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Effect of chondrocyte-derived early extracellular matrix on chondrogenesis of placenta-derived mesenchymal stem cells

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Keywords: cartilage, chondrocyte-derived extracellular matrix, chondrogenesis, decellularization, human placenta-derived mesenchymal stem cell

Supplementary material for this article is available [online](#)

Abstract

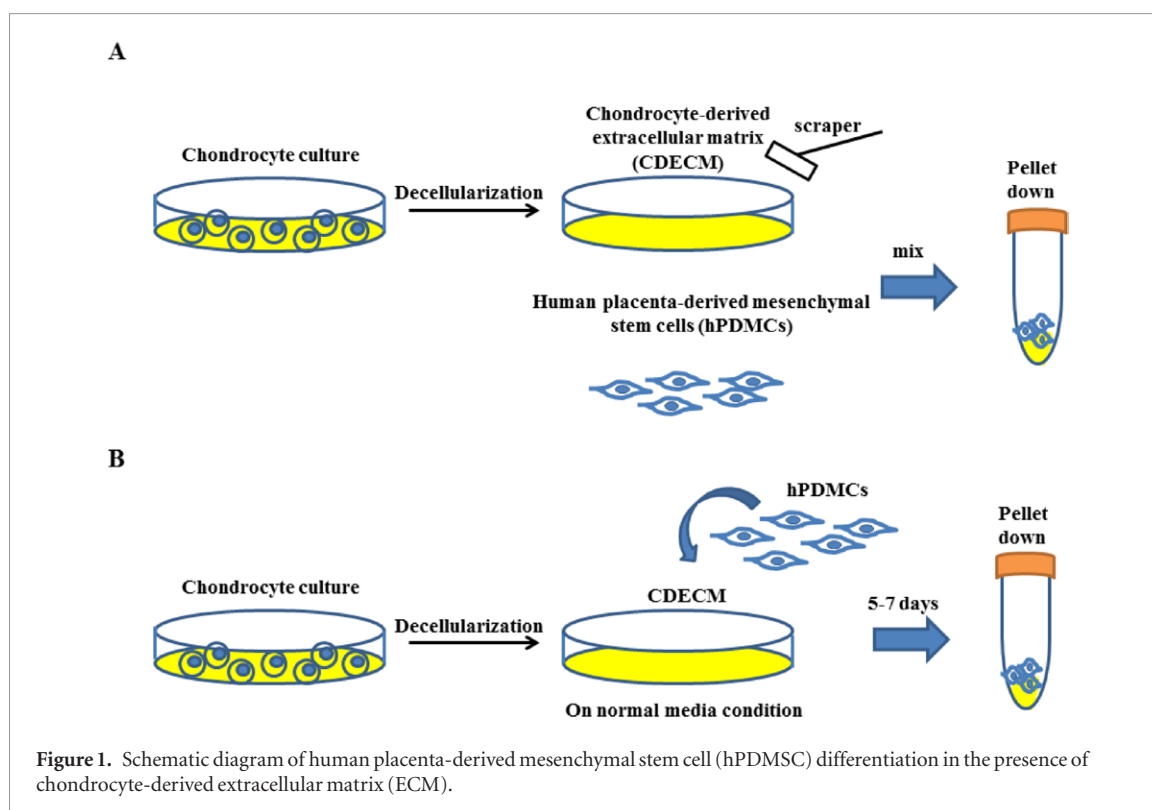
The extracellular matrix (ECM) surrounding cells contains a variety of proteins that provide structural support and regulate cellular functions. Previous studies have shown that decellularized ECM isolated from tissues or cultured cells can be used to improve cell differentiation in tissue engineering applications. In this study we evaluated the effect of decellularized chondrocyte-derived ECM (CDECM) on the chondrogenesis of human placenta-derived mesenchymal stem cells (hPDMSCs) in a pellet culture system. After incubation with or without chondrocyte-derived ECM in chondrogenic medium for 1 or 3 weeks, the sizes and wet masses of the cell pellets were compared with untreated controls (hPDMSCs incubated in chondrogenic medium without chondrocyte-derived ECM). In addition, histologic analysis of the cell pellets (Safranin O and collagen type II staining) and quantitative reverse transcription-PCR analysis of chondrogenic markers (aggrecan, collagen type II, and SOX9) were carried out. Our results showed that the sizes and masses of hPDMSC pellets incubated with chondrocyte-derived ECM were significantly higher than those of untreated controls. Differentiation of hPDMSCs (both with and without chondrocyte-derived ECM) was confirmed by Safranin O and collagen type II staining. Chondrogenic marker expression and glycosaminoglycan (GAG) levels were significantly higher in hPDMSC pellets incubated with chondrocyte-derived ECM compared with untreated controls, especially in cells precultured with chondrocyte-derived ECM for 7 d. Taken together, these results demonstrate that chondrocyte-derived ECM enhances the chondrogenesis of hPDMSCs, and this effect is further increased by preculture with chondrocyte-derived ECM. This preculture method for hPDMSC chondrogenesis represents a promising approach for cartilage tissue engineering.

