Suppression of discoidin domain receptor 1 expression enhances the chondrogenesis of adipose-derived stem cells

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Wu SC, Hsiao HF, Ho ML, Hung YL, Chang JK, Wang GJ, Wang CZ. Suppression of discoidin domain receptor 1 expression enhances the chondrogenesis of adipose-derived stem cells. Am J Physiol Cell Physiol 308: C685–C696, 2015. First published February 11, 2015; doi:10.1152/ajpcell.00398.2014.—Effectively directing the chondrogenesis of adipose-derived stem cells (ADSCs) to engineer articular cartilage represents an important challenge in ADSC-based articular cartilage tissue engineering. The discoidin domain receptor 1 (DDR1) has been shown to affect cartilage homeostasis; however, little is known about the roles of DDR1 in ADSC chondrogenesis. In this study, we used the three-dimensional culture pellet culture model system with chondrogenic induction to investigate the roles of DDR1 in the chondrogenic differentiation of human ADSCs (hADSCs). Real-time polymerase chain reaction and Western blot were used to detect the expression of DDR1 and chondrogenic genes. Sulfated glycosaminoglycan (sGAG) was detected by Alcian blue and dimethylmethylene blue (MBB) assays. Terminal deoxy-nucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was used to assess cell death. During the chondrogenesis of hADSCs, the expression of DDR1 but not DDR2 was significantly elevated. The depletion of DDR1 expression in hADSCs using short hairpin RNA increased the expression of chondrogenic genes (SOX-9, collagen type II, and aggrecan) and cartilaginous matrix deposition (collagen type II and sGAG) and only slightly increased cell death (2–8%). DDR1 overexpression in hADSCs decreased the expression of chondrogenic genes (SOX-9, collagen type II, and aggrecan) and sGAG and enhanced hADSC survival. Moreover, DDR1-depleted hADSCs showed decreased expression of the terminal differentiation genes runt-related transcription factor 2 (Runx2) and matrix metalloproteinase 13 (MMP-13). These results suggest that DDR1 suppression may enhance ADSC chondrogenesis by enhancing the expression of chondrogenic genes and cartilaginous matrix deposition. We proposed that the suppression of DDR1 in ADSCs may be a candidate strategy of genetic modification to optimize ADSC-based articular cartilage tissue engineering.

Damaged articular cartilage caused by trauma or disease has a limited capacity for self-repair (30, 47), which eventually leads to the progressive deterioration into chondral lesions and osteoarthritis (47). Although current methods of cartilage repair such as microfracture, mosaicplasty, and autologous chondrocyte implantation (ACI) are somewhat successful, they also have significant limitations such as fibrocartilage formation, donor site morbidity, and graft failure (62). Mesenchymal stem cells (MSCs) have attracted considerable interest for clinical use due to their self-renewing potential and chondrogenic differentiation capability (11, 29, 46, 74). MSCs offer many advantages compared with chondrocytes for the repair of damaged cartilage (11, 46, 58, 74, 75). Adipose-derived stem cells (ADSCs) and bone marrow-derived mesenchymal stem cells (BMSCs) are presently the most investigated and best characterized MSCs used to engineer articular cartilage (25, 36). ADSCs have more advantages over BMSCs because the ADSC harvesting procedure is easy and less invasive and yields higher numbers of ADSCs (36, 37). Although the chondrogenic differentiation potential of ADSCs has been reported, an unmet need still exists for the development of strategies to enhance the survival rate of ADSCs and effectively direct ADSC differentiation into the chondrogenic lineage of hyaline cartilage for ADSC-based tissue engineering of articular cartilage (15, 17, 32).

