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# Application of Chondrocyte Sheets for Cartilage Regeneration

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## ABSTRACT

Osteoarthritis (OA) is a degenerative disease of cartilage that is common in elderly people. OA becomes progressively worse, and late-stage OA patients have no choice but to undertake total knee arthroplasty as a radical cure. This paper reviews the current conventional medical treatments and novel therapies aimed at inducing cartilage regeneration. Transplantation of layered chondrocyte sheets is a promising novel option for patients with cartilage lesions including OA. Layered chondrocyte sheets have been shown to exhibit a cartilage-restoring effect in experimental animal models of cartilage defects. The safety and efficacy have been examined in humans. This review discusses the mode of action of cell sheets in cartilage restoration and future prospects.

**KEYWORDS**: Cell sheet, Articular cartilage, Regenerative medicine.

**ABBREVIATIONS**: OA: Osteoarthritis; TKA: Total Knee Arthroplasty; ACI: Autologous Chondrocyte Implantation; hESC: Human Embryonic Stem Cell; MMP: Matrix Metalloprotease; iPSC: Induced Pluripotent Stem Cell; MSC: Mesenchymal Stem Cell; $PGE_2$: Prostaglandin $E_2$; TGF: Transforming Growth Factor; MIA: Melanoma Inhibitory Activity.

## INTRODUCTION

Articular cartilage bears the body's weight and may wear away as a result of daily activities. The main components of articular cartilage are water, which comprises 70%–80% of the total weight, collagen (50%–70% of the dry weight), and proteoglycan (~30% of the dry weight). Articular chondrocytes maintain hyaline cartilage by producing extracellular matrix, which comprises collagens, proteoglycans, and enzymes essential for cartilage tissue metabolism. However, articular chondrocytes comprise less than 5% of articular cartilage tissue by volume [1]. Because of the absence of blood vessels and low density of chondrocytes, damaged cartilage can be only minimally repaired, especially in elderly patients.

Osteoarthritis (OA) affects 30%–50% of people aged 65 years or older and is considered to be a degenerative disease of cartilage [2]. Overweight, obesity, female gender, and knee injury are recognized risk factors for OA. The onset of OA is associated with previous joint injury in 5% of cases and with weight gain or obesity in 25% of cases [3]. Body weight management is an effective intervention to prevent or slow disease progression. Restoration of damaged cartilage should be considered from the early stage of OA.

Joint injury eventually causes OA. Malalignment of bones and joint instability cause inappropriate load-bearing contact in the joint, which causes the articular cartilage to wear out [4]. Injury to knee cartilage causes gradual loss of the extracellular matrix and disruption of the cartilage structure, which lead to subchondral bone exposure and the onset of knee pain. The changing microenvironment disrupts chondrocyte function and worsens the cartilage defect.

Late OA patients often receive total knee arthroplasty (TKA). Ninety-three percent of patients are generally satisfied 5 years postoperatively; 87% are satisfied with the relief of pain and 80% are satisfied with the improvement in physical function at that time.

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However, patients' preoperative expectations may be higher than their postoperative ability to undertake leisure activity and walking [5].

Novel therapeutic applications for the treatment of OA are needed to meet patients' expectations of medical treatment and postoperative daily life.

**Conventional Regenerative Medicine for Cartilage Damage**

Joint trauma and osteochondritis dissecans are other pathological conditions that can cause cartilage damage. Surgical interventions aim to reestablish the joint surface. The choice of the surgical procedure is based on the size of the damaged area, joint stability, and the patient's age and symptoms.

Microfracture is one procedure performed to stimulate the damaged cartilage to fill with tissue made by migrating mesenchymal stem cells (MSCs) derived from the bone marrow [6,7]. However, the repaired cartilage exhibits characteristics of fibrous cartilage and not hyaline cartilage, and the procedure has poor clinical outcomes on a long period of time [8].

Autologous osteochondral mosaicplasty can be applied to small and medium-sized osteochondral lesions. The cartilaginous surface is reconstructed using osteochondral grafts obtained from autologous non weight-bearing cartilaginous parts. Grafts provide a hyaline cartilage surface, but the intergraft spaces tend to be filled with fibrous cartilage [9-11].

