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Single-stage cell-based cartilage repair in a rabbit model: cell tracking and *in vivo* chondrogenesis of human umbilical cord blood-derived mesenchymal stem cells and hyaluronic acid hydrogel composite[1]

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SUMMARY

Objective: Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) have gained popularity as a promising cell source for regenerative medicine, but limited *in vivo* studies have reported cartilage repair. In addition, the roles of MSCs in cartilage repair are not well-understood. The purpose of this study was to investigate the feasibility of transplanting hUCB-MSCs and hyaluronic acid (HA) hydrogel composite to repair articular cartilage defects in a rabbit model and determine whether the transplanted cells persisted or disappeared from the defect site.

Design: Osteochondral defects were created in the trochlear grooves of the knees. The hUCB-MSCs and HA composite was transplanted into the defect of experimental knees. Control knees were transplanted by HA or left untreated. Animals were sacrificed at 8 and 16 weeks post-transplantation and additionally at 2 and 4 weeks to evaluate the fate of transplanted cells. The repair tissues were evaluated by gross, histological and immunohistochemical analysis.

Results: Transplanting hUCB-MSCs and HA composite resulted in overall superior cartilage repair tissue with better quality than HA alone or no treatment. Cellular architecture and collagen arrangement at 16 weeks were similar to those of surrounding normal articular cartilage tissue. Histological scores also revealed that cartilage repair in experimental knees was better than that in control knees. Immunohistochemical analysis with anti-human nuclear antibody confirmed that the transplanted MSCs disappeared gradually over time.

Conclusion: Transplanting hUCB-MSCs and HA composite promote cartilage repair and interactions between hUCB-MSCs and host cells initiated by paracrine action may play an important role in cartilage repair.

Introduction

Articular cartilage has very limited capacity for repair, probably because of its avascular nature and its specialized structure and composition. Current clinical strategies to repair articular cartilage defects are implanting *ex vivo*-expanded autologous chondrocytes or promoting an endogenous healing mechanism by stimulating bone marrow (microfracture technique)[1–3]. However, the quality of the repaired tissue is still far from ideal because it contains a large percentage of fibrocartilage rather

[1] This study was performed at Samsung Medical Center.

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than hyaline cartilage[4]. Therefore, a means of bringing about predictable and durable of cartilage regeneration remains an unmet clinical need.

Mesenchymal stem cells (MSCs) have gained popularity as a promising source for regenerative medicine because of their capacity for self-renewal, multi-lineage differentiation potential, and immunomodulatory[5–8]. MSCs can be isolated from various tissues[9–12], including the human umbilical cord. Human umbilical cord blood-derived MSCs (hUCB-MSCs) are easy to obtain, can be non-invasively collected, and have a good capacity for expansion[13,14]. In addition, some evidence suggests immunomodulatory functions and the presence of the nursing effect[15,16]. Based on these findings, hUCB-MSCs may be an appropriate source of MSCs for allogeneic transplantation.

A limited number of *in vivo* studies have reported cartilage repair with hUCB-MSCs[17–20]. Some studies have investigated the chondrogenic differentiation potential of hUCB-MSCs *in vitro*[21–25]. We previously demonstrated that transplanted hUCB-MSCs and hyaluronic acid (HA) hydrogel composite repaired articular cartilage remarkably well in a rat model[18,19]. Considering the application of this novel cartilage regenerative option to a human clinical trial, it was necessary to confirm the results in larger animals. In addition, the fate of transplanted hUCB-MSCs in repair tissue of defect area was not been investigated before. Despite growing information regarding MSCs and their use in cell-based cartilage repair, the roles of MSCs in cartilage repair are not well-understood and remain to be investigated.

Here, we investigated whether transplanting an hUCB-MSCs and HA hydrogel composite resulted in favorable cartilage repair in a rabbit model. In addition, we investigated the fate of transplanted hUCB-MSCs in repaired tissue in the defect area using an antihuman nuclear antibody. We hypothesized that transplanting an hUCB-MSCs and HA hydrogel composite would promote cartilage repair and would produce significantly better results compared to no treatment or transplanting the HA hydrogel alone. We also hypothesized that the cells would disappear as time passed.

Methods

Isolation and culture of hUCB-MSCs

hUCB was collected from umbilical veins after neonatal delivery by an independent cord blood bank with informed consent from the pregnant mother. The hUCB-MSCs were prepared according to the proper manufacturing practices at an approved cord blood bank. The MSCs were isolated and cultured as described previously[10] and donated for this animal study. The MSCs used for the present study was selected not based on the donor characteristics but on the characteristics of the cells. Approximately a quarter of the UCB harvests yielded MSCs under the culture conditions[10]. Among the many population of MSCs thus yielded, the MSCs population which showed good proliferation property as well as good potential in tri-lineage differentiation was selected for the present study. The selection criterion was recently set with the secretion level of TSP-2 during expansion culture based on the results of later studies[26,27]. The MSCs population used for the present study was found to be adequate by the TSP-2 criterion as well. The delivering mother of the MSCs for this study was 35 years old. In the present study, we used MSCs from a single donor to avoid the donor related variation. Mononuclear cells were isolated by density-gradient centrifugation at 550 × *g* for 30 min using Ficoll–Hypaque (density, 1.077 g/ml; Sigma, St. Louis, MO, USA). The separated mononuclear cells were cultured in α-minimum essential medium (α-MEM, Gibco BRL, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; HyClone, Logan, UT, USA). The culture media was changed twice weekly. Fibroblast-like adherent cells were observed 1–3 weeks after initial culture, when the cells reached 80% confluency. At that point, the cells were trypsinized (0.25% trypsin, HyClone) and resuspended in culture medium (α-MEM supplemented with 10% FBS, 1% antibiotics). All hUCB-MSCs used were at passage 6.

Preparation of hUCB-MSCs and the HA composite

The HA hydrogel was prepared by dissolving HA (Hyal 2000®, LG Life Science, Daejeon, South Korea) in α-MEM. A composite of 4% HA and hUCB-MSCs (0.5×10^7 cells/mL) was mixed thoroughly and transplanted into the experimental knees, whereas HA alone was used for the control knees. The cell concentration used in the present study was selected based on the results of our previous studies using various cell concentrations, because it showed the best result in terms of cartilage repair. The cells expressed CD105 and CD73, but did not express CD34, CD45, CD14, or HLA-DR, as is characteristic of the desired cell population.[10]

Animals

Forty healthy New Zealand white male rabbits (weight, 3.0–3.5 kg) were used in this study. All animals were obtained 1 week before the experiment and were raised in the same environment. All procedures and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at our institution (Samsung Medical Center, Seoul, Korea). This study followed the National Institutes of Health guidelines regarding the care and use of laboratory animals.

