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The interaction between co-cultured human nucleus pulposus cells and mesenchymal stem cells in a bioactive scaffold

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ABSTRACT

Mesenchymal stem cells (MSCs) can differentiate into nucleus pulposus (NP) cells upon being co-cultured with NP cells. Important growth factors and morphogens secreted by MSCs during the differentiation process also enhance the biological properties of NP cells. In this study, the interactions between human NP cells and MSCs co-cultured in different cell-ratio (100% NP, 75% NP with 25% MSCs, 50% NP with 50% MSCs, 25% NP with 75% MSCs, and 100% MSCs) in a three-dimensional gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold were examined. Results showed that the cell proliferation was increased when NP and MSCs were co-cultured. Real-time PCR and immunohistochemical staining revealed that all co-culture groups produced type II collagen which represent normal NP cells but not type I collagen secreted by degenerated NP cells. FADD expression, which modulates cell survival and extracellular matrix homeostasis, was maintained in a stable status for co-cultured groups. The cultures containing 75% NP cells with 25% MSCs showed high level of collagen production and glycosaminoglycan content. Moreover, 75% NP cells with 25% MSCs had upregulated SOX9 that contributes to the improvement in type II collagen mRNA expression and protein production. These findings showed the NP/MSC cell-ratio influenced the cell functions dramatically. The co-culture of NP/MSC cells in a bioactive scaffold is a promising treatment for intervertebral disc diseases.

1. Introduction

Intervertebral disc (IVD) degeneration is a major cause of lower back pain and lumbar disc herniation. Although the exact pathological mechanisms are not fully understood, IVD degeneration is considered to be a pathologic condition that is induced mechanically and mediated biologically [1]. Decreasing production of extracellular matrix (ECM) in aging nucleus pulposus (NP) cells may also contribute to IVD degeneration [2]. In addition to the currently available conservative and operative treatments, novel cell-based tissue engineering approaches have been proposed for the treatment of IVD diseases [3,4]. Such approaches include transplantation of viable and productive NP cells or NP-like cells into the degenerated disc to restore architecture and function [5,6]. Therefore, having a feasible source of NP cells is critical for this treatment modality.

Many strategies have been developed to obtain NP or NP-like cells. Harvesting NP cells from degenerated disc tissue during discectomy or herniation surgery has been proposed [7,8]. However, cells harvested via this approach are unhealthy and have abnormal phenotypes [8–10]. Harvesting autologous NP cells from other healthy intervertebral disc (IDs) has also been proposed; however, this approach is not clinically practical because the additional surgery is required [11]. Moreover, harvesting NP cells will accelerate degeneration of donor IDs [12]. Allogeneic cell transplantation also has ethical issues and can be associated with infection. The possibility of propagating a human NP cell line to provide a ready supply of cells has also been considered [13].

Mesenchymal stem cells (MSCs) are multipotent cells that have the capacity to differentiate to osteoblasts, adipocytes,

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neural cells, chondrocytes, and even NP-like cells [14,15]. Various methods including hypoxia, stimulation with growth factors, three-dimensional (3D) scaffold, and co-culture have been used to induce differentiation of MSCs to NP-like cells [16–19]. Indeed, several articles have reported rapid proliferation of viable NP cells with superior ECM productivity after co-culture with MSCs [20,21]. Svanvik found that human NP cells from degenerated disks co-cultured with MSCs result in increased ECM production [22]. Moreover, many important growth factors and morphogens are secreted by MSCs during the differentiation process. For example, bone morphogenetic proteins, transforming growth factor-beta, platelet-derived growth factor, insulin-like growth factors, and fibroblast growth factors are secreted when MSCs differentiate [23–27]. Strassburg et al. reported that a co-culture model induces human MSCs differentiation and modulates the phenotype of degenerate NP cells [28]. These growth factors and morphogens enhance the biological properties of NP cells and could potentially be used in IVD treatment [29].

In a previous study, we co-cultured NP cells with MSCs using a monolayer model and found that cell proliferation, ECM production, and critical gene expression were regulated in NP cells [30]. Other researchers have reported that 3D culture environment improves the biological functions of NP cells [31,32]. Previous work has also demonstrated that disc cells in 3D culture display a different proliferative response to exogenous growth factors [33]. In addition, cells harvested from degenerated discs show modified gene expression in 3D culture [34]. Kim et al. further found that mechanical stimulation in combination with a 3-D co-culture fashion enhances differentiation of MSCs to NP-like cells [35]. Allon et al. developed a spherical pellet culture model and reported that MSCs/NP cells co-culture pellets formed bilaminar organization with either MSCs inside and NP cells outside or NP cells inside and MSCs outside [36]. All these studies reveal that MSCs/NP cells in combination with a bioactive scaffold may be favorable for IVD regeneration. Therefore, the purpose of present study was to investigate the interactions between NP cells and MSCs co-cultured in a 3D environment using a gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold. This tri-copolymer scaffold showed promising results when applied in cartilage, tooth, and NP regeneration [27,31,32,37]. We evaluated the optimal co-culture ratio of NP cells to MSCs and examined cell proliferation, ECM production, and cytokine expression.

