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# Thrombospondin-2 Secreted by Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells Promotes Chondrogenic Differentiation

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**Key Words:** Human umbilical cord blood-derived mesenchymal stem cells • Paracrine action • Osteoarthritis • Secretome • Synovial fluid • Thrombospondin-2

ABSTRACT

**Increasing evidence indicates that the secretome of mesenchymal stem cells (MSCs) has therapeutic potential for the treatment of various diseases, including cartilage disorders. However, the paracrine mechanisms underlying cartilage repair by MSCs are poorly understood. Here, we show that human umbilical cord blood-derived MSCs (hUCB-MSCs) promoted differentiation of chondroprogenitor cells by paracrine action. This paracrine effect of hUCB-MSCs on chondroprogenitor cells was increased by treatment with synovial fluid (SF) obtained from osteoarthritis (OA) patients but was decreased by SF of fracture patients, compared to that of an untreated group. To identify paracrine factors underlying the chondrogenic effect of hUCB-MSCs, the secretomes of hUCB-MSCs stimulated by OA SF or fracture SF were analyzed using a biotin label-based antibody array. Among the proteins increased in response to these two kinds of SF, thrombospondin-2 (TSP-2) was specifically increased in only OA SF-treated hUCB-MSCs. In order to determine the role of TSP-2, exogenous TSP-2 was added to a micromass culture of chondroprogenitor cells. We found that TSP-2 had chondrogenic effects on chondroprogenitor cells via PKCα, ERK, p38/MAPK, and Notch signaling pathways. Knockdown of TSP-2 expression on hUCB-MSCs using small interfering RNA abolished the chondrogenic effects of hUCB-MSCs on chondroprogenitor cells. In parallel with in vitro analysis, the cartilage regenerating effect of hUCB-MSCs and TSP-2 was also demonstrated using a rabbit full-thickness osteochondral-defect model. Our findings suggested that hUCB-MSCs can stimulate the differentiation of locally presented endogenous chondroprogenitor cells by TSP-2, which finally leads to cartilage regeneration.** STEM CELLS *2013;31:2136–2148*

Disclosure of potential conflicts of interest is found at the end of this article.

## INTRODUCTION

Cartilage is a highly differentiated and avascular tissue and consequently has a low self-regeneration capacity; full regeneration of damaged cartilage, such as in osteoarthritis (OA), is therefore challenging. Many research groups have attempted to increase the regeneration potential of damaged cartilage using mesenchymal stem cells (MSCs) including our phase I/IIa clinical trial using MSCs for cartilage repair (Clinical Trials Gov Identifier: NCT01733186) under approval of the Food and Drug Administration.

MSCs can be isolated from various tissues and can differentiate into chondrogenic lineage cells in vitro and in vivo [1,2]. Human umbilical cord blood-derived MSCs (hUCB-MSCs) are an alternative stem cell source with several advantages, including noninvasive collection methods, hypoimmunogenicity, superior tropism, and differentiation potential [3–5].

Many studies have reported the therapeutic activity of the MSC secretome. The paracrine effects of MSCs promote

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fracture healing and restore new bone formation [6], and factors released from MSCs recruit macrophages and endothelial lineage cells into the wound, thus enhancing wound healing [7]. Furthermore, autologous MSC transplantation attenuates left ventricular remodeling and improves cardiac performance via the paracrine action of the engrafted cells [8]. Moreover, we have shown that hUCB-MSCs reduce neuronal cell death by secreting galectin-3 [9] and decrease A$\beta$ plaques by inducing neprilysin [10]. Such paracrine actions of MSCs can affect cartilage repair [11–15].

Despite these possible beneficial effects, the mechanism by which MSCs and their secreted factors influence cartilage repair remains unclear, necessitating analysis of the hUCB-MSC secretome. However, in clinical trials, the effector molecules produced by MSCs under in vitro conditions are often not related to the therapeutic effects observed under clinical conditions [16]. Such discrepancies between in vitro and in vivo effects of MSCs may result from their interactions with different microenvironments. Synovial fluid (SF) acquired from osteoarthritic joints (OA SF) can represent the in vivo disease microenvironment [17,18]; OA SF modulates MSC activity in vitro in a manner representative of the clinical condition [19]. Therefore, experiments that closely mimic disease microenvironments, such as OA SF, should be useful.

Here, we demonstrated a chondrogenic differentiation effect via the paracrine action of hUCB-MSCs using a coculture system of chondrogenic progenitor cells (CPC) derived from mouse limb buds. To determine whether hUCB-MSC paracrine molecules responded to SF, we analyzed the secretome profile of media from hUCB-MSCs conditioned with SF from OA and fracture patients, using a biotin label-based antibody array. One of the differentially expressed proteins, thrombospondin-2 (TSP-2), was selected and its effect on chondrogenic differentiation of progenitor cells was validated using micromass culture and an in vivo osteochondral-defect model. This is the first report to identify factors secreted from hUCB-MSCs in response to OA SF and to demonstrate that TSP-2 is a hUCB-MSC paracrine factor with chondrogenic effects.

## Materials and methods

### Culture of hUCB-MSCs

This study was approved by the Institutional Review Board of MEDIPOST Co., Ltd. Neonatal hUCB was collected from umbilical veins, with informed maternal consent. Mononuclear cells were isolated from hUCB by centrifugation on a Ficoll-Hypaque gradient (density: 1.077 g/cm$^3$; Sigma, St. Louis, MO). Separated mononuclear cells were washed, suspended in $\alpha$-minimum essential medium ($\alpha$-MEM; Gibco, Carlsbad, CA, http://www.invitrogen.com) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco) and 50 μg/mL gentamicin (Gibco), and seeded at a concentration of $5 \times 10^5$ cells per centimeter square in culture flasks. Cultures were maintained at 37°C in a humidified 5% $CO_2$ atmosphere with a twice-weekly medium change. After 1–3 weeks, when the monolayer of fibroblast-like adherent cell colonies had reached 80% confluence, the cells were detached with TrypLE Express (Gibco), washed, resuspended in culture medium ($\alpha$-MEM supplemented with 10% FBS and 50 μg/ml gentamicin), and subcultured. In all experiments, hUCB-MSCs used were at passage 6.

### Acquisition and Treatment of Cells with SF

All patients provided appropriate written informed consent. SF was withdrawn from the knee joints of four female and two male patients (mean [SD]: 60.5 [11.5] years) diagnosed with OA, grade 4, and a knee joint of two female patients (age: 31 and 36 years), each of which had fractured a knee in an accident. Radiographs were taken of all OA knees and scored for Kellgren and Lawrence (KL) grading (0–4). SF samples were centrifuged at 300$g$ for 5 minutes to sediment macromolecules and cell debris, and the supernatants were stored at −80°C until required for analysis. hUCB-MSCs were cultured in $\alpha$-MEM containing 10% (v/v) FBS and 50 μg/mL gentamicin. For the antibody array, the culture medium from hUCB-MSCs at 80% confluence was replaced with 10% (v/v) SF diluted with serum-free $\alpha$-MEM containing 50 μg/mL gentamicin, and the cells were cultured for 6 hours. To eliminate SF contamination and to confirm the increased expression of TSP-2, culture medium from hUCB-MSCs at 80% confluence was replaced with 0.2% (v/v) SF diluted with serum-free $\alpha$-MEM containing 50 μg/mL gentamicin, and cells were cultured for 6, 12, and 24 hours.

### Micromass Culture of Chondroprogenitor Cells

Chondroprogenitor cells were isolated from the limb buds of 11.5 dpc ICR mouse embryos and maintained as micromass cultures to induce chondrogenesis. Isolated chondroprogenitor cells were suspended, without expansion (passage 0), in Dulbecco's modified Eagle's medium (DMEM, Gibco), containing 2% (v/v) FBS and 50 μg/mL gentamicin at $2.0 \times 10^7$ cells per milliliter and were spotted as 15-μL drops onto culture dishes to induce chondrogenesis for 6 days. Human recombinant TSP-2 (R&D Systems, Minneapolis, MN, http://www.rndsystems.com) was prepared and applied to chondroprogenitor cells in serum-free DMEM. As positive control, insulin-like growth factor-1 (100 ng/mL; Merck Millipore, Billerica, MA, http://www.millipore.com) was added to micromass culture medium [20].

