Original article

Differentiation of adipose-derived stem cells toward nucleus pulposus-like cells induced by hypoxia and a three-dimensional chitosan-alginate gel scaffold in vitro

Zhang Zhicheng, Li Fang, Tian Haiquan, Guan Kai, Zhao Guangmin, Shan Jianlin and Ren Dajiang

Keywords: intervertebral disc degeneration; adipose-derived stem cells; tissue engineering; chitosan-alginate gel

Background Injectable three-dimensional (3D) scaffolds have the advantages of fluidity and moldability to fill irregular-shaped defects, simple incorporation of bioactive factors, and limited surgical invasiveness. Adipose-derived stem cells (ADSCs) are multipotent and can be differentiated toward nucleus pulposus (NP)-like cells. A hypoxic environment may be important for differentiation to NP-like cells because the intervertebral disc is an avascular tissue. Hence, we investigated the induction effects of hypoxia and an injectable 3D chitosan-alginate (C/A) gel scaffold on ADSCs.

Methods The C/A gel scaffold consisted of medical-grade chitosan and alginate. Gel porosity was calculated by liquid displacement method. Pore microstructure was analyzed by light and scanning electron microscopy. ADSCs were isolated and cultured by conventional methods. Passage 2 BrdU-labeled ADSCs were co-cultured with the C/A gel. ADSCs were divided into three groups (control, normoxia-induced, and hypoxia-induced groups). In the control group, cells were cultured in 10% FBS/DMEM. Hypoxia-induced and normoxia-induced groups were induced by adding transforming growth factor-β1, dexamethasone, vitamin C, sodium pyruvate, proline, bone morphogenetic protein-7, and 1% ITS-plus to the culture medium and maintaining in 2% and 20% O₂, respectively. Histological and morphological changes were observed by light and electron microscopy. ADSCs were characterized by flow cytometry. Cell viability was investigated by BrdU incorporation. Proteoglycan and type II collagen were measured by safranin O staining and the Sircol method, respectively. mRNA expression of hypoxia-inducing factor-1α (HIF-1α), aggrecan, and Type II collagen was determined by reverse transcription-polymerase chain reaction.

Results C/A gels had porous exterior surfaces with 80.57% porosity and 50–200 μm pore size. Flow cytometric analysis of passage 2 rabbit ADSCs showed high CD90 expression, while CD45 expression was very low. The morphology of induced ADSCs resembled that of NP cells. BrdU immunofluorescence showed that most ADSCs survived and proliferated in the C/A gel scaffold. Scanning electron microscopy showed that ADSCs grew well in the C/A gel scaffold. ADSCs in the C/A gel scaffold were positive for safranin O staining. Hypoxia-induced and normoxia-induced groups produced more proteoglycan and Type II collagen than the control group (P<0.05). Proteoglycan and Type II collagen levels in the hypoxia-induced group were higher than those in the normoxia-induced group (P<0.05). Compared with the control group, higher mRNA expression of HIF-1α, aggrecan, and Type II collagen was detected in hypoxia-induced and normoxia-induced groups (P<0.05). Expression of these genes in the hypoxia-induced group was significantly higher than that in the normoxia-induced group (P<0.05).

Conclusion ADSCs grow well in C/A gel scaffolds and differentiate toward NP-like cells that produce the same extracellular matrix as that of NP cells under certain induction conditions, which is promoted in a hypoxic state.

Lower back pain is very common and intervertebral disc (IVD) degeneration plays an important role in its epidemiology. Surgical approaches, such as spinal arthrodesis, sacrifice IVD function and increase the risk of adjacent segment degeneration. The ideal treatment for IVD degeneration is biological repair to restore their structure and functions. With the recent progress in molecular biology and biomaterials, many novel therapeutic interventions have been reported to repair IVDS, such as growth factor injection, gene transfection, cell transplantation, and tissue engineering.

Tissue engineering is an emerging field in modern medicine. It involves the combination of seed cells and scaffold materials, which can be combined with bioactive factors to replace and regenerate lost or damaged IVD tissues. Recently, nucleus pulposus (NP) tissue engineering has undergone rapid development of critical techniques. In tissue engineering, it is essential to select the appropriate seed cells and scaffolds. Multiple cell sources have been investigated for their possible applicability in NP tissue engineering. Although the lineages of different cell types

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in IVDs are not known definitively, it is agreed that chondrocytes and NP cells share many common features. Adipose-derived stem cells (ADSCs) have a multi-lineage differentiation potential and the advantage of easy harvesting from a rich source. Another crucial aspect of NP tissue engineering is the creation of an optimal scaffold that has the properties of strength, toughness, biological degradation, no inflammatory response, and formability.