Chondrogenesis is the complex process that results in the formation of a cartilage intermediate (27, 70, 75). During the early stages of chondrogenesis, the mesenchyme expresses various collagens such as collagen type I and IIa (27, 50, 73, 70). Collagens are also widely used as natural scaffold materials for the repair of damaged articular cartilage tissue (51, 56). The discoidin domain receptor tyrosine kinases (DDRs), DDR1 and DDR2, are collagen-binding receptors with a single-pass transmembrane domain that are classified according to the motif in the extracellular domain that is homologous to the...
Dictyostelium discoideum protein discoidin-I (63). DDRs regulate a wide range of cell functions ranging from migration and proliferation to cytokine secretion and extracellular matrix homeostasis/ remodeling (59). Although high similarity is present in the amino acid sequences between DDR1 and DDR2, the several cellular functions of DDR1 and DDR2 are distinct, especially in cartilage and skeletal development (6, 59). Collagens are candidate ligands for both DDRs. Specifically, DDR1 is activated by collagen type I to type V; however, DDR2 is activated primarily by fibrillar collagen type I and type III (57, 63). Although recent studies have indicated that DDR1 plays critical roles in cartilage homeostasis and skeletal development (38, 54, 64), little is known about the roles of DDR1 in the chondrogenesis of ADSCs for articular cartilage tissue engineering. In this study, we test the hypothesis that DDR1 may regulate the chondrogenic differentiation and cell survival of human (h)ADSCs.

MATERIALS AND METHODS

Isolation and culture of hADSCs. The hADSCs were isolated from the subcutaneous adipose tissue as previously described (12). Adipose tissue was obtained from patients with approval from the ethical committee at the Kaohsiung Medical University Hospital (KMUH-IRB-980564). After written informed consent was obtained, subcutaneous adipose tissue was excised from the gluteal area from patient during surgery, minced with scissors, and digested with 1 mg/ml type IA collagenase at 37°C under 5% CO2 for 1-h. The digested tissue was centrifuged at 1,000 rpm for 5 min, and the pellet was washed twice with PBS. The pellet was then resuspended in K-NAC medium and plated in a 100-mm culture dish. The K-NAC medium used in this study helps isolate and expand hADSCs (12). After confluence was achieved, the cells were trypsinized, suspended, washed twice with PBS, and renewed 24 h later and changed every 2 days thereafter.

Transfection with DDR1-specific short hairpin RNA and DDR1 plasmids into hADSCs using microporation. The DDR1-specific short hairpin (sh)RNAs (catalog no. RHS1764-921745; Open Biosystems, Pittsburgh, PA) or nonspecific control shRNA (mock shRNA) as well as control plasmid (control) or wild-type DDR1b plasmid (DDR1 plasmid) (18, 66) were transfected into hADSCs by microporation using a OneDrop MicroPorator (MP-100; BTX Harvard Apparatus, Holliston, MA) according to the manufacturer’s protocol. Briefly, the hADSCs were trypsinized, suspended, washed twice with PBS, and resuspended in resuspension buffer R (BTX Harvard Apparatus) at a concentration of 10^6 cells per 100 μl. Three micrograms of mock shRNA, DDR1-specific shRNA, control plasmid, or DDR1 plasmid suspended in 300 μl of resuspension buffer R were added into each microporation tube. Then, 100 μl of the cell suspension (10^6 cells/100 μl) were added to each microporation tube. The cell suspensions were subsequently microporated at 1,250 V with a 20-ms pulse width and 1-pulse poration. After microporation, the hADSCs were cultured at 37°C under 5% CO2 in K-NAC medium without antibiotics until induction of chondrogenic differentiation using a pellet culture.

Induction of chondrogenic differentiation using a pellet culture. To induce chondrogenic differentiation of the hADSCs, a pellet culture was used (7, 10, 31, 58, 73). The hADSCs that transfected with or without mock shRNA, DDR1-specific shRNA, control plasmid, or DDR1 plasmid were trypsinized and resuspended in basal medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% nonessential amino acid, and 100 U/ml penicillin/streptomycin (GIBCO) at a cell density of 4 × 10^5 cells/ml. hADSC (4 × 10^5 cells) aliquots suspended in 1 ml of basal medium were added in 15-ml polypropylene conical tubes and were centrifuged at 500 g for 5 min to form cell pellets. Then, the cell pellets in 15-ml polypropylene conical tubes were placed into an incubator for 24 h to allow them to become spherical (defined as day 0). Subsequently, the basal medium was removed, and the cell pellets were cultured in chondrogenic medium consisting of basal medium supplemented with 6.25 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), 10 ng/ml TGF-β1 (Sigma-Aldrich), and 50 μM l-ascorbate-2-phosphate (41). The chondrogenic medium was changed every 2 days. At each indicated time point, the cell pellets were collected for further experimental analysis.

