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Intra-articular administration of EP2 enhances the articular cartilage repair in a rabbit model

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Abstract

We have reported the usefulness of chondrocyte sheets on articular cartilage repair in animal experiments. Here, we investigated the regenerative effects of EP2 signalling with or without chondrocyte sheets. Forty-five rabbits were used, with six rabbits in each of the six groups and nine rabbits for chondrocytes and synovial cells harvesting to fabricate triple-layered chondrocyte sheets: osteochondral defect only (control, Group A), EP2 agonist (Group B), EP2 antagonist (Group C), chondrocyte sheets (Group D), EP2 agonist and chondrocyte sheets (Group E), and EP2 antagonist and chondrocyte sheets (Group F). After surgery, the weight distribution ratio was measured as an indicator of pain alleviation. Injections of the EP2 agonist or EP2 antagonist were given from 4 weeks after surgery. The rabbits were sacrificed at 12 weeks, and the repaired tissues were evaluated for histology. The weight distribution ratio and International Cartilage Repair Society grading were as follows: Group A: 40.5% ± 0.2%, 14.8 ± 0.5; Group B: 43.4% ± 0.7%, 25.4 ± 0.8; Group C: 38.7% ± 0.7%, 13.7 ± 0.3; Group D: 48.6% ± 0.6%, 40.2 ± 0.5; Group E: 49.1% ± 0.3%, 40.5 ± 0.4; and Group F; 46.8% ± 0.4%, 38.7 ± 0.5. Significant differences in histology and pain alleviation were observed between groups except between Groups A and C, between Groups D and E, and between Groups D and F. These findings show that the intra-articular administration of an EP2 agonist achieved pain alleviation and tissue repair. However, no synergistic effect with chondrocyte sheets was observed.

KEYWORDS

cartilage regeneration, chondrocyte sheet, transplantation

1 | INTRODUCTION

Osteoarthritis (OA) is the most common disease in older adults, is often refractory to treatment, and can lead to articular cartilage degeneration (Peat, McCarney, & Croft, 2001). With progression, cartilage degeneration causes dysfunction of the knee joint and makes activities of daily living difficult. Post-traumatic articular cartilage defects are treated mainly by microfracture (Mithoefer et al., 2006; Steadman, Rodkey, & Briggs, 2002; Steadman, Rodkey, & Rodrigo, 2001), mosaicplasty (Hangody, Feczko, Bartha, Bodó, & Kish, 2001; Hangody, Kish, Karpati, & Szerb, 1997; Szerb, Hangody, Duska, & Kaposi, 2005), or autologous chondrocyte implantation (Ochi, Uchio, Kawasaki, & Iwasa, 2002). There are other treatments for OA caused by articular cartilage degeneration and damage, such as high tibial osteotomy, unicompartmental knee arthroplasty, and total knee arthroplasty.

The process of OA development involves various factors, including cartilage degeneration, repair, and inflammation (Buckwalter, Saltzman, & Brown, 2004), but there is no intention to prevent human articular cartilage degeneration and damage by these treatments (Hunziker & Rosenberg, 1996; Kaneshiro et al., 2006). As treatments

for damaged articular cartilage, a variety of autologous chondrocyte implantation methods are used throughout the world using chondrocytes recovered from patients and then cultured in vitro and transplanted to articular cartilage defects. Post-traumatic articular cartilage defects are the main indications for this treatment but OA is not. Such untreated cartilage defects progress to OA because chondrocytes have poor regenerative ability and metabolic activity (Anderson et al., 2011; Otsuka et al., 2009). The possibility of exacerbating OA by using conventional cell therapies is a concern because fibrous cartilage is mixed in with the regenerated tissue.

We have previously reported the usefulness of layered chondrocyte sheets as a treatment for degenerated articular cartilage in animal experiments. Kaneshiro et al. reported the effects of layered chondrocyte sheets obtained from temperature-responsive culture dishes as a treatment for partial-thickness defects, which are considered difficult to treat conventionally (Kaneshiro et al., 2006). The characteristics of the humoral factors produced by layered chondrocyte sheets have also been clarified (Hamahashi et al., 2015). Experiments in humans have shown that synovial cell cocultures provide an optimal environment for the preparation of such sheets for tissue transplantation and are particularly beneficial for shortening the required culture period (Hamahashi et al., 2015; Kokubo et al., 2013; Mitani et al., 2009). Moreover, using experiments in minipigs (Ebihara et al., 2012), rabbits (Ito et al., 2012), and rats (Takaku et al., 2014), we achieved good treatment outcomes by transplanting chondrocyte sheets into models of full-thickness defects. These approaches are effective in treating both partial-thickness defects (damage confined to cartilage) and full-thickness defects (damage reaching the subchondral bone). Studies have suggested that chondrocyte sheets have therapeutic effects on both types of cartilage damage. Because there is always a mix of partial- and full-thickness defects in OA, chondrocyte sheets provide a promising option for inducing cartilage regeneration.