2. Materials and methods

2.1. Preparation of gelatin/chondroitin-6-sulfate/hyaluronan scaffold

The gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold was prepared with a modified, previously described method [38]. Briefly, 500 mg of gelatin (G2500, Sigma–Aldrich, St. Louis, MO, USA), 100 mg of chondroitin-6-sulfate (27043, Fluka, USA), and 10 mg of sodium hyaluronate (Seikagaku, Japan) were mixed and dissolved in 10 mL of double distilled water in a 55 °C water bath for 10 min. The mixture was placed at 4 °C for 2 h and frozen overnight at −20 °C. The frozen mixture was then lyophilized for 24 h to obtain a scaffold. The scaffold was further cross-linked with a 0.1% glutaraldehyde solution (3802, Sigma–Aldrich) overnight and then immersed in a 0.1 M glycine solution (55046, Sigma–Aldrich) to bind any non cross-linked glutaraldehyde. After additional lyophilization for 24 h, the scaffold was cut in the transverse direction to make a disc, 5 mm thick by 12 mm wide (Fig. 1a and b). This pore size of scaffold was in the range between 150 and 200 μm and porosity around 75% which was used for further study [39].

2.2. Human nucleus pulposus cell isolation and culture

Retrieval and use of human tissue and cells were approved by the Research Ethical Committee at NTUH (9361700959). Human NP tissue was obtained aseptically from 6 young adult patients who underwent spinal surgery for disorders of lumber IDs (mean age 25, range 23–29 y/o). NP cells obtained from each donor were used in experiments separately. Human NP cells were isolated by digesting minced NP tissue in DMEM/F-12 medium (D8900, Sigma–Aldrich) containing 0.2% collagenase (C0130, Sigma–Aldrich) at 37 °C overnight. Digested NP tissue was then washed with phosphate buffered saline. NP cells were resuspended in medium and expanded by monolayer culture in DMEM/F-12 medium containing 10% fetal bovine serum (04-001-1A, Biological Industries, Israel), 1% antibiotic (P4083, Sigma–Aldrich), and 25 mg/mL L-ascorbic acid (A5960, Sigma–Aldrich). NP cells were cultured in an incubator set at 5% CO_2 and 37 °C. The donor-to-donor variability influences cell's morphology and proliferation. Therefore, NP cells were examined regularly to ensure that fibroblastic transformation had not occurred. NP cells in passage 4–8 were used for this examination.

Human bone marrow-derived MSCs were purchased from Lonza (Walkersville, MD, USA). MSCs were routinely cultured in MSC-growth medium (MSC-GM, Lonza) and propagated as monolayers. Culture medium was changed every 2–3 days.

2.3. Seeding and co-culture of cells in tri-copolymer scaffolds

Monolayer-cultured NP cells and MSCs were trypsinized, collected and counted. Five groups with different proportions of NP cells and MSCs (group 1: 100% NP cells; group 2: 75% NP cells with 25% MSCs; group 3: 50% NP cells with 50% MSCs; group 4: 25% NP cells with 75% MSCs; group 5: 100% MSCs; studying groups were summarized