Introduction

The extracellular matrix (ECM) is an important component of the tissue microenvironment, and includes soluble factors, cell–cell interactions, and mechanical stimulation. The various structural and functional proteins of the ECM contribute to the structural integrity of the tissue and its unique shape. These proteins also regulate cell survival, proliferation, adhesion, migration, and differentiation through cell–matrix interactions [1]. ECM adhesion receptors and ligands regulate cell signaling, influencing various cellular functions [1–4]. For these reasons, ECM plays

a critical role in tissue engineering using mesenchymal stem cells (MSCs). The ECM can be isolated from tissues or cultured cells using physical, chemical, or enzymatic decellularization methods [5–7] to produce functional tissues and organs including heart [8, 9], heart valve [10, 11], trachea [12, 13], lung [14, 15], bladder [16, 17], and cartilage tissues [18, 19].

Cartilage tissue engineering involves the chondrogenic differentiation of MSCs. Recent studies have demonstrated the ability of ECM to direct stem cells toward specific fates [20–22] and ECM obtained from the decellularization of cultured cells has been shown to induce MSC differentiation for cartilage regeneration



[23–28]. One study compared an ECM scaffold derived from porcine chondrocytes with a polyglycolic acid scaffold, demonstrating that MSC chondrogenesis was more rapid and the chondrogenic phenotype was maintained longer with the ECM scaffold [23]. In addition, chondrogenesis of MSCs was successful using 3D collagen microspheres produced by decellularization of porcine chondrocytes [24] and an acellular electrospun poly (ϵ -caprolactone)/ECM composite scaffold [27].

ECM has previously been used in cartilage tissue engineering to promote the differentiation of MSCs derived from bone marrow or adipose tissue but not those derived from placenta, which is a good source of MSCs and normally discarded after delivery, minimizing ethical concerns. We hypothesized that the effect of chondrocyte-derived ECM (CDECM) would be different by way of ECM application to the chondrogenesis of human placenta-derived MSCs (hPDMSCs). Therefore, we compared the hPDMSCs precultured with chondrocyte-derived ECM for 7 d before chondrogenic differentiation with the same cells without this pre-culture step to determine the most effective technique for cartilage tissue engineering.

Materials and methods

Preparation of chondrocyte-derived extracellular matrix

Human chondrocytes obtained from ATCC (CRL-2847, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotic–antimycotic solution and cultured at 37 °C in a 5% CO₂ incubator. When cells exceeded 80% confluence, they

were detached with TrypLE™ Express (Gibco), counted, and seeded into 100 mm cell culture dishes (1×10^5 cells/dish). The cells were cultured for 7 or 8 d until 100% confluent, washed twice with phosphate buffered saline, and treated with decellularization solution containing 0.1% Triton X-100 (Amresco) and 40 mM ammonium hydroxide (Sigma). After incubation, the solution was removed, and DNase I ($50 \text{ U} \cdot \text{mL}^{-1}$; Invitrogen) and RNaseA ($25 \mu\text{g}$ per 10 mL; Invitrogen) were added. After 2 h of incubation at 37 °C, the chondrocyte-derived ECM was washed, treated with 0.1 M glycine (Sigma), and stored at -70°C until use [29]. 21 ± 2.5 mg of ECM protein amount was used for experiments. We used the same amount of ECM in all experiments. To confirm decellularization, the chondrocyte-derived ECM was stained with 4',6-diamidino-2-phenylindole (DAPI).