In the case of autologous chondrocyte sheet transplantation, we confirmed the improvement of symptoms and magnetic resonance imaging findings for cartilage at 1 year after surgery according to our previous studies. By contrast, the problems are that results at 1 month after surgery are inconsistent in autologous chondrocyte sheet transplantation. Furthermore, the structural and temporal differences in the formation of chondrocyte sheets between animal species (Kokubo et al., 2013) remain to be studied. To address these problems, new treatments that can be used in combination with chondrocyte sheet transplantation are needed to provide stable results from a very early stage after chondrocyte sheet transplantation. Chondrocyte sheets possess barrier functions that defend against invasion by catabolic factors in synovial fluid, prevent leakage of proteoglycans from the extracellular matrix, and preserve bone marrow cells in the damaged cartilage. These sheets also release anabolic factors to the defect, physically block catabolic factors in synovial fluid, promote the differentiation of bone marrow cells while protecting the damaged part, and eventually enable cartilage regeneration (Ebihara et al., 2012; Hamahashi et al., 2015; Ito et al., 2012; Kaneshiro et al., 2006; Mitani et al., 2009).

In OA, catabolic activity is increased in chondrocytes, the proliferation of chondrocytes is decreased, and the subchondral structure is weak (Otsuka et al., 2009). In normal cartilage, catabolic and anabolic activities are in dynamic equilibrium (Mitsui et al., 2011). In chondrocyte sheet transplantation, articular cartilage repair is achieved in a more normal environment, which shows the importance of the extracellular environment to articular cartilage repair.

Hamahashi et al. have reported that the abundant secretion of factors such as transforming growth factor-β (TGFβ) and prostaglandin E2 (PGE2) accelerates the effects of chondrocyte sheets in cartilage repair (Hamahashi et al., 2015). PGE2 works through four isoforms of the EP receptor, EP1 to EP4, and then EP2 receptor-mediated PGE2 signalling stimulates the growth of chondrocytes and promotes the regeneration of articular cartilage in rabbits (Aoyama et al., 2005; Mitsui et al., 2011; Otsuka et al., 2009). Signals through EP1 and EP3, coupled by Gi protein, increase the intracellular Ca^{2+} concentration, and those through EP2 and EP4, coupled by Gs protein, increase cyclic adenosine monophosphate (cAMP). Although they have a common second messenger, the amide acid identity between EP2 (362 amino acids) and EP4 (513 amino acids) is only 31% (Sugimoto & Narumiya, 2007). In addition, a specific agonist for EP2 but not EP4 accelerated the growth of mouse and human chondrocytes by upregulating the expression of growth-accelerating genes such as the cyclin D gene, thereby stimulating an increase in cAMP (Aoyama et al., 2005). Sato et al. demonstrated that EP2 signalling was responsible for the down-regulation of metalloproteinase-13 (MMP13) in protein level (Sato et al., 2010). It has been reported that MMP13 causes destruction of the matrix network (Goldring & Goldring, 2004; Goldring & Goldring, 2007), that inhibitors of MMP13 prevent the degradation of articular cartilage (Billinghurst et al., 1997). Hamahashi et al. measured the amount of humoral factors produced by layered chondrocyte sheets, and they confirmed that COL II and PGE2 secretion increased, and MMP13 secretion was inhibited, suggesting that layered chondrocyte sheets maintain an intact Extracellular matrix (ECM) and exert a chondroprotective action (Hamahashi et al., 2015). Using a rabbit model in which the animals received transection of the anterior cruciate ligament, partial resection of the medial meniscus, and intra-articular administration of a gelatin hydrogel containing an EP2 agonist, Mitsui et al. reported that EP2 agonists may exert a protective effect on articular cartilage by inhibiting MMP13 (Mitsui et al., 2011). Using a rabbit model of femoral chondral and osteochondral defects and a similar gelatin hydrogel, Otsuka et al. found that an EP2 agonist promoted regeneration of cartilage tissues (Otsuka et al., 2009). However, there are concerns about the biocompatibility of the scaffolds used in some studies (Hunziker, 2001; Mainil-Varlet et al., 2001) and the repeated administration and concomitant use of other treatments such as intra-articular injection of EP2 agonists. On the other hand, novel treatments that can be used in concomitant with chondrocyte sheet transplantation are necessary to provide stable results from a very early stage after chondrocyte sheet transplantation. The effects of a selective EP2 receptor antagonist were reported (Af Forselles et al., 2011), and it may be useful to elucidate the mechanisms underlying these effects by comparing EP2 receptor antagonists and EP2 agonists.

Here, we examined the efficacy of intra-articular administration of an EP2 agonist and EP2 antagonist after chondrocyte sheet transplantation in treating full-thickness articular defects in the knees of rabbits.

2 | MATERIALS AND METHODS

All procedures using animals in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 2010) published by the National Institutes of Health, USA, and the Guidelines of Tokai University on Animal Use (Authorization No. 141013).

Forty-five Japanese white rabbits, aged 16 to 18 weeks and weighing about 3 kg, were used in this study. These rabbits were purchased from a professional breeder (Tokyo Jiken Dobutsu, Tokyo, Japan). Nine Japanese white rabbits were used as the source of articular chondrocytes and synovial cells. After the chondrocytes and synovial cells had been harvested and isolated enzymatically, the chondrocytes were seeded onto temperature-responsive inserts and the synovial cells into temperature-responsive culture dishes, and the cells were cocultured for 2 weeks. Each retrieved cell sheet was then layered to prepare triple-layered chondrocyte sheets, which were cultured for 1 week (Ebihara et al., 2012; Ito et al., 2012; Kokubo et al., 2013).