First reported by Brittberg et al. [12], autologous chondrocyte implantation (ACI) is now the most commonly used cell-based therapy for the treatment of cartilage defects in young patients and has been applied to over 20,000 patients worldwide [13]. Lynch et al. [14] reported superior clinical results of mosaicplasty compared with microfracture. They reported a higher rate of return to sport and maintenance of patients' sports ablity after surgery, and a lower rate of reoperation. Compared with ACI, the prognostic superiority of mosaicplasty is not conclusive, and mosaicplasty has a higher failure rate. A high incidence (49%) of a subsequent surgical procedure has been reported [15]. The cartilage tissue morphology generated after ACI had been found to be predominantly hyaline in 22% of biopsy specimens, mixed in 48%, and predominantly fibrocartilage in 30% [16]. Because hyaline cartilage restoration is very important to joint function, the effects of ACI [17,18] and the outcomes of all available therapies for damaged cartilage are insufficient. In addition, the effectiveness of these therapies in treating damaged cartilage associated with OA has not been confirmed, and thus there is no authorized treatment for cartilage restoration in OA patients. To address these issues, a novel therapy using cell sheet technology to treat damaged cartilage has been developed.

**Cartilage Regeneration Using Cell Sheets**

Cell sheet technologies have been applied to many cell types and therapeutic applications [19] including the cornea [20], esophagus [21], myocardium [22], and periodontium [23]. Kaneshiro et al. [24] introduced cell sheet technologies in the treatment of cartilage regeneration. Cell sheets can be created using poly (N-isopropylacrylamide), a thermoresponsive polymer and grafting in a culture dish [25,26]. The thermoresponsive surface of the culture dish allows for the noninvasive harvesting of intact sheets of cells within their deposited extracellular matrix. Using this approach, cell sheets can be transplanted into host tissues without the use of scaffolding or carrier materials [27].

Chondrocytes can adhere to and proliferate on the thermoresponsive polymer-grafted plate surface. When cells become confluent, they produce chondrogenic extracellular matrix and the cell sheets become thick. Chondrocyte sheets can be readily detached from these surfaces by lowering the incubation temperature without the need for enzymes to digest the extracellular matrix. Incorporating the cells within the extracellular matrix allows the chondrocytes in the cell sheet to retain their adherent molecules, receptors, cell–cell contact, and tissue microenvironment.

Multilayered chondrocyte sheets can be created by simply stacking three cell sheets and cultivating them for 1 additional week. The triple-layered chondrocyte sheets provide a fused monolithic structure with sufficient strength to be transplanted [28].

Transplantation of layered chondrocyte sheets onto a partial-thickness defect created in the knee cartilage of Japanese white rabbit prevented cartilage tissue degeneration [24]. In a rabbit total-thickness defect model, layered chondrocyte sheets seemed to alleviate pain and stimulate tissue repair. Sheet transplantation has produced excellent results for both defect-filling rates and subchondral bone formation. The graft cartilage layer exhibits a columnar arrangement showing repair with hyaline cartilage [29]. Cartilage restoration has also been reported for layered chondrocyte sheets applied to full-thickness cartilage defects in a minipig model [30]. The cartilage-regenerating effects achieved with cell sheets were the same as those achieved with tissue-engineered cartilage with a scaffold [31,32] or scaffold less cartilage discs [33,34].

The pathogenesis of OA includes a mix of full- and partial-thickness cartilage defects. Generally, partial-thickness cartilage defects are more difficult to restore because of the lack of chondrogenic progenitor cells. Layered chondrocyte sheets can induce cartilage-restoring effects in both partial- and total-thickness defect models, as mentioned above. This suggests that the sheets may be effective in treating cartilage lesions caused by OA.

Human articular chondrocytes have low proliferative capacity. The poor availability and yield of cells from patients limit the development of feasible therapies. Because coculture with synovial cells promotes the proliferation of human articular chondrocytes,to overcome this difficulty, human articular chondrocytes are cocultured with synovial cells to create human layered chondrocyte sheets [35].

Based on these encouraging results in experimental cartilage defect models and the establishment of cell sheet preparation procedures, a clinical study of the transplantation of human layered chondrocyte sheets into cartilage defects, including those caused by OA, has been conducted and completed safely. This study has shown the efficacy of this procedure (**Figure 1**). A manuscript is in preparation and the results will appear elsewhere.

