve better quality of repair cartilage, including improvement of culture condition (growth factor, hypoxia, co-culture), introduction of gene therapy, 3D printing, and iPS (induced pluripotent stem) cells. Clinical application of these procedures is expected after confirmation of safety and ethical issue.

11. Homologous use of stem cell therapy

Homologous use of stem cell therapy is the strategy to transplant artificial hyaline cartilage generated by stem cells to the chondral lesion. MSCs can be differentiated into chondrocyte using three-dimensional culture at high density, such as micromass cultures[71] or pellet cultures,[72] and differentiated chondrocyte deposits cartilage matrices to generate hyaline cartilage. However, these methods cannot be directly applied to most clinical situations because of limitations in the mass size of the materials.[55] Bhardwaj et al. reviewed current strategy to generate tissue-engineered cartilage in 2015 and mentioned that despite tremendous growth and progress in the field of cartilage tissue engineering, the properties and structure of native cartilage have not been entirely mimicked by any tissue-engineered replacement till date.[21]

It is notable that recently Yamashita et al. succeeded in generating hyaline cartilaginous tissue from iPS cells without use of exogenous scaffold. iPS cells are an attractive cell source because of its unlimited self-renewal capacity. The average diameter of the hyaline cartilaginous particles is 1.4 ± 0.5 mm and they were transplanted to the chondral defect of the miniature pig. Although only short-term results were evaluated, the particles integrated with the native cartilage.[33] However, there have not been much evidence using these cells in terms of safety, and thus further studies are likely necessary.

We recently demonstrated the generation of pure hyaline cartilaginous tissue of approximately 1 cm in diameter by differentiation of basic TEC. While chondrogenic-differentiated TEC cultured under conventional normal oxygen was a mixture of hyaline-like and fibrocartilaginous tissue, chondrogenic-differentiated TEC cultured under low oxygen tension was pure hyaline cartilaginous tissue without fibrous tissue.[73] This was the first demonstration of in vitro development of a hyaline-like cartilaginous tissue of an implantable size to chondral defect that was generated by human MSCs without the use of exogenous scaffolds. The low oxygen tension culture at physiological range is a safe procedure with low cost, and thus, may be a clinically relevant option to repair cartilage.

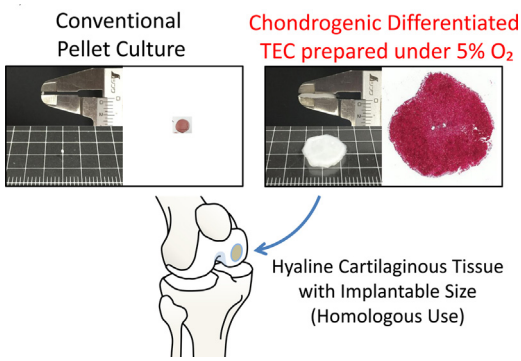

Fig. 6. Schematic representation of homologous use of stem cell therapy using chondrogenic-differentiated TEC under low oxygen tension.

These homologous uses of stem cell therapies are expected to overcome the limitations of nonhomologous use of stem cell therapies and thus further studies are necessary (Fig. 6).

12. Conclusion

Stem cell therapy for cartilage repair is a promising method of cell-based therapy without use of chondrocytes, which has limitation regarding the dedifferentiation and donor site morbidity. In the majority of stem cell-based cartilage repair, exogenous scaffolds made of chemical or animal-derived biomaterials are widely used to provide an appropriate three-dimensional environment for subsequent cell proliferation and differentiation. However, exogenous scaffold-free approach has advantage in terms of long-term safety. We have developed novel exogenous scaffold-free TEC-mediated cartilage repair as discussed above. Native ECM within the TEC, synthesized by MSCs, must play an important role as internal 3D scaffolds, providing MSCs appropriate microenvironment to differentiate into chondrocyte and generate cartilage matrices. TEC can be generated by MSC from other tissue, and differentiate into mesenchymal lineage, and thus, TEC methodology could be introduced to variety of therapeutic approaches in regenerative medicine.

Conflicts of interest

The authors have none to declare.

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