The rabbits were anaesthetized by exposure to isoflurane, N2O, and O2. Under sterile conditions, a medial parapatellar approach on the right side was used to release the joint capsule and to create an osteochondral defect (diameter 5 mm; depth 3 mm) on the patellar groove of the femur using a drill and biopsy punch (REF-BP-50F; Kai Industries, Seki, Japan). Bleeding from the bone was observed, confirming that the osteochondral defects had been produced.

The rabbits underwent the following six treatments, with six animals in each group: Group A had an osteochondral defect only (defect/no treatment); Group B received an EP2 agonist (EP2 Ag); Group C received an EP2 antagonist (EP2 An); Group D received chondrocyte sheets (ChSh); Group E received an EP2 agonist and chondrocyte sheets (EP2 Ag + ChSh); and Group F received an EP2 antagonist and chondrocyte sheets (EP2 An + ChSh). These operations were performed in six unilateral knees of six rabbits in each group. After the surgery, all of the rabbits were returned to the cage without splinting or immobilization.

The first injection of the EP2 agonist or EP2 antagonist was given 4 weeks after the surgery; the second and subsequent injections were administered once every 2 weeks up to 10 weeks (Figure 1). The weight distribution ratio of the damaged limb was observed as a measure of pain evaluation. The rabbits were sacrificed at 12 weeks by an intravenous pentobarbital overdose. The transplanted tissues were sectioned and stained for histological evaluation using the International Cartilage Repair Society (ICRS) grading system.

2.1 | Temperature-responsive culture dishes

The temperature-responsive culture dishes (UpCell; provided by CellSeed, Tokyo, Japan) were coated with poly (N-isopropylacrylamide), which can change between hydrophilic and hydrophobic properties depending on the temperature and were developed by Okano et al. (Okano, Yamada, Okuhara, Sakai, & Sakurai, 1995; Okano, Yamada, Sakai, & Sakurai, 1993). This polymer facilitates cell adhesion and growth in normal culture conditions at 37 °C. Reducing the temperature of the culture to <30 °C causes the surface to hydrate and swell rapidly, which prompts the complete detachment of adherent cells without the need for typical treatment using proteolytic enzymes or trypsin. The culture dishes were sterilized using ethylene oxide gas (Sekiya, Shimizu, Yamato, Kikuchi, & Okano, 2006).

2.2 | Cell culture using temperature-responsive culture dishes

The harvested cartilaginous and synovial tissues were finely sliced with scissors and incubated in Petri dishes in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco, Grand Island, NY, USA) containing 0.5% (*w*/*v*) collagenase type 1 (Worthington, Lakewood, NJ, USA) at 37 °C and 5% CO2 in humidified air for 4 hr while being stirred with a stirrer to degrade the proteins. The isolated chondrocytes and synovial cells were passed through a cell strainer (BD Falcon Labware, Franklin Lakes, NJ, USA) with a pore size of 100 μm, and the cells were retrieved by centrifugation at 1,500 rpm for 10 min. The chondrocytes were incubated in a culture medium of DMEM/F12 supplemented with 20% fetal bovine serum (Gibco) and 1% antibiotics–antimycotics (Gibco). From Day 7 onward, the culture was maintained by adding a further 50-μg/ml ascorbic acid (Wako Junyaku Kogyo Corp, Osaka, Japan), and the synovial cells were maintained in a culture medium of DMEM/F12 supplemented with 10% fetal bovine serum and 1% antibiotics–antimycotics. From Day 4 onward, the culture was maintained by adding a further 50-μg/ml ascorbic acid. All culturing was performed at 37 °C in 5% CO2 and 95% humidified air. The chondrocytes were seeded on temperature-responsive inserts (4.2 cm^2; CellSeed), and the synovial cells were seeded in temperature-responsive culture dishes (9.6 cm^2; CellSeed) and cocultured for 14 days. The chondrocytes were seeded at a density of 50,000 cells/cm^2, and the

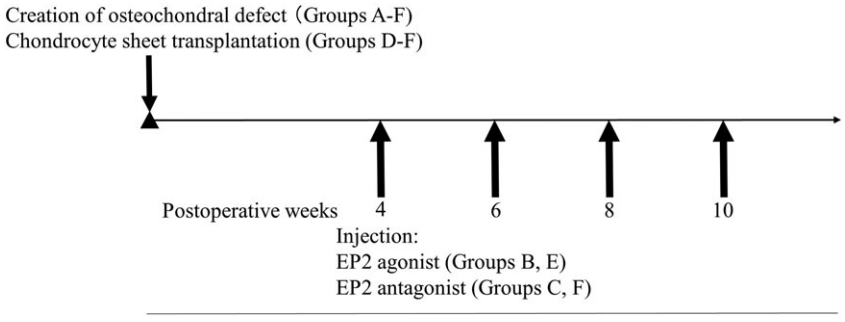

FIGURE 1 Timing of chondrocyte sheet transplantation and intra-articular administration. The first injection of the EP2 agonist or EP2 antagonist was given 4 weeks after surgery; the second and subsequent injections were administered once every 2 weeks up to 10 weeks. The rabbits were sacrificed at 12 weeks. Both of the concentrations were 100 nM and both of the volumes were 0.5 ml

synovial cells were seeded at a density of 10,000 cells/cm^2 (Hamahashi et al., 2015; Ito et al., 2012).

