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Enhanced Chondrogenic Differentiation of Embryonic Stem Cells by Co-Culture with Hepatic Cells

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ABSTRACT

Enhancing the specific differentiation of pluripotent embryonic stem (ES) cells has been a challenge in the field of tissue engineering. Previously, hepatic cells have been shown to secrete various soluble morphogenic factors to direct mesodermal differentiation of ES cells. In this study, we hypothesized that factors secreted by hepatic cells possess chondrogenic-differentiating effects, and, therefore, the co-culture of hepatic cells would enhance chondrogenesis of ES cells. ES-derived cells (ESDCs) were co-cultured with hepatic cells (HEPA-1C1c7) in three-dimensional bilayered hydrogels. After 3 weeks culture, the histological and biochemical analysis of the HEPA-co-cultured ESDCs revealed a four-fold increase in glycosaminoglycan (GAG) compared to ESDCs cultured alone. This result was supported by real-time PCR analysis, which demonstrated an 80-fold increase in *aggrecan* expression in co-cultured ESDCs. Additionally, *type IIB collagen* expression was observed only with co-cultured ESDCs, and immunohistochemical analysis resulted in significantly more positive type II collagen staining with co-cultured ESDCs. Moreover, at day 21, gene expression of other lineages in HEPA-co-cultured ESDCs was either comparable to or lower than those of ESDCs cultured alone. These results indicated that co-culture of ESDCs with hepatic cells significantly enhanced specific chondrogenic differentiation of ESDCs.

INTRODUCTION

STEM CELLS HAVE GENERATED a great deal of excitement and promise as a potential source of cells for cell-based therapeutic strategies, primarily owing to their intrinsic ability to self-renew and differentiate into functional cell types that constitute the tissue. In particular, embryonic stem (ES) cells, derived from the inner cell mass (ICM) of a blastocyst, are capable of differentiating into cells of all three germ layer lineages [1,2]. These cells are capable of unlimited symmetrical self-renewal, thus providing an unlimited cell source for tissue engineering applications. One of the challenges in stem cell regenerative therapy, however, is the control of the dif-

ferentiation commitment of these cells. Additionally, another limitation is the poor survival of human ES cells after cell dissociation, hindering manipulations such as subcloning [3].

A variety of morphogenic factors are known to provide instructive signals that promote cellular proliferation or differentiation. Differentiation of ES cells in vitro into chondrocytes, for example, has been induced by supplementing the culture medium with members of the transforming growth factor- β (TGF- β) family, such as TGF- β and bone morphogenic proteins (BMPs) [4,5]. Another method to control the differentiation of ES cells is to use cell-secreted morphogenic factors from fully differentiated cells by co-culture or conditioned medium

[6–9]. Previously, the conditioned medium from hepatic cells was shown to enhance mesodermal differentiation and, furthermore, osteogenesis of murine ES cells [10–12]. However, chondrogenic differentiation of ES cells induced by soluble factors secreted by hepatic cells has not yet been investigated.

Cartilage is an avascular tissue, which cannot regenerate itself. Moreover, upon multiple monolayer in vitro passage, primary chondrocytes dedifferentiate and become fibroblastic [13]. Therefore, engineering cartilage requires an appropriate cell source that can provide an adequate number of functional cells. ES cells have been studied as an unlimited source for chondrocytes, but further research is required for specific chondrogenic differentiation of ES cells. We hypothesized that morphogenic factors secreted by hepatic cells possess chondrogenic differentiating effects and, therefore, the co-culture of hepatic cells helps chondrogenesis of murine ES cells. In the present study, hepatic cells were co-cultured with ES-derived cells (ESDCs) in a bilayered hydrogel system. We investigated the effect of hepatic cell-secreted soluble factors on chondrogenesis of ES cells.

METHODS

Preparation and culture of ESDCs and HEPA cells

Mouse ES-D3 GL cell line (American Type Culture Collection) was cultured over monolayers of mitomycin C-inactivated mouse fibroblasts in a medium supplemented with leukemia inhibitory factor (LIF) following the methods described previously [14]. Embryoid bodies (EBs) were formed and cultured in liquid suspension culture for 4 days [5]. Then, EBs were plated, dissociated, and cultured on tissue culture dishes for 3

days in ES medium without LIF, and these cells were referred to as ESDCs. The mouse hepatic cell line HEPA-1C1c7 (ATCC, Manassas, VA) was cultured in α -minimum essential medium (α -MEM) containing 10% fetal bovine serum (FBS) in a humidified CO₂ incubator at 37°C.