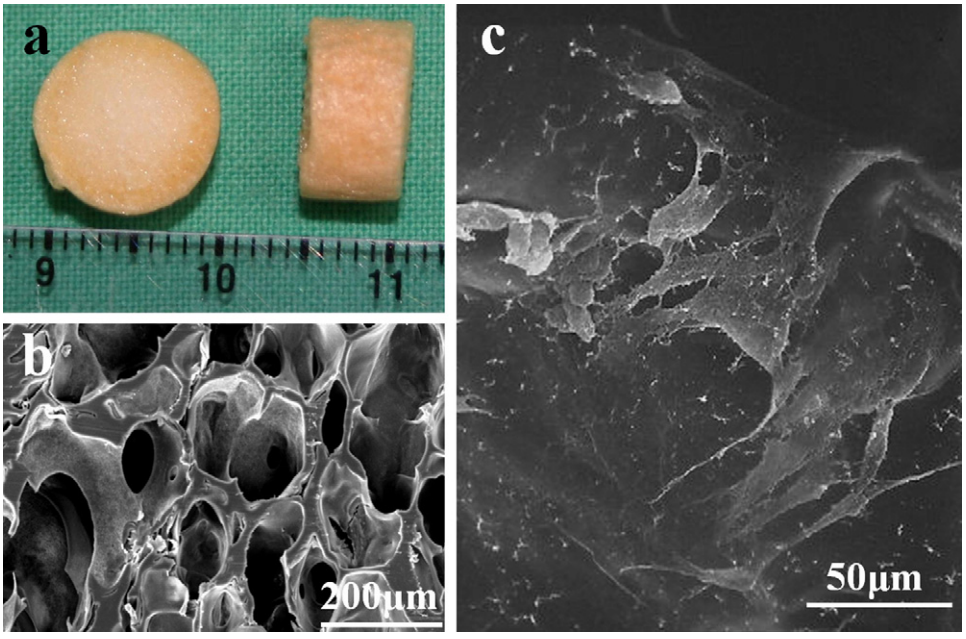

Fig. 1. (a) The macroscopic view of a gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold. (b) The internal cross-section of scaffold showed that the scaffold had pore size in the range between 150 and 200 μm. (c) A scaffold with NP/MSC cells observed using a scanning electron microscope after 2 weeks culture.

Table 1
NP cells and MSCs in different proportions for study group.

Group	NP cell number	MSC number	NP:MSC (%)
1	1.0E+06	0.0E+00	100:0
2	7.5E+05	2.5E+05	75:25
3	5.0E+05	5.0E+05	50:50
4	2.5E+05	7.5E+05	25:75
5	0.0E+00	1.0E+06	0:100

in Table 1) were suspended in 0.5 mL of culture medium. A total of 1×10^6 cells were injected into one scaffold. The NP/MSC-scaffold construct was placed in a 6-well culture plate for 60 min for cell adhesion, and then the culture medium was added. Culture medium was changed every 2–3 days (Fig. 1c).

2.4. DNA quantification for cell proliferation

As NP cells and MSCs penetrated deep into the scaffolds and formed complex arrangements, we were unable to isolate and evaluate the cell populations separately. After co-culture for 2 and 4 weeks, the NP/MSC-scaffold constructs were digested in 0.1% papain/Hank's balanced salt solution (P4762, Sigma–Aldrich) at 60 °C for 16 h. Double-stranded DNA was quantified (Quant-iT™ PicoGreen® dsDNA Reagent and Kits, Invitrogen, USA) to assess cell proliferation. In the preliminary study, we found that the DNA content of NP cell is slight higher (about 1.17 folds) than that of MSCs in the same cell number [30]. Therefore, NP and MSCs were used to establish the standard curve, respectively. NP and MSCs standard curves were then used to normalize the DNA content in each group to estimate the cell number.

2.5. Hydroxyproline assay for collagen content

A hydroxyproline assay was used to evaluate the collagen content of NP/MSC-scaffold constructs cultured for 2 and 4 weeks. Constructs were digested in a papain solution first; an aliquot of digested product was used to determine the DNA content as Section 2.4. Other digested product was then hydrolyzed in 6 N hydrochloric acid at 115 °C. Digested samples were then reacted with *p*-dimethylamino benzaldehyde and chloramines-T hydrate [40]. Trans-4-hydroxy-L-proline (H5534, Sigma–Aldrich) was diluted sequentially to construct a standard curve. Absorbance was measured at 550 nm using a microplate reader (Sunrise remote, Tecan, Switzerland). Since gelatin and chondroitin-6-sulfate were two major components of the scaffold, the hydroxyproline content of blank scaffolds undergone the identical culture periods as the studying group was used for background subtraction. Finally, the hydroxyproline content of each group was normalized based on the DNA content.

2.6. Glycosaminoglycan content measurement

Glycosaminoglycan (GAG) is a major component of the ECM in NP cells. After co-culture for 2 or 4 weeks, the GAG content of NP/MSC-scaffold constructs was evaluated using a 1,9-dimethyl-methylene blue (DMMB, 341088, Sigma–Aldrich) assay. Cultured specimens were digested in papain solution and the DNA content of each group were determined as previously described. The digested specimen was reacted with DMMB reagent. The GAG content of blank scaffolds undergone the identical culture periods as studying groups was used for background subtraction. Chondroitin-6-sulfate (C4384, Sigma–Aldrich) was used to construct a standard curve. Absorbance was measured at 595 nm using a microplate reader. The GAG content of each group was normalized based on the DNA content.

2.7. RNA extraction and real-time polymerase chain reaction

After co-culture for 2 and 4 weeks, total RNA was isolated from NP/MSC-scaffold constructs using an RNeasy mini kit (Qiagen, USA). An aliquot RNA was used to quantify the RNA yield using an ultra violet/visible/near infrared spectrophotometer (DU 7500, Beckman, USA). cDNA was synthesized from RNA by reverse-transcription polymerase chain reaction (RT-PCR) using a SuperScript III First-Strand Synthesis System (18080-051, Invitrogen) and stored at −80 °C. RNA extracted from scaffolds seeded with NP/MSC cells not further co-cultured was used as a control.