Chondrogenesis was determined by staining of sulfated proteoglycans and examining the expression of collagen type II, aggrecan, hyaluronan and proteoglycan like protein 1 (HAPLN1), and Sox-9 using Western blotting. Accumulation of sulfated proteoglycans was detected by Alcian blue staining. During coculture, hUCB-MSCs or human embryonic kidney (HEK-293) cells were cultured in the upper phase of transwells. hUCB-MSCs or HEK-293 ($9 \times 10^4$ cells per 2 milliliter) were added to the upper chamber, and 2 mL of DMEM containing 10% serum was added to the lower chamber. After 24 hours of incubation at 37°C and 5% $CO_2$, cells on the upper surface of the chamber (which had not penetrated the insert) were applied to progenitor cells in a different culture plate. Alcian blue-stained cells were extracted and quantified by measuring absorbance at 590 nm.

### Gross Findings from Osteochondral-Defect Animal Model

The animal experiment protocol was approved by the Institutional Animal Care and Use Committee of Chungbuk National University. Thirty 10-week-old male New Zealand White (NZW) rabbits, each weighing 2–2.5 kg, were used. Knee joints of rabbits were sterilely draped and were opened through a parapatellar arthrotomy with general anesthesia. The patella was dislocated laterally, and full-thickness articular osteochondral defects (5-mm diameter; 5-mm depth) were induced in the trochlear groove by carefully perforating the respective areas on the cancellous bone using a biopsy punch. After elimination of cartilage, bone fragments, and thrombi, the border of the perforated site was trimmed using a surgical knife and cleansed from blood. Each group was untreated, transplanted with hyaluronic acid (HA), HEK-293, hUCB-MSCs, TSP-2 protein, and hUCB-MSCs treated with TSP-2 small interfering RNA (siRNA), respectively. Mixtures of hUCB-MSCs ($1 \times 10^6$ cells per 200 μL) or HEK-293 ($1 \times 10^6$ cells per 200 μL) with 4% sodium HA gel composite were applied into the area of the full-thickness defect using a syringe. TSP-2 (25 μg/200 μL) was also administered into the defect area along with 4% sodium HA gel composite. The untreated group received only a osteochondral defect. Finally, in all animals, the patellar retinaculum and the overlaying skin were sutured. An

intramuscular injection of antibiotics and analgesics was given immediately after surgery and once daily for 3–5 days afterward. After surgery, clinical signs were observed once daily during the study period. The animals were sacrificed at 10 weeks after surgery to evaluate the degree of cartilage regeneration. The histological scoring analysis was with reference to the modified O'Driscoll scoring scale [21].

### Statistical Analysis

Quantification of extraction of Alcian blue, gene expression, Western blotting intensity, and ELISA data were analyzed using Student's *t* test in Microsoft Excel (Microsoft, Redmond, WA). *p* values $<.05$ were considered significant. Histological scoring data were analyzed using the Kruskal–Wallis test to investigate the differences in scores among the six groups. A Mann–Whitney *U* test was used for pairwise comparison. Statistical analysis was performed using SPSS software version 17.0 (IBM Corporation, Armonk, NY) and *p* values $<.05$ were considered statistically significant.

## Results

### Coculture of hUCB-MSCs Promotes Differentiation of Chondroprogenitor Cells

Micromass culture of mouse embryonic chondroprogenitor cells is an accessible model for studying spontaneous cartilage differentiation in vitro [22]. To examine the paracrine effect of hUCB-MSCs on endogenous chondroprogenitor cells, hUCB-MSCs were cocultured with chondroprogenitor cells as a micromass culture. This promoted a 2.8-fold increase in the expression of sulfated proteoglycans in chondroprogenitor cells (Fig. 1A) compared to a culture group consisting only of chondroprogenitor cells, as evaluated by Alcian blue destaining (Fig. 1B). Additionally, cartilage-specific markers, that is, collagen type II, aggrecan, HAPLN1, and chondrogenic transcription factor, Sox-9, were also increased by coculture with hUCB-MSCs in chondroprogenitor cells (Fig. 1C, 1D).

We then hypothesized that pathological conditions could modulate this paracrine effect of transplanted hUCB-MSCs. Thus, hUCB-MSCs were treated with SF of fracture or OA patients and cocultured with chondroprogenitor cells; we observed a 3.4-fold increased sulfated proteoglycan synthesis by chondroprogenitor cells in OA SF-activated hUCB-MSCs. Interestingly, coculture of hUCB-MSCs with fracture SF reduced proteoglycan synthesis compared to untreated hUCB-MSCs. Similarly, fracture SF-treated hUCB-MSCs demonstrated a reduced expression of cartilage-specific markers, including collagen type II, aggrecan, HAPLN1, and Sox-9, as compared to naïve hUCB-MSCs. (Fig. 1C, 1D).

The three-dimensional structures generated in the pellet of chondrogenic cells are similar to hyaline cartilage [23]. To further validate the paracrine effects of hUCB-MSCs on three-dimensional chondrogenic differentiation, murine chondroprogenitor cells, ATDC5, were cultured with modified pellet culture media (see Supporting Information Materials and Methods). After 4 weeks, size of ATDC5 pellets treated with conditioned media of the micromass culture groups other than the fracture SF-treated group (hUCB-MSCs and OA SF-treated hUCB-MSCs) increased compared to control group (Fig. 1E, 1F). Histologically, lacunae were seen throughout sections of pellets treated with conditioned media of hUCB-MSCs or OA SF-treated hUCB-MSCs. Collagen type II was expressed in only these two groups (Fig. 1G), and the sulfated glycosaminoglycan contents of these groups were higher than that in the control (Fig. 1H). These result indicated that three-dimensional chondrogenic differentiation of ATDC5 cells was also enhanced by the secretome of hUCB-MSCs and OA SF-treated hUCB-MSC micromass-conditioned media; thus, hUCB-MSCs exert chondrogenic effects via paracrine action in vitro.

### Identification of Secreted Proteins from hUCB-MSC Induced by OA SF

Next, we analyzed the secretome of hUCB-MSCs to identify paracrine factors associated with this chondrogenic effect using a biotin label-based antibody array with conditioned media from each group. Proteins with a ratio of more than 1.0 were listed as proteins of which secretion by hUCB-MSC was increased by SF treatment (Supporting Information Table 1). A heat map revealed that protein expression in OA SF-stimulated hUCB-MSC was higher and more diverse than that of fracture SF-treated hUCB-MSCs (Fig. 2A); only three proteins were upregulated in fracture SF-treated cells, whereas 146 proteins (from two independent experiments) were upregulated in OA SF-treated samples (Supporting Information Table 1).

We used GO annotations to explore the biological categories of proteins upregulated in OA SF-treated hUCB-MSC-derived clusters; significantly ($p < .05$) overrepresented annotations are shown in Figure 2. Several annotations were related to immune reaction, proliferation, and the defense response (Fig. 2B). Twenty-seven proteins commonly upregulated in OA SF-treated hUCB-MSCs were chosen for expression validation (Supporting Information Table 2). These proteins included inflammatory cytokines in the interleukin family, chemokine receptors, trophic factors, anti-inflammatory cytokines, and soluble adhesion molecules.

To compensate for the limitations of the antibody array experiment, using the label-based detection method [24], and to determine quantitative changes, the mRNA levels of the 27 selected proteins was analyzed by real-time polymerase chain reaction (PCR) (data not shown). Among these, six genes showing increased expression in OA SF-treated compared to untreated groups were chosen for further analysis (Fig. 2C). Of these, the expressions of five molecules, including brain-derived neurotrophic factor, follistatin, transforming growth factor-$\beta$2, toll-like receptor 4, and TSP-1 were increased by both fracture and OA SF-treatment of samples. However, TSP-2 increased only in OA SF-treated samples (Fig. 2C). We then further studied OA SF-specific expression of TSP-2.

### Specific Expression of TSP-2 in hUCB-MSCs by OA SF

To confirm whether the expression of TSP-2 specifically increased with OA SF treatment, hUCB-MSCs treated with SFs from fracture and OA patients were harvested, and the protein level of TSP-2 was verified by Western blotting and ELISA. TSP-2 levels in hUCB-MSCs treated with OA SF were higher than those in fracture SF-treated hUCB-MSCs (Fig. 3A). In addition, the level of TSP-2 secreted into the conditioned media by OA SF-treated hUCB-MSCs increased time-dependently, compared to that of fracture SF-treated hUCB-MSCs (Fig. 3B). In contrast, TSP-2 levels of hUCB-MSCs as well as of fracture SF-treated hUCB-MSCs were lower than that in the untreated group. These results indicated that expression of TSP-2 in hUCB-MSCs was increased specifically by OA SF. With preliminary clinical data, we confirmed that TSP-2 in SF of OA patients increased markedly after transplantation with hUCB-MSCs (Supporting Information Fig. 3C).