In vivo transplantation

The rabbits were anesthetized, both knee joints were draped sterilely and opened using a medial parapatellar approach, and the patella was dislocated laterally. The intra-articular structures were thoroughly inspected to detect any abnormalities, such as infection or deformities. Full-thickness osteochondral defects (3 mm in diameter and 3 mm in depth, which is a critical-sized defect) were created in the trochlear groove of the femur by careful drilling in a vertical direction. To avoid thermal denaturation, we applied normal saline irrigation while making the defects with the motorized drill. After removing cartilage and bone debris, the boundaries of the drilled holes were trimmed using a surgical knife, and the defect sites were carefully washed. A composite of hUCB-MSCs (0.5×10^7 cells/mL) and 4% HA hydrogel was transplanted into the full-thickness defect in the experimental knee (right knee), whereas 4% HA hydrogel without hUCB-MSCs was transplanted into the control knee (left knee) ($n = 10$ for evaluation after 8 weeks, $n = 10$ for evaluation after 16 weeks). The defect was left untreated as a control ($n = 5$ for 8 weeks and $n = 5$ for 16 weeks). For cell tracking evaluation at 2 and 4 weeks, a composite of hUCB-MSCs (0.5×10^7 cells/mL) and 4% HA hydrogel was transplanted into both knees ($n = 5$ for 2 weeks and $n = 5$ for 4 weeks). Following transplantation, patellae were re-located, and the soft tissues were closed in layers. All rabbits were allowed to move their knee joints freely in their cages without restriction, and clinical signs were observed daily. Animals were sacrificed at 8 and 16 weeks post-transplantation to evaluate articular cartilage repair (10 rabbits at 8 weeks and 10 at 16 weeks); some were instead sacrificed at 2 and 4 weeks to evaluate the fate of transplanted cells (5 rabbits at 2 weeks and 5 at 4 weeks). No animal was excluded owing to an abnormal clinical finding.

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Macroscopic evaluation

An arthrotomy was performed post-mortem in the same manner as during transplantation to re-inspect the intra-articular structure. The condition of the structure was assessed; we looked for evidence of rejection or infection, severe inflammation, extensive fibrosis, or any other abnormality in the joint. Then, the degree of articular cartilage repair was grossly assessed using the International Cartilage Repair Society (ICRS) macroscopic evaluation system[28]. The parameters included degree of defect repair, integration into the border zone, and macroscopic appearance.

Histological and immunohistochemical evaluation

Full-thickness samples (cartilage and bone) were taken from each group at 8 and 16 weeks post-transplantation for histological analysis. The samples were fixed in 10% formalin, decalcified in Immunocal™ (Decal Corp., Tallman, NY, USA) for 3 days, dehydrated in a graded ethanol series, and embedded in paraffin wax. The paraffin-embedded sections (4 μm) were cut, deparaffinized, and stained with hematoxylin and eosin (H & E) staining, Masson's trichrome, Safranin-O, and Sirus red, and subjected to immunohistochemistry for type-II collagen. Detailed descriptions of the staining are provided in the online Supplemental material.

The sections were semi-quantitatively analyzed with a modified O'Driscoll score[29]. The nature of the predominant tissue (cellular morphology and safranin O staining of the matrix), structural characteristics (surface regularity, structural integrity, thickness, and extent of bonding to the adjacent cartilage), freedom from cellular changes of degeneration (hypocellularity and chondrocyte clustering) and freedom from degenerative changes in adjacent cartilage were analyzed. All samples were scored independently by two observers.

Cell tracking using anti-human nuclear antibody

Full-thickness samples were taken from each group at 2, 4, 8, and 16 weeks post-transplantation for cell tracking analysis. The paraffin was removed from the slides and the specimens were rehydrated. The slides were washed three times with 0.5% Triton-X100 in PBS for 15 min and then with proteinase K (Dako, Carpentaria, CA, USA) for 20 min. The slides were then washed three times with 0.5% Triton-X100 in PBS for 5 min and incubated with 2% bovine serum albumin in PBS for 30 min. A mouse anti-human nuclei monoclonal antibody (MAB1281; Chemicon, Temecula, CA, USA) was applied to tissues, which were then incubated in a moist chamber for 1 h, and washed three times with 0.1% Tween-20 in PBS for 5 min. Anti-mouse IgG was applied and the slides were incubated in a moist chamber for 1 h. The Slides were mounted and scanned using ScanScope AT (Leica Biosystems Nussloch GmbH, Nussloch, Germany). Human placental tissue was incubated with mouse IgG after preprocessing the mouse anti-human nuclei antibody in the positive control, whereas human placenta tissue was incubated with mouse IgG without preprocessing the mouse anti-human nuclei antibody in the negative control. Images were captured by bright field microscopy at 20× microscopic magnification, and five images from randomly selected visual fields were captured from each slide for quantification. Human nuclei positive cells were counted using Image J v1.45 software (http://rsb.info.nih.gov/ij/) by three observers blinded to the samples.

Statistical analysis

The two-tailed Mann–Whitney test was used to compare the macroscopic and histological evaluations between the transplanted and control groups using SAS 9.3 software (SAS Institute, Cary, NC, USA). A P-value < 0.05 was considered significant.

Results

Macroscopic findings

No abnormal findings suggesting rejection or infection, such as severe inflammation or extensive fibrosis, were observed. The experimental knees were more transparent and brighter in appearance than were the control knees [Fig. 1(A) and (B)]. The surfaces of the repaired tissue in the experimental knees were relatively smooth and had fewer depressions compared to those in the control knees. The repaired tissue filled the defect areas poorly, and numerous spaces and cleavages were observed throughout the repaired tissue in the control knees. The ICRS gross repair assessment scores of the experimental knees at 8 and 16 weeks post-transplantation were higher than those of the control knees [4.5 (95% confidence interval 2.4–6.6) in the control group vs 6.6 (95% confidence interval 6.0–7.5) in the hUCB-MSCs group at 8 weeks; $P = 0.110$, 5.0 (95% confidence interval 3.2–6.3) in the control group vs 7.8 (95% confidence interval 7.1–8.4) in the hUCB-MSCs group at 16 weeks; $P < 0.003$, Fig. 1(C)]. In experimental knees, the surfaces of the repaired tissue examined at 16 weeks compared to those examined at 8 weeks showed fewer depressions and less distinct borders with the normal surrounding articular cartilage [Fig. 1(A) and (B)].