Gene expression levels of type I collagen, type II collagen, aggrecan, Fas-associated death domain protein (FADD), and SOX-9 were determined. Primers were designed as previously described [30]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous housekeeping gene. The mRNA expression for each target gene was normalized to that of the housekeeping gene GAPDH. The ΔC_T was calculated by subtracting C_T for GAPDH from C_T for each target gene, where C_T was the cycle threshold. The ΔC_T for each co-culture group was further normalized to the control group (NP cells and MSCs without co-culture period, the cell ratio was identical to the studying groups) to obtain $\Delta\Delta C_T$. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

2.8. Histologic examination and immunohistochemistry

After 4 weeks culture, NP/MSC-scaffold constructs were fixed in 10% formalin in neutral buffer. Samples were then dehydrated in a graded series of ethanol solutions and embedded in paraffin wax. Consecutive 5 μm sections were cut from the paraffin blocks and mounted on slides. Sections were deparaffinized and stained with hematoxylin and eosin (H&E, 3008-1&3204-2, Muto, Japan) and examined with an optical microscope to assess general construct morphology. Alcian blue staining (Muto, Japan) was performed to evaluate the production of GAG in NP/MSC-scaffold constructs.

For the immunohistochemical (IHC) staining, sections were immersed in a methanol solution with 3% H_2O_2 for 10 min to quench endogenous peroxidase activity and then incubated with serum blocking solution for 30 min to block non-specific binding. The sections were labeled with streptavidin-biotin (85-8943, Histostain-Plus, Invitrogen) following incubated overnight with anti-type I collagen antibody (251221, Abbiotec, CA, USA), anti-type II collagen antibody (250484, Abbiotec), or anti-S-100 antibody (N1573, Dako, CA, USA). Antigen presence was indicated with the 3,3′-diaminobenzidine (00-2014, Invitrogen). Sections were further counterstained with hematoxylin. Negative controls were processed in the same manner, except that rabbit IgG replaced the primary antibody.

2.9. Statistical analysis

All data are expressed as mean ± SEM. Analysis of variance (ANOVA) was used for statistical evaluation. Differences were considered statistically significant when p value was less than 0.05. For the figure symbols, * represent $p < 0.05$ and ** represents $p < 0.01$.

3. Results

3.1. DNA quantification for cell proliferation

In spite of difficulties in estimating the exact cell proportions in a co-culture model, all groups showed significant difference when compared with the initial cell number. When standard curve obtained from NP cells was applied (Fig. 2a), the cell number for group 2 and group 3 was significantly higher than that of NP cell culture (group 1) at week 2 ($p < 0.05$ for group 2 and group 3; and $p > 0.05$ for group 4). All co-culture groups had significant difference when compared with NP cell at week 4 ($p < 0.01$ for group 2, group 3, and group 4). The cell number of group 3 was significantly higher than MSC cell culture (group 5) at week 2 ($p > 0.05$ for group 2; $p < 0.05$ for group 3; and $p > 0.05$ for group 4). At week 4, the cell number of group 3 was significantly higher than MSC (group 5) ($p > 0.05$ for group 2; $p < 0.01$ for group 3; and $p > 0.05$ for group 4).

When standard curve obtained from MSCs was applied (Fig. 2b), similar results were found.

3.2. Hydroxyproline assay for collagen content

There was no significant difference between group 2 and group 3 for collagen content (Fig. 3). However, the collagen content in group 2 and group 3 was significantly higher than group 1 and group 4 at week 2 ($p < 0.05$); the collagen content in group 5 was significantly lower than that in groups 1 through 4 ($p < 0.01$). At week 4, the collagen content in group 2 was significantly higher than that in group 1, group 3, group 4, and group 5 ($p < 0.05$ for group 3; $p < 0.05$ for group 1, group 4, and group 5). There was no significant difference between group 1 and group 4 at week 4.

3.3. GAG content measurement

GAG content was higher in group 2 (2.48 ± 0.59 μg/μg) and group 3 (2.81 ± 0.56 μg/μg) compared with group 1 (1.61 ± 0.32 μg/μg) and group 4 (1.62 ± 0.50 μg/μg); however, the differences were not statistical significant (Fig. 4). GAG content was significantly higher in group 1, group 2, group 3, and group 4 compared with group 5 (1.21 ± 0.25 μg/μg) after 2 weeks culture ($p < 0.01$ for group 2, and group 3; $p < 0.05$ for group 1, and group 4).