2.3 | Chondrocyte sheet retrieval

After the cells had been cultured for 2 weeks, they reached a confluent state and the temperature-responsive inserts were removed from the incubator and left for 30 min at 25 °C. After removal of the culture medium, a polyvinylidene fluoride (PVDF) membrane was used to retrieve a chondrocyte sheet, as described (Yamato, Utsumi, Kushida Konno, Kikuchi, & Okano, 2001). Briefly, the chondrocyte sheet was covered with a PVDF membrane, and both were harvested carefully as one unit. This method facilitated good retrieval of the cultured chondrocyte sheets. Next, the PVDF membrane covering the chondrocyte sheet was harvested by rolling the membrane up at the edge of the culture dish and then overlaying the membrane on top of another chondrocyte sheet. This operation was performed three times to fabricate a triple-layered chondrocyte sheet. Because the layered chondrocyte sheet floated in the culture fluid, a cell strainer (BD Falcon Labware) was placed on top. The layered chondrocyte sheets were cultured for 1 week further in temperature-responsive culture dishes.

2.4 | Intra-articular administration of the EP2 agonist and EP2 antagonist

ONO-2750481 (Ono Pharmaceutical Co., Ltd., Tokyo, Japan) was administered as the EP2 agonist. PF-04418948 (Cayman Chemical Co., Ltd., Ann Arbor, MI, USA) was used as the EP2 antagonist. Both were used at a concentration of 100 nM in reference to the past papers (Af Forselles et al., 2011; Aoyama et al., 2005; Mitsui et al., 2011; Otsuka et al., 2009). Briefly, 0.4-mg ONO-2750481 was dissolved in 0.5 ml of distilled water, and 0.02 μg of PF-04418948 was dissolved in 0.025 ml of ethanol and 0.475 ml of phosphate-buffered saline (PBS). The solution was administered intra-articularly with a 27-gauge needle on a 1.0-ml syringe through the lateral infrapatellar area toward the intercondylar space of the femur with each animal in a deep knee-flexed position. The first injection of the EP2 agonist or EP2 antagonist was given 4 weeks after the surgery; the second and subsequent injections were administered once every 2 weeks up to 10 weeks (Figure 1).

2.5 | Pain evaluation

One week after surgery, an incapacitance tester (Linton Instrumentation, Norfolk, UK) was used to determine any trends in differences in the ratio of weight distribution between the undamaged and damaged limbs, and these trends served as the gauge for evaluating pain. This device facilitates automatic and reproducible pain evaluation by measuring the distribution of load between the hind limbs using a dual-channel weight-averaging technique, which is used widely to investigate pain amelioration in animal models (Ito et al., 2012; Mihara, Higo, Uchiyama, Tanabe, & Saito, 2007). To acclimatize the animals to the incapacitance tester, each day for 7 days after they were delivered, each animal was placed in the main container (holder) of the device and held still for 5 s. The measurements were performed when the animals were immobile, after they had been transferred into the rabbit holder, and when they were immobile after being removed from, and then returned to, the holder. This operation was performed five times, and the weight distribution ratio between the hind legs was calculated using the following formula and is expressed here as a percentage:

Damaged limb weight distribution ratio (%) = damaged limb load (g)/(undamaged limb load [g] + damaged limb load [g]) × 100.

This measurement was recorded as the mean of five repeats. After surgery, the measurements were performed eight times at Weeks 1, 2, 3, 4, 6, 8, 10, and 12.

2.6 | Histological evaluation of cartilage repair

The rabbits were sacrificed at 12 weeks by an intravenous pentobarbital overdose. The transplanted tissue was removed from the distal portions of the unilateral femur, fixed in 4% paraformaldehyde for 1 week, and then decalcified for 2–3 weeks using distilled water (pH 7.4) containing 10% ethylenediaminetetraacetic acid. Next, the tissues were embedded in paraffin wax and sectioned perpendicularly (8-mm sections) through the centre of the defect. Each section was stained with Safranin O for glycosaminoglycans. Immunostaining