Co-culture of ESDCs with HEPA cells

Macromer solution was prepared by mixing 0.05% (wt/vol) photoinitiator (Irgacure 2959; Ciba Speciality Chemicals, Tarrytown, NY) and 10% (wt/vol) poly(ethylene glycol) diacrylate (PEODA, molecular weight, 3,400; SunBio, South Korea) in sterile phosphate-buffered saline (PBS). ESDCs or HEPA-1C1c7 were resuspended in the macromer solution (40 μ l) at a concentration of 60×10^6 or 10×10^6 cells/ml, respectively. Then, the solution was photopolymerized by ultraviolet (UV) exposure (EXFO Acticure 4000; wavelength, 365 nm; intensity, 5 mW/cm²). For a co-culture sample, 20 μ l of acellular and 40 μ l of ES-containing macromer solutions were polymerized on top of the HEPA-1C1c7-containing construct in the order shown in Fig. 1. The hydrogel construct was transferred to a well in a 12-well culture plate containing 2 ml of chondrogenic medium. Chondrogenic medium is composed of 10 ng/ml TGF- β 1, 100 nM dexamethasone, 40 mg/L Proline (Sigma), 50 mg/L ascorbate-2-phosphate (Sigma), 100 mg/L Sodium Pyruvate (Gibco), 50 mg/ml ITS-Premix (Collaborative Biomedical, San Jose, CA), and 1% penicillin-streptomycin in high-glucose Dulbecco modified Eagle medium (DMEM). Constructs were cultured at 37°C for 1 or 3 weeks in 5% CO₂ atmosphere and medium was replaced every 2–3 days.

Cell viability test

Viability of encapsulated cells in PEODA hydrogel was determined on the basis of the integrity of cellular membrane using the Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR) that contains calcein-AM (“Live” dye)

FIG. 1. Experimental scheme. Bilayered PEODA hydrogels with HEPA cells or ESDCs in each layer were created by photopolymerization. Control hydrogels were also photopolymerized with ESDCs alone.

and ethidium homodimer-1 ("Dead" dye). Dye solution was made with 0.5 μ l of calcein-AM dye and 2 μ l of ethidium homodimer-1 dye in 1 ml of DMEM. Constructs were washed twice with PBS. A slice of the construct was incubated in 500 μ l of the "Live/Dead" dye solution for 30 min. Fluorescence microscopy was performed using a fluorescein optical filter (485 ± 10 nm) for calcein-AM and a rhodamine optical filter (530 ± 12.5 nm) for ethidium homodimer-1.

Biochemical assays of ESDC-encapsulated hydrogel

After 3 weeks of culture, a bilayered construct was cut in half to separate layers of different cells. Hydrogels were lyophilized for 48 h, and their dry weights were measured. Next, dry constructs were digested with papain (Worthington Bio-medical, Lakewood, NJ) overnight [15]. Hoechst 33258 dye (Molecular Probes, Eugene, OR) was used for the DNA assay as previously described [16]. Briefly, the solution (0.1 μ g/ml) was prepared in 1 $\times$ TNE buffer (10 mM Tris, 1 mM EDTA, 0.2 M NaCl, pH 7.4). Papain-digested samples (60 μ l) or standards were mixed with the prepared dye solution. Fluorescence intensity of the dye-conjugated DNA solution was measured on a fluorometer (365 nm excitation and 458 nm emission), and the DNA content was calculated from a standard curve generated with calf thymus DNA. The GAG content was measured using the dimethylmethylene blue (DMMB) dye [17]. Absorbance was measured on a UV-Vis spectrophotometer (525 nm). The DNA and GAG contents from the biochemical assays were normalized against the construct dry weights. The value is presented as mean $\pm$ SD. Statistical significance was determined by unpaired Student *t*-test and set as $p < 0.001$.