Chondrogenic differentiation of hPDMSCs with or without chondrocyte-derived ECM

Placentas (38–40 week gestation) were collected from three women who gave birth after a normal full-term pregnancy. Informed consent was obtained from the donors, and the protocol was approved by the institutional review board of Samsung Medical Center (Seoul, Korea). The hPDMSCs were isolated as previously described [30, 31]. Differentiation of the hPDMSCs in the presence of chondrocyte-derived ECM was tested using two different methods. In the first method (figure 1(A)), the decellularized chondrocyte-derived ECM was harvested using a scraper, mixed with 3×10^5 hPDMSCs in a 15 mL polypropylene tube (Corning Inc., NY, USA), and centrifuged (at 3000 rpm) to form pellets. In the second method (figure 1(B)), 1×10^5 hPDMSCs were seeded into plates containing

Table 1. Primer sequences for quantitative RT-PCR.

Gene	Forward sequence	Reverse sequence
GAPDH	5'-ACACCCACTCCTCCACCTTTG-3'	5'-TCCACCACCCTGTTGCTGTAG-3'
Aggrecan	5'-CTACGACGCCATCTGCTACA-3'	5'-GGCTTCACCCTCAGTGATGT-3'
Collagen 2A1	5'-GTCTGGCTGGTCAGAGAGG-3'	5'-GGACCTCTGTCTCCAGATGC-3'
SOX9	5'-AGCGAACGCACATCAAGAC-3'	5'-GCTGTAGTGTGGGAGGTTGAA-3'

chondrocyte-derived ECM and pre-cultured for 7 d until >80% confluent. After detachment of cells with TrypLE™ Express (Gibco) and chondrocyte-derived ECM with a scraper, 3×10^5 cells and chondrocyte-derived ECM were transferred to a 15 mL polypropylene tube and pelleted. To serve as a control, 3×10^5 hPDMSCs alone (without chondrocyte-derived ECM) were also pelleted. The cell pellets (with or without chondrocyte-derived ECM) were then cultured in chondrogenic medium containing 500 ng · mL⁻¹ bone-morphogenetic protein-6 (R&D Systems, Inc.), 10 ng · mL⁻¹ transforming growth factor- β_3 (Sigma), 0.1 μ M dexamethasone (Sigma, USA), 50 μ g · mL⁻¹ ascorbic acid (Sigma), 40 μ g · mL⁻¹ L-proline (Sigma, USA), and 50 mg · mL⁻¹ ITS+ Premix (Sigma, USA) for 1 or 3 weeks. The medium was replaced every 4 d.

Histology analysis

The hPDMSC pellets were fixed in 10% formalin for 24 h and embedded in paraffin. The paraffin blocks were then cut into 5 μ m sections, which were stained with Safranin O (ALPHA Chem) to quantify glycosaminoglycans (GAGs). For immunohistochemistry analysis, samples were treated with peroxidase blocking solution for 1 h and then incubated with primary antibodies against type II collagen (Millipore, MA, USA) overnight at 4 °C. Bound antibody was detected by using the REAL™ EnVision™ + Detection System (Dako, CA, USA) according to the manufacturer's instructions.

Quantitative reverse transcription-PCR

Total RNA was extracted from the cell pellets using TRIzol reagent (Ambion, CA, USA) following the manufacturer's instructions. Reverse transcription was carried out using CycleScript RT PreMix (Bioneer, Korea). Quantitative reverse transcriptase-PCR (RT-PCR) was performed on an ABI 7000 Detection System (Applied Biosystems) using SYBR Green Premix (Takara) and gene-specific primers (table 1). The relative expression of aggrecan, collagen type II, and SOX9 was determined by the $2^{-\Delta\Delta Ct}$ method as previously described [32], using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

Glycosaminoglycan analysis

The cell pellets were digested by incubating with 0.1 M phosphate buffer (pH 6.0) containing 250 μ g · mL⁻¹ papain, 10 mM Na₂-EDTA (Sigma, USA), and 10 mM L-cysteine hydrochloride (Sigma, USA) at 57 °C. After 16 h the cell suspension was mixed with an equal volume of 1,9-dimethylmethylene blue zinc chloride

double salt solution (Sigma, USA), a dye that specifically binds to sulfated GAGs. Absorbance was measured in a 96-well plate at 530 nm using an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, 168–1150).