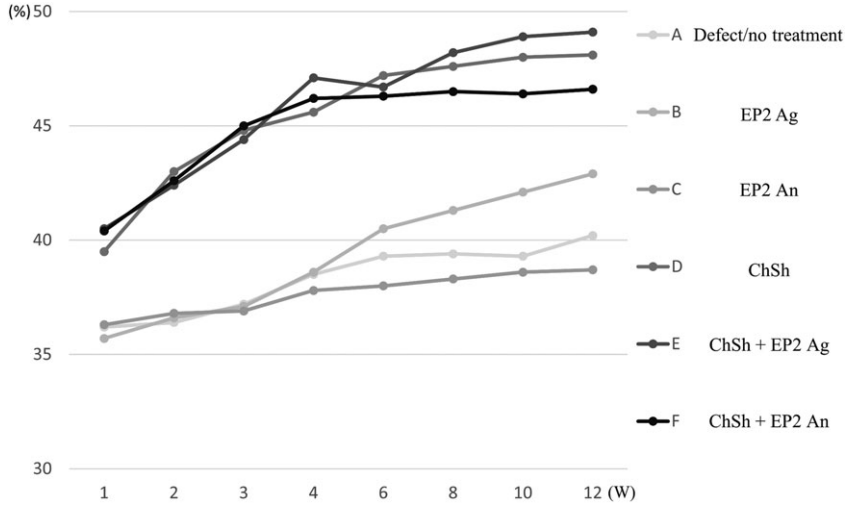

FIGURE 2 Damaged limb weight distribution ratios at various weeks (w) after surgeryGroup A had an osteochondral defect only (defect/no treatment); Group B received an EP2 agonist (EP2 Ag); Group C received an EP2 antagonist (EP2 An); Group D received chondrocyte sheets (ChSh); Group E received an EP2 agonist and chondrocyte sheets (ChSh + EP2 Ag); and Group F received an EP2 antagonist and chondrocyte sheets (ChSh + EP2 An). The weight distribution ratio (%) was used to evaluate pain and was calculated as damaged limb load (g)/(undamaged limb load [g] + damaged-limb load [g]) × 100. Groups D, E, and F exhibited a significantly higher ratio than Groups A, B, and C at 1 week after surgery (*$p < 0.05$)

was performed as described (Ito et al., 2012; Nagai et al., 2008). Briefly, the sections were deparaffinized using standard procedures, immunostained, and treated with 0.005% proteinase (type XXIV; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 30 min. After washing in PBS, the sections were treated with 0.3% hydrogen peroxide/methanol solution at room temperature for 15–20 min to activate endogenous peroxidase. The sections were then reacted for 30 min in normal goat serum diluted 1:20 in PBS. Mouse primary monoclonal antibodies to human types I and II collagen (Daiichi Fine Chemical Co., Toyama, Japan) were diluted 1:200 with PBS plus 1% bovine serum albumin (Sigma-Aldrich). The sections were left in the solution at 4 °C for one night, washed 10 times with PBS, and reacted at room temperature for 1 hr with goat antimouse biotin-conjugated secondary antibodies that had been diluted 1:100 with 1% bovine serum albumin in PBS. The sections were then treated for 1 hr with horseradish peroxidase, stained with streptavidin–horseradish peroxidase, and immersed for 2–4 min in Tris–HCl buffer (pH 7.6) containing 0.05% diaminobenzidine and 0.005% hydrogen peroxide. After

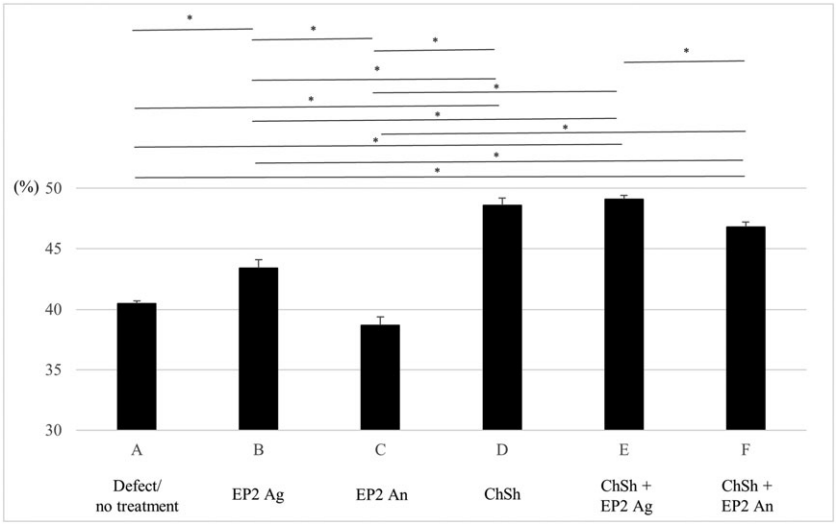

FIGURE 3 Weight distribution ratio (%) at 12 weeks after surgery. Group A, osteochondral defect only (defect/no treatment); Group B, EP2 agonist (EP2 Ag); Group C, EP2 antagonist (EP2 An); Group D, chondrocyte sheets (ChSh); Group E, chondrocyte sheets and an EP2 agonist (ChSh + EP2 Ag); and Group F, chondrocyte sheets and an EP2 antagonist (ChSh + EP2 An). Significantly different weight distribution ratios were found in Group A compared with Groups B, D, E, and F; in Group B compared with Groups C, D, E, and F; in Group C compared with Groups D, E, and F; and in Group E compared with Group F (all $p < 0.05$)