Histology and immunostaining

Cell-encapsulated PEODA constructs were fixed overnight in a 10% formalin solution and stored in 70% ethanol. The fixed construct was dehydrated and embedded in paraffin, sectioned, and stained with Safranin-O [18]. Immunohistochemistry was performed using rabbit polyclonal antibody against collagen type II (Research Diagnostics, Flanders, NJ) by 1:100 dilution factor. After extensive washing, fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibodies (Jackson ImmunoResearch Laboratory, West Grove, PA) were used for fluorescent detection of the specific collagen. Finally, DAPI solution (0.1 mg/ml, Millipore, Temecula, CA) was applied to dye the cell nuclei. Images were taken by confocal microscopy (Zeiss LSM 510 Meta Confocal Microscope).

Reverse transcription-polymerase chain reaction

Total RNAs were isolated from three cellular PEODA hydrogels per group and from ESDCs cultured in monolayer at the time of encapsulation using TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. The first-strand cDNA synthesis was performed using SuperScript First-Strand Synthesis System (Invitrogen). Two microliters of the reverse transcriptase product were amplified in a total volume of 20 μ l for PCR amplification with specific primer sets (Table 1) using ExTaq DNA polymerase premix (Invitrogen). The cycling

parameters for this reaction were as follows: hold at 95°C for 2 min; 95°C for 30 sec, primer-specific temperature for 1 min, and 72°C for 1 min (34 cycles). The PCR product was analyzed by separating 4 μ l of the amplicon and 1 μ l of loading buffer in a 2% agarose gel in TAE buffer.

RESULTS

Photoencapsulation of ESDCs and HEPA cells in a hydrogel co-culture system

ESDCs and HEPA cells were encapsulated in PEODA hydrogels as a bilayered system by photopolymerization of macromer and cell mixture solutions. As shown in Fig. 1, the two layers of HEPA cells and ESDCs in the co-culture system were separated by an acellular PEODA layer. This separation was created to ensure that there would be no significant cell migration between layers. The absence of cells in the blank PEODA layer after 3 weeks of culture confirmed that the cells remained in their respective layers. The viability results after 24 h of culture in the chondrogenic medium demonstrated that 80% and 84% of the HEPA cells and ESDCs, respectively, remained homogeneously viable throughout the constructs in the PEODA gels (Fig. 2), which indicated that photoencapsulation and culture in PEODA hydrogels did not have any toxic effect on embedded cells.

HEPA cell co-culture increases DNA content and ECM production of ESDCs

The DNA and cartilage-specific extracellular matrix (ECM) contents of ESDC hydrogels co-cultured with HEPA cells and ESDCs cultured alone were evaluated after 3 weeks of culture. DNA analysis using Hoechst dye demonstrated a significant increase in cell proliferation of co-cultured ESDCs compared to ESDCs cultured alone (Fig. 3a). Histological evaluation verified the increased cell number and the homogeneity of encapsulated cells in the hydrogel (Fig. 3c).

GAG is one of the major ECM components of cartilage. Co-culturing of ESDCs with HEPA cells significantly increased GAG content of ESDCs (Fig. 3b). The GAG content of ESDC hydrogel co-cultured with HEPA was approximately four times higher than that of ESDCs cultured alone. We also observed a greater intensity of Safranin-O staining for GAG in co-cultured hydrogels compared to control hydrogels (Fig. 3c).

Type II collagen is another major ECM component of cartilage, and its presence in hydrogels has been shown by immunohistochemistry (Fig. 3d). Co-cultured ESDC constructs exhibited significantly more type II collagen compared to those cultured alone. Type II collagen was

