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Autocrine Action of Thrombospondin-2 Determines the Chondrogenic Differentiation Potential and Suppresses Hypertrophic Maturation of Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells

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Key Words. Chondrogenic differentiation • Hypertrophy • Notch signaling • Thrombospondin-2 • Umbilical cord blood-derived mesenchymal stem cell

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ABSTRACT

Previous studies have shown that mesenchymal stem cell (MSC)-based therapies have varying efficacies for the treatment of various diseases, including cartilage defects. In this study, we demonstrated that the chondrogenic differentiation potential of human umbilical cord blood-derived MSCs (hUCB-MSCs) obtained from different individual donors varies, and we investigated the molecular basis for this variation. Microarray gene expression analysis identified thrombospondin-2 (*TSP2*) as a candidate gene underlying the interindividual variation in the chondrogenic differentiation potential of hUCB-MSCs. To assess the association between TSP-2 and the differentiation potential, we evaluated chondrogenic differentiation of hUCB-MSCs treated with *TSP2* siRNA. In addition, we studied the effect of supplementing exogenous recombinant TSP-2 on *TSP2* siRNA-treated hUCB-MSCs. We found that TSP-2 autocrinally promoted chondrogenic differentiation of hUCB-MSCs via the Notch signaling pathway, which was confirmed in MSCs from other sources such as bone marrow and adipose tissue. Interestingly, we observed that TSP-2 attenuated hypertrophy, which inevitably occurs during chondrogenic differentiation of hUCB-MSCs. Our findings indicated that the variable chondrogenic differentiation potential of MSCs obtained from different donors is influenced by the TSP-2 level in the differentiating cells. Thus, the TSP-2 level can be used as a marker to select MSCs with superior chondrogenic differentiation potential for use in cartilage regeneration therapy. STEM CELLS *2015;33:3291–3303*

SIGNIFICANCE STATEMENT

This study demonstrated MSCs obtained from different donors vary in their chondrogenic differentiation potential, which is influenced by TSP-2 levels. It also provided further data showing that autocrine action of TSP-2 promotes chondrogenic differentiation of MSCs via Notch signaling pathway and suppresses hypertrophy of hUCB-MSC by inhibiting the osteogenic pathway that involves the WNT/β-catenin and TGF-β/BMP signal cascades. Based on this dual role of TSP-2, this study suggested that TSP-2 is a new marker for prediction and selection of MSCs with superior chondrogenic differentiation potential for cartilage regeneration therapy.

INTRODUCTION

Articular cartilage disorders commonly cause restricted mobility and disability in older adults [1]. Articular cartilage degeneration affects cartilage, synovium, and subchondral bone, and progressive breakdown of joint surfaces leads to degenerative disorders such as osteoarthritis. Furthermore, cartilage degeneration is accompanied by pain and loss of function. Cartilage tissue has low self-regeneration potential because it is not vascularized and thus has limited access to progenitor cells or factors in the blood and bone marrow (BM) that facilitate regeneration [2]. Therefore, development of cartilage regeneration and repair treatments remains a challenge.

Mesenchymal stem cells (MSCs) can differentiate into cells of mesodermal lineages such

as cartilage, bone, and fat cells [3, 4]. MSCs can be isolated from various adult tissues including BM [5], adipose tissue (AT) [6], Wharton's jelly [7], and umbilical cord blood (UCB) [8, 9]. Human UCB-derived MSCs (hUCB-MSCs) are preferred because they can be obtained using anon-invasive procedure and have hypoimmunogenicity [10], high differentiation potential [11], high proliferation rate [12], and superior tropism [13, 14]. These characteristics of hUCB-MSCs are particularly attractive for clinical application. Therapeutic use of hUCB-MSCs for cartilage repair has been approved by the Korea Food and Drug Administration (KFDA). A phase I/IIa clinical trial (ClinicalTrials.gov identifier: NCT01733186) is currently ongoing under approval of the FDA.

Similarly, based on the cell proliferation and chondrogenic differentiation characteristics of MSCs, potential preclinical and clinical applications of MSCs for treating damaged cartilage have been investigated [15]. However, cartilage regeneration treatments using MSCs are not consistently efficacious in animal models and clinical trials. For instance, new cartilage structure was not produced after transplantation of BM-MSCs in a gelatin sponge containing transforming growth factor beta 1 (TGF-β1) into an ovine model of growth plate cartilage injury [16]. After injecting injured porcine discs with allogeneic MSCs and juvenile chondrocytes, proteoglycan matrix synthesis in the MSC-treated group was lower than that in the juvenile chondrocyte-treated group [17]. Furthermore, Wakitani et al. [18] reported that autologous BM-MSCs did not differentiate into hyaline cartilage in the cartilage lesions of some patients.

We speculated that failure of cartilage regeneration after MSC transplantation is related to insufficient chondrogenic differentiation of MSCs in the transplanted site. Adult tissue-derived MSCs vary in their chondrogenic potential, which is influenced by the age and genetic makeup of the donor [19, 20]. Thus, interindividual differences in MSC chondrogenic potential may be an important factor influencing the success of MSC-based treatments for damaged cartilage. Therefore, a method for selecting MSCs with superior chondrogenic differentiation potential would be useful to improve outcomes of cartilage repair. However, no such markers have been characterized to date. In this study, we evaluated the variability in the chondrogenic differentiation potential of hUCB-MSCs obtained from different donors, and we investigated the underlying mechanism responsible for this variation by using comparative transcriptome analysis.

Materials and Methods

MSC Isolation and Culture

Neonatal UCB samples were collected from the umbilical vein after delivery of the infant. All samples were obtained after obtaining informed consent from the mothers. In all cases, UCB harvests were processed within 24 hours of collection, and the cell viability in all samples was above 90%. Mononuclear cells (MNCs) were isolated by layering the UCB samples on a Ficoll-Hypaque gradient (Sigma, St Louis, MO, http://www.sigmaaldrich.com) followed by centrifugation at 300*g* at 25°C. The separated MNCs were suspended in α-minimum essential medium (α-MEM, Gibco BRL, Carlsbad, CA, http://www.gibcobrl.com) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, http://www.hyclone.com). The cells were seeded in a culture plate at a density of 5×10^5 cells per cm^2 and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was replaced with fresh medium twice a week. Adherent MNCs with fibroblast-like morphology were observed. After 1–3 weeks of growth, the monolayer colonies reached 80% confluence and the cells were detached using 0.25% trypsin containing EDTA (HyClone), washed, resuspended in culture medium (α-MEM supplemented with 10% FBS) and subcultured at a concentration of 5×10^4 cells per cm^2. hUCB-MSCs were characterized by expression of surface markers (Supporting Information Table S1) and tested for mesodermal lineage differentiation potential (Supporting Information Table S2). Human BM (hBM)- and AT (hAT)-MSCs were isolated from BM aspirates and lipoaspirates, respectively, as described previously [21], and purchased from certified suppliers (Promocell, Heidelberg, Germany, http://www.promocell.com; ATCC, Manassas, VA, http://www.atcc.org; Life Technologies, Carlsbad, CA, http://www.lifetech.com; Lonza, Basel, Switzerland, www.lonza.com). Passage 5–7 MSCs were used for all experiments.