Additionally, we measured TSP-2 levels in the medium of hUCB-MSCs and chondroprogenitor cells cocultured with or

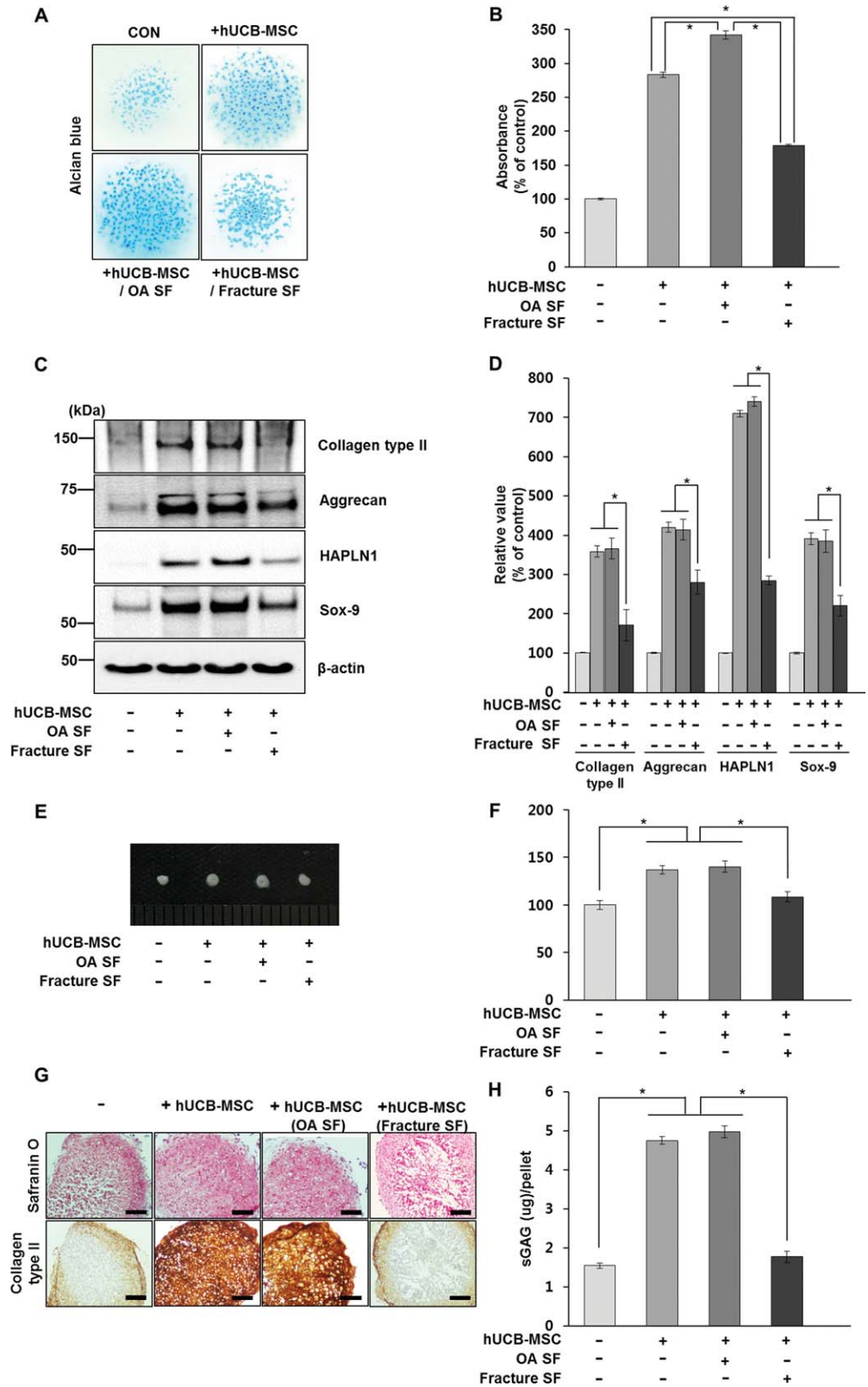


**Figure 1.** Enhanced differentiation of chondroprogenitor cells by coculture with hUCB-MSCs. Mouse limb bud chondroprogenitor cells were prepared from 11.5 dpc mouse embryos and maintained as micromass culture in 2% fetal bovine serum-containing medium for 6 days. hUCB-MSCs and chondroprogenitor cells were separately cocultured by a transwell approach in a culture plate. Effects of hUCB-MSCs on differentiation of chondroprogenitor cells were monitored by synthesis of sulfated proteoglycans and expression of chondrogenic markers. **(A):** Chondroprogenitor cells that had differentiated into mature cartilage were stained by Alcian blue dye. **(B):** The stained dye was extracted and quantified by absorbance at 590 nm. **(C):** The expression of the chondrogenic markers, collagen type II, aggrecan, HAPLN1, and chondrogenic transcription factor, Sox-9 was determined by Western blotting and quantified **(D)** using ImageJ software. **(E):** The photo of chondrogenic differentiated ATDC5 pellets was represented. **(F):** Relative size difference among the pellets was measured using IMT i-SolutionTM software. **(G):** The frozen pellets in OCT compound were cut and stained with Safranin O and anticollagen type II antibody. **(H):** The sulfated glycosaminoglycan contents were extracted and determined. Data are shown as the mean (SD) of at least six independent experiments. *, $p < .05$. Scale bars = 250 μm. Abbreviations: hUCB-MSC, human umbilical cord blood-derived mesenchymal stem cell; OA, osteoarthritis; HAPLN1, hyaluronan and proteoglycan link protein 1; SF, synovial fluid.