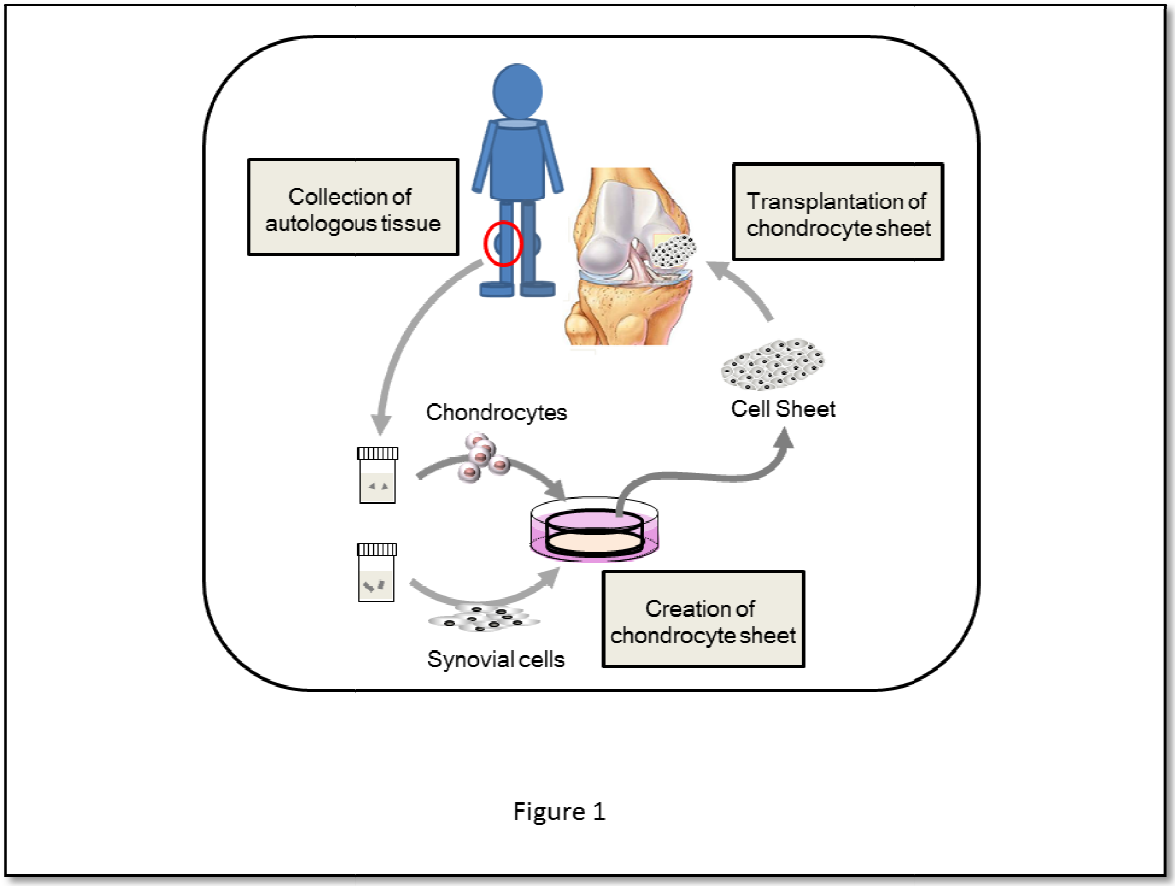


**Figure 1. Regeneration of articular cartilage using autologous chondrocyte sheets.** Chondrocytes and synovial cells are prepared from the patient's tissue. Synovial cells are cultured in a carrier plate, and chondrocytes are cultured in a temperature-responsive polymer-grafted culture insert for 2–3 weeks. Cell sheets can be detached readily and used to create layered chondrocyte sheets. The layered chondrocyte sheets are transplanted into the cartilage defect in the patient's knee.

## Mode of Action of Chondrocyte Sheets in Cartilage Regeneration

Triple-layered chondrocyte sheets express genes that are critical to cartilage maintenance, including those encoding type II collagen, aggrecan-1, and tissue metallopeptidase inhibitor 1, but not those encoding type I collagen, matrix metalloproteinase (MMP)-3, MMP-13, and A-disintegrin and metalloproteinase with thrombospondin motifs 5 [35]. Expression of the gene encoding the adhesion factor fibronectin-1 has also been reported [35]. Mitani et al. [28] reported the increased expression of SOX9, collagen type 27, and integrin alpha 10 in triple-layered chondrocyte sheets compared with monolayer cultures. This finding suggests that the layered structure contributes to the maintenance of the cartilaginous characteristics.

Hamahashi et al. [36] evaluated the secretion of humoral factors by layered chondrocyte sheets. Production of collagen type 1, collagen type 2, MMP-13, transforming growth factor-β (TGFβ), melanoma inhibitory activity (MIA), and prostaglandin $E_2$ ($PGE_2$) were detected by enzyme-linked immunosorbent assays. Higher

concentrations of $PGE_2$ and TGFβ were detected in the supernatants from cell sheets compared with those from ordinary cell cultures.