Statistical analysis

Results were expressed as mean $\pm$ standard deviation (SD). Groups were compared by one-way analysis of variance (ANOVA). Statistical analyses were carried out using SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA); * $p < 0.05$ and ** $p < 0.01$ were considered significant.

Results

Characterization of chondrocyte-derived ECM

Decellularization of the chondrocyte-derived ECM was confirmed by bright field microscopy (figure 2(D)), using untreated chondrocytes as a control (figure 2(A)). The decellularized chondrocyte-derived ECM showed no cellular structures, which were clearly present in the control. Also, no nuclei were present in the chondrocyte-derived ECM, as assessed by DAPI staining and fluorescence microscopy (figure 2(E)), whereas blue-stained nuclei were present in the untreated chondrocytes (figure 2(B)). In addition, the amount of DNA on the CDECM was measured and confirmed that DNA concentration was less than 1 ng · mL⁻¹ (see supplementary figure 1, available at stacks.iop.org/BMM/10/035014/mmedia) and GAPDH expression was not detected by PCR.

Growth of hPDMSC pellets during chondrogenesis

Our results demonstrated that chondrocyte-derived ECM increased the growth of hPDMSC pellets during differentiation. The compact round pellets (incubated with or without chondrocyte-derived ECM) were larger after incubation in chondrogenesis medium for 3 weeks, compared with their size at 1 week (figure 3(A)), with an increase of 115% in cells that were not pre-cultured with chondrocyte-derived ECM and 114% in cells that were without pre-culturing (figure 3(B)). In contrast, pre-culture had no significant effect on pellet wet mass compared to the untreated control. Similarly, wet mass increased 116% when pre-cultured with chondrocyte-derived ECM and 126% with no pre-culturing (figure 3(C)).

Histologic analysis

Results of histologic analysis demonstrated that chondrogenesis of hPDMSCs was enhanced by the

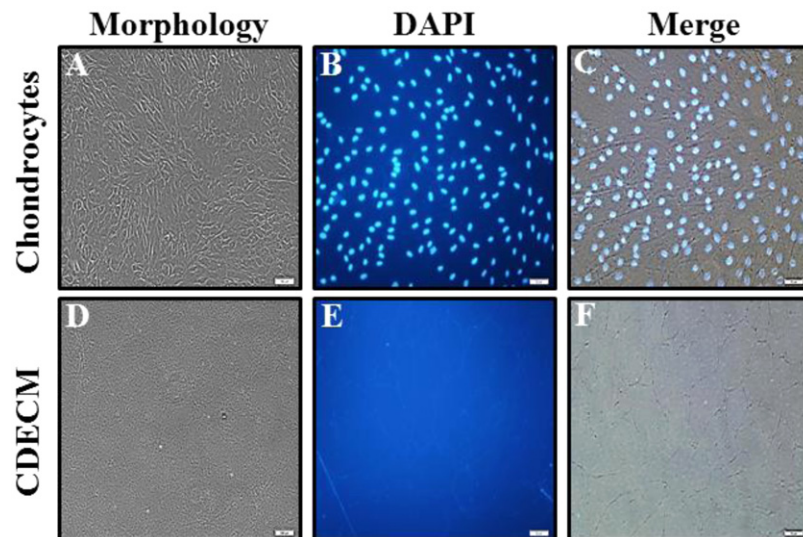


Figure 2. Decellularization of chondrocyte-derived extracellular matrix was evaluated by bright field microscopy (A), (D), DAPI staining (B), (E), and merged images (C), (F) ($\times 200$, scale bar = $100\ \mu\text{m}$). CDECM, chondrocyte-derived extracellular matrix.