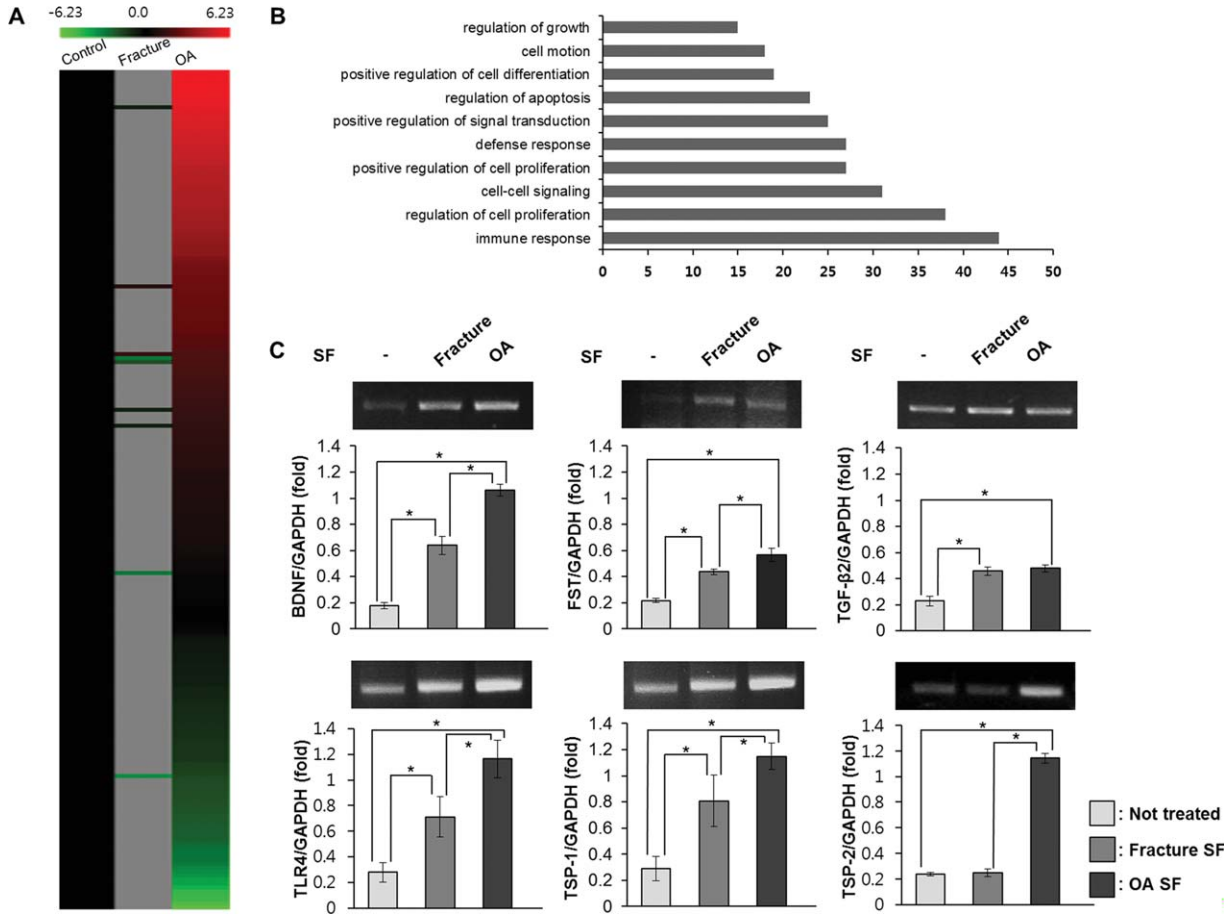


**Figure 2.** Proteins upregulated in human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) treated with OA or fracture SF. **(A):** Heat map analysis showing the proteins upregulated in the secretome of hUCB-MSCs treated with 10% (v/v) OA or fracture SF. **(B):** For the 146 proteins upregulated in hUCB-MSCs treated with OA SF, functional categories were analyzed by biological process annotation (gene ontology). **(C):** To validate the upregulated protein expression in the biotin label-based antibody array, reverse transcription-polymerase chain reaction was performed for the mRNA corresponding to six proteins present in hUCB-MSCs treated with 10% SF for 6 hours. GAPDH was used as loading control. Values are presented as mean (SD) expression levels relative to GAPDH of at least four independent experiments. *, $p < .05$. Abbreviations: BDNF, brain derived neurotrophic factor; FST, follistatin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OA, osteoarthritis; SF, synovial fluid; TLR4, toll-like receptor 4; TSP-1, thrombospondin-1; TGF-$\beta$2, transforming growth factor $\beta$2.

without OA SF or fracture SF using ELISA. As expected, the TSP-2 level in the medium of naïve hUCB-MSCs was markedly increased as compared to that of cultures containing only progenitor cells. Expression of TSP-2 in the hUCB-MSC medium was increased by OA SF treatment. However, in the fracture SF-treated hUCB-MSC medium, TSP-2 decreased to 65% of that in the medium of untreated hUCB-MSCs (Fig. 3C). Furthermore, coculture between chondroprogenitor cells and hUCB-MSCs derived from 10 different donors showed that hUCB-MSCs with a high level of TSP-2 expression resulted in better chondrogenic differentiation of chondroprogenitor cells (Supporting Information Fig. 1A, 1B). Collectively, the expression pattern of TSP-2 in hUCB-MSCs was not only related to the enhanced differentiation of chondroprogenitor cells but was also pathological condition-dependent.

## Effect of Exogenous TSP-2 on Differentiation of Chondroprogenitor Cells

Because we observed that the expression pattern of TSP-2 in hUCB-MSCs was related to increased differentiation of chondroprogenitor cells, we examined its role in differentiation of chondroprogenitor cells. Exogenous TSP-2 was applied to chondroprogenitor cells maintained as micromass cultures. Exogenous addition of TSP-2 enhanced differentiation of chondroprogenitor cells as the concentration of TSP-2 increased, as validated by Alcian blue staining (Fig. 4A, 4B) and the increased expression of collagen type II, aggrecan, HAPLN1, and Sox-9 (Fig. 4C, 4D).

Thus, to confirm the signaling pathways involved in TSP-2-induced chondrogenic differentiation, the effects of specific inhibitors for previously reported pathways [25–27] were examined. TSP-2-induced accumulation of sulfated proteoglycans was enhanced by inhibition of extracellular signal-regulated protein kinase (ERK) with PD98059, whereas inhibition of p38 mitogen-activated protein (MAP) kinase with SB203580, protein kinase C (PKC) $\alpha$ with Go6976, and $\gamma$-secretase with *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-(*S*)-phenylglycine *t*-butyl ester (DAPT) suppressed TSP-2-induced sulfated proteoglycan accumulation (Fig. 4E, 4F). Similarly, inhibition of ERK with PD98059 promoted TSP-2-induced collagen type II, aggrecan, HAPLN1, and Sox-9 expression, whereas expression of these proteins was blocked by inhibition of p38 MAP kinase with SB203580, of PKC $\alpha$ with Go6976, or of $\gamma$-secretase with DAPT (Fig. 4G, 4H). In the absence of TSP-2, these expression patterns were retained,

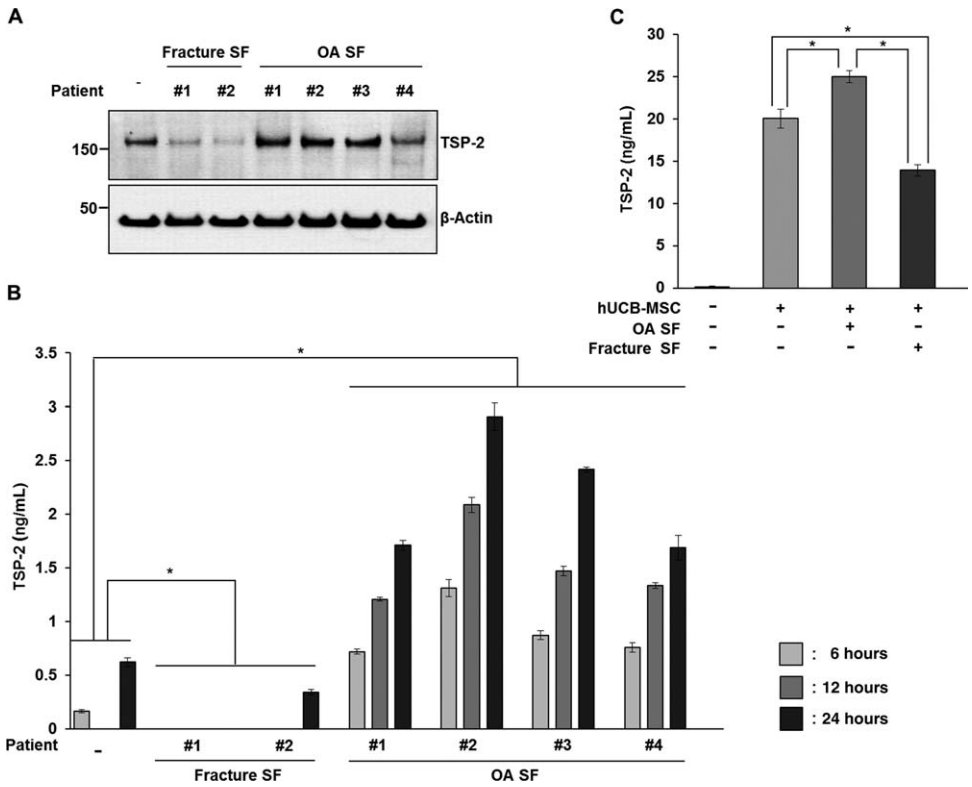


**Figure 3.** TSP-2 expression by hUCB-MSCs responded to treatment with SF from fracture and OA patients. **(A):** Western blots of cell lysates from hUCB-MSCs treated with 10% SF from two fracture patients and four OA patients for 6 hours. **(B):** ELISA analysis on expression of TSP-2 in culture media from hUCB-MSCs treated with 0.2% SF samples from two fracture patients and four OA patients for 6, 12, and 24 hours. **(C):** TSP-2 concentrations in supernatants of micromass culture media. Data are shown as the mean (SD) of at least four independent experiments. *, $p < .05$. Abbreviations: hUCB-MSC, human umbilical cord blood-derived mesenchymal stem cell; OA, osteoarthritis; SF, synovial fluid; TSP-2, thrombospondin-2.

but expressions of cartilage-specific markers were lower than those in the presence of TSP-2 (Fig. 4E–4H). These results clearly demonstrated that TSP-2 has chondrogenic effects, which are mediated through PKC α/ERK, p38/MAPK, and Notch signaling pathways.

## Knockdown of TSP-2 Abolishes Chondrogenic Effects of hUCB-MSC on Differentiation of Chondroprogenitor Cells

Since we had observed chondrogenic effects of TSP-2 in hUCB-MSCs, we attempted to block expression of TSP-2 for further validation. After TSP-2 siRNA treatment, expression of TSP-2 was completely blocked (Fig. 5E) and knockdown of TSP-2 was confirmed up to 28 days (Supporting Information Fig. 4). siRNA-mediated knockdown of TSP-2 expression led to decreased synthesis of sulfated proteoglycans (Fig. 5A, 5B) and expression of collagen type II, aggrecan, HAPLN1, and Sox-9 (Fig. 5C, 5D), indicating reduced chondrogenesis. However, use of HEK-293 cells as non-stem cells in coculture did not replicate the findings in hUCB-MSCs (Fig. 5A–5D). Collectively, these results suggested that the chondrogenic effects of hUCB-MSCs are mediated via TSP-2.