RNA isolation and quantitative real-time PCR. At the indicated time points, four cell pellets were collected from the 15-ml polypropylene conical tubes in each experimental group. TRizol reagent (Invitrogen, Carlsbad, CA) was used to extract the total RNA from these cells following the manufacturer’s instructions. The RNA quality was confirmed by detecting the absorbance ratio at 260 and 280 nm using the Thermo Scientific NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). According to the manufacturer’s instructions, the absorbance ratio at 260 and 280 nm ranging from 1.8 to 2.0 was considered as no DNA contamination. Subsequently, 0.5–1 μg of total RNA per 20 μl of reaction volume were reverse transcribed into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen). Real-time PCR reactions were performed and monitored using the iQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA) and a quantitative real-time PCR detection system (Bio-Rad Laboratories). The cDNA samples (2-μl samples in a total volume of 25 μl per reaction) were amplified for the genes of interest. The previously published primers (71, 72) were used for detecting SOX-9, collagen type II (COL-II), aggrecan, collagen type I (COL-I), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The primers used for detecting DDR1, DDR2, matrix metalloproteinase 13 (MMP-13), and runt-related transcription factor 2 (Runx2) were also used. All primers used in this study are listed in Table 1. After the real-time PCR reaction, a dissociation (melting) curve was generated to determine the specificity of the reaction. The relative mRNA expression levels for each target gene were calculated from the threshold cycle (Ct) value of each PCR product and normalized to the expression of GAPDH using the comparative Ct method (44). For each gene of interest, the readings of four cell pellets of each experimental group in every indicated time point were collected.

Western blot. At each indicated time point, the cell pellets were washed twice with ice-cold PBS with 1 mM sodium vanadate and lysed in modified radio immunoprecipitation assay buffer (RIPA); 150 mM NaCl, 1 mM EGTA, 50 mM Tris, pH 7.4, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics, Mannheim, Germany) and 1 mM sodium vanadate. The lysates were cleared by centrifugation at 14,000 rpm for 15 min at 4°C. The expression levels were analyzed by Western blot using antibodies against DDR1 (catalog no. sc-532; Santa Cruz Biotechnology, Santa Cruz, CA), DDR2 (catalog no. AF2538; R&D Systems, Minneapolis, MN), COL-II (catalog no. MAB1330; Chemicon International, Temecula, CA), and β-actin (catalog no. MAB1501; Chemicon International), and they were monitored by enhanced chemiluminescence analysis (ECL system; GE Healthcare, Piscataway, NJ).

Alcian blue staining for the detection of sulphated glycosaminoglycan deposition. To assess the presence of a cartilage-specific matrix, the cells pellets were fixed overnight using 4% paraformaldehyde in PBS and transferred to 70% ethanol until processing. For evaluation, the cell pellets were embedded in paraffin and cut into 5-μm sections. The sections were stained with Alcian blue (Fluka, Buchs, Switzerland) to assess sulphated glycosaminoglycan (sGAG)
Table 1. Primer sequences and cycling conditions for real-time PCR