MIA is recognized as a marker of chondrocytes. MIA and collagen type II mRNA expression correlates specifically with chondrogenic differentiation and is not induced by osteoblastic differentiation [37]. By modulating the actions of bone morphogenetic protein-2 and TGFβ3 during mesenchymal stem cell differentiation, MIA supports the chondrogenic phenotype while inhibiting osteogenic differentiation [38]. Nishitani et al. [39] demonstrated that $PGE_2$ inhibits IL-1β-induced MMP-1 and MMP-13 production via prostaglandin E receptor 4 by suppressing the mitogen-activated protein kinase - Jun N terminal kinase pathway.

These results suggest that the humoral factors produced by layered chondrocyte sheets may contribute to cartilaginous tissue repair. Kaneshiro et al. [40] demonstrated that layered chondrocyte sheets adhered firmly to porcine cartilage after 1 day of culture. Histological analysis showed reduced safranin-O staining intensity of partially damaged cartilage tissue, whereas good staining intensity was observed in the damaged tissue covered by the layered cell sheet. This finding suggests that leakage of proteoglycans and cartilage degeneration occur in partial cartilage defects and that layered chondrocyte sheets can prevent these effects.

Another hypothesis is that cell sheets may provide chondrogenic progenitor cells for cartilage regeneration at the transplanted site. To investigate the cell fate in recipient animals, Takaku et al. [41] established a method for tracking cell sheets noninvasively and consecutively using luciferase-expressing chondrocyte sheets created from transgenic Lewis rats. The luciferase-expressing chondrocytes were monitored continuously using bioluminescence imaging. They found that the transplanted cells remained in the joint after 21 months and did not migrate to other parts of the body. However, the intensity of the luciferase signal decreased rapidly after transplantation, which suggests that the transplanted sheets were less likely to act as the main source of chondrocytes in the restored cartilage tissue.

Taken together, these findings suggest that chondrocyte sheets can contribute to cartilage regeneration by providing anabolic factors for chondrogenesis, by protecting against catabolic factors in the joint cavity, and by preventing loss of the extracellular matrix.

### Future Cell Sources for Cell Sheet Technology

Cell sourcing is one obstacle to the development and clinical application of regenerative therapy using cell sheets. The proliferative capacity and characteristics of autologous cells can vary, which may affect the reliability of cell sheet therapy and clinical outcomes. Patients must undertake two surgical procedures—one to collect autologous tissue and a second to transplant the cell sheets. Other cell sources have been explored to overcome these problems.

Cartilage is considered an immune-privileged tissue, and allogeneic cartilage tissue is now used as a cell source. Allogenic juvenile articular cartilage grafts (DeNovo® NT Natural Tissue Graft; Zimmer, Warsaw, IN) have been used in more than 7500 patients with cartilage defects. Because primary adult chondrocytes have limited proliferative capacity and their long-term cultivation causes dedifferentiation [42], stem cells are considered as a possible source of chondrocyte progenitor cells.

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) are reasonable candidates as a cell source. These cells have infinite proliferative capacity and can provide enough cells for therapeutic applications. However, the use of hESCs raises ethical concerns. Theoretically, iPSCs can be established from any individual. Considering the immune-privileged characteristics of cartilage, certain iPSC cell lines may be applicable to all patients. However, iPSCs require multistep, long-term procedures to obtain properly differentiated chondrocytes or chondrogenic progenitor cells [43,44]. Another concern relating to the risks associated with the tumorigenic potential of iPSCs needs to be addressed [45].

Multipotent MSCs exhibit potential for chondrogenic differentiation and have been found in various tissues such as bone marrow, synovial tissue, adipose tissue, umbilical cord, and skin. Many procedures for chondrogenic differentiation of MSCs have been reported [46]. Except for umbilical cord MSCs, these cells can be prepared from individual patients. Allogeneic MSCs may also be applicable. However MSCs have a finite proliferative capacity.

The possible methods for preparing the cell source for cartilage regeneration using cell sheet technology need further evaluation. The safety, characteristics of the chondrocytes obtained, and costs of preparation must also be considered.

## CONCLUSION

Restoration of damaged cartilage using chondrocyte sheets is a promising novel regenerative therapy for OA or cartilage lesions. The use of allogeneic chondrocytes as a cell source for chondrocyte sheets needs further evaluation before this therapy can be offered as standard treatment. The multistep, long-term procedure required for preparation of chondrocyte sheets directly affects the feasibility of regenerative therapy. The need for quality differentiated cells and the establishment of feasible procedures will determine which cell sources are used in this technology.

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