In Vitro Chondrogenic Differentiation and Hypertrophy-Inducing Culture

To induce chondrogenic differentiation, approximately 2×10^5 cells were suspended in chondrogenic medium in a 15-mL polypropylene tube and centrifuged at 500*g* for 5 minutes at room temperature. The cell pellet was cultured in chondrogenic medium for 4 weeks; the medium was replaced with fresh medium twice a week. Chondrogenic medium consisted of high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 0.6 μg/mL dexamethasone (Sigma), 50 μg/mL L-ascorbic acid (Sigma), 100 μg/mL sodium pyruvate (Sigma), 40 μg/mL L-proline (Sigma), 10 ng/mL transforming growth factor-β3 (TGF-β3, R&D Systems, Minneapolis, MN, http://www.rndsystems.com), 500 ng/mL bone morphogenic protein-6 (BMP-6, R&D Systems), and 1% insulin-transferrin-selenium (ITS+) Premix (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL bovine serum albumin (BSA), and 5.35 μg/mL linoleic acid; Becton Dickinson, Franklin lakes, NJ, http://www.bd.com). After 14 days, chondrogenic medium was replaced with hypertrophy-inducing medium composed of high-glucose DMEM (Gibco BRL), 50 μg/mL L-ascorbic acid, 40 μg/mL L-proline, 1% ITS+ premix, and 1 nM triiodothyronine (T3, Sigma). The cells were then cultured for an additional 2 weeks.

Determination of Sulfated Glycosaminoglycan Contents

The chondrogenic hUCB-MSC, hBM-MSC, and hAT-MSC pellet was digested with papain in 100 mM sodium acetate (Sigma), 10 mM EDTA (Gibco BRL), 5 mM L-cysteine (Sigma) at 65°C for 16 hours. The sulfated glycosaminoglycan (sGAG) contents were measured using a Blyscan sGAG assay kit (Biocolor, Carrickfergus, U.K., http://www.biocolor.co.uk) following the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction

Relative transcript expression levels of 13 Notch signaling-related genes were analyzed by quantitative real-time polymerase chain reaction (PCR) method using TaqMan probes. Gene-specific primers were designed using the ProbeFinder software provided by the Universal ProbeLibrary Assay Design Center

(Roche Diagnostics, Indianapolis, IN, http://www.roche-applied-science.com). All real-time PCR reactions were performed using the LightCycler 480 II (Roche Diagnostics) and LightCycler 480 Probes Master Mix (Roche Diagnostics). Relative quantification of gene expression was determined using the $2^{-\Delta\Delta Ct}$ method [22] and normalized to an internal control gene (*GAPDH*).

Histological and Immunohistochemical Analysis

Chondrogenic pellets of hUCB-, hBM-, and hAT-MSCs cultured under each experimental condition were frozen in OCT compound (Tissue-Tek, Torrance, CA, http://www.sakura-americas.com), and 5-μm sections were cut. For histological evaluation, sections were stained with Safranin-O (Sigma). For immunohistochemistry, frozen sections were fixed for 10 minutes in ethanol after brief immersion in distilled water to remove the OCT compound. Nonspecific antibody-binding sites were blocked by incubating the slides in phosphate-buffered saline with 5.0% BSA for 1 hour. The slides were incubated overnight at 4°C with primary antibody against type II collagen (Millipore, Billerica, MA, http://www.millipore.com) diluted in 0.05 M Tris-HCl with 1.0% BSA. Reactivity was detected by using the DAKO EnVisionSystem Peroxidase (DAB) kit (K4006, DAKO, Carpinteria, CA, http://www.dako.com) according to the manufacturer's instructions.

Statistical Analysis

Gene expression, protein expression, and ELISA data were analyzed using Microsoft Excel (Microsoft, Redmond, WA, www.microsoft.com) and Student's *t* test. p values $<.05$ were considered significant. Values are expressed as the mean $\pm$ SD of at least four independent experiments.

Results

Variation in Chondrogenic Differentiation of hUCB-MSCs Obtained from Different Donors

We assessed interindividual differences in the chondrogenic differentiation potential of hUCB-MSCs obtained from 24 donors. After 28 days of chondrogenic differentiation, we evaluated the pellet cultures for size, Safranin-O staining intensity, and lacunae formation. These characteristics varied in hUCB-MSCs derived from different donors (Supporting Information Fig. S1). To investigate the differentiation potential of the hUCB-MSC samples further, we selected eight pellets on the basis of size (four large and four small pellets) and the extent of Safranin-O staining (Fig. 1A, 1B). The pellets were assessed for proteoglycan content and localization of collagen type II, which represent the chondrogenic differentiation status. Lacunae structures, which are seen in hyaline cartilage, were also monitored (Fig. 1B, 1C). The results indicated that the large pellets consisted of highly chondrogenic-differentiated MSCs (HC-MSCs).

For validation of the chondrogenic differentiation of the hUCB-MSCs, the expression of cartilage-specific markers was evaluated. Collagen type II, aggrecan, hyaluronan and proteoglycan link protein 1 (HAPLN1), and the chondrogenic transcription factor SOX-9 were highly expressed in the HC-MSC group (Fig. 1E, 1F). These results were corroborated by mRNA expression analysis results (Fig. 1G, 1H). In contrast, the protein and mRNA expression of the cartilage-specific markers in the small pellet group were lower than those in the HC-MSC group. Additionally, the sGAG content of the small pellets was considerably lower than that of the large pellets (Fig. 1D). These findings indicated that the small-pellet samples consisted of MSCs with low chondrogenic differentiation potential (LC-MSCs).

Furthermore, we assessed the chondrogenic differentiation potential of MSCs from other sources including BM and AT. The results confirmed donor-dependent potential difference (Supporting Information Fig. S2), similar to that of the hUCB-MSCs. Taken together, our results showed that the chondrogenic differentiation potential of hUCB-MSCs varied among different donors.

TSP2 Expression in hUCB-MSCs with High Chondrogenic Differentiation Potential

To identify the factors related to the chondrogenic differentiation potential of hUCB-MSCs, we performed microarray analysis to assess gene expression in HC-MSC pellets and LC-MSC pellets that had been allowed to differentiate for 28 days. Heat map analysis revealed that the gene expression pattern of HC-MSCs differed from that of LC-MSCs (Fig. 2A). To determine the fold-change ratio, the signal of HC-MSCs was divided by that of LC-MSCs. Genes with a ratio higher or lower than 2 were selected as differentially expressed between both groups (Supporting Information Table S3).

We previously reported that chondrogenic differentiation of chondroprogenitor cells was enhanced by TSP-2 from hUCB-MSCs through paracrine action [23]. Based on this fact and the potential role of autocrine action in chondrogenic differentiation, this study focused on the TSP family. Among the five TSP subtypes (TSP-1, 2, 3, 4, and 5), the level of TSP-2 was higher in HC-MSCs than in LC-MSCs (Supporting Information Table S4). The remaining four TSP subtypes were not significantly differentially expressed.

To validate quantitative changes, the TSP-2 level in the supernatants was quantified using ELISA. The secreted TSP-2 level was higher in the HC-MSCs than in the LC-MSCs (Fig. 2B). Additionally, the TSP-2 protein and mRNA levels in the HC-MSC group were higher than those in the LC-MSC group (Fig. 2C–2F). Similarly, we found that TSP-2 expression in hBM- and hAT-MSCs was higher in pellets with high chondrogenic differentiation potential (Supporting Information Fig. S3). Taken together, the data indicated that TSP-2 expression differed between HC-MSCs and LC-MSCs. Interestingly, when we analyzed *TSP-2* expression in the MNC fraction of whole hUCB, the MNCs unable to form MSC colonies did not express *TSP-2*, while MNCs containing an MSC population did (Supporting Information Fig. S4). This result indicated that TSP-2 is specifically expressed in the MSC population of hUCB cells.

TSP-2 Mediates Chondrogenic Differentiation of hUCB-MSCs

We studied the correlation between TSP-2 expression and the chondrogenic potential of hUCB-MSCs by inhibiting the expression of *TSP2* with siRNA. Additionally, we evaluated the chondrogenic potential of *TSP2* siRNA-treated hUCB-MSC pellet cultures in the presence of exogenous recombinant human TSP-2 (rhTSP-2) during differentiation.