<table>
<thead>
<tr>
<th>Gene/PCR Primer Sequence (Forward and Reverse)</th>
<th>Annealing Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOX-9</td>
<td>55</td>
</tr>
<tr>
<td>Forward: 5’-CTG CCG GCA GCT GCA CAT-3’</td>
<td>61</td>
</tr>
<tr>
<td>Reverse: 5’-GTT GGG CAG GTA CTC-3’</td>
<td>61</td>
</tr>
<tr>
<td>Collagen type II (COL-II)</td>
<td>61</td>
</tr>
<tr>
<td>Forward: 5’-CAA CAG TGC CAA GCT CCA GAT-3’</td>
<td>61</td>
</tr>
<tr>
<td>Reverse: 5’-TGG TGC ATT GGT AGG CGG TTA TCT-3’</td>
<td>55</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>61</td>
</tr>
<tr>
<td>Forward: 5’-ACA GCT GGG GAC ATT GTG G-3’</td>
<td>61</td>
</tr>
<tr>
<td>Reverse: 5’-TGG GAA TGC AGT GGT TT-3’</td>
<td>61</td>
</tr>
<tr>
<td>Collagen type I (COL-I)</td>
<td>61</td>
</tr>
<tr>
<td>Forward: 5’-GGG TCC TGC TGC TCT TAG-3’</td>
<td>61</td>
</tr>
<tr>
<td>Reverse: 5’-CAG TCC TGC TGC TGC CTA-3’</td>
<td>61</td>
</tr>
<tr>
<td>Discoid domain receptor I (DDR1)</td>
<td>60</td>
</tr>
<tr>
<td>Forward: 5’-AAC AAT TCT TCT CCG GCA CTG-3’</td>
<td>61</td>
</tr>
<tr>
<td>Reverse: 5’-CAT GAG GGC GAT GAG CAG-3’</td>
<td>60</td>
</tr>
<tr>
<td>Discoid domain receptor II (DDR2)</td>
<td>60</td>
</tr>
<tr>
<td>Forward: 5’-GGA GCC TGG TGC CCT GAG-3’</td>
<td>60</td>
</tr>
<tr>
<td>Reverse: 5’-CAG GGA TCA TCA CCA GTC-3’</td>
<td>60</td>
</tr>
<tr>
<td>Matrix metalloproteinase 13 (MMP-13)</td>
<td>60</td>
</tr>
<tr>
<td>Forward: 5’-TCT TCA GCT GGA CTC ATT GT-3’</td>
<td>61</td>
</tr>
<tr>
<td>Reverse: 5’-AGC CTC TCA GTC GGT CTA-3’</td>
<td>55</td>
</tr>
<tr>
<td>Run-related transcription factor 2 (Runx2)</td>
<td>55</td>
</tr>
<tr>
<td>Forward: 5’-AGA TGA GAG TCT GTG TAG-3’</td>
<td>55</td>
</tr>
<tr>
<td>Reverse: 5’-GTAGCTATCCGGGCAGATT-3’</td>
<td>55</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)</td>
<td>61</td>
</tr>
<tr>
<td>Forward: 5’-TTC CTT GCT CTT GCT TCC AG-3’</td>
<td>61</td>
</tr>
<tr>
<td>Reverse: 5’-GCG TCG TGC TGC TCC AAAT C-3’</td>
<td>61</td>
</tr>
</tbody>
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Cycling conditions were as follows: denature: 95°C for 30 s, 95°C for 4 min, followed by 35 cycles of 95°C for 10 s, 55–61°C (shown in column of Annealing Temperature) for 15 s and 72°C for 15 s.

deposition and were counterstained with nuclear Fast Red to identify the nuclei.

Dimethylmethylen blue assay for the quantification of sGAG deposition. At each indicated time interval, the DNA content and sGAG deposition in the cell pellets were quantified spectrophotometrically using 33258 Hoechst dye and dimethylmethylen blue (DMMB), respectively (19, 69). A standard curve for the DMMB assay was generated using an aqueous solution of chondroitin sulphate C (Sigma-Aldrich), with concentrations ranging from 0 to 25 μg/ml.

Terminal deoxy-nucleotidyl transferase-mediated dUTP nick end labeling staining to assess cell death. We measured cell death in the cell pellets via the terminal deoxy-nucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining method, using the In Situ Cell Death Detection Kit TMR Red (Roche Diagnostics). Following the manufacturer’s guidelines, sections of the cell pellets were deparaffinized and then rehydrated. The sections were rinsed twice with PBS and then permeabilized by incubation in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. The TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and rhodamine (the labeling dye) was added to the slides and incubated at 37°C in a humidified chamber in the dark for 60 min. The reaction was stopped by blocking buffer (0.1% Triton X-100/0.5% bovine serum albumin in PBS). The cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The slides were observed under a fluorescence microscope with an 800-nm excitation wavelength for rhodamine and 365 nm for DAPI. The cell nuclei were stained blue with DAPI, and only dead cells were stained red with rhodamine. The number of stained cells was counted, and the data were analyzed using Image-Pro Plus analysis software (Media Cybernetics, Silver Spring, MD). The rate of cell death in the cell pellets was defined as the ratio of red-stained cells (dead cells) to blue-stained cells (total cells).

Statistical analysis. Each experiment was repeated at least three times, and the data are expressed as the means ± SE of the combined data from each experimental replicate. Statistical significance was evaluated by a one-way ANOVA, and multiple comparisons were performed using Scheffe’s method. A P < 0.05 was considered significant.