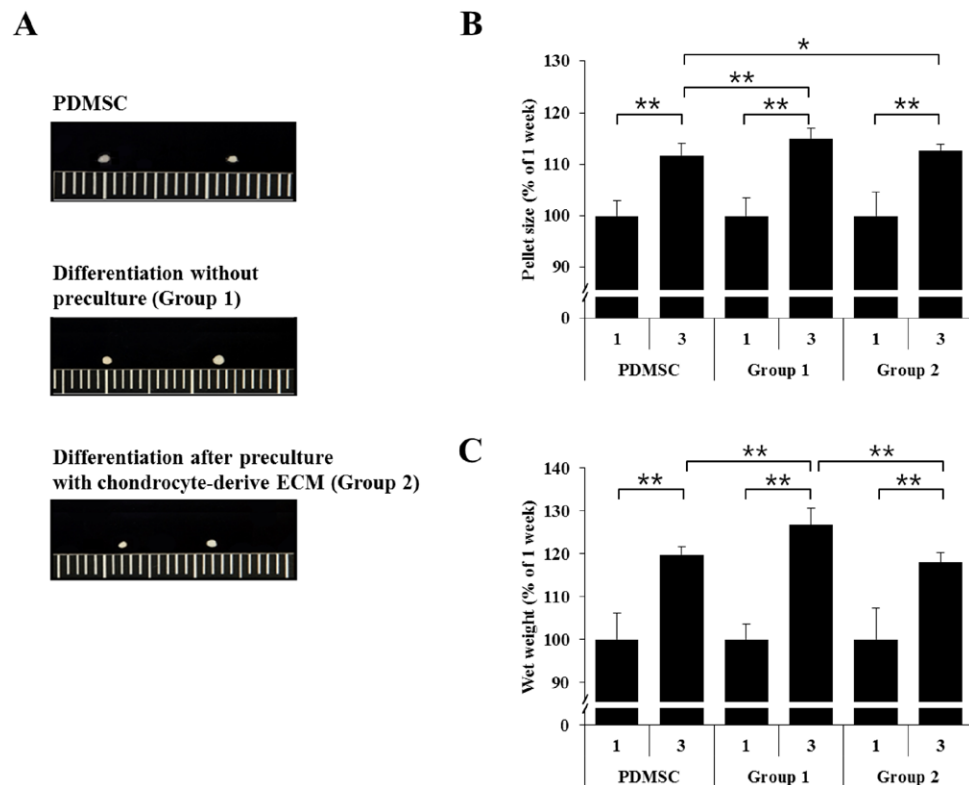


Figure 3. Growth rate of human placenta-derived mesenchymal stem cells (hPDMSCs) differentiated in the presence or absence of chondrocyte-derived extracellular matrix (ECM). (A) Macroscopic images of pellets cultured in chondrogenic medium with or without chondrocyte-derived ECM for 1 or 3 weeks (scale bar = 1 mm). (B) Size and (C) wet mass of hPDMSC pellets differentiated in the absence or presence of chondrocyte-derived ECM, without or with the preculture step. Results are expressed as mean $\pm$ SD ($n=6$); * $p < 0.05$ and ** $p < 0.01$.

presence of chondrocyte-derived ECM. Safranin O staining for GAG content, a marker of chondrogenesis, was positive in cells differentiated with or without chondrocyte-derived ECM (figures 4(A), (B), (E), (F), (I) and (J)). Similarly, all hPDMSC pellets showed positive staining for type II collagen (figures 4(C), (D), (G), (H), (K) and (L)). Type II collagen staining was more intense in hPDMSCs differentiated in the presence

of chondrocyte-derived ECM, and precultured with chondrocyte-derived ECM did appear to more affect differentiation than in untreated controls.

Analysis of chondrogenic markers

Our results showed that chondrocyte-derived ECM upregulated chondrogenic marker expression during the chondrogenesis of hPDMSCs. Aggrecan and