Histological and immunohistochemical findings

The defects repaired with the hUCB-MSCs and HA composite exhibited a restoration of normal articular contours to the level of the surrounding normal cartilage at 8 weeks post-transplantation [Fig. 2(A) and (Ai)]. The deep portion of the repaired tissue was not completely replaced by subchondral bone, which contributed to the appearance of a thick cartilage layer at the repair site. The cells in the repaired tissue were round with lacunae in the deep portion but somewhat flat in the superficial zone [Fig. 3(A) and (Ai)]. The border area was fully filled with repaired tissue without any significant gaps [Fig. 2(A)]. The defects in the control knees were also restored to the level of the surrounding normal tissues, although a significant gap was noted in the border area. The cells in the repaired tissue in control knees were smaller, with fewer lacunae, and were not morphologically different in the deep and superficial areas [Fig. 3(Ai)].

The overall architecture of the repaired tissue in the experimental knees was similar to that of normal cartilage at 16 weeks post-transplantation [Fig. 2(B)]. The deep areas were mostly replaced by subchondral bone, and the articular surface was smooth without any gaps with the surrounding normal articular cartilage [Fig. 2(Bi)]. The cellular architecture and arrangement were similar to those of the surrounding normal cartilage. Round cells were grouped, had lacunae, and were perpendicular to the subchondral bone in the deep zone, whereas flat cells were parallel to the articular surface in the superficial zone [Fig. 3(B) and (Bi)]. The overall contours of the repaired tissue in the control knees was restored at 16 weeks post-transplantation, but the surfaces of the repaired tissue were irregular, and a significant gap was noted in the border area [Fig. 2(B)]. The cells in the repaired tissue were irregularly arranged and the deep repaired tissue had not been replaced by subchondral bone, as it had in the experimental knees [Fig. 3(Bi)].

Type II collagen immunohistochemical staining revealed weakly positive staining in the repaired tissue of the experimental knees at 8 weeks post-transplantation, whereas the control knees were

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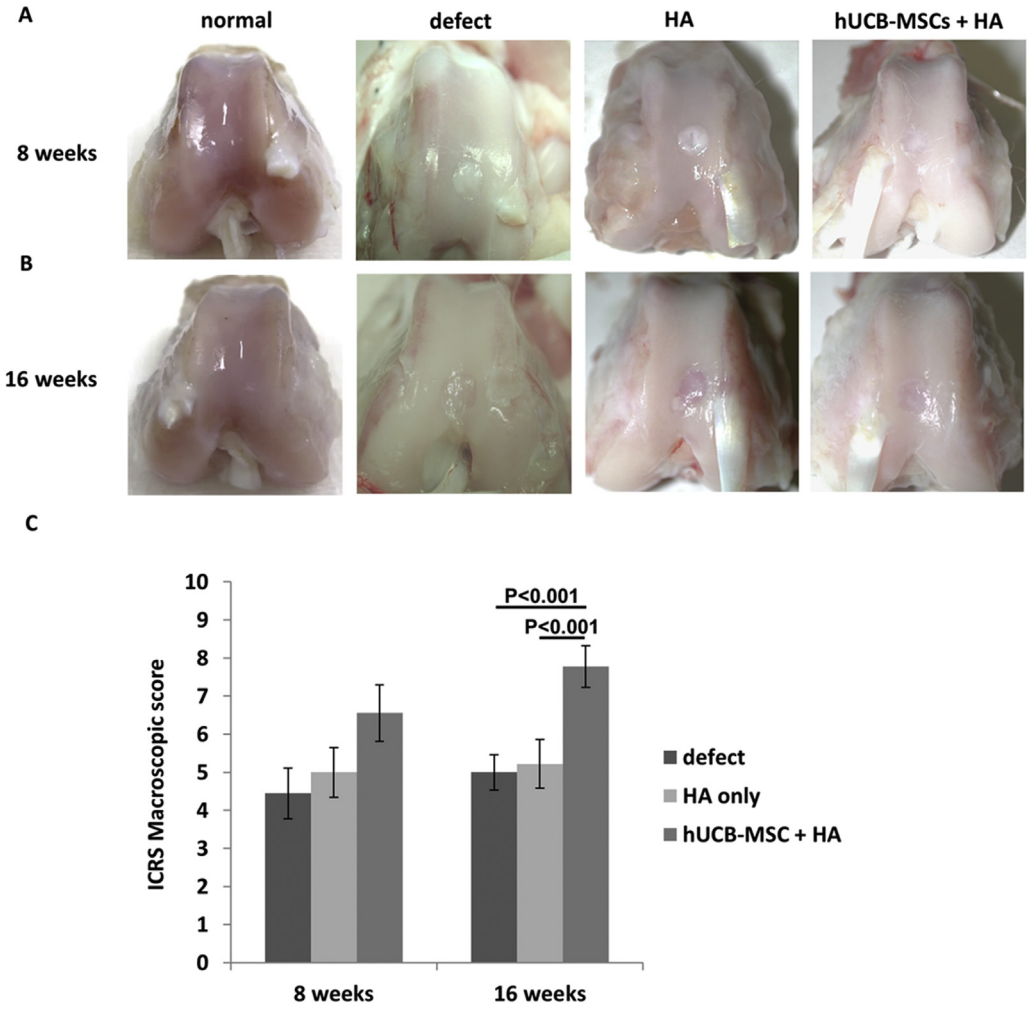

Fig. 1. Gross appearance and assessment results of articular cartilage defects in a rabbit model at 8 and 16 weeks post-transplantation. **(A)**: Experimental knees with hUCB-MSCs + HA, control knees with HA only, defect only, and normal knee without defect at 8 weeks. **(B)**: Experimental knees with hUCB-MSCs + HA, control knees with HA only, defect only, and normal knee without defect at 16 weeks. **(C)**: ICRS macroscopic cartilage repair assessment for hUCB-MSCs + HA, HA only, and defect only (10 knees/group). Error bars represent 95% confidence interval.

negative for type II collagen [Fig. 4(A) and (Ai)]. Strong positive type II collagen immunohistochemical staining was noted in the repaired tissue of experimental knees at 16 weeks post-transplantation [Fig. 4(B) and (Bi)], and its density was similar to that of the surrounding normal cartilage. The repaired tissues in the control knees were weakly positively stained, compared to the surrounding normal cartilage tissue [Fig. 4(A) and (B)].

Safranin-O staining revealed chondral differentiation in the repaired tissue. Denser and more reddish staining was noted, without a remarkable gap, in the repaired tissue of experimental knees, whereas less red repaired tissue was observed and there was a remarkable gap in control knees [Fig. 5(A), (Ai), (B) and (Bi)]. The overall collagen arrangement, as shown by Sirius Red staining in the repaired tissue of the experimental knees, was nearly identical to the that of the surrounding normal cartilage tissue (Fig. 6). Most of the collagen fibers were arranged perpendicularly, whereas those in the superficial area were horizontal and parallel to the articular cartilage.