RESULTS

DDR1 expression was increased during hADSC chondrogenesis. To determine the expression levels of DDR1 and DDR2 during the chondrogenic differentiation of hADSCs, the hADSCs were cultured in the three-dimensional pellet culture system with basal medium (Control group) or chondrogenic medium (chondro-induction group; Ch-I group). First, the chondrogenic differentiation of hADSCs was confirmed by using Alcian blue staining and DMMB assay to detect and quantify sGAG deposition by hADSCs. Alcian blue staining results showed intense sGAG staining in the Ch-I group at day 11 compared with the Control group (Fig. 1A). The DMMB quantification assay showed that the total amount of sGAG deposition (Total sGAG; Fig. 1B) and the average amount of sGAG per cell (sGAG/DNA; Fig. 1B) were significantly higher in the Ch-I group compared with the Control group, which confirms the chondrogenic differentiation of hADSCs in the Ch-I group. No significant difference in the total DNA content (Total DNA) was observed between these two groups (Fig. 1B). In the Ch-I group, the real-time PCR results showed that DDR1 expression was significantly increased at day 2 and decreased on day 3 compared with the Control group (Fig. 1C). In contrast, no significant difference in DDR2 mRNA expression was observed between the Ch-I group and Control group in hADSCs (Fig. 1C). DDR1 protein expression in the Ch-I group was significantly increased compared with the Control group (Fig. 1D). The DDR2 protein expression level was not significantly different between the Ch-I group and the Control group (Fig. 1D).

DDR1 depletion enhanced hADSC chondrogenic differentiation. To deplete DDR1 expression in hADSCs, the microporation method was used to transfer DDR1-specific shRNA (DDR1-depleted group) or nonspecific control oligonucleotides (Mock group) into the hADSCs. The hADSCs were transfected with plasmids encoding green fluorescent protein (GFP) as a reporter gene to evaluate the microporation transfection efficacy. Figure 2A depicts that via the microporation technique; the GFP plasmids were efficiently transfected into hADSCs with a transfection rate of ~80% (data not shown). DDR1 gene expression in the DDR1-depleted group was significantly decreased after treated with chondrogenic medium in pellet culture for 24 and 72 h compared with the Mock group (Fig. 2B). DDR1 protein expression was also significantly decreased in the DDR1-depleted group compared with the Mock group after being treated with chondrogenic medium in pellet culture for 3 and 5 days (Fig. 2C).

To investigate whether DDR1 is involved in hADSC chondrogenic differentiation, chondrogenic marker gene expression and cartilaginous matrix synthesis in the Mock group and DDR1-depleted group were evaluated after culturing in the three-dimensional pellet cultures system with chondrogenic medium. The real-time PCR results showed that SOX-9, COL-II, and Aggrecan gene expression significantly increased in the DDR1-depleted group at day 5 compared with the Mock group (Fig. 3A). Higher COL-II protein expression was found in the DDR1-depleted group compared with the Mock group (Fig.
Fig. 1. Expression pattern of the discoidin domain receptors (DDRs) and sulphated glycosaminoglycan (sGAG) deposition during human adipose-derived stem cell (hADSC) chondrogenic differentiation. hADSCs were grown as cell pellets and were treated with chondrogenic (Ch-I group) or basal medium (Control group). A: sGAG synthesis of hADSCs assessed with Alcian blue staining for the Control and Ch-I groups on day 11. Blue: Alcian blue stained. Red: cell nuclei stained. Scale bars = 300 and 50 μm. B: total sGAG synthesis by hADSCs in the Control and Ch-I groups at days 9 and 11 were quantified using the dimethylmethylene blue (DMMB) assay. sGAG synthesis normalized to the total amount of DNA in each group is expressed as sGAG/DNA. The total sGAG, sGAG/DNA, and total DNA are expressed relative to the control group at day 9, which is defined as 1. C: mRNA expression levels of DDR1 and DDR2 in the Control and Ch-I groups from 1 to 5 days. The mRNA expression of DDRs in hADSCs is expressed relative to the control group at day 0, which is defined as 1. D: protein expression of DDR1, DDR2, and β-actin in the Control and Ch-I groups from 1 to 5 days. The ratio of DDRs/β-actin in hADSCs is expressed relative to the Control group on day 1, which is defined as 1. Values are means ± SEM (n = 3–4). *P < 0.05 and **P < 0.01, respectively, compared with the Control group.
3B). The Alcian blue staining results showed more sGAG deposition by hADSCs in the DDR1-depleted group compared with the Mock group (Fig. 3C). The DMMB assay quantification results showed that the average amount of sGAG deposition (sGAG/DNA) by hADSCs in the DDR1-overexpressed group was significantly decreased compared with the Control group at day 5 compared with the Control group (Fig. 4B). The Alcian blue staining results showed that the sGAG staining in the DDR1-overexpressed group was lighter than the staining in the Control group (Fig. 4C). The DMMB assay quantification results showed that the average amount of sGAG deposition (sGAG/DNA) by hADSCs in the DDR1-overexpressed group was significantly decreased compared with the Control group at day 11 (Fig. 4D). These results suggest that the overexpression of DDR1 decreases the chondrogenic differentiation of hADSCs.