To verify downregulation of *TSP2* expression by *TSP2* siRNA treatment, we measured secreted TSP-2 levels on day

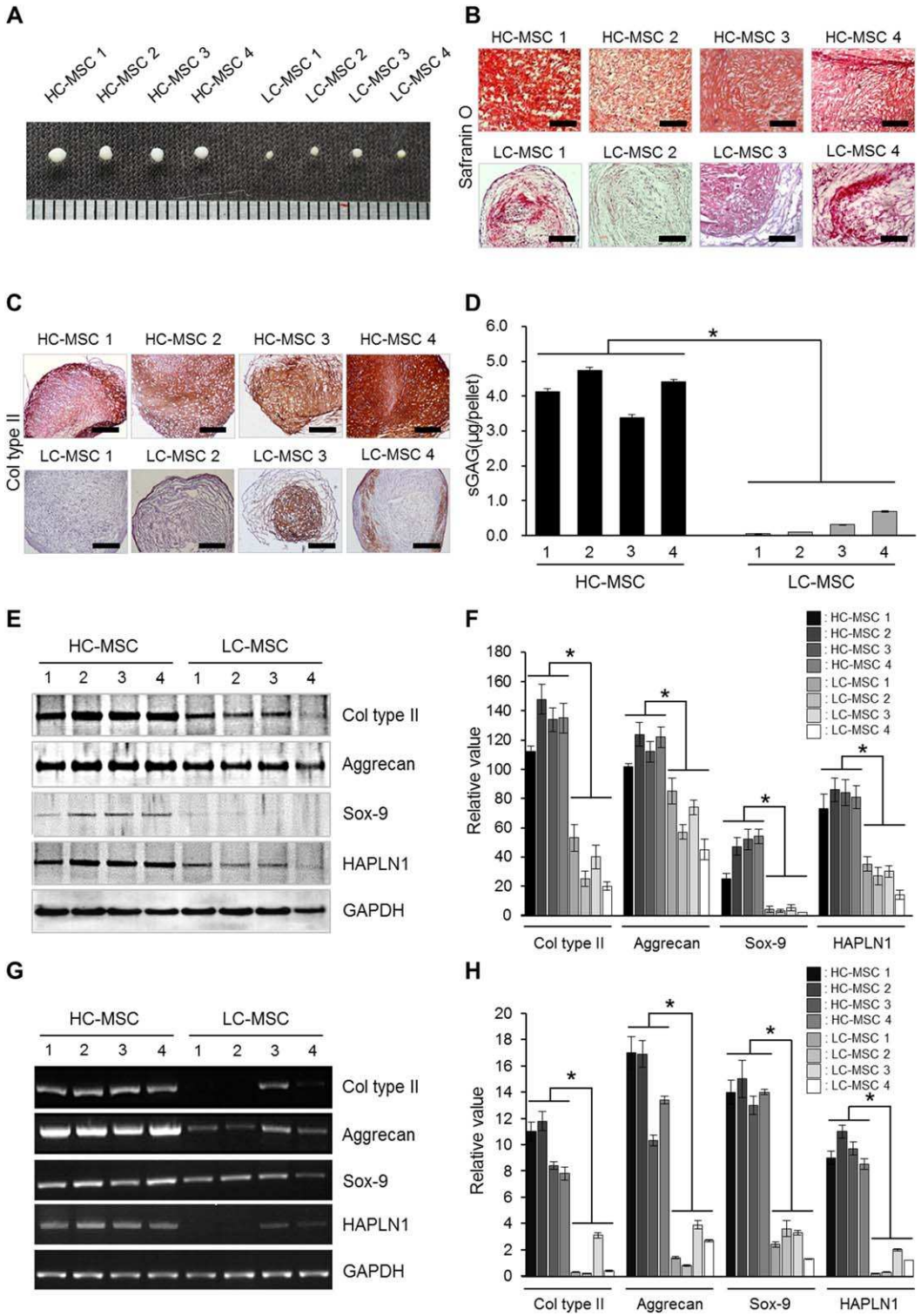


Figure 1. Donor-dependent chondrogenic differentiation potential of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs). hUCB-MSC pellets obtained from different neonates were cultured in chondrogenic medium for 28 days. **(A):** An image depicting eight chondrogenic-differentiated hUCB-MSC pellets. **(B):** Pellets frozen in OCT compound were sectioned and stained with Safranin-O and hematoxylin. **(C):** To assess chondrogenic differentiation, expression of collagen type II was analyzed by immunohistochemical staining. **(D):** The contents of sulfated glycosaminoglycan were assayed. **(E):** The expression of the chondrogenic markers collagen type II, aggrecan, and HAPLN1, and the chondrogenic transcription factor SOX-9 was determined using Western blotting and quantified **(F)** using ImageJ software. **(G):** To validate the expression of the four chondrogenic markers, RT-PCR was performed and **(H)** quantified using ImageJ software. Data are shown as the mean ± SD of at least five independent experiments. *, $p < .05$. Scale bars = 200 μm (in HC-MSC group), 500 μm (in LC-MSC group). Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAPLN1, hyaluronan and proteoglycan link protein 1; HC-MSC, highly chondrogenic-differentiated-MSC; LC-MSC, lowly chondrogenic-differentiated-MSC.