TABLE 1. SEQUENCES OF PRIMERS AND CONDITIONS USED IN RT- AND REAL-TIME PCR

	PCR primers	
	Sequences (forward and reverse)	Annealing temperature
<i>Aggrecan</i>	5'-TCCTCTCCGGTGGCAAAGAAGTTG-3' 5'-CCAAGTTCCAGGGTCACTGTTACCG-3'	60°C
<i>Cbfa1</i>	5'-ATCCATCCACTCCACCACGC-3' 5'-AAGGGTCCACTCTGGCTTTGG-3'	63°C
<i>Collagen type II</i>	5'-AGGGGTACCAGGTTCTCCATC-3' 5'-CTGCTCATCGCCGCGGTCCGA-3'	60°C
<i>Collagen type X</i>	5'-ATGCCTTGTTCTCCTCTTACTGGA-3' 5'-CTTTCTGCTGCTAATGTTCTTGACC-3'	61°C
<i>GATA6</i>	5'-GCAATGCATGCGGTCTCTAC-3' 5'-CTCTTGGTAGCACCAGCTCA-3'	55°C
<i>HNF4α</i>	5'-TGCCCTCTCACCTCAGCAATG-3' 5'-CCCCTCAGCACACGGTTTTG-3'	55°C
<i>Keratin 1-15</i>	5'-CACCACATTCTTGCAAAC-3' 5'-ATTAAGGTTCTGCATGGTC-3'	55°C
<i>Nestin</i>	5'-CGGCCACGCATCCCCCATCC-3' 5'-AGCGGCCTTCCAATCTCTGTTCC-3'	60°C
<i>Scleraxis</i>	5'-GTGGACCCTCCTCCTTCTAATTTCG-3' 5'-GACCGCACCAACAGCGTGAA-3'	60°C
<i>Sox-9</i>	5'-TGGCAGACCAGTACCCGCATCT-3' 5'-TCTTTCTTGTGCTGCACGCGC-3'	57°C
β -Actin	5'-CGCACCCTGGCATTGTCAT-3' 5'-TTCTCCTTGATGTCACGCAC-3'	55°C
β -Actin	5'-TGGCACCACACCTTCTACAATGAG-3' 5'-GCACAGCTTCTCCTTAATTGTCACGC-3'	60°C

only minimally detected in single layer constructs containing ESDCs alone.

Cartilage-relevant markers of ESDCs are increased with HEPA cell co-culture

RT-PCR analysis showed a time-dependent increase in *type II collagen* expression of ESDCs in hydrogels. *Type IIA collagen* was expressed as early as day 3 of HEPA co-culture, whereas such expression in ESDCs cultured alone was detected only on day 21. RT-PCR analysis also demonstrated that production of *type IIB collagen* splice variant was initiated at day 12 of the culture, and its expression was up-regulated in a time-dependent manner. However, we did not detect any *type IIB collagen* expression in ESDCs cultured alone even at day 21.

Chondrogenesis of ESDC was further evaluated by real-time PCR to quantify its time-dependent changes in gene expression. The relative expression levels of a particular chondrogenic marker gene were normalized to ESDC gene expression at day 0 and a housekeeping gene, β -actin. Co-

culture of ESDCs with HEPA cells resulted in an increased expression of the chondrogenic markers, *aggrecan* and *sox-9*, compared to control ESDCs without co-culture from day 9. *Aggrecan* expression constantly increased in both co-cultured and individually cultured ESDCs, but a more rapid increase was observed with HEPA co-cultured ESDCs (Fig. 4a). The difference between co-cultured ESDCs and control ESDCs in their *aggrecan* expression is significantly increased from a two-fold increase at day 9 to an 80-fold increase at day 21. Additionally, the expression of *sox-9*, a transcription factor involved in regulation of *type II collagen* [19,20], peaked at day 9 and 21 for HEPA co-cultured ESDCs, corresponding with its previously described positive type IIB collagen expression at day 9 (Fig. 4b). Overall RT-PCR results confirmed the increased chondrogenic gene expression of HEPA co-cultured ESDCs compared to control ESDCs cultured alone. Histological, immunohistochemical, and gene expression analysis confirmed that the HEPA co-culture systems enhanced chondrogenic differentiation of ESDCs and the corresponding matrix secretion.

FIG. 2. Fluorescence micrographs of HEPA cells (a) and ESDCs (b) encapsulated in PEODA. The percentage of viable cells was determined by counting the live cells, which were stained with dye, relative to the total cells presented in three different fields.

Other lineage markers of ESDCs are decreased with HEPA cell co-culture

Other mesodermal, ectodermal, and endodermal markers were also evaluated by real-time PCR to verify specific chondrogenic differentiation of ESDCs. Expression of *Scleraxis*, a muscular marker [21,22], was increased

in ESDCs co-cultured with HEPA cells at day 12 and 15 (Fig. 4c). However, the level was decreased significantly by day 18 and became comparable to that of control ESDCs without co-culture. Additionally, expression of *cbfal*, a transcription factor that binds to the promoters of several osteogenic genes [23,24], was rapidly increased in HEPA co-cultured ESDCs between days 6 and

FIG. 3. ECM secretions of ESDCs were evaluated by biochemical assays ($n = 6$, *: $p < 0.001$): (a) DNA content, (b) GAG content. Histological sections of hydrogels seeded with MPCs were evaluated by Safranin O staining (c) and type II collagen immunostainings (d) after 3 weeks of culture. RT-PCR analysis of individually cultured and HEPA co-cultured ESDCs support the immunostaining results (e).