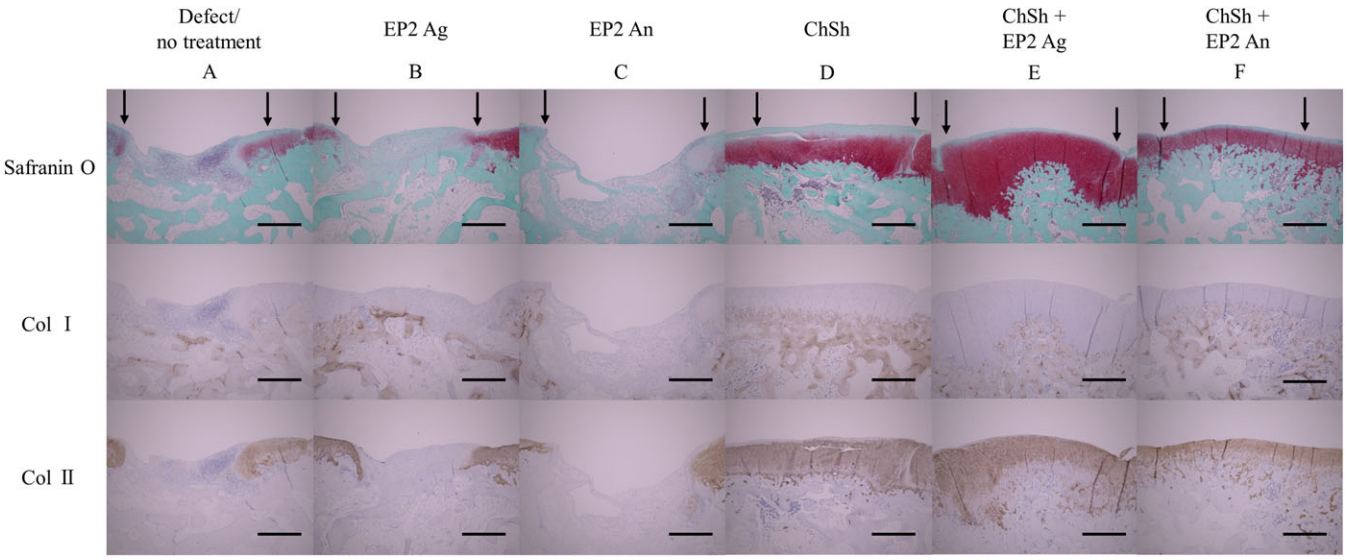

FIGURE 4 Histology of repaired tissues at 12 weeks after surgery after immunostaining for collagen I (Col I) and II (Col II; middle and lower rows) and staining with Safranin O (upper row; bars = 1,000 μm). Group A, osteochondral defect only (defect/no treatment); Group B, EP2 agonist (EP2 Ag); Group C, EP2 antagonist (EP2 An); Group D, chondrocyte sheets (ChSh); Group E, chondrocyte sheets and an EP2 agonist (ChSh + EP2 Ag); and Group F, chondrocyte sheets and an EP2 antagonist (ChSh + EP2 An). In Group A, stainability with Safranin O was minimal, and the defects were repaired with fibrous tissue. In Group B, the implants were partially replaced with fibrous tissue and bone. Group C shows no staining and fibrous tissue repair. In Groups D and E, repair with hyaline cartilage was achieved, and the condition of the graft tissue, structural consistency, defect filling rate, and subchondral bone formation were all good. In Group F, the formation of convex repaired tissue was inferior to that of Groups D and E, but it was considered good repair. In Groups D, E, and F, type II collagen expression (brown) was observed in tissues that also stained with Safranin O. Type II collagen was expressed uniformly in the surrounding cells (images in the lower rows), which made the cartilaginous repair tissue borders clearer. By contrast, in Groups A, B, and C, no type II collagen expression was observed in the part of the defect that had been replaced with fibrous tissue. Significant differences were found in Group A compared with Groups B, D, E and F; in Group B compared with Groups C, D, E, and F; and in Group C compared with Groups D, E, and F (all $p < 0.05$) [Colour figure can be viewed at wileyonlinelibrary.com]

immunostaining, the slides were counterstained with Mayer's hematoxylin to increase cell visibility.

In the histological evaluation, scoring was performed by three independent blinded examiners using a modified form for Safranin O staining outcome as reported (O'Driscoll, Keeley, & Salter, 1998) and the ICRS grading system (Brehm et al., 2006; Mainil-Varlet, Aigner, Brittberg, et al., 2003). This system can be used to evaluate repaired tissue based on 11 items: tissue morphology, matrix staining, structural integrity, cluster formation, tidemark opening, bone formation, histological appraisal of surface architecture, histological appraisal of the degree of defect filling, lateral integration of defect-filling tissue, basal integration of defect-filling tissue, and histological signs of inflammation. The total score range is from 11 (no repair) to 45 (normal articular cartilage).

2.7 | Statistical analysis

Analysis of variance was used to analyse the weight distribution ratio 12 weeks after surgery and the histological appraisal scores. The Tukey–Kramer method was used for post hoc testing. The results are expressed as the mean ± standard error, and $p < 0.05$ was considered significant.

3 | RESULTS

3.1 | Rate of loading

Figure 2 shows the weight distribution ratio (mean ± standard error) at Weeks 1, 2, 3, 4, 6, 8, 10, and 12 after surgery. Pain-alleviating effects were observed from the onset in the chondrocyte sheet transplantation groups (Groups D, E, and F). Groups A and C showed little improvement: for Group A, from 36.2% ± 0.4% to 40.5% ± 0.2% and for Group C, from 36.3% ± 0.4% to 38.7% ± 0.7% in Week 12 compared with Week 1 after surgery. Group B exhibited some improvement: 35.7% ± 0.6% to 43.4% ± 0.7%. Groups D, E, and F showed greater improvement in the ratios: Group D, 39.5% ± 0.5% to 48.6% ± 0.6%; Group E, 40.5% ± 0.4% to 49.1% ± 0.3%; and Group F, 40.4% ± 0.3% to 46.8% ± 0.4%. Groups D, E, and F exhibited a significantly higher ratio than Groups A, B, and C at 1 week after surgery. Figure 3 shows the damaged limb weight distribution ratios at Week 12 after surgery. At 12 weeks after surgery, pain-alleviating effects were also observed in the chondrocyte sheet transplantation groups.