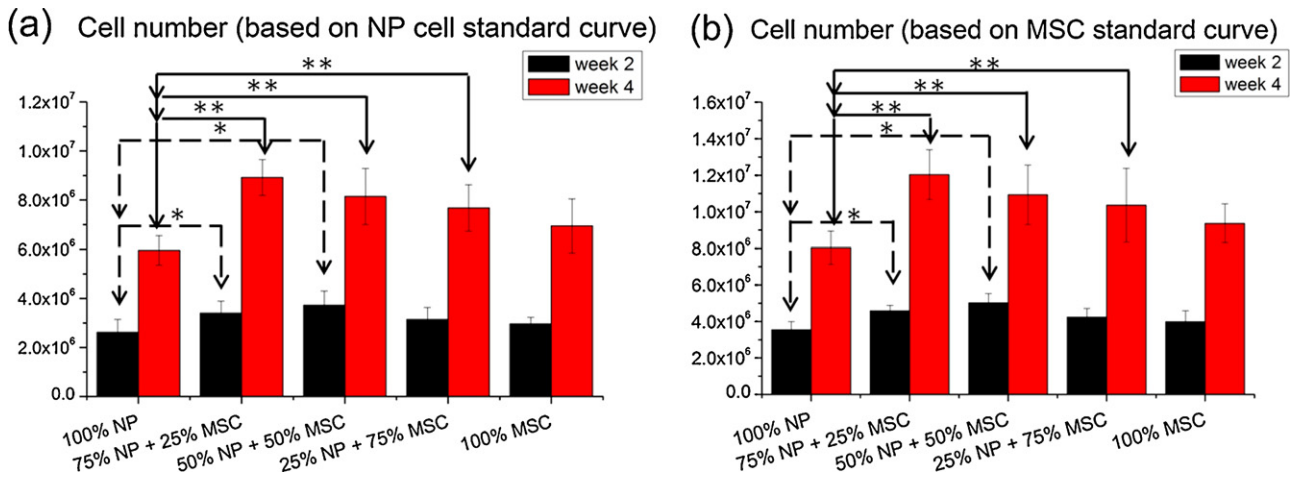

Fig. 2. Cell number of NP, NP/MSCs, and MSC groups. (a) When NP cells standard curve was applied, the cell number for group 2 and group 3 was significantly higher than that of NP cell culture at week 2. All co-culture groups had significant difference when compared with NP cell at week 4. The cell number of group 3 was significantly higher than MSC cell culture at week 2. At week 4, the cell number of group 3 was significantly higher than MSC group. (b) Similar results were found when MSCs standard curve obtained was applied.

Results were similar at week 4. There was no significant difference in GAG content between group 2, and group 3, but was significantly higher when compared with group 1, group 4 and group 5 ($p < 0.01$ for group 1 and group 3; $p < 0.05$ for group 5).

3.4. Gene expression

The RNA expression of aggrecan was downregulated for all groups at week 2 (Fig. 5a) and week 4 (Fig. 5b). For type I collagen, there was no significant change for group 1, group 2, group 3 and group 4; however, type I collagen was highly expressed in group 5 after 2 and 4 weeks culture. Expression of type II collagen was upregulated in NP cells (group 1) and co-culture NP/MSCs (group 2, group 3, and group 4). The FADD expression was slightly decreased in group 2; however, there was no significant difference between group 1, group 2, group 3 and group 4 at week 2 or 4. MSC group showed relatively high level FADD at week 2 and week 4 when compared with other groups. SOX9 expression was upregulated in group 2, group 3, and group 4 at week 2 and 4.

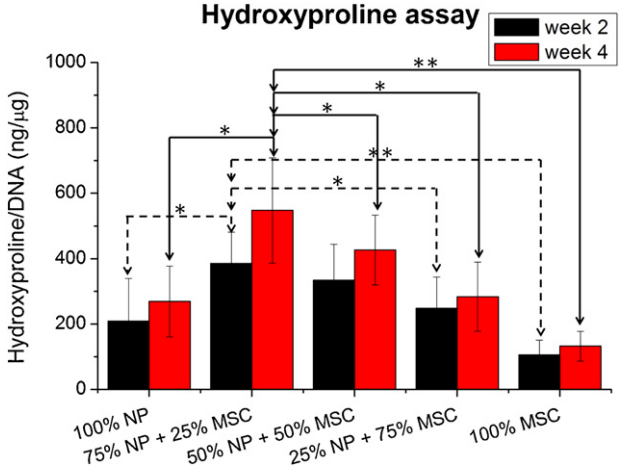

Fig. 3. Results of hydroxyproline assay (collagen content) for NP (group 1), NP/MSC co-cultured groups (groups 2–4), and MSC (group 5). The collagen content for group 2 and group 3 was significant higher compared with group 1 and group 4 at week 2; the collagen content in group 5 was significantly lower than that in groups 1 through 4. At week 4, the collagen content in group 2 was significantly higher than that in group 1, group 3, group 4, and group 5. The result for hydroxyproline assay was normalized to DNA content.