The semi-quantitative O'Driscoll score analysis revealed that the repaired tissue in the experimental knees was histologically superior to that in the control knees (Fig. 7). At 16 weeks post-transplantation, the scores from experimental knee were statistically higher overall (10.7 (95% confidence interval 9.6–11.9) in controls vs 16.9 (95% confidence interval 15.6–18.2) in hUCB-MSCs-treated knees; $P < 0.001$, and 11.6 (95% confidence interval 10.4–12.6) in HA-treated knees vs 16.9 in hUCB-MSCs-treated knees, $P < 0.001$).

The track of the grafted cells was evaluated by immunohistochemistry using anti-human nuclei antibody after transplanting the cells in the articular cartilage defect region. Anti-human nuclei antibody levels decreased gradually at 2, 4, and 8 weeks post-transplantation [Fig. 8(A)–(C)]. After 16 weeks, anti-human nuclei antibody staining had disappeared [Fig. 8(D)]. The number of human nuclei-positive cells decreased as time went by (1,296.2 (95% confidence interval 1,125.7–1,466.7) at 2 weeks, 538.4 (95% confidence interval 334.2–742.6) at 4 weeks, and 128.4 [95% confidence interval 74.9–181.9) at 8 weeks; Fig. 8(E)]. No signal was detected in the negative control tissue [Fig. 8(F)].

Discussion

Here, we demonstrated that an hUCB-MSCs and 4% HA hydrogel composite resulted in favorable cartilage repair grossly and histologically compared to HA only treatment and no treatment in a rabbit model. The repaired tissue following hUCB-MSCs and HA transplantation was more similar to the surrounding normal

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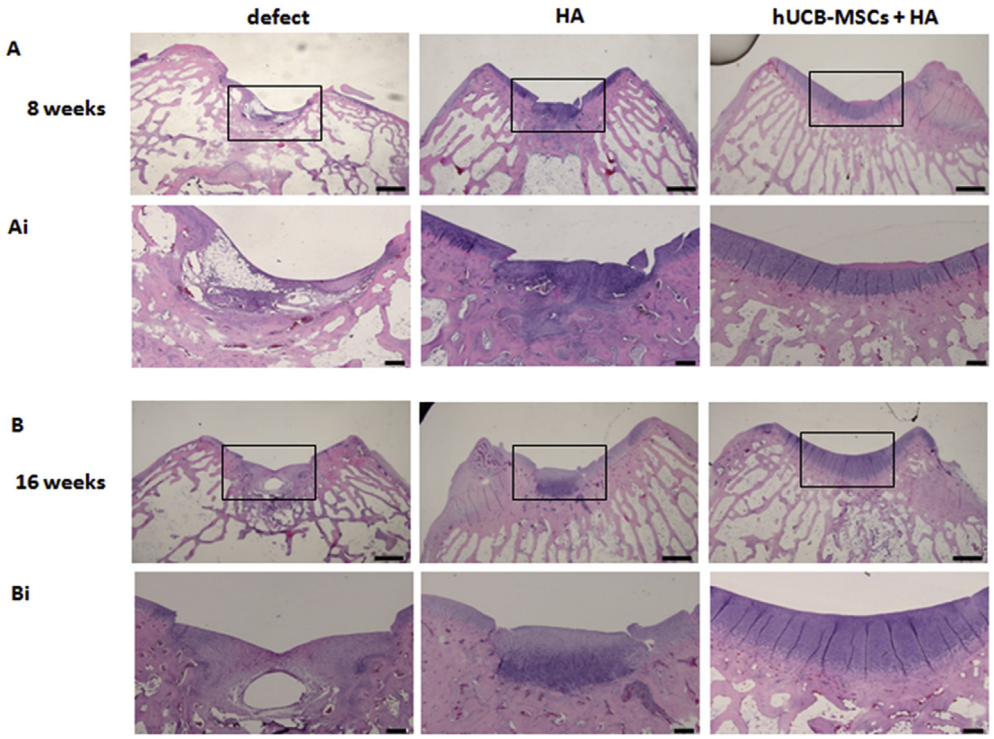

Fig. 2. Microscopic findings of the repair tissue at articular cartilage defect sites in a rabbit (10 knees/group). **(A)**: H & E staining at 8 weeks; ×12.5. Scale bars = 1 μm. **(B)**: H & E staining at 16 weeks; ×12.5. Scale bars = 1 μm. **(Ai, Bi)**: Higher magnification views of the areas boxed in (A, B) respectively; ×40. Scale bars = 200 μm.