FIG. 4. Real-time PCR analysis data showed significantly increased gene expressions of chondrogenic markers (a,b) in co-cultured ESDCs compared to ESDCs cultured alone. Temporary increase in muscular and osteogenic markers (c-f) was also observed between days 9 and 18.

9, exceeding that of ESDCs cultured alone at day 9 (Fig. 4d). However, *cbfa1* expression of HEPA co-cultured ESDCs decreased soon after to below the level found in control ESDCs cultured alone at day 18. The peak of *cbfa1* expression at day 9 was followed by a peak in the expression of *type X collagen* at day 12 in the co-cultured ESDCs (Fig. 4e). The level of *type X collagen* expression of HEPA co-cultured ESDCs changed throughout the 3 weeks of culture and was comparable to that of control ESDCs without co-culture at day 21. The highest level of *type I collagen* expression (day 15) was followed by the peak of *type X collagen* expression for both co-cultured and singly cultured ESDCs (Fig. 4f). The level of *type I collagen* expression of co-cultured ESDCs was not significantly different from that of control ESDCs co-cultured alone, except at day 9.

Levels of endodermal and ectodermal differentiation markers were also measured after 3 weeks of culture and normalized to levels at day 0. Expressions of a hepatogenic differentiation marker (*HNF4*) [25], another endoderm marker (*GATA6*) [26,27], and an ectoderm marker (*Krt1-15*, *Keratin 15*) [28] in ESDCs were all increased by day 21 compared to day 0, but such increases were significantly reduced by co-culture of HEPA cells. *Nestin* expression of HEPA co-cultured ESDCs was comparable to that of control ESDCs without co-culture. Therefore, increase in chondrogenic (a mesodermal tissue)

gene expression was specific, with no increase in expression of markers for ectodermal or endodermal lineages observed.

DISCUSSION

ES cells are a potential cell source for applications in tissue engineering and regenerative medicine because they can differentiate into diverse cell types. One in vitro technique to initiate the differentiation of ES cells is via EB formation. Once cultured in suspension without LIF, ES cells aggregate and form clusters, mimicking the structure of natural blastocysts. Conventionally, EBs are plated onto tissue culture dishes, and the dissociated EBs are differentiated into various specialized cells in a monolayer [14,29,30]. Bilayer co-culture systems, alternatively, provide three-dimensional environments to the cells, mimicking the native tissue environment. Specifically, PEODA scaffolds have been used for cartilage engineering, where photopolymerization of PEODA has been used to encapsulate chondrocytes, mesenchymal stem cells and ES cells [4,31,32]. Experimental results indicate that a PEODA hydrogel serves as a three-dimensional support and permits the chondrogenic differentiation of encapsulated cells. In particular, Hwang et al. compared the chondrogenic potential of ES cells in

FIG. 5. Real-time PCR analysis data showed significantly decreased gene expressions of nonchondrogenic markers (**a,b,d**) in co-cultured ESDCs compared to ESDCs cultured alone, except *Nestin* (**c**), after 3 weeks of co-culture.

three-dimensional environments with their monolayer culture (5). They observed enhanced chondrogenesis of ES cells in the 3D PEODA hydrogel system as compared to the conventional monolayer system.

Another advantage of using the three-dimensional bilayer co-culture system over conventional monolayer co-culture is that it can prevent cells from one layer migrating to another layer by creating a simple acellular space between layers. The volume of the acellular space between the bilayer, however, needs to be optimized properly, as we have observed the distance-dependent response of co-cultured cells. In our bilayer PEODA hydrogel system with approximate 10-nm pore size, when 40 μ l of acellular space, instead of 20 μ l, was used in between co-cultured cells, no significant effect of co-culture has been observed (data not shown) [33]. This result indicates the importance of close proximity between co-cultured cells. In most cases, paracrine signaling is effective in a short range of distance, and, therefore, it is advantageous to have the cells in close proximity as the morphogenic signals break down rapidly over great distances. Such a close proximity between the cells may not be easily achieved in conventional co-culture systems such as the Transwell system. In the bilayer system, on the other hand, the distance can be easily controlled by the volume of acellular space between co-cultured cells. Furthermore, the diffusion of secreted molecules from co-cultured cells may be modulated by the pore size of the scaffolding polymer.