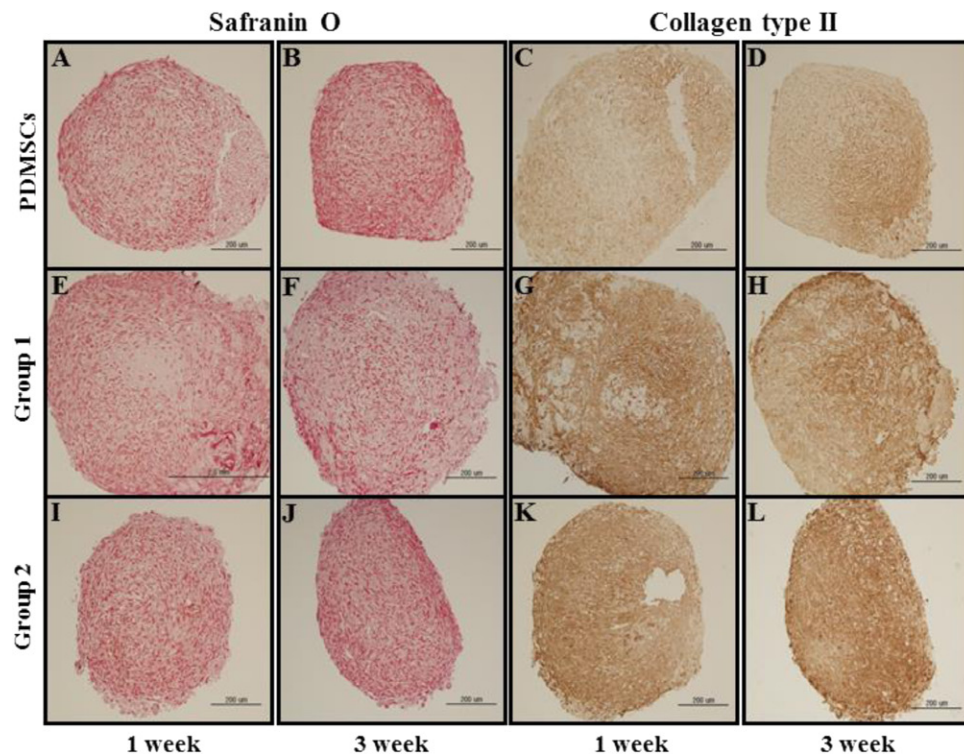


Figure 4. Histologic and immunohistochemistry analysis. Chondrogenic differentiation of human placenta-derived mesenchymal stem cells (hPDMSCs) in the absence and presence (group 1: without preculture of MSCs and group 2: with preculture of MSCs) of chondrocyte-derived extracellular matrix was confirmed by Safranin O staining (A), (B), (E), (F), (I), and (J) and collagen type II staining (C), (D), (G), (H), (K), and (L); $\times 100$, scale = $200\ \mu\text{m}$).

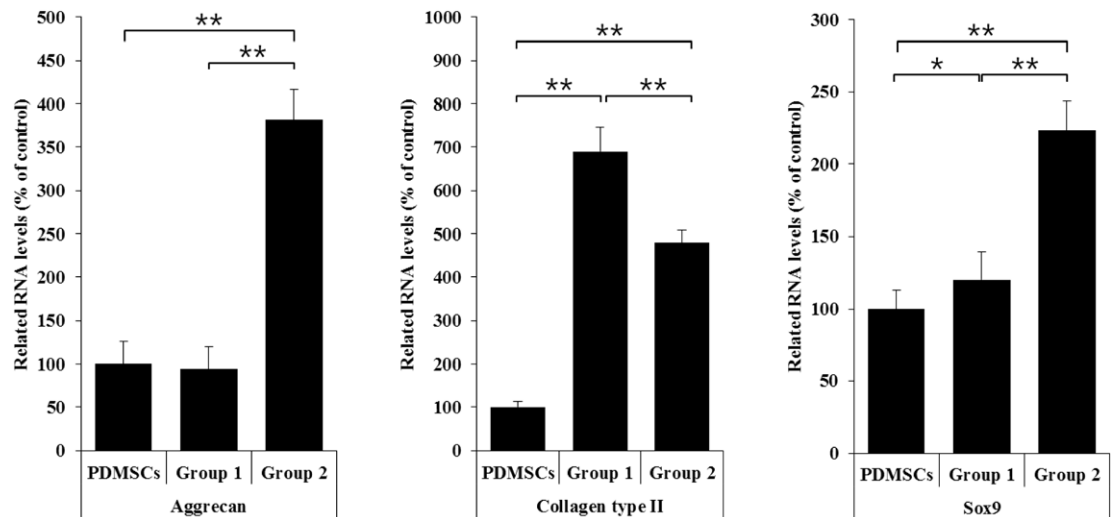


Figure 5. Expression of chondrogenic markers in human placenta-derived mesenchymal stem cell (hPDMSC) pellets differentiated in the absence (PDMSCs) and presence (group 1: without preculture of MSCs and group 2: with preculture of MSCs) of chondrocyte-derived extracellular matrix was evaluated by quantitative RT-PCR at 3 weeks. Relative expression of aggrecan (A), collagen type II (B), and SOX9 (C) was determined by using the $2^{-\Delta\Delta\text{CT}}$ method. Results are expressed as mean $\pm$ SD ($n = 6$); * $p < 0.05$ and ** $p < 0.01$.

collagen type II, which are major components of cartilage, and the cartilage-specific transcription factor SOX9 [33, 34] were evaluated by quantitative RT-PCR at 3 weeks (figure 5). The mRNA level of collagen type II was higher in hPDMSC pellets differentiated in the presence of chondrocyte-derived ECM and precultured cells than in untreated controls (figure 5(B)). In contrast, mRNA expression levels of aggrecan and

SOX9 were increased only in hPDMSCs precultured with chondrocyte-derived ECM (figures 5(A) and (C)).