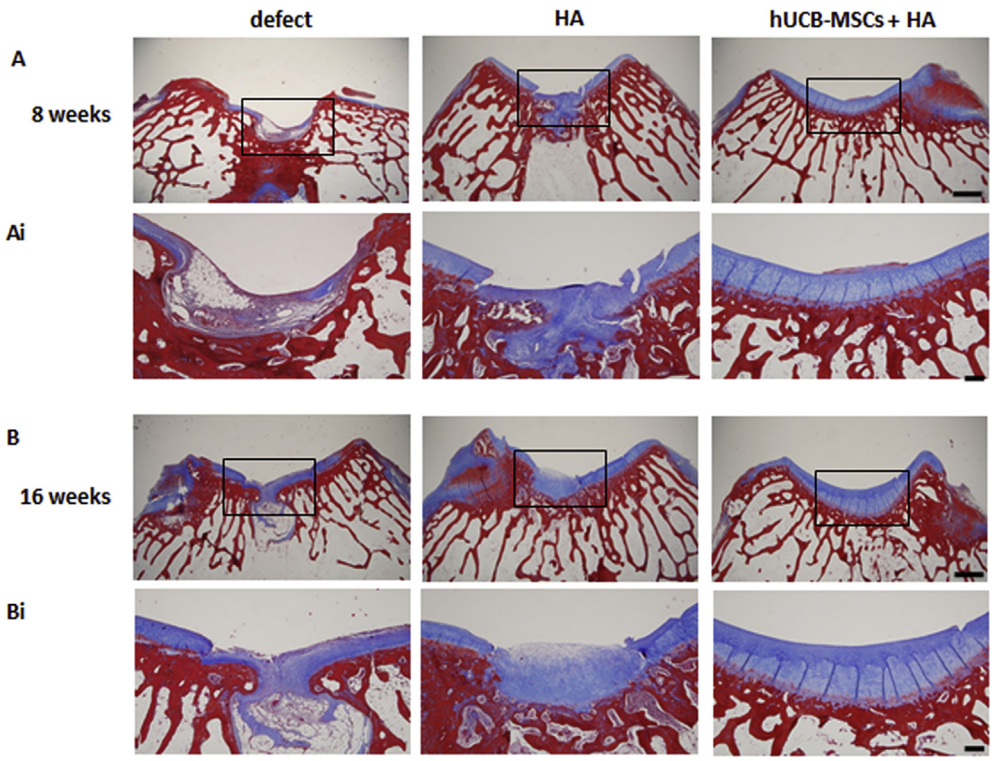

Fig. 3. Microscopic findings of the repair tissue at articular cartilage defect sites in a rabbit (10 knees/group). **(A)**: Masson's trichrome stain at 8 weeks; ×12.5. Scale bars = 1 μm. **(B)**: Masson's trichrome stain at 16 weeks; ×12.5. Scale bars = 1 μm. **(Ai, Bi)**: Higher magnification views of the areas boxed in (A, B) respectively; ×40. Scale bars = 200 μm.

articular cartilage than was the repaired tissue in the controls (HA alone or defect only). The transplanted cells disappeared from the repaired tissue over time, which seems to support paracrine action rather than chondrogenic differentiation of the transplanted MSCs.

Transplanting an hUCB-MSCs and HA hydrogel composite repaired full thickness cartilage defects, as was evidenced both qualitatively and quantitatively. At 16 weeks post-transplantation, cellular architecture and collagen arrangement were almost

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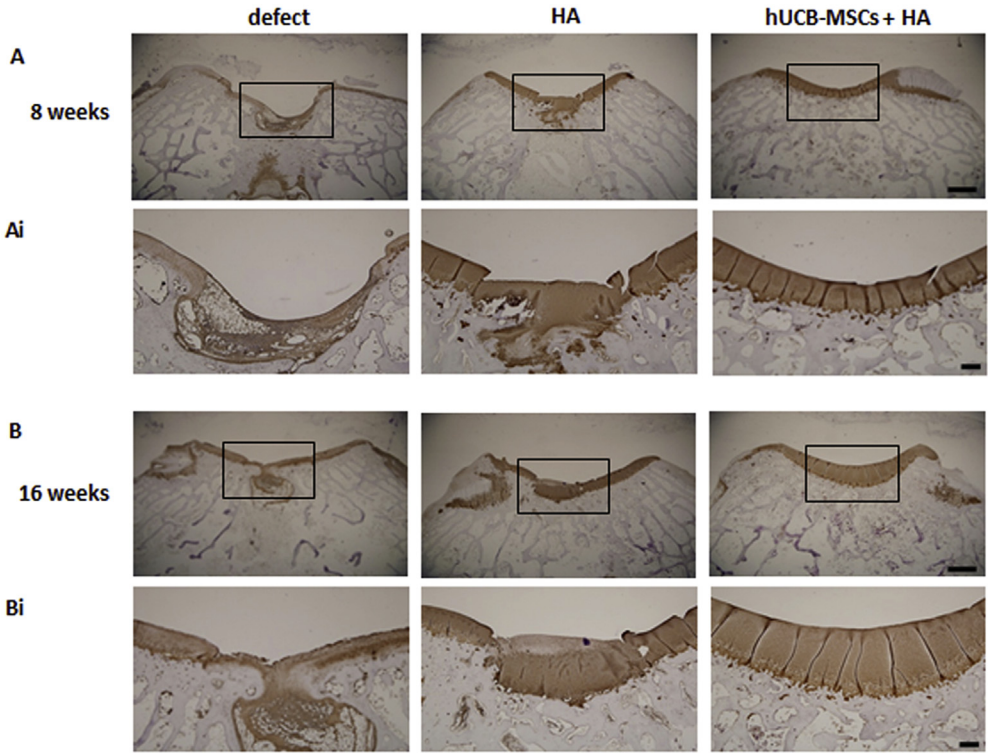

Fig. 4. Microscopic findings of repair tissue at the articular cartilage defect sites in a rabbit (10 knees/group). **(A)**: Type II collagen immunostaining at 8 weeks; ×12.5. Scale bars = 1 μm. **(B)**: Type II collagen immunostaining at 16 weeks; ×12.5. Scale bars = 1 μm. **(Ai, Bi)**: Higher magnification views of the areas boxed in (A, B) respectively; ×40. Scale bars = 200 μm.

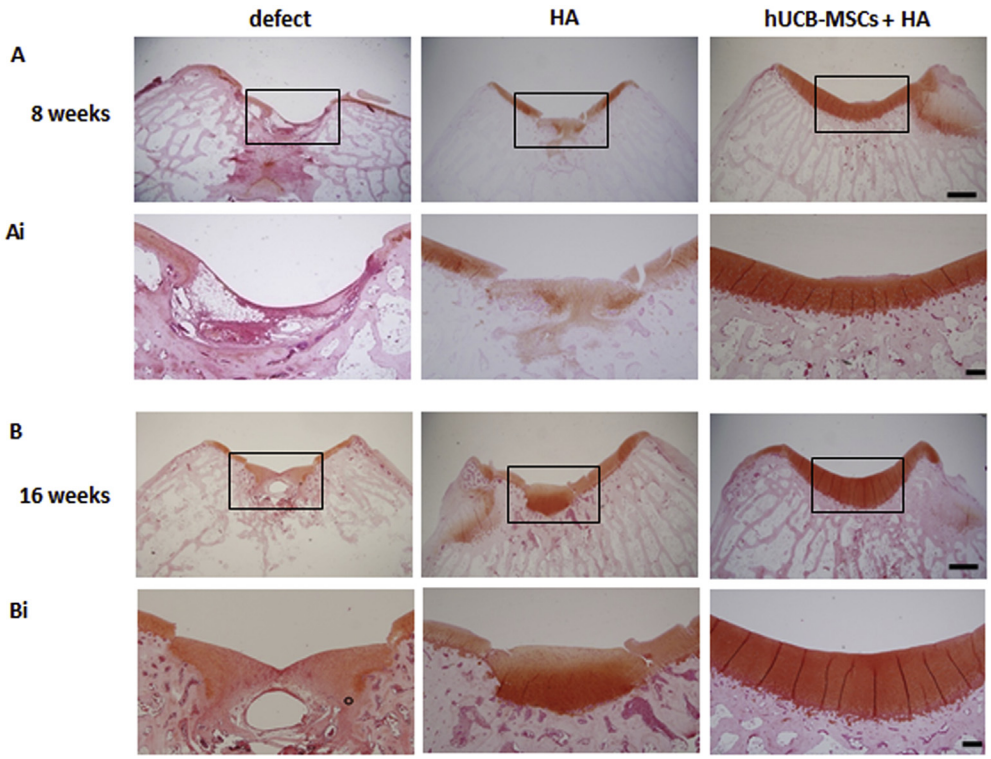

Fig. 5. Microscopic findings of repair tissue at the articular cartilage defect sites in a rabbit (10 knees/group). **(A)**: Safranin-O staining at 8 weeks; ×12.5. Scale bars = 1 μm. **(B)**: Safranin-O staining at 16 weeks; ×12.5. Scale bars = 1 μm. **(Ai, Bi)**: Higher magnification views of the areas boxed in (A, B) respectively; ×40. Scale bars = 200 μm.