## Gross Findings and Histological Observations of Effects of hUCB-MSCs and TSP-2 in an Osteochondral-Defect Animal Model

To further validate the role of TSP-2 in cartilage regeneration in an in vivo system, we made osteochondral defects in the trochlear groove of rabbits and applied either hUCB-MSCs or only TSP-2 into the defects. In addition, given our previous in vitro data that hUCB-MSCs can promote differentiation of endogenous chondroprogenitor cells, we used a full-thickness cartilage defect model that exposed chondroprogenitor cells from the marrow [28]. At 10 weeks after surgery, the gross morphology of joints was observed and sections were stained with hematoxylin and eosin and Safranin O. Upon gross examination, the margins of the defects of untreated or HA-treated groups were clearly recognizable and their surfaces were irregular (Fig. 6Aa, 6Ab). The surfaces of defects transplanted with HEK-293 were depressed and most parts of the defects were not filled with any tissue (Fig. 6Ac). The reparative tissues of untreated and HA-treated groups were filled with fibrous tissue and remained concave (Fig. 6Ag, m, 6Ah, n). In the HEK-293-treated group, thin fibrous tissue was observed on the subchondral bone (Fig. 6Ai, 6Ao). In contrast, the hUCB-MSC-treated group showed good lateral integration, macroscopically (Fig. 6Ae), and a hyaline-like cartilage matrix was evident with Safranin O. The tissue was hyaline-like, with good integration, thickness, and surface regularity. An enlarged image of the regenerated tissue showed the lacunae structure (Fig. 6Ak, 6Aq). There was marked improvement in the quality of the repaired tissue seen in the hUCB-MSC-treated group compared with the untreated, HA, and HEK-293-treated groups.

The surfaces of regenerated tissue in the TSP-2-treated group were mostly not depressed, and the defects were covered with white opaque tissue compared with the hUCB-MSC-treated group (Fig. 6Ad). Histologically, a magnified view of the repaired tissue represented a partially hyaline-like

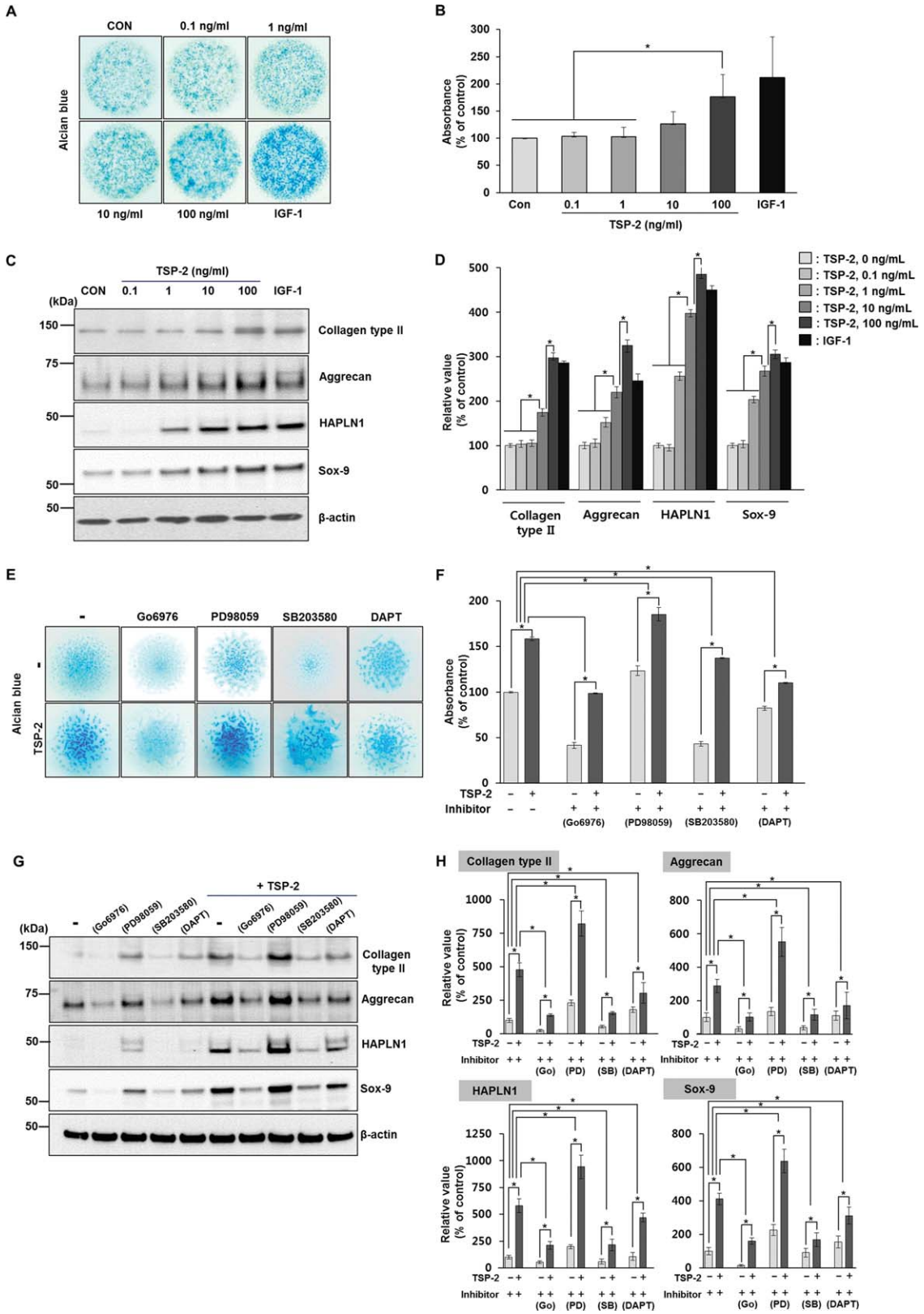


**Figure 4.** Effects of exogenous TSP-2 on differentiation of chondroprogenitor cells. **(A):** Alcian blue staining of mouse limb bud chondroprogenitor cells which had been maintained as micromass culture for 6 days in the presence of TSP-2 (0.1–100 ng/mL) under serum-free conditions. IGF-1 (100 ng/mL) was used as positive control for induction of chondrogenic differentiation. **(B):** Alcian blue-stained cells were extracted and quantified by measuring absorbance. **(C):** Expression levels of collagen type II, aggrecan, HAPLN1, and Sox-9 protein were determined by Western blotting; $\beta$-actin was used as loading control. **(D)**: Expression of collagen type II protein was quantified by ImageJ software. **(E):** Chondroprogenitor cells were maintained as micromass culture up to 6 days in the absence or presence of TSP-2 (100 ng/mL). Cells were concurrently exposed to 1 μM Go to inhibit PKC$\alpha$, 10 μM of PD to inhibit ERK, 10 μM of SB to inhibit p38/MAPK, or 50 nM DAPT to inhibit $\gamma$-secretase. Accumulation of sulfated proteoglycans was detected by Alcian blue staining. **(F):** Alcian blue-stained cells were extracted and quantified by measuring absorbance. **(G):** Effects of inhibitors on expression of collagen type II, aggrecan, HAPLN1, and Sox-9 protein were detected by Western blotting. $\beta$-actin was used as loading control. **(H):** Expressions of collagen type II, aggrecan, HAPLN1, and Sox-9 protein were quantified by ImageJ software. Data are presented as the mean (SD) result from four independent experiments. *, $p < .05$. Abbreviation: DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-(*S*)-phenylglycine *t*-butyl ester; HAPLN1, Hyaluronan and proteoglycan link protein 1; Go, Go6976; IGF-1, insulin-like growth factor-1; PD, PD98059; SB, SB203580; TSP-2, thrombospondin-2.