Glycosaminoglycan analysis

To further analyze the effect of chondrocyte-derived ECM on hPDMSC chondrogenesis, the GAG content in hPDMSC pellets differentiated with or without chondrocyte-derived ECM was quantified (figure 6).

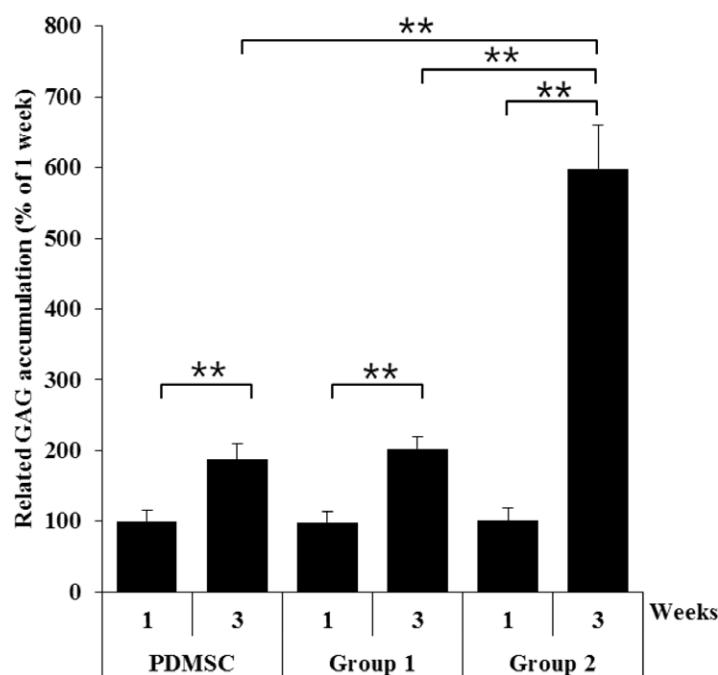


Figure 6. Analysis of glycosaminoglycan (GAG) contents in human placenta-derived mesenchymal stem cells (hPDMSCs) in the absence (PDMS) and presence (group 1: without preculture of MSCs and group 2: with preculture of MSCs) of chondrocyte-derived extracellular matrix. Results are expressed as mean $\pm$ SD ($n = 6$); * $p < 0.05$ and ** $p < 0.01$.

The rate of GAG deposition was significantly higher in hPDMSCs precultured with chondrocyte-derived ECM compared with untreated hPDMSC pellets or those differentiated with chondrocyte-derived ECM. Therefore the increased amount of the GAG provided by the ECM did not affect the result.

Discussion

We found that preculturing cells in chondrocyte-derived ECM before chondrogenesis resulted in higher aggrecan and SOX9 mRNA levels at 3 weeks, compared to cells differentiated in the presence of chondrocyte-derived ECM without the preculture step. We identified that the preculture step was confirmed to significantly increase the amount of GAG in the differentiation process of the MSC. Our results suggest that MSCs precultured with CDECM are most effective method. However, cells that were not precultured had higher wet mass and collagen type II mRNA levels at 3 weeks. It is known well that MSC proliferation is downregulated once differentiation is initiated [35, 36]. The decreased proliferation rate of precultured hPDMSCs may therefore be due to increased chondrogenesis. Because hPDMSCs that were not precultured may have had more time to proliferate, they were able to form larger and heavier pellets.