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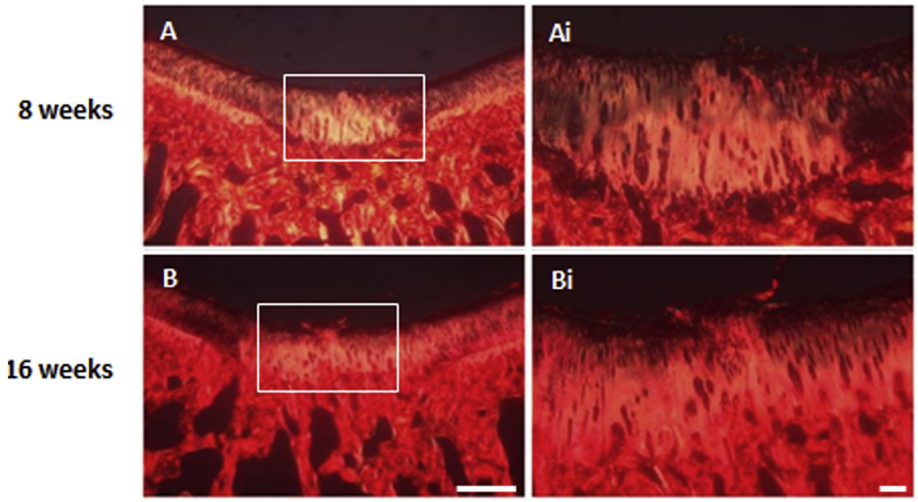

Fig. 6. Microscopic findings of repair tissue at the articular cartilage defect sites in a rabbit (10 knees/group). **(A)**: Sirius Red staining at 8 weeks; ×40. Scale bars = 200 μm. **(B)**: Sirius Red staining at 16 weeks; ×40. Scale bars = 200 μm. **(Ai, Bi)**: Higher magnification views of the areas boxed in (A, B) respectively; ×100. Scale bars = 100 μm.

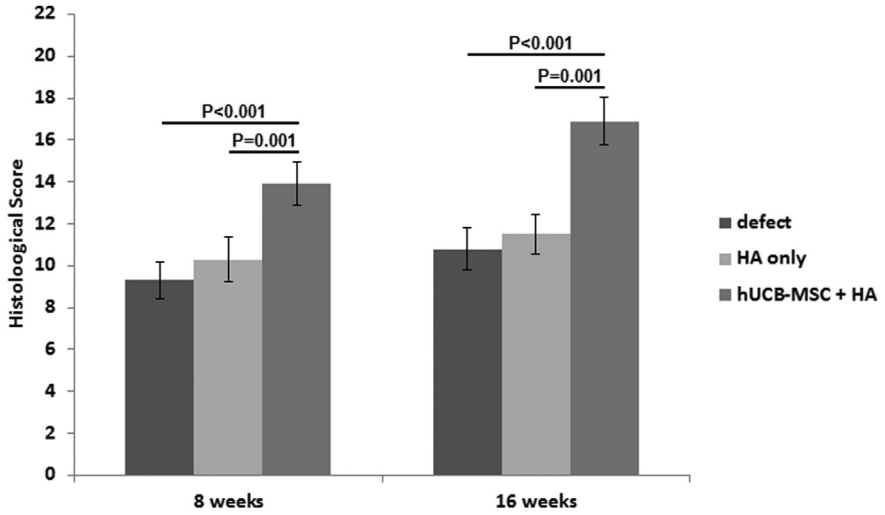

Fig. 7. Semiquantitative analysis of repair tissue at the articular cartilage defect sites in rabbit knee at 8 and 16 weeks (10 knees/group). Sections were histologically evaluated based on a modified O'Driscoll score). Error bars represent 95% confidence interval.

identical to those of the surrounding normal articular cartilage tissue, and the repaired tissue contained a considerable amount of type II collagen. The repaired tissue maintained a rather smooth surface contour without notable gaps with the surrounding normal articular cartilage. The deep portion of the repaired tissue was replaced by subchondral bone. These findings supported the bone and cartilage regeneration potentials of hUCB-MSCs. The transplanted cells in the defect site disappeared gradually. Other studies have investigated the fate of transplanted or injected MSCs in full-thickness cartilage defects or animal osteoarthritis models[11,26]. These studies showed that labeled transplanted MSCs disappear gradually in repaired tissue with time after transplantation. One study reported that transplanted hUCB-MSCs are detectable 4 but not 8 weeks after transplantation[26]. These results are in agreement with our results. The transplanted cells were detectable in the repaired tissue until 8 weeks, but were hardly detectable in the repaired tissue at 16 weeks. Another study reported that the number of transplanted cells disappears gradually, but they are detectable in the cartilage zone at 24 weeks post-transplantation[11]. This long-term observation of transplanted cells does not agree with our results. One possible explanation for the disappearance of the cells is that immune responses after allogeneic or xenogeneic transplantation could lead to clearance of the transplanted MSCs. However, we did not observe any rejection or inflammation-like responses. In addition, the hUCB-MSCs have low immunogenicity and are immunomodulatory *in vitro* and *in vivo*[5,7,30].