DDR1-depletion decreases the mRNA expression of the terminal differentiation-related genes Runx2 and MMP-13 in hADSCs. To investigate whether DDR1-depletion in hADSCs directs the formation of hyaline cartilage rather than fibrocartilage during chondrogenic differentiation, the mRNA expression of COL-I, which is significantly expressed in fibrocartilage, was detected in the DDR1-depleted and Mock groups after culturing in the three-dimensional pellet culture system with chondrogenic medium. The results showed that COL-I mRNA expression did not significantly change in the DDR1-depleted group compared with the Mock group at days 1–5 (Fig. 5A). The expression of the terminal differentiation-related genes Runx2 and MMP-13 was also investigated. Runx2 mRNA expression was significantly decreased on day 1 compared with the Mock group (Fig. 5B). MMP-13 mRNA expression in the hADSCs also significantly decreased in the DDR1-depleted group compared with the Mock group at days 1–5 (Fig. 5C). These results show that the suppression of DDR1 expression during hADSC chondrogenic differentiation did not alter COL-1 expression but decreased the expression of the terminal differentiation genes Runx2 and MMP-13.

DDR1 depletion increased cell death, whereas DDR1 overexpression decreased cell death during the chondrogenic differentiation of hADSCs. To investigate the effect of DDR1 on the viability of hADSCs during chondrogenic differentiation, we measured hADSC cell death in the DDR1-depleted and DDR1-overexpressed groups after chondroinduction using the TUNEL staining method. The TUNEL staining results showed that the rate of hADSC cell death increased (2–8%) in the DDR1-depleted group at days 3 and 5 compared with the Mock group (Fig. 6A). In contrast, lower hADSC cell death was found in the DDR1-overexpressed group at days 3 and 5 than in the Control group (Fig. 6B). These results suggest that DDR1 depletion slightly increased cell death, whereas DDR1 overexpression decreased cell death during the chondrogenic differentiation of hADSCs.