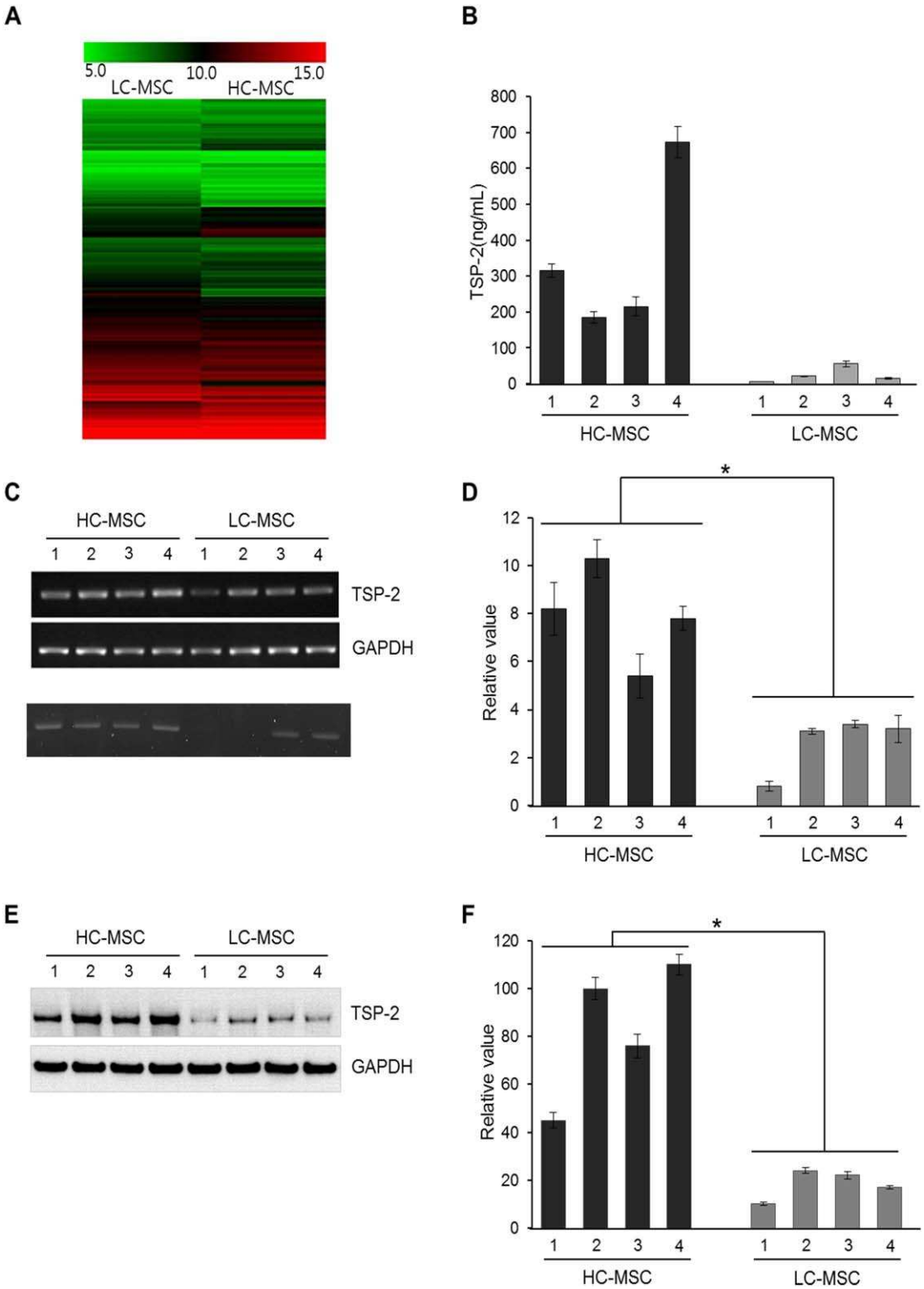
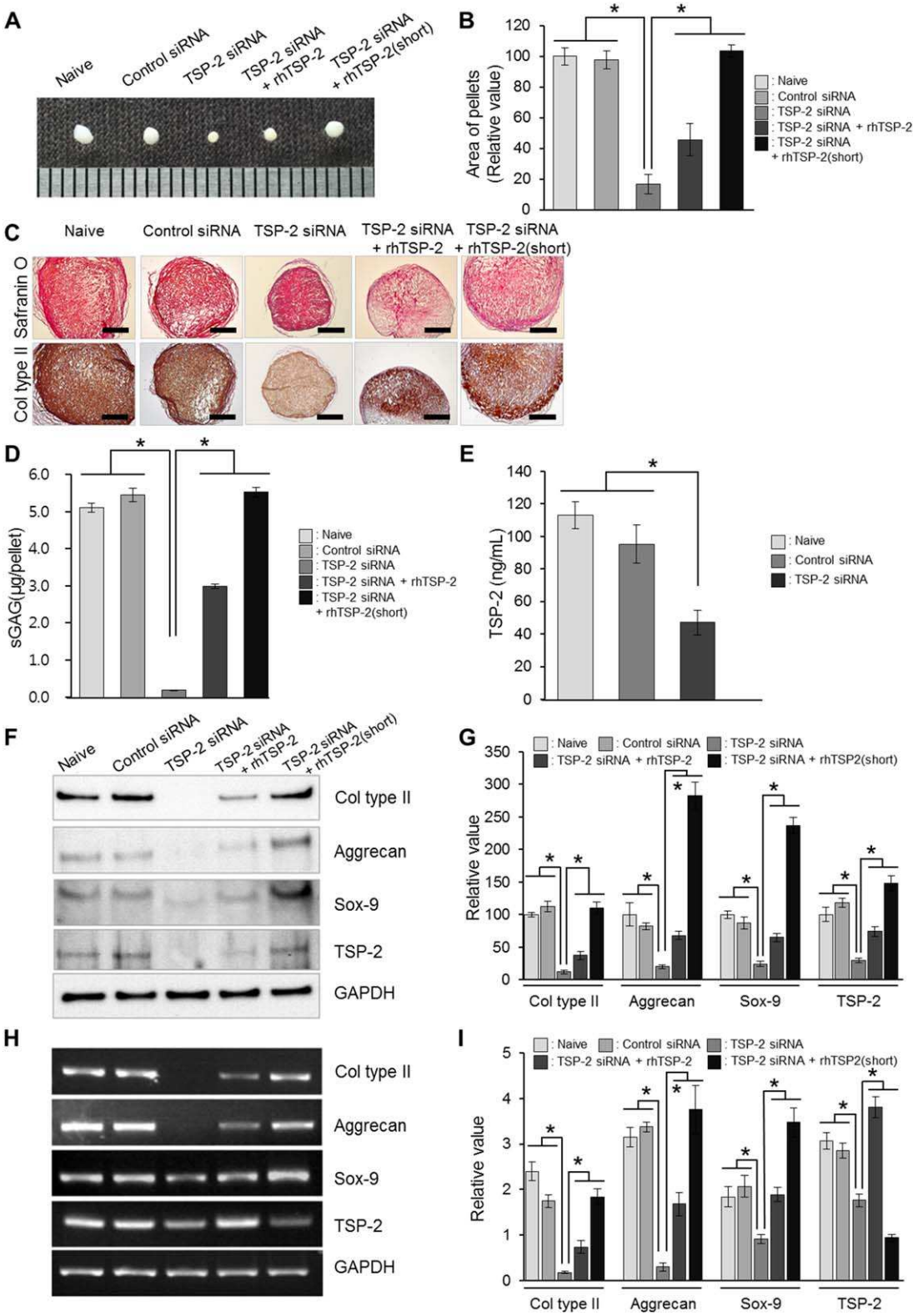


Figure 2. Validation of *TSP-2* expression in hUCB-MSCs with high or low chondrogenic differentiation potential. **(A):** To assess *TSP2* expression in high- and low-chondrogenic-potential human umbilical cord blood-derived mesenchymal stem, cell differentiated for 28 days, RNA microarray analysis was performed. The data are depicted as a heatmap. **(B):** Secreted TSP-2 was quantified by ELISA. **(C):** Expression of *TSP2* mRNA in HC-MSCs and LC-MSCs was analyzed by reverse transcriptase polymerase chain reaction and **(D)** the data were quantified using ImageJ software. **(E):** Western blotting was performed to evaluate TSP-2 expression in lysates of pellet and the data were quantified **(F)** with ImageJ software. Data are shown as the mean ± SD of at least four independent experiments.*, $p < .05$. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HC-MSC, highly chondrogenic-differentiated-MSC; LC-MSC, lowly chondrogenic-differentiated-MSC; TSP-2, thrombospondin-2.

28 of chondrogenic differentiation. The TSP-2 level in the conditioned medium of *TSP2* siRNA-treated pellets was 42% ±9.7% of that in the naïve pellets (Fig. 3E). The degree of chondrogenic differentiation was determined by comparing the pellet sizes. On day 28, *TSP2* siRNA-treated pellets were smaller (16% ± 3%) than the naïve pellets, while the size of the rhTSP-2-treated pellets was 45% ± 7.8% of the pellets grown under naïve conditions (Fig. 3A, 3B).



A previous study showed that the period of initial differentiation affects chondrogenic differentiation of BM-MSCs [24]. Therefore, we treated chondrogenic-differentiating pellets with rhTSP-2 only during the initial 7 days of differentiation. Interestingly, the size of these pellets recovered markedly and was similar to that of the naïve pellets (Fig. 3A, 3B).

To analyze the degree of chondrogenic differentiation, cryosectioned pellets were stained with Safranin-O and collagen type II antibody. The proteoglycan content of the *TSP2* siRNA-treated pellets was lower than that of the naïve pellets. Collagen type II expression was significantly reduced in the sections of siRNA-treated pellets (Fig. 3C), and lacunae were virtually absent. Pellets treated with rhTSP-2 for 28 days showed lacunae structures and proteoglycan expression. The pellets treated with rhTSP-2 during the initial 7 culture days also showed lacunae structures and proteoglycan expression similar to that of the naïve pellet (Fig. 3C).