Chondrogenesis is a complex process that is controlled by cellular interactions with the surrounding matrix, growth and morphogenic factors, and other environmental cues that modulate cellular signaling pathways and the transcription of specific genes in a temporal-spatial manner [34–36]. Chondrogenic differentiation of stem cells requires microenvironments composed of a multifaceted direction for differentiation, which cannot be provided by simply adding chemicals or growth factors. Previous studies presented the specific differentiation of ES cells by conditioned medium or co-culture with a different cell line, which might provide a more diverse range of morphogenic factors [8,9,12]. For example, Sui et al. co-cultured limb bud progenitor cells with ES cells in monolayer culture and showed increased chondrogenic-specific genes, such as type II collagen and proteoglycans [9]. In this study, hepatic cells were co-cultured with ESDCs, and detailed gene expression profiles of ESDCs co-cultured with hepatic cells were generated by RT- and real-time PCR. The results showed the increased chondrogenic-specific gene expressions of co-cultured ESDCs, as compared to those of control ESDCs cultured alone. In particular, *type IIB collagen* expression was observed only with co-cultured ESDCs, and *aggrecan* expression of ESDCs was increased 80-fold at day 21 by the co-culture, even in the chondrogenic medium supplemented by dexamethasone and TGF- β . The gene expression result was also supported by four-fold increased GAG contents and significantly more positive type II col-

lagen immunohistochemical staining in ESDC constructs co-cultured with hepatic cells in the chondrogenic medium. This demonstrates the importance of a co-culture system in providing a combination of various morphogenic factors.

The detailed gene expression profile of co-cultured ESDCs also revealed a temporary increase in osteogenic markers, such as *cbfa-1* and *type X collagen*, between days 9 and 15 of the hepatic cell co-culture. These results support the experiment of Hwang et al. in which *cbfa-1* expression of ES cells was enhanced after treatment with hepatic cell-conditioned medium at EB formation period [10]. *cbfa-1* plays a crucial role in stages of cartilage mineralization and bone formation, and it is known to promote the hypertrophic chondrocyte marker *type X collagen* [23,24]. The gene expression of type I collagen was also slightly increased by the co-culture. Overall gene expression profile appeared to parallel the order of hypertrophic markers by showing the peaks of *cbfa-1* at day 9, *type X collagen* at day 12, and *type I collagen* at day 15. By day 18, however, all of these hypertrophic markers in co-cultured ESDCs were decreased significantly. Levels of *cbfa-1* and *type X collagen* were lower than those of control ESDCs cultured alone. Such decrease in hypertrophic markers may be due to the presence of TGF- β in the co-culture medium, which is known to inhibit terminal chondrocyte maturation. Previously, Wang et al. studied hypertrophic markers during the cartilage differentiation of limb bud cell condensation in response to different growth factors and showed a gradual decrease in *cbfa-1* and *type X collagen* expression in the presence of TGF- β [24].

The conditioned medium study by Hwang et al. also showed not only increased osteogenic markers but also increased the number of ES cells by morphogenic factors secreted from hepatic cells [10]. In the present study, co-culture with hepatocarcinoma cells resulted in more than three-fold increased DNA contents, indicating significantly higher cell survival/proliferation with the co-culture. Therefore, hepatic cell co-culture may improve the limitation of slow human ES cell proliferation, and the increased cell survival/proliferation as well as the enhanced chondrogenic differentiation of ES cells by hepatic cell co-culture in a bilayer system promises a strong potential for cartilage tissue engineering.

CONCLUSION

We have demonstrated the enhanced chondrogenic differentiation of ESDCs with hepatic cell co-culture in a bilayer hydrogel system by histology, biochemical analysis, and gene expression. The results indicate that morphogenic factors secreted by hepatic cells have a potential to improve the chondrogenesis of ES cells. Further

studies to identify the combination of hepatic cell morphogenic factors involved in the co-culture may help us understand the mechanism of stem cell chondrogenesis and facilitate in vivo therapies for cartilage defects.

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