**DISCUSSION**

Effectively enhancing stem cell chondrogenesis to engineer a hyaline cartilage is still an unmet need in stem cell-based articular cartilage tissue engineering (8, 15, 17, 24, 30, 32, 33). The expression of collagen receptors, including DDR1 and DDR2, has been indicated as an important factor in the homeostasis of cartilage; however, little is known about the roles of DDRs, especially DDR1, in ADSC chondrogenesis (38, 64). In this study, we showed that DDR1 expression increased...
during hADSC chondrogenesis, while DDR2 remained unchanged. Both the DDR1 loss of function and gain of function experiments were performed to investigate the roles of DDR1 in hADSC chondrogenesis. The suppression of DDR1 expression during hADSC chondrogenesis increased chondrogenic gene expressions and cartilaginous matrix production, while DDR1 overexpression had an inhibitory effect on hADSC chondrogenesis. The enhancement effect on chondrogenesis
Fig. 4. Effect of DDR1 overexpression on the chondrogenic differentiation of hADSCs. Control plasmid (Control group) and DDR1 plasmid (DDR1-overexpressed group)-transfected hADSCs were cultured in the pellet culture system with chondrogenic medium. A: protein expression levels of DDR1 and β-actin in the Control and DDR1-overexpressed groups after cultured in chondrogenic medium for 3 and 5 days. B: mRNA expressions levels of chondrogenic genes, SOX-9, COL-II, and aggrecan, in the Control and DDR1-overexpressed groups at days 3 and 5. The mRNA expression level of each gene is expressed relative to the Control group at day 3, which is defined as 1. C: Alcian blue staining of sGAG produced by hADSCs in the Control and DDR1-overexpressed groups on day 11. Blue: Alcian blue stained. Red: cell nuclei stained. Scale bars = 300 and 50 μm. D: total sGAG synthesis by hADSCs in the Control and DDR1-overexpressed groups on days 9 and 11 was quantified by the DMMB assay. The sGAG synthesis normalized to total DNA for each group was expressed as sGAG/DNA. Values of sGAG/DNA are expressed relative the Mock group at day 9, which is defined as 1. Values are means ± SE (n = 4). *P < 0.05 and **P < 0.01, respectively, compared with the Control group.
chondrogenesis, the hADSC DDR1 mRNA and protein levels were elevated at approximately days 1 to 3 after chondrogenic induction; however, the DDR2 mRNA and protein levels remained unchanged (Fig. 1, C and D). Based on these results, we speculate that the expression of DDR1 during chondrogenesis may affect chondrogenic genes expression of SOX-9, COL-II, and aggrecan as well as sGAG deposition in hADSCs. In the loss and gain of function experiments, the depleted of DDR1 in hADSCs enhanced chondrogenic genes on day 5 and sGAG on days 9 and 11 (Fig. 3); on the contrary, the overexpression of DDR1 in hADSCs decreases chondrogenic genes on day 5 and sGAG on day 11 (Fig. 4). These results suggest that DDR1 may regulate the ADSC chondrogenesis by regulated chondrogenic gene expression of SOX9, COL-II, and aggrecan as well as sGAG deposition in hADSCs; however, the mechanism needs further investigation.

The miR-199a* is a microRNA that has been reported as an inhibitor of early chondrogenic differentiation (40). DDR1 is a direct target of miR-199a, which shares the same pre-miRNA hairpin with miR-199a* (55). In this study, we showed that the mRNA expression of DDR1 was significantly decreased on day 3 compared with the Control group (Fig. 1C). We speculate that DDR1 mRNA expression may be regulated by miR-199a during ADSC chondrogenesis. Furthermore, DDR1 protein expression in the Ch-I group was significant increased at days 1–3 compared with the Control group (Fig. 1D). Because the DDR1 mRNA expression was not increased at days 1 and 3, it is possible that the posttranslational regulation may involve in the upregulation of DDR1 protein levels at days 1–3 during ADSC chondrogenesis. Ectodomain shedding, the proteolytic cleavage of extracellular domains of membrane-anchored protein, is an important posttranslational regulatory mechanism for modifying the function of cell surface proteins (61). It has been reported that DDR1 “shedding” can be mediated by transmembrane MMPs (such as MT1-MMP, MT2-MMP, and MT3-MMP) (20). We suppose that the increase of DDR1 protein expression at days 1 and 3 during ADSC chondrogenesis may be due to the inactivation of transmembrane MMPs. However, further studies are needed to clarify these associations.

Viral vectors are highly efficient as delivery systems (1, 2, 52); however, the potential for oncogenic activation, inflammatory, immunogenic, and mutagenic effects of viral methods make them a safety risk (4). Although both viral and nonviral methods have been reported, transferring genes into ADSCs by nonviral methods is difficult (13, 68). Microporation is a nonviral electroporation method that possesses high transfection efficacy for gene delivery into hADSCs without impairing their stem cell properties (3, 39, 67). Therefore, in this study, we used the microporation method to transfect DDR1-specific shRNA or DDR1 plasmids into hADSCs to respectively deplete or overexpress DDR1 in hADSCs. Our results show that DDR1 expression in hADSCs at the mRNA and protein levels can be efficiently downregulated on the first 3 and 5 days after the delivery of DDR1-specific shRNA using microporation (Fig. 2). Regarding the delivery of the DDR1 plasmid, DDR1 protein the expression was efficiently increased in hADSCs (Fig. 4A). Thus our data suggest that microporation is a good method for the genetic modification of DDR1 expression in hADSCs.