The sGAG content of the *TSP2* siRNA-treated group significantly decreased (5% of that of the naïve pellets). In contrast, in *TSP2* siRNA-treated pellets supplemented with rhTSP-2 for 28 days, the sGAG concentration was restored to 56% of that of the naïve pellet. In pellets treated with rhTSP-2 during the initial 7 days, the sGAG content significantly recovered to the level in naïve pellets (Fig. 3D).

TSP2 siRNA treatment decreased the expression of chondrocyte-specific factors including collagen type II, aggrecan, and SOX-9 (Fig. 3F, 3G). Treatment with rhTSP-2 for 28 days increased the expression of these factors to 30%–65% of the levels in the naïve pellets. Addition of rhTSP-2 during the initial 7 days augmented the expression levels of these factors to those in naïve pellets or higher (Fig. 3F, 3G). The mRNA expression data corroborated the protein expression data and indicated that chondrogenic marker expression was lowered by *TSP2* siRNA treatment and was reconstituted by rhTSP-2 treatment (Fig. 3H, 3I). From these results, we concluded that chondrogenic differentiation of hUCB-MSCs is regulated by TSP-2. Moreover, similar findings were obtained for hBM- and hAT-MSCs (Supporting Information Fig. S5).

TSP-2 Mediates Chondrogenic Effects through Notch Signaling During Chondrogenic Differentiation of hUCB-MSCs

Previous reports have indicated that Notch signaling is associated with initial chondrogenic differentiation of BM-MSCs [24]; thus, we assessed Notch signaling during early chondrogenic differentiation (initial 8 days) of hUCB-MSCs. Among the four Notch receptors tested, only *NOTCH3* showed increased expression during the first 4 days of differentiation, while the expression of the other receptors did not increase (Supporting Information Fig. S6). Of the five Notch ligands tested, only *JAGGED-1* was gradually induced during chondrogenic differentiation (Supporting Information Fig. S7).

To confirm the involvement of the Notch pathway in early chondrogenic signaling, chondrogenic hUCB-MSC pellets were cultured in chondrogenic medium containing DAPT (*N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-(*S*)-phenylglycine *t*-butyl ester), a γ-secretase inhibitor, for the initial 8 days of the differentiation period. Subsequently, the pellets were further cultured in chondrogenic medium without DAPT for 20 days. The histological and immunohistochemical analyses of the pellets showed that chondrogenic differentiation was inhibited by DAPT (Fig. 4A). DAPT-treated pellets were poorly formed as compared to the naïve pellets; lacunae structures were absent and collagen type II expression was reduced. Additionally, the sGAG level was decreased to 17% of that in the naïve pellets (Fig. 4B).

To evaluate DAPT-induced changes in the expression of downstream targets of Notch signaling, we focused on *HEY1*. *HEY1* mRNA expression increased during the initial differentiation period in chondrogenic pellets; DAPT treatment inhibited this response (Fig 4C). Since Notch signaling was related to chondrogenic differentiation of the hUCB-MSCs, we assessed whether TSP-2 expression also affected *HEY1* expression. *TSP2* siRNA treatment reduced *HEY1* expression and the decrease in expression was similar to that observed in cells treated with DAPT during early differentiation (Fig. 4C). However, addition of rhTSP-2 recovered the *HEY1* expression level to 61% of that of the naïve pellets at day 8. These results indicated that TSP-2 mediates chondrogenic differentiation of hUCB-MSCs via Notch signaling, which was confirmed in hBM- and hAT-MSCs (Supporting Information Fig. S8).

TSP-2 Inhibits Hypertrophy-Associated Marker Expression in hUCB-MSCs Undergoing Hypertrophic Differentiation In Vitro

Our data indicated that the secreted TSP-2 levels decreased during the first 14 days of chondrogenic differentiation of hUCB-MSCs, after which they continuously increased (Fig. 5A). We speculated that consistent expression of TSP-2 might induce hypertrophy because of its chondrogenic promotional effect. We investigated this hypothesis by assessing the expression of TSP-2 in hUCB-MSCs under hypertrophy-inducing conditions in vitro. We observed increased TSP-2 levels throughout the experimental period. Interestingly, the TSP-2 level in hUCB-MSCs cultured underhypertrophy-inducing conditions were higher than that in hUCB-MSCs cultured under chondrogenic differentiation-inducing conditions, from 7 days after induction of hypertrophy (Fig. 5A, Day 21). By 17 days after hypertrophy induction (Day 31), theTSP-2 levels in both naïve and control

Figure 3. Effects of TSP-2 on chondrogenic differentiation of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs). **(A):** Images of chondrogenic differentiated pellets of hUCB-MSCs treated with *TSP2* siRNA or with *TSP2* siRNA plus exogenous TSP-2. The recombinant TSP-2 was added to the chondrogenic medium during 28 days or initial 7 days (Short). **(B):** The pellet size was measured using IMT i-solution software. **(C):** Pellets of hUCB-MSCs cultured under each experimental condition were assessed during a 28-day differentiation period. The pellets were sectioned and stained with Safranin-O. Expression of collagen type II was analyzed using immunohistochemical staining. **(D):** The sGAG contents were measured using the Blyscan sGAG assay kit. **(E):** Except in case of exogenous TSP-2 treatment, the concentration of secreted TSP-2 was measured using commercially available ELISA kits. **(F):** The expression of the chondrogenic markers collagen type II and aggrecan, and the chondrogenic transcription factor SOX-9 was determined by Western blotting and **(G)** quantified using ImageJ software. **(H):** To validate transcript expression of the four chondrogenic markers, reverse transcriptase polymerase chain reaction was performed and **(I)** quantified using ImageJ software. Data are shown as the mean $\pm$ SD of at least five independent experiments. *, $p < .05$. Scale bar = 250 μm. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; sGAG, sulfated glycosaminoglycan; TSP-2, thrombospondin-2.