3.2 | Histology of repaired tissues

The operations were uneventful and all of the rabbits were returned to the cage and allowed to act freely. There were no signs of infection. Twelve weeks after surgery, six knees from each group were evaluated after the animals had been sacrificed. Figure 4 shows a histological image of repaired tissue stained with Safranin O.

In these rabbits, the total score range using the ICRS histological grading system was 13–41 (Table 1). Table 2 shows the ICRS grading system results 12 weeks after surgery. The results were Group A, 14.8 ± 0.5; Group B, 25.4 ± 0.8; Group C, 13.7 ± 0.3; Group D, 40.2 ± 0.5; Group E, 40.5 ± 0.4; and Group F, 38.7 ± 0.5. The chondrocyte sheet transplantation groups exhibited significantly higher scores than Groups A, B, and C for every item except the histological signs of inflammation.

TABLE 1 ICRS Histological grading system

Item	Description
Ti: Tissue morphology	1: Exclusively not cartilage 2: Mostly not cartilage 3: Mostly fibrocartilage 4: Mostly hyaline cartilage
Matx: Matrix staining	1: None 2: Slight 3: Moderate 4: Strong
Stru: Structural integrity	1: Severe disintegration 2: Cysts or disruptions 3: No organization of chondrocytes 4: Beginning of columnar organization of chondrocytes 5: Normal, similar to healthy mature cartilage
Clus: Chondrocyte clustering in implant	1: 25–100% of the cells clustered 2: <25% of the cells clustered 3: No clusters
Tide: Intactness of the calcified cartilage layer, formation of tidemark	1: <25% of the calcified cartilage layer intact 2: 25–49% of the calcified cartilage layer intact 3: 50–75% of the calcified cartilage layer intact 4: 76–90% of the calcified cartilage layer intact 5: Completely intact calcified cartilage layer
Bform: Subchondral bone formation	1: No formation 2: Slight 3: Strong
SurfH: Histological appraisal of surface architecture	1: Severe fibrillation or disruption 2: Moderate fibrillation or irregularity 3: Slight fibrillation or irregularity 4: Normal
FilH: Histological appraisal defect filling	1: <25% 2: 26–50% 3: 51–75% 4: 76–90% 5: 91–110%
Latl: Lateral integration of implanted material	1: Not bonded 2: Bonded at one end or partially both ends 3: Bonded at both sides
Basl: Basal integration of implanted material	1: <50% 2: 50–70% 3: 70–90% 4: 91–100%
InfH: Inflammation	1: No inflammation 3: Slight inflammation 5: Strong inflammation
Maximum total	45 points

Note. This system evaluates repaired tissue based on 11 items: tissue morphology (Ti); matrix staining (Matx); structural integrity (Stru); cluster formation (Clus); tidemark opening (Tide); bone formation (Bform); histological appraisal of surface architecture (SurfH); histological appraisal of the degree of defect filling (FilH); lateral integration of defect-filling tissue (Latl); basal integration of defect-filling tissue (Basl); and histological signs of inflammation (InfH). The total score range was 11–45. ICRS: International Cartilage Repair Society.

TABLE 2 ICRS grades 12 weeks after surgery

	A Defect/no treatment	B EP2 Ag	C EP2 An	D ChSh	E ChSh + P2 Ag	F ChSh + EP2 An
Tissue morphology (Ti)	1.11 ± 0.07	2.39 ± 0.18	1.06 ± 0.06	3.95 ± 0.06	3.84 ± 0.09	3.78 ± 0.10
Matrix staining (Matx)	1.06 ± 0.05	2.22 ± 0.17	1.00 ± 0.00	3.89 ± 0.08	3.84 ± 0.09	3.78 ± 0.10
Structural integrity (Stru)	1.33 ± 0.16	2.61 ± 0.18	1.17 ± 0.09	4.44 ± 0.22	4.56 ± 0.19	4.11 ± 0.10
Cluster formation (Clus)	1.06 ± 0.05	2.00 ± 0.00	1.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00
Tidemark opening (Tide)	1.28 ± 0.10	2.61 ± 0.19	1.22 ± 0.10	4.22 ± 0.21	4.11 ± 0.14	4.00 ± 0.00
Bone formation (Bform)	1.22 ± 0.10	2.00 ± 0.00	1.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00
Histologic appraisal of surface architecture (SurfH)	1.11 ± 0.07	2.06 ± 0.06	1.00 ± 0.00	3.78 ± 0.10	3.84 ± 0.11	3.22 ± 0.10
Histologic appraisal of the degree of defect filling (FilH)	1.44 ± 0.18	2.44 ± 0.17	1.22 ± 0.18	3.95 ± 0.10	4.28 ± 0.09	3.95 ± 0.10
Lateral integration of defect-filling tissue (Latl)	1.11 ± 0.09	2.11 ± 0.07	1.00 ± 0.00	3.33 ± 0.20	3.50 ± 0.20	3.56 ± 0.12
Basal integration of defect-filling tissue (Basl)	1.11 ± 0.08	2.00 ± 0.00	1.00 ± 0.00	3.67 ± 0.21	3.61 ± 0.21	3.11 ± 0.08
Histologic signs of inflammation (InfH)	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00
Total scores (Hgtot)	14.8 ± 0.54	25.4 ± 0.82	13.7 ± 0.29	40.2 ± 0.51	40.5 ± 0.42	38.7 ± 0.50