Native articular cartilage is hyaline cartilage that is rich in COL-II and sGAG but negative for COL-I (9, 30). Current...
techniques for cartilage repair usually form fibrocartilage, which is characterized by containing significant amounts of COL-I, that possesses inferior mechanical properties compared with native hyaline articular cartilage (30, 45, 49). The expressions of terminal differentiation genes such as Runx2 and MMP-13 was found in damaged cartilage chondrocytes (21, 22, 42, 60). Moreover, the expression of Runx2 in chondrocytes results in the production of MMP-13, which is induced during chondrocyte terminal differentiation (14, 28). In this study, we showed that the depletion of DDR1 does not change COL-I expression (Fig. 5A) but decreases terminal differentiation genes (Runx2 and MMP-13) expression (Fig. 5, B and C) when induced into chondrogenic differentiation, which suggest that DDR1 depletion

Fig. 6. Effect of DDR1 modification on hADSC cell survival during chondrogenic differentiation. A: mock shRNA (Mock group) and DDR1-specific shRNA (DDR1-depleted group)-transfected hADSCs were cultured in pellet culture system with chondrogenic medium for 3 and 5 days. Representative images of bright field, DAPI-stained, and terminal deoxy-nucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-stained images of Mock and DDR1-depleted hADSCs cultured in chondrogenic medium for 5 days are shown. Scale bars = 300 μm. Rate of cell death was quantified. Values are means ± SE (n = 4). *P < 0.05 and **P < 0.01, respectively, compared with the Mock group. B: control plasmid (Control group) and DDR1 plasmid (DDR1-overexpressed group)-transfected hADSCs were cultured in the pellet culture system with chondrogenic medium for 3 and 5 days. The representative images of DAPI-stained and TUNEL-stained images of Control and DDR1-overexpressed hADSCs cultured in chondrogenic medium for 5 days are shown. Scale bars = 300 μm. Rate of cell death was quantified. *P < 0.05 and **P < 0.01, respectively, compared with the Control group.
in hADSCs may not promote terminal differentiation when induced into chondrogenic differentiation. Cell death in implanted reparative tissue makes reparative tissue fail to integrate into host cartilage, causing cartilage repair to fail clinically (34). DDR1 suppression showed a beneficial effect on enhancing chondrogenesis; however, preventing hADSC death is also a crucial issue for effective ADSC-based articular cartilage repair. Therefore, we investigated hADSC cell death after DDR1 modification. Our results showed that the DDR1 depletion in hADSCs only slightly enhances cell death (Fig. 6A) and the overexpression of DDR1 increases cell survival (Fig. 6B). These results suggest that DDR1 depletion in hADSCs may be a beneficial genetic modification strategy for more effective ADSC-based articular cartilage tissue engineering through enhanced chondrogenesis and a minor effect on decreased hADSC survival.

The limitation of this study is that we did not test the DDR1 depletion in hADSCs for cartilage repair in vivo. Repair of articular cartilage defect and maintaining the repaired cartilage for a long time is still an unsolved problem. The transplantation of chondrocytes or MSCs with biomaterials could stimulate the formation of a new articular surface in vivo and could effectively repair the defects of articular cartilage over a long-term follow-up (9). However, complications like hypertrophic change of chondrocytes and graft failure are also indicated (46). Further in vivo studies are needed to determine whether this DDR1 depletion in hADSCs is beneficial for long-term articular cartilage repair. On the other hand, although we found that the DDR1 depletion in ADSCs could promote chondrogenesis, the detailed molecular mechanism is still not defined. Studies have also indicated that there are mutual antagonistic action between DDR1 and integrins (22, 57). It has been reported that α1β1, α2β1, and α3β1-integrins are activated during the chondrogenesis of MSCs and that this activation produces signal transduction factors that further induced chondrogenesis (16, 25). A previous study showed that integrins are critical for vascular smooth muscle survival (56). Therefore, we speculate that DDR1 and integrins may be mutually regulated by antagonistic action to control chondrogenesis and survival of hADSCs. The molecular mechanism of the interaction between DDR1 and integrin on promoting chondrogenesis and survival of hADSCs needs to be further investigated.

REFERENCES
Discoid Domain Receptor 1 on Adsc Chondrogenesis

C695