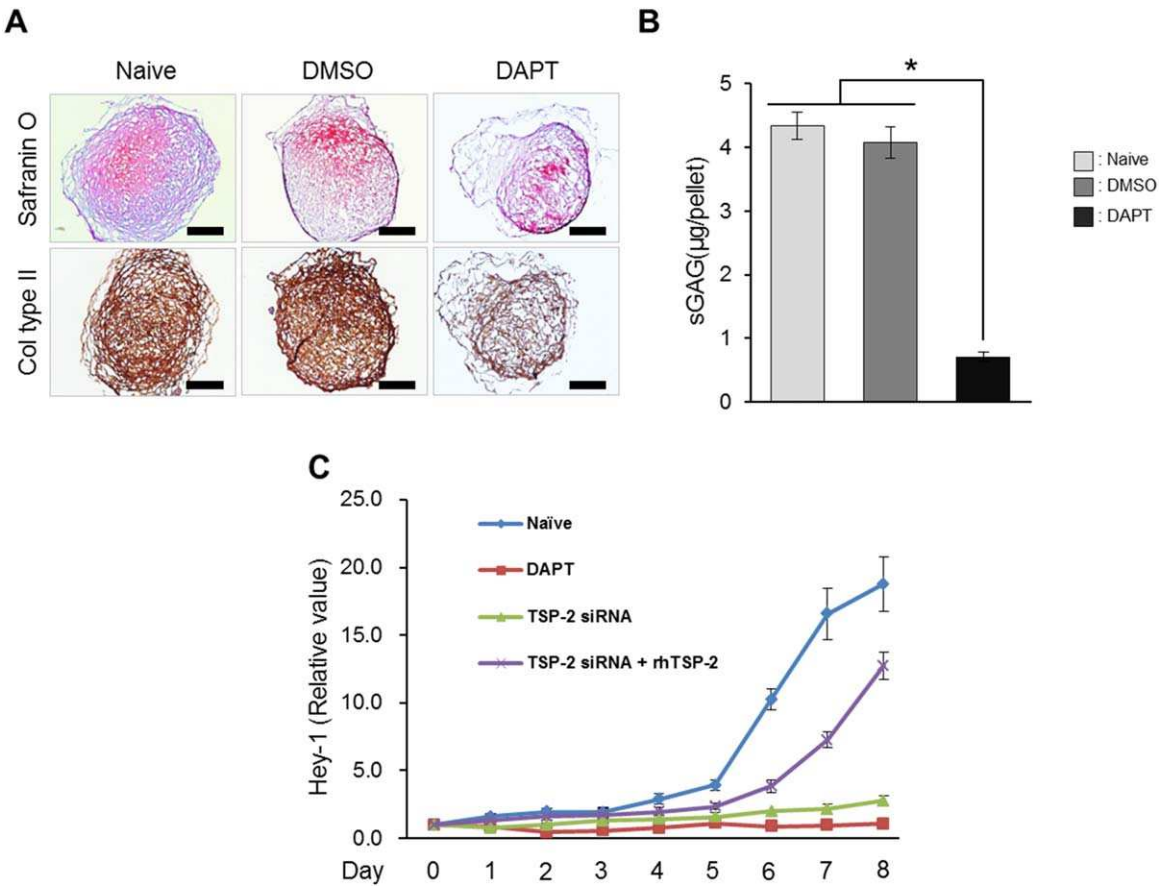


Figure 4. TSP-2 affects Notch signaling in differentiating hUCB-MSCs. **(A):** 50 nM DAPT, a γ-secretase inhibitor of Notch signaling, was added to chondrogenic medium for the initial 8days of differentiation. On day 28 of chondrogenic differentiation, the pellets were sectioned and analyzed using Safranin-O dye and collagen type II antibody. **(B):** On day 28, chondrogenic pellets were digested by papain and the sGAG contents were measured using the Blyscan sGAG assay kit. **(C):** Chondrogenic differentiation of hUCB-MSCs was induced for 8 days. During the culture period, pellets were harvested daily. Of the downstream target genes, *HEY1* showed decreased expression in pellets with*TSP2* suppression and increased expression in pellets supplemented with exogenous TSP-2. Data are shown as the mean $\pm$ SD of at least five independent experiments. *, $p < .05$. Scale bar = 250 μm. Abbreviations: DMSO, dimethyl sulfoxide; DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-(*S*)-phenylglycine *t*-butyl ester; sGAG, sulfated glycosaminoglycan; TSP-2, thrombospondin-2.

siRNA-treated cells under hypertrophy conditions decreased gradually (Fig. 5A).

To assess hypertrophy in the hUCB-MSC pellets, expression of collagen type X was analyzed using a collagen type X antibody. Naïve pellets obtained under chondrogenic conditions showed low collagen type X expression (Fig. 5B). However, in pellets cultured in hypertrophy-inducing medium, the level was increased in all cross-sections. Under hypertrophy-inducing conditions, siRNA-mediated inhibition of TSP-2 expression enhanced collagen type X expression. Addition of rhTSP-2 to *TSP2*siRNA-treated pellets significantly inhibited this increase; the collagen type X expression level was similar to that in the naïve pellets (Fig. 5B).

For further investigation of the effect of TSP-2 on hypertrophy of chondrogenic-differentiated hUCB-MSCs, we measured the protein expression of the hypertrophy markers runt-related transcription factor 2 (RUNX2) and matrix metallopeptidase-13 (MMP-13). Under hypertrophy-inducing conditions, the level of the osteogenic transcription factor RUNX2 was 2.2-fold higher than that in naïve pellets grown under chondrogenic conditions (Fig. 5C, 5D), while the level in *TSP2* siRNA-treated pellets was 2.1-fold higher than that in the naïve pellets. Addition of rhTSP-2 reduced the RUNX2 content to a level comparable to that in the naïve pellets cultured under hypertrophy-inducing conditions (Fig. 5C, 5D). The MMP-13 level in pellets of hUCB-MSCs treated with *TSP2* siRNA was also markedly increased (ninefold higher than those of the naïve pellets cultured under hypertrophy-inducing conditions). Addition of rhTSP-2 to the hypertrophy-inducing medium resulted in recovery of the MMP-13 level to that in the naïve pellets. These expression patterns were paralleled by the mRNA expression results (Fig. 5E, 5F). Taken together, these data indicated that TSP-2 attenuated the expression of hypertrophy-associated markers in hUCB-MSCs that were cultured under hypertrophy-inducing conditions.

Inhibition of Osteogenesis Signaling by TSP-2 During Hypertrophic Differentiation of hUCB-MSCs

To characterize TSP-2-mediated suppression of hypertrophy markers in hUCB-MSCs further, we investigated the signaling pathways associated with inhibition of TSP-2 during hypertrophy. We evaluated the expression of phosphorylated (p)GSK-3β, β-catenin, and pSMAD1 in the WNT/β-catenin and TGF-β/BMP osteogenic signaling pathways. The level of pGSK-3β in