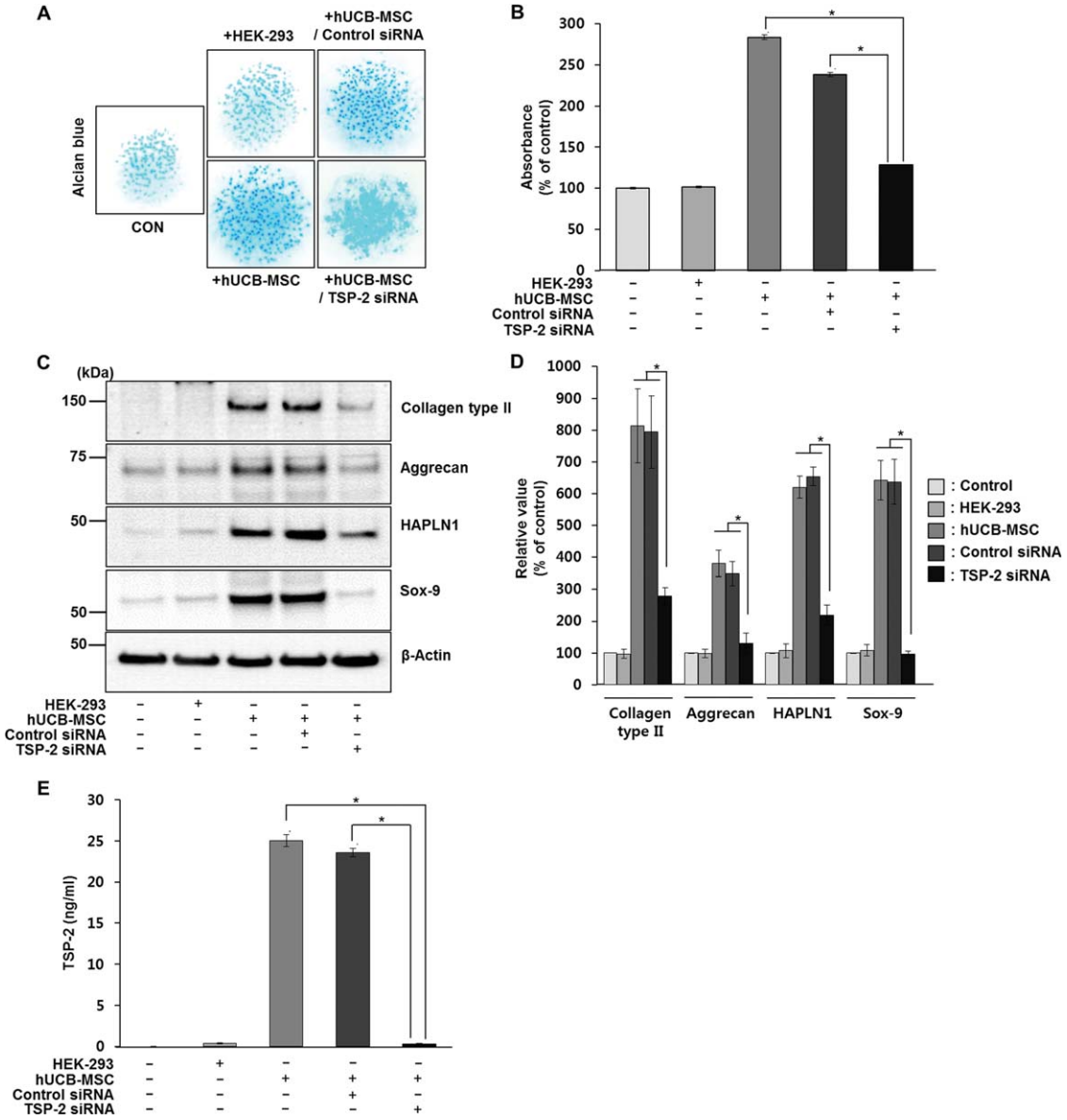


**Figure 5.** Reduced differentiation of chondroprogenitor cells by inhibition of TSP-2 in hUCB-MSCs. hUCB-MSCs were transfected for 24 hours with negative control siRNA (25 nM) or TSP-2 siRNA (25 nM). After a 24-hour incubation, transfected hUCB-MSCs were applied to chondroprogenitor cells in a coculture system. Chondroprogenitor cells were cultured with hUCB-MSCs or HEK-293 (non-stem cells) cells up to 6 days in 2% fetal bovine serum-containing medium. **(A):** Chondroprogenitor cells stained with Alcian blue at day 6. **(B):** Alcian blue stain was extracted from the cells and quantified by measuring absorbance. **(C):** Protein levels of collagen type II, aggrecan, HAPLN1, and Sox-9 were determined by Western blotting; $\beta$-actin was used as loading control. **(D):** Expression of collagen type II protein was quantified by ImageJ software. **(E):** TSP-2 concentrations were measured in the supernatants of each culture condition. Data are shown as the mean (SD) of at least four independent experiments. *, $p < .05$. Abbreviations: hUCB-MSCs, human umbilical cord blood derived mesenchymal stem cells; HAPLN1, hyaluronan and proteoglycan link protein 1; siRNA, small interfering RNA; TSP-2, thrombospondin-2.

structure (Fig. 6Aj, 6Ap). In defects transplanted with hUCB-MSCs treated with TSP-2 siRNA, the surfaces were irregular (Fig. 6Af). Defects showed dense fibrous tissue rather than cartilage tissue (Fig. 6Al, 6Ar). Upon immunohistochemical analysis, untreated, HA-treated, and HEK-293-transplanted groups rarely expressed collagen type II (Fig. 6As–6Au), whereas collagen type II was distributed throughout the neocartilage in the hUCB-MSC-treated group (Fig. 6Aw). Defects treated with TSP-2 were well repaired in deep zone of cartilage, but the superficial zone was partly unrepaired (Fig. 6Av). However, collagen type II expression in defects transplanted with hUCB-MSCs treated with TSP-2 siRNA was observed at periphery of the defect sites (Fig. 6Ax). Histological observations were quantified according to the modified O'Driscoll scoring scale [21]. The mean modified O'Driscoll scores of each experimental group correlated well with both macroscopic and histological analyses (Fig. 6B). Both TSP-2- and hUCB-MSC-treated groups had higher scores than those of the control groups, including the untreated, HA-treated, and HEK-293-treated groups ($p < .01$). The TSP-2-treated group achieved a lower score compared with the hUCB-MSC-treated group ($p < .05$). The score in the hUCB-MSCs