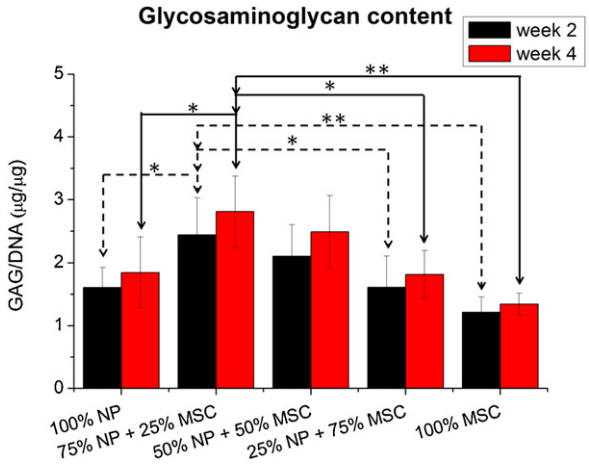

Fig. 4. GAG content for NP (group 1), NP/MSC co-cultured groups (the groups 2–4), and MSC (group 5). GAG content was significantly higher in group 1, group 2, group 3, and group 4 compared with group 5 after 2 weeks culture; similar results were found at week 4. The GAG content was normalized to DNA content.

3.5. Histologic examination

Cell population within scaffolds was higher in group 2, group 3, and group 4 (H&E, Fig. 6a2–4) compared with group 1 (Fig. 6a1) and group 5 (Fig. 6a5). Clusters of cells were observed inside pores of scaffold, especially in center of the scaffold. All groups were positive for Alcian blue staining, and abundant ECM was observed in co-culture groups (Fig. 6b2–4).

IHC staining revealed that cells in group 1, group 2, group 3, and group 4 were negative for type I collagen (Fig. 6c1–4), but positive for type II collagen (Fig. 6d1–4). In contrast, cells in group 5 were positive for type I collagen but negative for type II collagen (Fig. 6c5 and d5). IHC also revealed that cells in group 1, group 2, group 3, and group 4 were positive for S-100 staining (Fig. 6e1–4), whereas cells were negatively stained for S-100 in group 5 (Fig. 6e5).

4. Discussion

Many pathologic changes, including altered collagen production, reduced proteoglycan and water content, degradation of the ECM, and decreased cell density within degenerated discs [41–43].

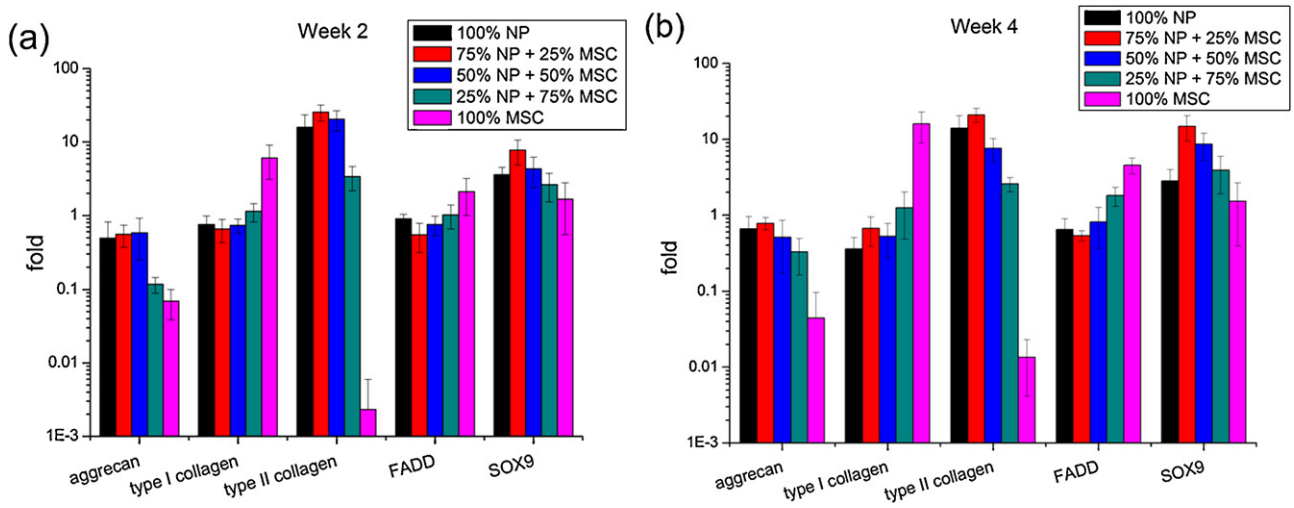

Fig. 5. Gene expression profiles of NP (group 1), NP/MSC co-cultured groups (groups 2–4), and MSC (group 5) after (a) 2 weeks and (b) 4 weeks culture. Aggrecan expression was downregulated for all groups at week 2 and week 4. Type I collagen was highly expressed in group 5 after 2 and 4 weeks culture. Expression of type II collagen was upregulated in NP cells and co-culture NP/MSCs groups. MSC group showed relatively high level FADD at week 2 and week 4 when compared with other groups. SOX9 expression was upregulated in group 2, group 3, and group 4 at week 2 and 4.