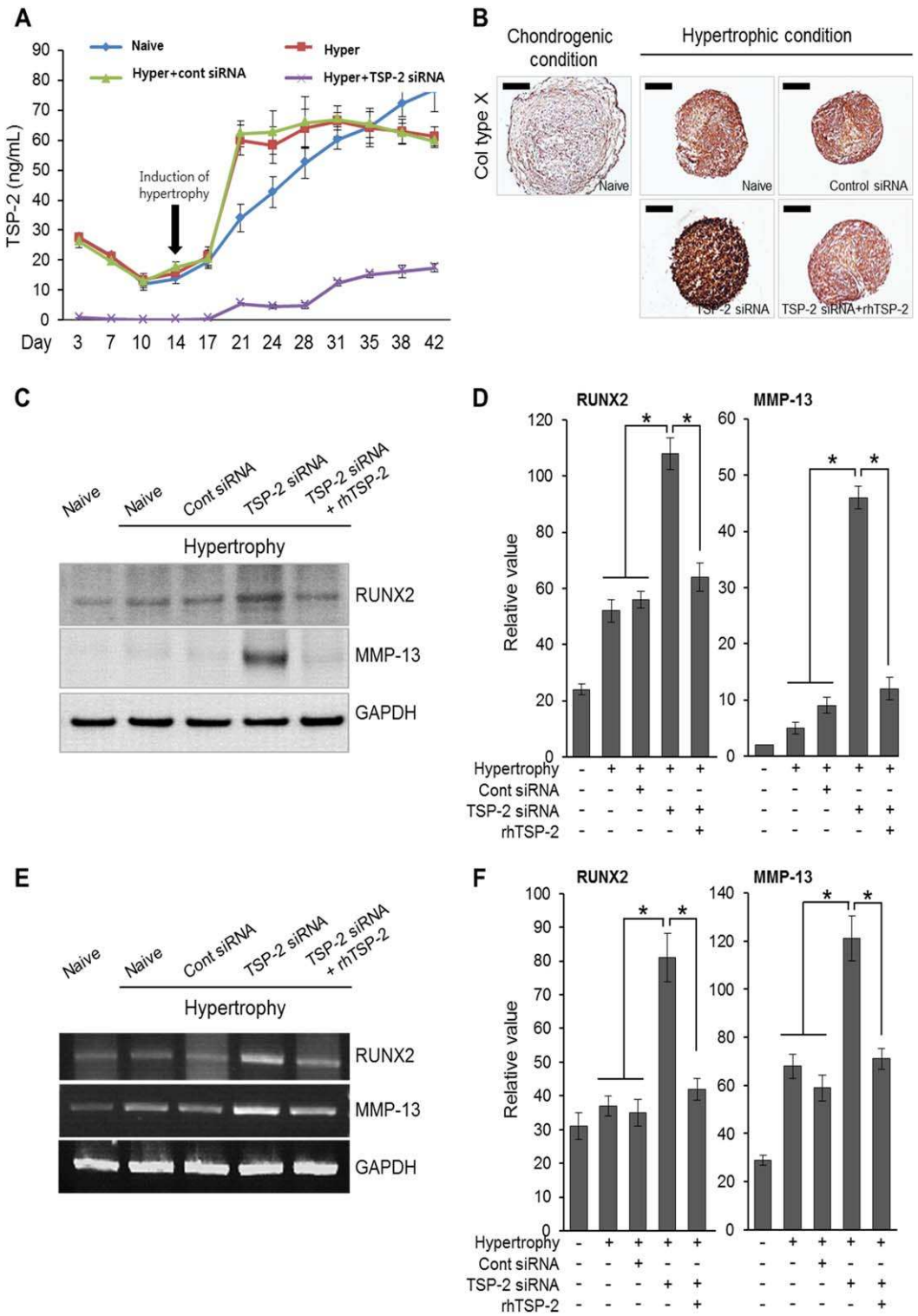


Figure 5. Effect of TSP-2 in in vitro hypertrophic differentiation of human umbilical cord blood-derived MSCs (hUCB-MSCs). For 2 weeks, hUCB-MSC pellets were cultured in chondrogenic medium and then in hypertrophy-inducing medium for an additional 2 weeks. **(A):** The cell culture supernatants were collected twice a week. Secreted TSP-2 in the supernatants was quantified using an ELISA. **(B):** To validate the induction of hypertrophy in the hUCB-MSCs, the pellets were sectioned and the expression of the hypertrophy marker collagen type X was detected by immunohistochemical staining. **(C):** To investigate the effect of TSP-2 on hypertrophic differentiated hUCB-MSCs, two hypertrophy markers (RUNX2, MMP-13) were assessed using Western blotting and **(D)** quantified with ImageJ software. **(E):** Transcript expression of *RUNX2*and *MMP13* was determined using reverse transcriptase polymerase chain reaction and **(F)** quantified with ImageJ software. Data are shown as the mean ± SD of at least four independent experiments. *, $p < .05$. Scale bar = 250 μm. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP3, matrix metallopeptidase 3; RUNX2, runt-related transcription factor 2; TSP-2, thrombospondin-2.

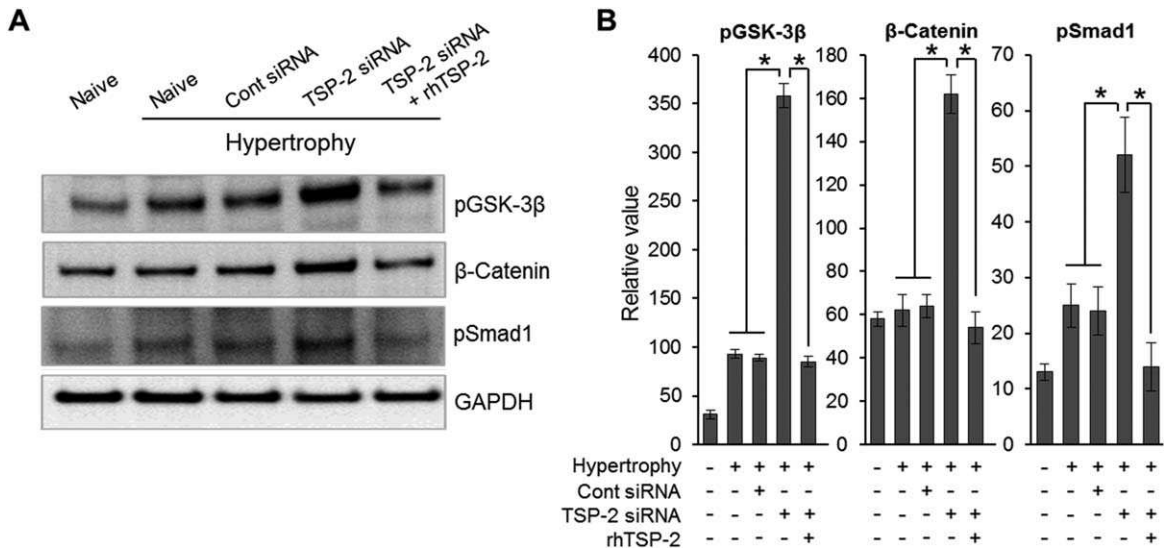


Figure 6. Attenuation effect of TSP-2 on osteogenic signaling pathway during hypertrophic differentiation of human umbilical cord blood-derived MSCs (hUCB-MSCs) in vitro. **(A):** hUCB-MSC pellets were cultured in chondrogenic medium for 2 weeks, and medium was replaced with hypertrophy-inducing medium. After an additional week of cultivation, the pellets were harvested and lysed. The expression of GSK-3β and β-catenin in WNT/β-catenin signaling and SMAD1 in TGF-β/BMP signaling was analyzed by Western blotting and **(B)** quantified with ImageJ software. Data are shown as the mean $\pm$ SD of at least four independent experiments. *, $p < .05$. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; pGSK-3β, phosphor GSK-3β; rhTSP-2, recombinant human TSP-2; TSP-2, thrombospondin-2.

hUCB-MSCs with hypertrophic differentiation was higher than that in the chondrogenic-differentiated pellet. pGSK-3β expression was 3.8-fold higher in hUCB-MSC pellets treated with *TSP2* siRNA than in naïve pellets cultured under hypertrophy-inducing conditions. In addition, in *TSP2* siRNA-treated pellets, the β-catenin and pSMAD1 levels were 2.6-fold and 2.1-fold higher, respectively, than those in naïve pellets cultured under hypertrophy-inducing conditions. The levels of pGSK-3β, β-catenin, and pSMAD1 in rhTSP-2-treated pellets were lower than those in the naïve pellets cultured under hypertrophy-inducing conditions (Fig. 6A, 6B). Taken together, these results demonstrated that TSP-2 can attenuate hypertrophy of hUCB-MSCs through the osteogenic WNT/β-catenin and TGF-β/BMP signaling pathways.

Discussion

Currently, MSC-based therapies are used to treat various diseases, including cartilage defects [25], myocardial infarction [26], liver failure [27], hyperoxic lung injury [28], and Alzheimer disease [29, 30], with varying success [31]. The stem cell differentiation potential, proliferation, and colony-forming capacity decreases with increasing donor age [32, 33], and MSCs with different genetic backgrounds have distinct gene expression profiles that determine the differentiation potential [34]. Therefore, variation in the therapeutic effects of MSCs may be attributed to donor-related variability in the adult stem cell proliferation rate, differentiation potential, and senescence [35]. Recently, there has been a focus on identifying MSCs with desirable characteristics to increase the therapeutic success rate. For instance, hUCB-MSCs with high levels of N-cadherin were found to be most effective for repairing tissue in a myocardial infarction animal model [36], and cell adhesion molecule1 has been identified as a predictive marker for MSCs that can promote effective bone formation [37]. However, reliable markers of the cartilage regeneration potential of MSCs have not yet been characterized. Here, we showed that there are considerable differences in chondrogenic differentiation potential of hUCB-MSCs, hBM-MSCs, and hAT-MSCs obtained from different donors and that this is affected by TSP-2; thus, TSP-2 expression levels in MSCs could be used as a marker for optimal MSC selection for effective cartilage regeneration.