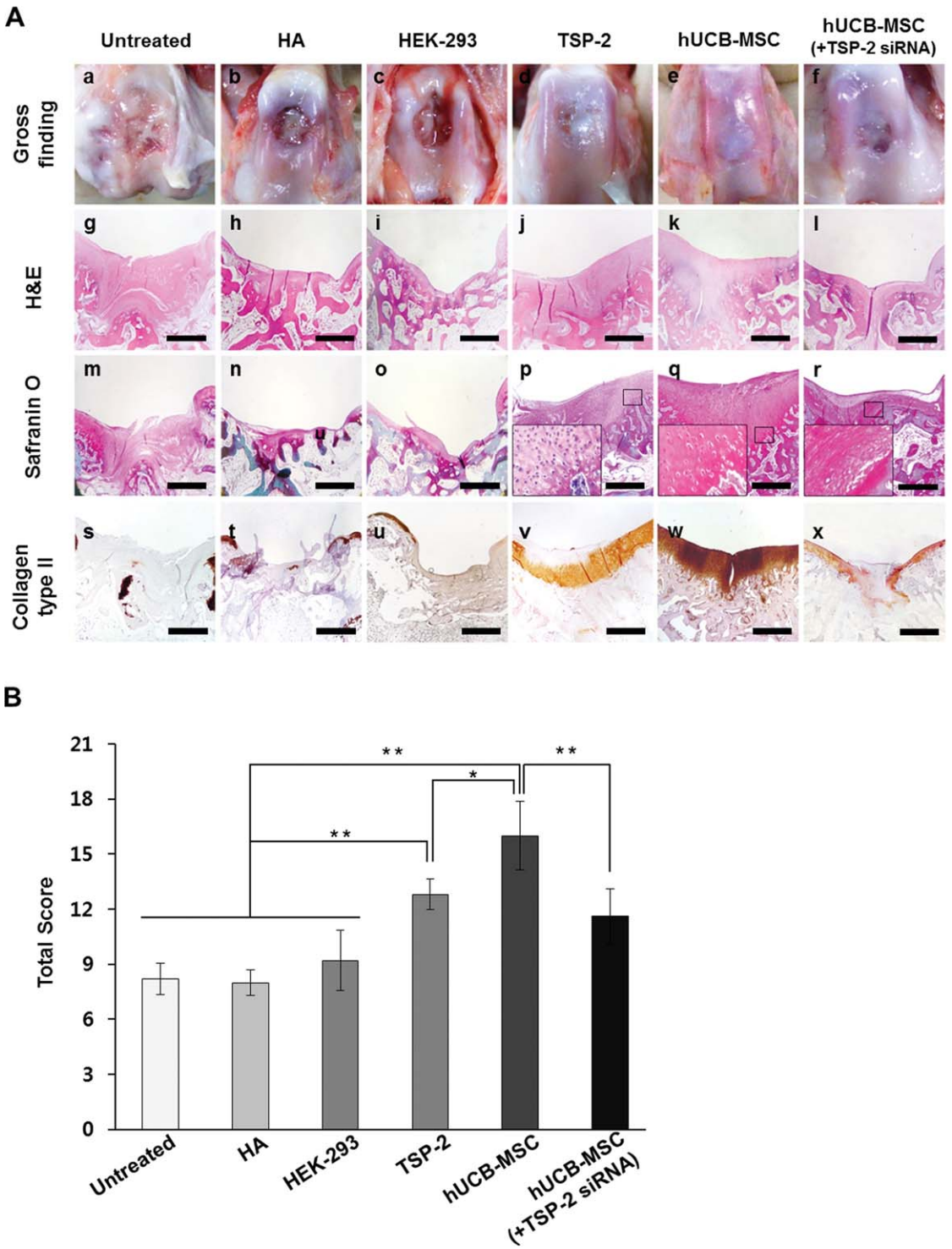


**Figure 6.** Macroscopic and histological analysis and immunostaining of hUCB-MSC-treated and TSP-2-injected knees in a full-thickness osteochondral-defect rabbit model. **(A):** Six experimental groups, including an untreated group ($n = 5$), and groups treated with 4% HA ($n = 5$), a composite of HEK-293 ($5 \times 10^6$ cells/ml) and 4% HA ($n = 5$), TSP-2 (25 μg) and 4% HA ($n = 5$), a composite of hUCB-MSCs ($5 \times 10^6$ cells/ml) and 4% HA ($n = 5$), and a composite of hUCB-MSCs treated with TSP-2 siRNA ($5 \times 10^6$ cells/ml) and 4% HA ($n = 5$) were used. At 10 weeks postoperatively, animals were sacrificed and the defect area was observed and stained for analysis. Gross findings are shown in **(A–F)**. Femoral condyles were sectioned and stained with hematoxylin and eosin **(G–L)** and Safranin-O **(M–R)**. For immunohistochemical analysis, collagen type II was detected as brown color **(S–X)**. The untreated group (saline treatment) showed poor healing (a, g, m, and s). The defects in the HA-treated group were rarely recovered (b, h, n, and t). The defects treated with HEK-293 exhibited irregular surfaces similar to the HA-treated group (c, i, o, and u). In the TSP-2-treated group, the regenerated tissue was slightly less recovered compared to the hUCB-MSCs-treated group but showed better-integrated cartilage tissue than in the untreated, HA-treated, and HEK-293 groups (d, j, p, and v). The hUCB-MSCs-treated animals demonstrated good defect filling, with a smooth surface and regenerated cartilage tissue (e, k, q, and w). The defects transplanted with hUCB-MSCs treated with TSP-2 siRNA represented poorly regenerated tissue compared with TSP-2- and hUCB-MSCs-treated groups (f, l, r, and x). Scale bars = 1 mm (G–X). (B): Using the modified O'Driscoll scoring scale, the integration rates were quantified in the six experimental groups at 10 weeks. Data are shown as the mean (SD) of each group ($n = 5$). Statistical significance was determined using Kruskal–Wallis test followed by Mann–Whitney $U$ test. *, $p < .05$ or **, $p < .01$. Abbreviations: hUCB-MSCs, human umbilical cord blood-derived mesenchymal stem cells; HA, Hyaluronate; siRNA, small interfering RNA; TSP-2, thrombospondin-2.

treated with TSP-2 siRNA decreased compared with that in the hUCB-MSC-treated group ($p < .01$). Collectively, these data indicated that hUCB-MSCs have cartilage regeneration effects in an osteochondral defect model, which are mainly mediated by the secreted TSP-2.

## Discussion

Here, we demonstrated that hUCB-MSCs promoted differentiation of chondroprogenitor cells by paracrine action. This chondrogenic effect was modulated by the surrounding environment, such as OA or fracture SF. Through secretome array analysis and in vitro evaluation, we found that hUCB-MSCs secreted trophic factors that varied with the pathological conditions, such as exposure to OA SF. Moreover, expression of TSP-2 by hUCB-MSCs was specific to OA SF treatment and enhanced cartilage regeneration in an osteochondral defect animal model.

When MSCs are transplanted into cartilage lesions, they are exposed to a complex microenvironment, including inflammatory cytokines, proteases, and degraded extracellular matrix secreted from damaged cartilage, which can modulate paracrine activities of MSCs. For instance, the proinflammatory cytokine, tumor necrosis factor-α, changes the secretome of human adipose tissue-derived MSCs [29]. In this study, OA SF-treated hUCB-MSCs increased the chondrogenic differentiation of chondroprogenitor cells, compared to a non-treated hUCB-MSC group, whereas fracture SF-treatment of hUCB-MSCs decreased chondrogenic differentiation (Fig. 1). Furthermore, secretome analysis of hUCB-MSCs induced by these SFs resulted in distinct expression patterns (Fig. 2; Supporting Information Table 2). These data indicated that the pathological condition can modulate the paracrine action of hUCB-MSCs. In elucidating this modulation, we found that, among factors secreted from hUCB-MSCs, only TSP-2 was specifically expressed upon OA SF-treatment (Fig. 3). Indeed, TSP-2 levels also markedly increased in the SF of OA patient two weeks after hUCB-MSCs transplantation (Supporting Information Fig. 3C).

To date, TSP-2 was known as an antiangiogenic multifunctional protein that interacts with diverse cellular regulatory factors [30,31]. In the context of skeletal function, the role of TSP-2 in bone remodeling has been intensively studied by Hankenson's group, who revealed that TSP-2 affects proliferation and osteogenic differentiation of MSCs [32,33]. Mice lacking TSP-2 display increased bone formation and MSCs numbers [32]. Moreover, TSP-2 is an autocrine inhibitor of MSCs proliferation [33]. Thus, TSP-2 plays an important role in bone homeostasis in several conditions, such as fracture healing [34], ovariectomy [35], and mechanical loading [36]. Although the biological processes of chondrogenesis and osteogenesis are tightly coupled, few reports have directly investigated the chondrogenic effects of TSP-2. Of the thrombospondin family, only TSP-2 and cartilage oligomeric matrix protein (TSP-5) are expressed in chondrocytes [37] and a lack of TSP-2 results in connective tissue abnormalities in mice [38]. However, these previous reports did not fully address the direct chondrogenic effects of TSP-2.

In our study, we confirmed that TSP-2 promoted differentiation of chondroprogenitor cells in a concentration-dependent manner (Fig. 4A–4D). Furthermore, treatment of an osteochondral defect model with TSP-2 resulted in cartilage regeneration (Fig. 6). Thus these data showed direct evidence of TSP-2-mediated chondrogenic effects. Interestingly, two isoforms of TSP-2 (molecular weights of 200 kDa and 125 kDa) were recently reported; 200 kDa species are secreted in osteoblasts [39]. In this study, we could not detect 200 kDa TSP-2, but lower sized TSP-2 was expressed in hUCB-MSCs with or without OA SF (data not shown). Therefore, the expression of the TSP-2 isoforms seems to be cell type-specific.

To understand the mechanism of action of TSP-2, the various signaling pathways related to the chondrogenic effects of TSP-2 must be investigated. This include the protein kinase signaling pathways, of PKCα, p38/MAPK, and ERK [25,26], as we also confirmed (Fig. 4E–4H); we showed that TSP-2 enhanced these signaling pathways synergistically. Recently, there have been reports that TSP-2 initiates chondrogenic differentiation of MSCs by potentiation of the Notch signaling pathway [27,40]. In our data, expression of TSP-2 rapidly increased during coculture between hUCB-MSCs and chondroprogenitor cells. Therefore, we examined and verified the involvement of the Notch signaling pathway in the TSP-2-mediated differentiation of chondroprogenitor cells. Additionally, it is possible that TSP-2 can also potentiate chondrogenic differentiation of hUCB-MSCs by autocrine action via the Notch signaling pathway.

Our findings suggested that the secretome of hUCB-MSCs, including TSP-2, can be regulated by the pathological condition, which may modulate the chondrogenic effects of transplanted hUCB-MSCs. Thus, finding factors in OA SF associated with the specific TSP-2 expression pattern is important for predicting the therapeutic effect of transplanted hUCB-MSCs, which may also guide selection of patients who have these factors related to TSP-2 expression.