Note. Group A, osteochondral defect only (defect/no treatment); Group B, EP2 agonist (EP2 Ag); Group C, EP2 antagonist (EP2 An); Group D, chondrocyte sheets (ChSh); Group E, chondrocyte sheets and an EP2 agonist (ChSh + EP2 Ag); and Group F, chondrocyte sheets and an EP2 antagonist (ChSh + EP2 An). ICRS: International Cartilage Repair Society.

3.3 | Immunohistochemistry

Figure 4 shows immunostained repaired tissue 12 weeks after surgery. In Groups D, E, and F, type II collagen expression (brown) was observed in tissue that also stained with Safranin O. No type II collagen expression was observed in the defect in Groups A, B, and C. Type I collagen expression was observed in the superficial portion of fibrocartilage and all layers of subchondral bone.

4 | DISCUSSION

In this study, we confirmed that intra-articular injection of the EP2 agonist achieved better pain alleviation and tissue repair, as shown by the significant differences in these variables between Groups A and B, and between Groups B and C, and the lack of significant differences between Groups D and E. These findings suggest that intra-articular administration of the EP2 agonist was inferior to chondrocyte sheet transplantation for both pain alleviation and tissue repair. Although the differences between Groups A and C were not significant, there were trends toward better pain alleviation and tissue repair for intra-articular injection of the EP2 antagonist.

PGE2 works through four isoforms of the EP receptor, EP1 to EP4, and EP2 receptor-mediated PGE2 signalling stimulates the proliferation of chondrocytes and promotes the regeneration of articular cartilage in rabbits using a gelatin hydrogel containing an EP2 agonist (Mitsui et al., 2011; Otsuka et al., 2009). Aoyama et al. have previously demonstrated that EP2 is the major PGE2 receptor in articular chondrocytes (Aoyama et al., 2005). In addition, EP2 agonists prevent the degeneration of articular cartilage by inhibiting MMP13 in rabbits (Mitsui et al., 2011; Otsuka et al., 2009). However, the use of such scaffolds has problems in terms of the long-term results and complications and involves many biocompatibility issues (Hunziker, 2001; Mainil-Varlet et al., 2001).

In this study, the repeated intra-articular injection of the EP2 agonist and concomitant use of the EP2 agonist (Group B) and chondrocyte sheet transplantation (Groups D, E, and F) produced significantly better pain alleviation and tissue repair compared with no treatment (Group A). The chondrocyte sheet transplantation groups (Groups D, E, and F) obtained pain relief from 1 week after surgery, which suggests that the early pain-alleviating effects resulted from the chondrocyte sheet transplantation.

One limitation of this study is that the results from the pain and histological evaluations used were inconclusive about whether the EP2 agonist had a synergistic effect with chondrocyte sheets in repairing articular cartilage. No significant differences were observed between Groups D and E in this study, and further studies are necessary to clarify the mechanism of EP2 agonists and whether a synergistic effect exists.

We have performed clinical studies of joint repair using autologous chondrocyte sheets and have achieved excellent results. However, we still do not understand fully the mechanisms responsible for the effects of chondrocyte sheets and the factors that reinforce or weaken these effects on pain alleviation and tissue repair. Hamahashi et al. have reported significantly greater secretory capacities of TGFβ and PGE2 in layered chondrocyte sheets fabricated using a coculture method. They found that the chondrocyte sheets provide a barrier function and that the abundant secretion of humoral factors produced by the sheets supports the efficacy of chondrocyte sheets in cartilage repair (Hamahashi et al., 2015). Signs of improved symptoms and magnetic resonance imaging findings indicating cartilage repair were evident from 3 months after surgery for autologous chondrocyte sheet transplantation in our previous studies (data not shown).

There are structural and temporal differences in the formation of chondrocyte sheets between animal species (Kokubo et al., 2013). New treatments that can be used in combination with chondrocyte sheet transplantation are needed to provide stable results from a very early stage after chondrocyte sheet transplantation. Our data suggest that intra-articular administration of an EP2 agonist may be effective in repairing articular cartilage while synergy with chondrocyte sheet transplantation needs to be further investigated. In the early

post-operative period, the barrier function of the chondrocyte sheets may provide some pain relief, but this needs to be confirmed by further studies. PGE2 and TGFβ also have an inhibitory effect on T cell proliferation (Aggarwal & Pittenger, 2005; Di Nicola et al., 2002). By counteracting immune rejection, such as graft-versus-host disease, this inhibitory effect may be an advantage and provide a clinical application. We plan to investigate further the protocols and safety of administration of EP2 agonists.

5 | CONCLUSIONS

We have confirmed the effectiveness of intra-articular administration of an EP2 agonist in articular cartilage repair. No synergistic effect with chondrocyte sheets was observed.

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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