To date, TSP-2 has been known as a multifunctional, anti-angiogenic protein that interacts with the extracellular matrix and various binding partners [38, 39]. Recent studies indicated that TSP-2 is also involved in cartilage biology. TSP-2 is expressed in chondrocytes [40] and its absence results in connective tissue abnormalities in mice [41]. In embryonic and adult mice, TSP-2 is distributed in areas of chondrogenesis [42]. Moreover, we have previously shown that TSP-2 from hUCB-MSCs promotes chondrogenic differentiation of chondroprogenitor cells through paracrine action [23]. Here, we demonstrated that siRNA-mediated *TSP2* knockdown, as well as recombinant TSP-2 supplementation, affects the chondrogenic differentiation of hUCB-MSCs via autocrine signaling. Interestingly, we observed that hUCB-MNCs without MSCs did not express TSP-2, while an MNC fraction containing MSCs did, indicating that TSP-2 is specifically expressed by MSCs in hUCB. In accordance with this result, Hankenson et al. [43] reported that TSP-2 is not expressed in hematopoietic lineage cells and MSCs are the primary source of TSP-2 in marrow.

Several studies have characterized signaling pathways involved in chondrogenic differentiation of MSCs [44, 45]. Various studies have shown that Notch signaling plays a role in chondrogenic differentiation [24, 46–49] and that Notch receptors are expressed in articular cartilage and are involved in chondrogenesis [50, 51]. Indeed, chondroprogenitor cells and MSCs have been confirmed to express Notch signaling

components [46, 47, 52]. Systemic inhibition of Notch led to reduced cartilage formation in vivo [53] and Notch inhibition by DAPT resulted in blockage of chondrogenesis in vitro [54, 55]. Similarly, we previously observed that TSP-2 enhances chondrogenic differentiation of chondroprogenitor cells via Notch signaling, which was inhibited by DAPT treatment [23]. Therefore, we assessed the association between chondrogenic differentiation of hUCB-MSCs and Notch signaling. We found that, similar to BM-MSCs, the Notch signaling pathway regulates chondrogenic differentiation of hUCB-MSCs, and that this differentiation is TSP-2-dependent. Moreover, recent reports have shown that TSP-2 potentiates Notch signaling [56, 57]. Unexpectedly, in the TSP-2 knockdown hUCB-MSCs, the chondrogenic differentiation of cells treated with exogenous TSP-2 during the first 7 days only was higher than that of the cells treated for 4 weeks. This seems to indicate that the role of Notch signaling is dependent on the chondrogenic differentiation status of MSCs. Oldershaw et al. [24] showed that, to complete chondrogenic differentiation of MSCs, Notch signaling must be turned off after the initiation of differentiation. Temporary activation of Notch signaling is also important during the early stages of chondrogenic differentiation of embryonic stem cells [49]. In contrast, continued activation of Notch signaling prevents chondrogenic differentiation of MSCs by interrupting SOX9 binding to *HEY1*on the *COL2A1* enhancer site [58]. These findings suggest that the potentiation effect of TSP-2 on Notch signaling is required in the initial period but must be reduced in the late stages of chondrogenic differentiation. In our study, the endogenous TSP-2 levels were actually reduced during the initial stages of chondrogenic differentiation of hUCB-MSCs. Thus, we speculated that the 4-week treatment with TSP-2 maintained Notch signaling, which interrupts chondrogenic differentiation throughout the differentiation period. Overall, we found that TSP-2 secreted from MSCs mediates their chondrogenic effect by activating the Notch signaling pathway during differentiation.

Induction of chondrogenic differentiation in MSCs inevitably induces hypertrophy in vitro [59]. Additionally, transplanted chondrogenic MSC pellets show a hypertrophic phenotype in vivo due to terminal differentiation through endochondral ossification of the chondrocytes [60]. To date, several factors such as TGF-β [61], BMP [62–64], and GDF-5 [65] have been used for accelerating chondrogenic differentiation of MSCs. These factors can induce hypertrophy by terminal differentiation after accelerating chondrogenic differentiation of MSCs. A previous study showed that coculture with chondrocytes promoted chondrogenic differentiation of MSCs without hypertrophy [66]. Additionally, a recent report demonstrated that intermittent treatment with parathyroid hormone-related protein (1–34) reduced hypertrophy of MSCs [67]. However, to our knowledge, it had not yet been demonstrated that an endogenous factor from MSCs could enhance chondrogenic differentiation and simultaneously attenuate hypertrophy of MSCs, as we have shown for TSP-2. In previous studies, TPS-2 has been partially suggested to have antihypertrophy or antiosteogenic activities [43, 68–70]. In this study, we provided direct evidence for these activities as TSP-2 inhibited the expression of the hypertrophy markers MMP-13 and RUNX-2 and downregulated the upstream WNT/β-catenin and TGF-β/BMP signaling pathways, which have previously been implicated in chondrogenic hypertrophy [71] and osteogenic differentiation of MSCs [72].

Our data indicated that TSP-2 expression was markedly higher during hypertrophy-inducing conditions than during chondrogenic conditions, from day 3 after hypertrophy induction, and decreased from day 17 after hypertrophy induction, possibly due to the role of TSP-2 in a rate-limiting step of the hypertrophic process. We propose that high levels of TSP-2 during the early hypertrophy-inducing period are required to maintain chondrogenic differentiation and to simultaneously inhibit initiation of hypertrophy. Several previous studies have shown that Notch signaling inhibits the transition from prehypertrophic to hypertrophic chondrocytes [46, 73, 74], in particular, via suppressing WNT/β-catenin signaling [75]. Thus, high levels of TSP-2 in the early hypertrophic stage may sustain Notch signaling, resulting in delay of hypertrophic progression. If hypertrophy-inducing conditions are maintained, TSP-2 expression may be downregulated by a negative feedback mechanism for hypertrophy induction. A similar reciprocity between TSP-2 expression and angiogenesis has been shown in tumor growth studies. For instance, TSP-2 is downregulated by a negative feedback mechanism during tumor growth in skin papillomas [76]. Taken together, TSP-2 not only promoted chondrogenic differentiation but also attenuated hypertrophy during in vitro chondrogenesis of hUCB-MSCs, thus regulating chondrogenic differentiation in hUCB-MSCs.

Conclusions

Our findings indicated that hUCB-MSCs obtained from various donors vary in their chondrogenic differentiation potential, which we found to be influenced by the TSP-2 level. TSP-2 promotes chondrogenic differentiation through Notch signaling and suppresses hypertrophy of hUCB-MSCs by inhibiting the expression of osteogenic signaling factors through autocrine action. The chondrogenic effects of TSP-2 were reconfirmed in MSCs from other sources such as BM and AT. Therefore, our results suggested that TSP-2 could be a marker for prediction and selection of MSCs with superior chondrogenic differentiation potential for regeneration of cartilage defects. Furthermore, induced expression of endogenous TSP-2 in MSCs with low chondrogenic differentiation potential may enhance cartilage regeneration.

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Author Contributions

S.Y.J.: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; J.E.H., M.Y.L., H.J.J., and D.H.K.: collection and/or assembly of data; S.J.C., W.I.O., J.-S.K., J.H.C., and D.-H.C.: data analysis and interpretation; Y.S.Y.: data analysis and interpretation and financial support; B.-G.K.: provision of study material and data analysis and interpretation; H.B.J.: conception and design,

data analysis and interpretation, manuscript writing, and final approval of manuscript.

Disclosures of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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