The cartilage repair mechanism in this study may be associated with chondrogenic differentiation, the paracrine action of hUCB-MSCs, and their immunomodulatory effects. Several studies have reported that hUCB-MSCs retain chondrogenic differentiation potential[10,15,20,25,31]. The hUCB-MSCs used in this study also showed multi-lineage differentiation potential *in vitro* (data not shown). Besides chondrogenic differentiation of hUCB-MSCs, an unidentified paracrine action between the transplanted hUCB-MSCs and the host may have stimulated chondrogenic differentiation or enhanced cartilage-specific extracellular matrix synthesis by MSCs, as suggested previously[32–34]. One study reported that hUCB-MSCs promote differentiation of chondroprogenitor cells via a paracrine action[26]. In this study, transplanted cells gradually disappeared until 16 weeks after transplantation, which seem to indicate that a

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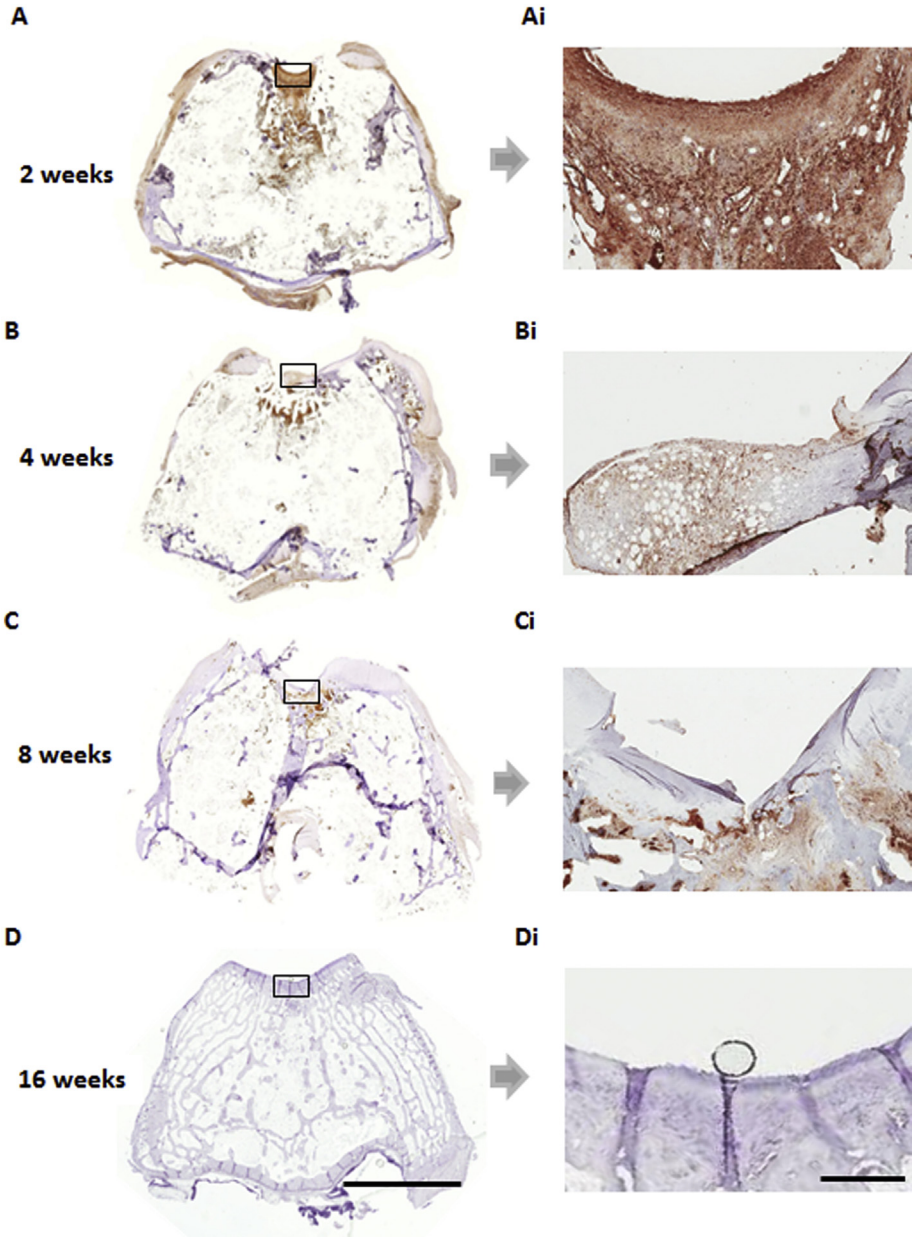

Fig. 8. Cell tracking images using anti-human nuclear antibody staining at 2, 4, 8, and 16 weeks post-transplantation (10 knees/week). **(A)**: 2 weeks. **(B)**: 4 weeks. **(C)**: 8 weeks. **(D)**: 16 weeks; X Fit. Scale bars = 6 mm. **(Ai, Bi, Ci, Di)**: Higher magnification views of the areas boxed in (A–D) respectively; ×4. Scale bars = 500 μm. **(E)**: Quantification of human nuclei staining in cartilage sections. **(F)**: As a negative control, human kidney tissues were incubated in mouse IgG instead of the mouse anti-human nuclei monoclonal antibody. **(F)**: Positive controls were incubated with anti-human nuclei monoclonal antibody. Error bars represent 95% confidence interval.

paracrine interaction between hUCB-MSCs and subchondral progenitor cells may play an essential role in cartilage regeneration. The immunomodulatory properties, particularly the anti-inflammatory effect of hUCB-MSCs, could provide suitable conditions in which the transplanted hUCB-MSCs produced more relevant repair tissue, even in a xenograft trial with immunocompetent animals[35]. Further studies are required to elucidate the details of the cartilage repair mechanism of hUCB-MSCs.

HA hydrogel provides several advantages as a scaffold for cartilage repair. The material used for successful cartilage repair should have appropriate viscoelastic properties, such as compressive strength, friction, and tensile strength, to bear the deformation and motion forces[36]. The mechanical strength of HA is approximately 29–149 KPa[37,38]. In addition, HA hydrogel can maintain transplanted cells at the site of the defect rather than allowing them to disperse. Therefore, HA can be a good scaffold and provide the proper environment for chondrogenesis. Previous studies have demonstrated that HA may inhibit inflammatory factors, reduce cartilage degradation, and suppress interleukin-1β-induced apoptosis of chondrocytes[39–41]. Furthermore, intra-articular HA has been approved for clinical use, promotoes cartilage regeneration, and has a role as a delivery vehicle *in vivo*[42]. Taken together, HA improves the microenvironment in articular cartilage defect sites; thus, enhancing the cartilage repair process following cell transplantation.