We also found that chondrocyte-derived ECM increased the growth of hPDMSC pellets during chondrogenesis (figure 3). Like other MSCs, hPDMSCs have multilineage differentiation potential, which is regulated by the ECM [31, 37–41]. However, this is the study to show that chondrocyte-derived ECM enhances chondrogenic differentiation of hPDMSCs in pellet

culture. MSC self-renewal potential is influenced by growth factors and cytokines such as leukemia inhibitory factor [42, 43], fibroblast growth factor [44, 45], and WNT signaling [35, 46]. Many growth factors bind to components of the ECM, which regulate their distribution, activation, and presentation [47] suggesting that chondrocyte-derived ECM may contain many growth factors that regulate hPDMSC differentiation. In this study, we compared on the increasing of collagen type II expression by chondrogenic differentiation of hPDMSCs in the absence of CDECM with presence of CDECM by using histology analysis. However, we found that chondrogenesis also occurred in hPDMSC pellets that were not exposed to chondrocyte-derived ECM. We confirmed that chondrocyte-derived ECM enhances chondrogenic differentiation of hPDMSCs in pellet culture (figure 4).

The transcription factor SOX9 and ECM proteins such as aggrecan, collagen type II, collagen type IX, and cartilage oligomeric matrix protein play important roles in chondrocyte proliferation and differentiation during cartilage development [48–50]. Chondrogenesis is also regulated by the ECM, growth factors, and other environmental factors [33, 51, 52]. The ECM consists of glycoproteins, collagens, GAGs, and proteoglycans, which mediate cell behaviors such as survival, proliferation, migration, and differentiation through signaling [1–4, 53, 54]. Various factors mediate each step of chondrogenesis, which include condensation, proliferation, differentiation, cartilaginous ECM deposition and cartilage formation. Proliferation and condensation are mediated by ECM proteins such as collagen type II [33, 49] and the transcription factor SOX9 [33, 34]. Aggrecan is produced during MSC differentiation

into chondrocytes [33, 34]. We found that expression of chondrogenic markers such as aggrecan, collagen type II, and Sox9 in the preculture of chondrocyte-derived ECM at 3 weeks were significantly higher than hPDMSCs alone. Aggrecan and Sox9 did not significantly increase, while collagen type II was found to increase significantly more without the precultured group than PDMSC (figure 5). During chondrogenesis, collagen type II is expressed primarily during MSC proliferation. Our results showed that collagen type II expression was highest in hPDMSC pellets differentiated with chondrocyte-derived ECM than when precultured with ECM; this finding indicates that these hPDMSCs were in the proliferation stage of differentiation and accounts for the greater increase in size and wet mass of cell pellets at 3 weeks of differentiation. In contrast, the expression of aggrecan and SOX9 in hPDMSCs precultured with chondrocyte-derived ECM was significantly higher than that of hPDMSCs that were not precultured with chondrocyte-derived ECM, indicating that precultured cells were in a later stage of chondrogenesis.

In this study, the signaling pathways that induce expression of these chondrogenic factors have not been completely elucidated. However, several signaling molecules including transforming growth factor- β , bone morphogenetic proteins, and WNT ligands have been identified by previous studies [55]. These factors bind to ECM proteins, which are present in chondrocyte-derived ECM leading to paracrine effects [51, 52] that stimulate proliferation and chondrogenic differentiation. This accounts for the increased GAG accumulation in hPDMSC pellets differentiated with chondrocyte-derived ECM in our study. We compared the amount of GAG in cell pellets in the absence of chondrocyte-derived ECM with the amount in the presence of chondrocyte-derived ECM. GAG contents of PDMSC pellets cultures are not significantly different between PDMSC (control) and PDMSC culture with CDECM (group 1 and group 2) at 1 week (figure 6). We figured out that the amount of GAG was increased significantly after preculture of MSCs with CDECM at 3 weeks.

One limitation of our study is that the exact mechanism underlying the effect of chondrocyte-derived ECM on hPDMSC chondrogenesis is unclear. However, chondrocyte-derived ECM is able to provide chondrocyte-specific factors, signaling receptors, growth factors, and cytokines that stimulate MSC self-renewal and differentiation. We assume that preculturing hPDMSCs with chondrocyte-derived ECM would be as effective in promoting chondrogenesis in 2D tissue culture as it is in 3D pellet cultures.

Conclusion

In conclusion, chondrogenesis of hPDMSCs in pellet culture is enhanced by the presence of chondrocyte-derived ECM. Furthermore, preculturing hPDMSCs in chondrocyte-derived ECM before differentiation accelerates chondrogenesis. Our results suggest that

hPDMSC chondrogenesis using the chondrocyte-derived ECM and preculture method represents a promising approach for cartilage tissue engineering.

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Author disclosure statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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