These factors should be examined to evaluate the effects of disc regeneration. Many studies have highlighted the benefits of MSCs to NP cells. However, most of these studies have demonstrated interactions between NP cells and MSCs using monolayer models [21,30,44,45]. Such models may not reflect the truly 3D environment in vivo. Therefore, we examined cell proliferation, collagen production, GAG content, and gene expression in NP cells co-cultured with MSCs in a 3D scaffold model.

Despite the discrepancy in cell number determined using different standard curves (NP and MSCs standard curves), all co-culture groups had significantly higher cell number than that of the NP and MSC groups, especially at week 4. The cell proliferation rate was significantly increased for MSCs compared with NP cells, while the cell proliferation rate was further increased in all co-culture groups compared with the MSC group. These findings are consistent with the H&E staining results, which demonstrated the presence of many cells within scaffolds in the co-culture groups. In contrast, H&E staining revealed that fewer cells were present within scaffolds in the NP and MSC groups after 4 weeks culture. All data indicated that the rate of cell proliferation was increased when NP cells were co-cultured with MSCs. This result is consistent with the finding reported by Watanabe et al., who reported that proliferation of NP cells was increased when co-cultured with MSCs [21].

We found that collagen content was higher in the NP group compared to the MSC group. Although all co-culture groups had high cell densities, collagen content was significantly higher in

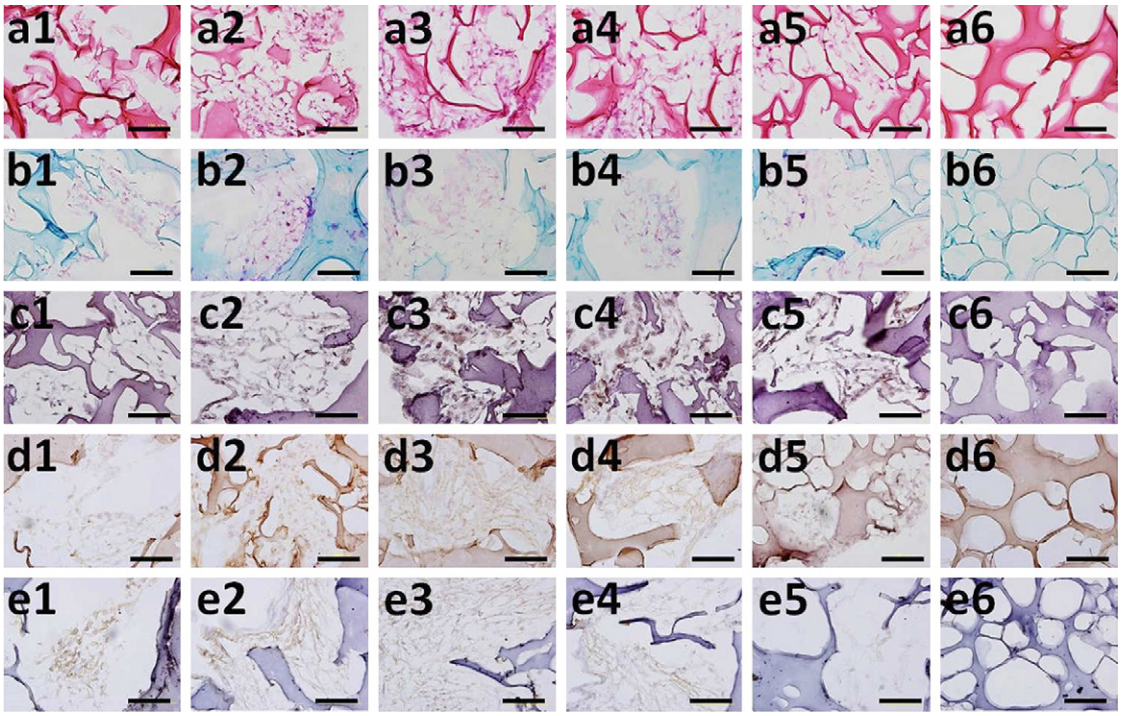

Fig. 6. (a) H&E, (b) alcian blue, (c) type I collagen, (d) type II collagen, and (e) S-100 staining for NP (1), NP/MSC co-culture groups (2–4), MSC (5), and empty scaffold (6) after 4 weeks culture (scale bar represented 100 μm).