In this study, we tried to evaluate the effect of hUCB-MSCs on cartilage repair in a rabbit model. We have been investigating cartilage regeneration potential of hUCB-MSCs and HA hydrogel

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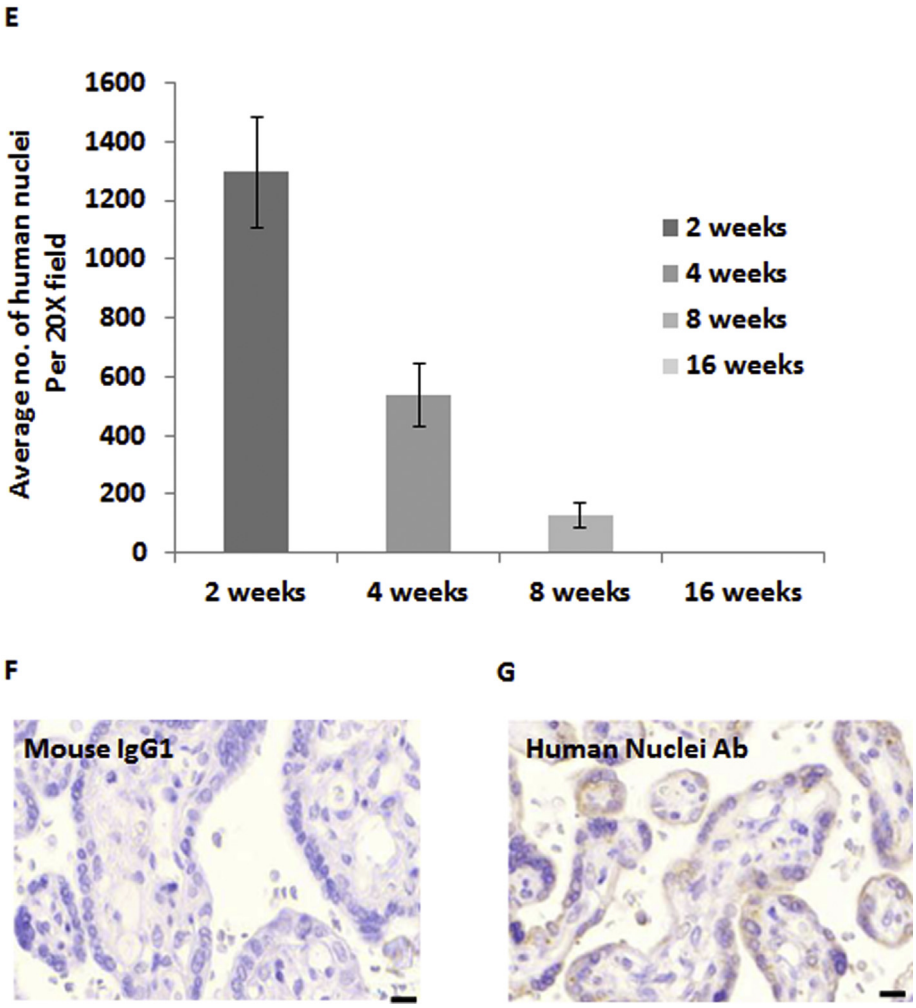

Fig. 8. (*continued*).

composite and attained remarkable results in a rat model[18,19]. In a rat model, hUCB-MSCs with 4% HA hydrogel showed superior cartilage repair grossly and histologically compared to control groups (HA only and defect). The MSCs used in a rat model was prepared by same good manufacturing practice at an approved cord blood bank and donated. Although the cells used for the rat studies were not from the same donor for the present rabbit study, we reached the same conclusion even the hUCB-derived MSCs from different donors were used. The cells used for the rat studies were also selected by the same way we mentioned in the Methods section of the present study, i.e., cells with good proliferation and differentiation potentials, not based on the donor characteristics. Although we recently set the selection criteria based on the TSP-2 secretion level of the MSCs population based on the results of later studies[26,27], this criteria (based on the proliferation and differentiation) for selecting the cells worked well for the rat and rabbit studies, although the cells were not from the same donor. The results of the present study shows effective cartilage repair even in a larger animal model. These consistent results in animal models warrant further investigations for a human clinical trial in the future.

Some limitations of this study need to be addressed. First, the MSCs used in this study were at passage 6, which may be a fairly high passage number. To acquire the hUCB-MSCs used in this study, a cryopreservation and thawing process was necessary. Before cryopreservation, the MSCs isolated from hUCB were cultured to passage 3. To obtain sufficient numbers of hUCB-MSCs after thawing, an additional 3 passages were required. However, UCB-MSCs are known to have a highly capacity for expansion compared to BM-MSCs[43], and the hUCB MSCs at the high passage number used in the present study demonstrated good activity. Based on the results of this study, hUCB-MSCs at passage 6 seem to be suitable for transplantation to treat cartilage defects. Second, the study period of 16 weeks may be short. In another study of ours, however, repaired tissues with an hUCB-MSCs and HA composite at 52 weeks were similar to those at 16 weeks as assessed by the O'Driscoll score (unpublished data) and did not display overgrowth, osseous metaplasia, or tumor formation. Many previous studies done to evaluate cartilage repair using a rabbit model have analyzed tissues at periods of less than 16 weeks[44–47]. Thus, we chose 16 weeks as the end point to evaluate the efficacy of cartilage repair following hUCB-MSCs transplantation. Third, as we performed the present study with only one dose of cells, the findings that the cells gradually disappeared over the course of 16 weeks cannot be generalized to different doses. Based on the results of our previous animal studies, however, we believe that transplanted cells will gradually disappear when administered at different doses. Finally, the mechanism by which MSCs enhanced cartilage repair remains unclear. It remains to be determined whether the transplanted MSCs restored

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cartilaginous tissue directly by chondrogenic differentiation, or whether they supported the cartilage restoration by host cells. These issues should be further investigated in future studies.

In conclusion, we showed that hyaline cartilage can be repaired using a composite of hUCB-MSCs and HA hydrogel. The cellular architecture, collagen arrangement, and quantity of type II collagen in the repaired tissue were very similar to those of the surrounding normal hyaline cartilage. Although further studies are needed to elucidate the precise underlying mechanisms of action, the finding that the transplanted cells disappeared at the defect site indicates that a paracrine interaction between hUCB-MSCs and host cells plays an essential role in cartilage repair. These findings suggest that transplanting hUCB-MSCs and 4% HA hydrogel composite may be a novel therapeutic modality to treat full-thickness cartilage defects. The system used in the present study may be of use to optimize conditions before attempting to repair cartilage defects in humans using such composites.

Author contributions

(1) The conception and design of the study (CWH), acquisition of data (JAK, WJH, JHR, YGP), analysis and interpretation of data (YBP, CWH, JAK, JHR, KJK, YGP), (2) drafting the manuscript (YBP, CWH, JYC), critically revising the manuscript for important intellectual content (YBP, HJL, CWH), and (3) final approval of the version to be submitted (All authors). YBP (whybe1122@gmail.com) and CWH (chulwon.ha@gmail.com) take responsibility for the integrity of this work.

Conflict of interest

All authors declared no conflicts of interest.

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Supplementary data

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