There are two available transplantation routes for delivery of MSC-based cartilage disease therapy, such as scaffold-assisted application or direct intra-articular injection of MSCs [41]. Here, we used 4% HA gel as a scaffold for delivery of hUCB-MSCs into defect sites with exposed marrow; this scaffold-assisted application of MSC is a prerequisite for the surgical process. On the other hand, there are several studies showing that delivery of MSCs in HA solution by intra-articular injection into damaged joints enhances cartilage regeneration [42–44]. These studies suggested that HA facilitates the migration and adherence of injected MSC into injured sites. Based on these studies, it is possible that direct intra-articular injection of hUCB-MSCs in HA solution allow greater response to the microenvironment, resulting in cartilage repair by paracrine action.

Because MSCs differentiate into cells of a mesodermal lineage, including cartilage, bone, tendon, and ligament [45], most studies using MSCs for repair of cartilage defects have focused on MSC-mediated cartilage formation [46]. Recently, it has been acknowledged that paracrine actions of MSCs play a role in regeneration of damaged cartilage [11], leading to trials for applying the paracrine action of MSCs in cartilage repair [11–15]. There have been several reports that the immunomodulatory effects of MSCs control expression of inflammatory cytokines in damaged cartilage tissue [12,47–50]. However, it is uncertain whether the cartilage newly formed after MSC transplantation is generated by paracrine actions of MSCs in vivo. To address this issue, we monitored the fate of transplanted hUCB-MSCs using reverse transcriptase PCR of human gene and PKH 26 labeling during cartilage repair. As a result, transplanted hUCB-MSCs were detected at 4 weeks, but not at 8 weeks, after transplantation (Supporting Information Fig. 2A, 2B). Our data indirectly indicated that the regenerated cartilage after hUCB-MSCs transplantation was of recipient origin, implying the involvement of host cells, such as CPC [51,52], in cartilage repair. Similarly, several studies demonstrated the

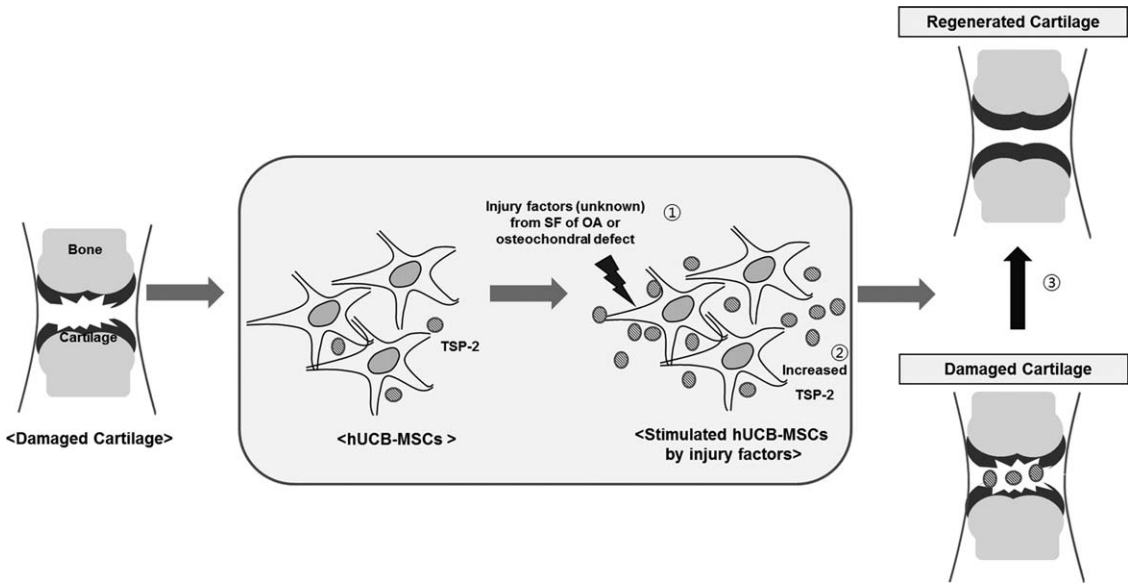


**Figure 7.** Schematic diagram of the chondrogenic effect of hUCB-MSCs on damaged cartilage. hUCB-MSCs transplanted into damaged cartilage were stimulated by unknown injury factors in the SF from OA patients to increased TSP-2 secretion. This TSP-2 diffused into synovia and induced differentiation of chondroprogenitor cells. By means of this paracrine action of at least TSP-2, hUCB-MSCs accomplishes regeneration of damaged cartilage. Abbreviations: hUCB-MSCs, human umbilical cord blood-derived mesenchymal stem cells; OA, osteoarthritis; SF, synovial fluid; TSP-2, thrombospondin-2.

fate of MSCs transplanted into osteochondral defects [53,54] or an OA animal model [42]; the labeled MSCs gradually disappeared in regenerated cartilage over time after transplantation. One possible explanation for these observations could be nonspecific immune responses by allogeneic or xenogeneic MSCs, leading to the clearance of the transplanted MSCs. Although cartilage is known to be immunologically privileged as an avascular tissue, pathological conditions can cause recruitment of immune cells [55–57]. Indeed, Yan et al. [56] observed that lymphocyte infiltration occurred in a rabbit osteochondral defect model after hUCB-MSCs implantation. Therefore, although we cannot completely exclude chondrogenic differentiation of grafted hUCB-MSCs due to the possible limitation of sensitivity for detecting a low-dose of labeled cells, given our results, it is possible that transplanted hUCB-MSCs promote cartilage regeneration mainly by paracrine action on endogenous chondroprogenitor cells.

In this study, our key hypothesis was that hUCB-MSCs exert a paracrine action on host cells leading to cartilage repair. Therefore, although lacking an OA microenvironment, such as OA SF, we selected the rabbit full-thickness osteochondral defect model to evaluate the cartilage repair potential of hUCB-MSCs, given the increased chance of interaction between hUCB-MSCs and marrow-originated endogenous progenitor cells in this model. Since TSP-2 expression of hUCB-MSCs is a prerequisite for mediating paracrine action for cartilage regeneration in osteochondral defect models, we examined the presence of TSP-2 in this animal model. We observed increased TSP-2 levels in the SF from this animal model after transplantation of hUCB-MSCs (Supporting Information Fig. 3A) and increased TSP-2 levels of hUCB-MSCs by SF treatment from the same model (Supporting Information Fig. 3B). Moreover, transplantation of hUCB-MSCs with knockdown of TSP-2 resulted in decreased TSP-2 levels in the SF from this animal model, leading to reduced effects of hUCB-MSCs on cartilage regeneration (Fig. 6). Thus, these data supported that the TSP-2-mediated paracrine action of hUCB-MSCs leads to cartilage regeneration in this animal model. However, further study using OA animal models are required to verify the mechanisms underlying the therapeutic effects of hUCB-MSCs in OA.

Recently, MSCs, with their multilateral paracrine effects, have been viewed as a "drugstore" [58] and are considered to have a better therapeutic effect than a single factor. In this study, we also observed that greater recovery ensued when a cartilage defect in a rabbit osteochondral-defect model was transplanted with hUCB-MSCs, than from treatment with TSP-2 protein alone (Fig. 6). It is likely that hUCB-MSCs secrete not only TSP-2, but also various other proteins, including anti-inflammatory, antiapoptotic, and mitogenic factors (Supporting Information Table 1), which may simultaneously improve the pathological environment of the cartilage defect. Thus, to fully understand the mechanisms of hUCB-MSCs in cartilage repair, it is necessary to also investigate the functions of trophic factors other than TSP-2, which we are currently pursuing.

## Conclusion

In conclusion, our findings indicated that hUCB-MSCs exert cartilage regeneration effects via paracrine action involving at least TSP-2, suggesting the following possible therapeutic mechanism (Fig. 7): (a) hUCB-MSCs transplanted into OA lesions are stimulated by unidentified factors in OA SF. (b) Activated hUCB-MSCs specifically increase the secretion of TSP-2 as a beneficial paracrine factor. (c) TSP-2 released into the synovia promotes the differentiation of endogenous chondroprogenitor cells. To select hUCB-MSCs with high TSP-2 expression or to manipulate hUCB-MSCs to overexpress TSP-2 by virus or engineering using zinc-finger nuclease technology may facilitate development of hUCB-MSC-based therapy with enhanced cartilage regeneration effects.

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## Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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