co-culture group 2 only. Previous research suggests that hypertrophic NP cells may highly produce type I collagen [46]. Therefore, we evaluated type I and type II collagen gene expression in this study. We found that mRNA expressions of type I collagen was increased and type II collagen expression was decreased in group 5. These findings suggest that fibrous transformation of MSCs occurred [47]. It is similar with the results in a previous monolayer culture study [30]. Hence, transplantation of MSCs alone to degenerated discs (i.e. without NP cells) may not be an optimal treatment for IVD disease. In spite of type II collagen also was upregulated in NP cells cultured in 3D environment (group 1), type II collagen mRNA expression was highest in group 2. Type II collagen is a critical component of the disc ECM. Our results indicate that co-culture with NP/MSCs increases type II collagen production. This result was verified by IHC staining. Specifically, all co-culture groups were negative for type I collagen, but positive for type II collagen. S-100 proteins have been implicated in a variety of intracellular and extracellular functions [48]. In general, S-100 protein is expressed in cells of ectodermal origin, such as neurogenic cells. NP cells, except those of mesenchymal origin, also express S-100 protein. Lack of S-100 expression is reported in degenerated IVDs indicating the NP cells lose their phenotype. An increase in the number of cells positively stained for S-100 was also noticed in group 2. In addition, although the cell proliferation rate also increased in group 4, the collagen production level was similar to group 1 (NP cells). These findings indicate that the NP/MSC cell ratio dramatically influences cell functions.

With regards to GAG content, it was difficult to identify difference between group 1 through 4 by Alcian blue staining, and the aggrecan mRNA expression also decreased slightly. However, we found that GAG production was increased in co-culture group 2 and group 3. Recently, Wang et al. developed a highly organized alginate scaffold for cartilage tissue engineering [49,50]. They reported that when chondrocytes seeded within the scaffold, the mRNA expression of aggrecan increased first but decreased later. In the current study, we found that the GAG production only increased slightly during week 2–4. This reveals the NP/MSCs may produce GAG in the first 2 weeks, and provides an explanation for the decreasing mRNA level of NP cells at the pre-determined examining time points. Svanvik et al. noticed GAG production in human NP cells from degenerated discs was increased when these cells were cultured with MSCs [44]. Similarly, Le Visage et al. also reported that MSCs significantly enhanced proteoglycan production in NP cells in a co-culture system. These authors concluded that direct cell-to-cell contact enhances the biological properties of NP cells [45]. Although the precise molecular mechanisms underlying these effects of co-culture are not fully understood, it seems likely that a highly conserved signaling pathway may be involved.

SOX9 is a direct transcriptional activator of the cartilage-specific type II collagen gene, and SOX9 protein also plays a role in chondrogenesis and in mediating type II collagen expression [51]. We found that SOX9 expression was upregulated in the co-culture groups. Several genes and transcription factors, including SOX9, are critical for ECM homeostasis [52]. Indeed, SOX9 stimulates expression of a number of ECM components, such as type II collagen and aggrecan [53]. In a previous study demonstrating the importance of SOX9, Paul et al. found that type II collagen expression was increased in chondroblastic and human NP cells after transfection with a SOX9 promoter [54]. In this present study, the increased expression of SOX9 in co-culture group 2 may have contributed to the observed increase in type II collagen gene expression and production. In group 5, the type II collagen was still inactive when the SOX9 was upregulated. The type II collagen gene in MSCs is inactive under normal culture condition [55]. Our finding reveals that the upregulation in SOX9, by itself, is insufficient to activate the type II collagen gene in MSCs.

Although the role of apoptosis in the pathogenesis of IVD degeneration remains unclear, there is some evidence to support involvement of the FasL-Fas signaling pathway, in particular several key proteins and caspases, such as FADD and caspase-3 [56,57]. In this study, we found that FADD expression was maintained in a stable status in the co-culture groups. Excessive programmed cell death has also been suggested to play a role in human IVD cellular loss and ECM degradation [58,59]. Therefore, static FADD expression may improve ECM homeostasis and exert a positive effect on NP tissue engineering.

For lack of vascularity, cell-based approaches to restore disc functions are believed reasonable. After expansion and optimization of retrieved NP cells with MSCs in the 3D scaffold, the obtained cells could be used to restore the degenerated IVDs [60,61]. In the meantime, using an injectable hydrogel to deliver cells to the injured disc may minimize the surgical risk of transplantation [62]. The bioreactor culture system is also being considered to further improve the ECM production and cell proliferation in the project [63]. Based on the previous literature, we have launched the in vivo project to evaluate the feasibility of co-cultured cells re-implantation for future clinical applications [36,62].

In summary, we have reported that cell proliferation was increased for the co-cultured NP/MSCs. PCR and IHC analysis demonstrated that the co-culture NP/MSCs produced type II collagen but not type I collagen secreted by unhealthy NP cells. Further, co-culture of 75% NP cells with 25% MSCs resulted in increased collagen production and GAG content, upregulation in SOX9 and a static FADD expression. The co-culture of NP cells and MSCs in a 3D scaffold may be a promising approach for disc regeneration.

Author contributions

Chang-Chin Wu: participated in grant apply, research design, data analysis, and paper writing, Teng-Le Huang: participated in research design and study performing, Chia-Ching Liu: participated in study performing, Dai-Hua Lu: Participated in data analysis and paper writing, Shu-Hua Yang: participated in research design and data analysis, Kai-Chiang Yang: participated in research design, study performing, data analysis, and paper writing, Feng-Huei Lin: participated in study design and grant apply.

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