Recently, injectable gel scaffolds have been reported as a tissue engineering approach. Injectable three-dimensional (3D) scaffolds have the advantages of fluidity and moldability to fill irregular-shaped defects, simple incorporation of bioactive factors, and limited surgical invasiveness. The aim of our study was to explore the induction effect of a new injectable 3D chitosan-alginate (C/A) gel scaffold and hypoxia on ADSC differentiation toward NP-like cells.

**METHODS**

**Isolation, culture, characterization, and BrdU labeling of ADSCs**

Subcutaneous adipose tissues were harvested from the neck of New Zealand white rabbits. The tissue was washed with phosphate buffered saline (PBS), small blood vessels were removed, and the outer membrane and obvious connective tissues were dissected out. The adipose tissue was minced to a fine slurry with iridectomy scissors. The slurry was then digested with trypsin and collagenase in PBS at 37°C with intermittent mechanical agitation. The cells were filtered through sterile gauze, placed in a culture medium, and then incubated at 37°C. The medium was replaced every 3 days. Flow cytometry was used to analyze the surface markers of ADSCs. A total of 1×10⁶ cells were washed twice with PBS and then incubated for 1 hour at room temperature with FITC-conjugated anti-rabbit CD45 (1:200) antibodies (both purchased from Abcam, Cambridge, UK). The cells were then washed twice with PBS and analyzed by flow cytometry. For cell tracking, ADSCs were labeled with 25 µmol/L BrdU for 48 hours before seeding in the C/A gel scaffold.

**Preparation of the C/A gel scaffold**

Medical-grade chitosan (relative molecular weight 209 000 and 87% deacetylation) was dissolved in 0.2 mol/L acetic acid at a final concentration of 3%. After filtering the solution, the pH was adjusted to 8.5 with 0.1 mol/L NaOH. After rinsing by centrifugation, the chitosan solution was digested with trypsin and collagenase in PBS at 37°C. The mixture was emulsified by sonication (60 seconds at 50 W) and then stored at 4°C.

**ADSC induction and culture in the 3D scaffold**

A 50 µl suspension of 5×10⁶ passage 2 ADSCs and a 150 µl C/A gel scaffold were mixed in 12-well plates at room temperature for 20 minutes. Three groups of cells were evaluated in this study. In the control group, cells were cultured in 10% FBS/DMEM (high glucose). Hypoxia-induced and normoxia-induced groups were induced with 1.25 g/L bovine serum albumin, 10 µg/L transforming growth factor-β1 (TGF-β1), 100 nmol/L dexamethasone, 50 mg/L vitamin C, 100 mg/L sodium acetate, 40 mg/L proline, 100 µg/L BMP-7, and 1% ITS-plus (10 g/L insulin, 6.7 g/L sodium selenite, 5.5 g/L ferritin, and 2 µg/L ethanolamine) in 10% FBS/DMEM and maintained in 2% and 20% O₂, respectively. The cells and scaffold were co-cultured in an incubator with saturated humidity. The medium was changed every 3 days. Cell-gel constructs were evaluated on Days 7, 14, and 21.

**Histological observation**

The porosity of the C/A gel scaffold was calculated by liquid displacement method. The pore microstructure was analyzed by light and scanning electron microscopy. The co-cultured samples were fixed with 40 µg/ml paraformaldehyde, embedded in paraffin, and then stained with safranin O. Histological and morphological changes were observed by light microscopy. After fixation in glutaraldehyde, the samples were sprayed with gold and scanned with an electron microscope.

**Assessment of ADSCs viability in the C/A gel scaffold**

The viability of ADSCs in the C/A gel scaffold was measured by BrdU immunofluorescence. Briefly, tissue sections were fixed with paraformaldehyde, pretreated with 2 N HCL at room temperature for 30 minutes, and then washed three times. The sections were permeabilized with 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) and then blocked with 5% fetal bovine serum for 2 hours at 4°C. The specimens were incubated with the primary antibody (1/200, Abcam) at 4°C for 12 hours and then with an FITC-conjugated goat anti-rabbit IgG (1/200, Abcam). Cells were counterstained with 0.1% DAPI and analyzed under a fluorescence microscope (Olympus, Tokyo, Japan).

**Proteoglycan testing**

Proteoglycan levels were evaluated by safranin O staining. Dilutions of a chondroitin sulfate stock solution (0.5 µg/ml) were used to prepare a standard curve. Absorbance values were measured at 600 nm with a microplate reader.

**Type II collagen measurement**

Type II collagen levels were measured by a Sircol assay kit according to the manufacturer’s instructions. Briefly, a 100 µl sample was combined with 1 ml Sircol dye reagent, oscillated for 30 minutes, and then centrifuged to remove unbound dye. Alkaline reagent (1 ml) was added to release the bound dye, and 200 µl samples were transferred to a 96-well plate to measure the absorbance value at 540 nm.

**Measurement of mRNA expression**

On Day 21, mRNA expression of hypoxia-inducing factor-α (HIF-1α), aggrecan, and Type II collagen was determined by reverse transcription-polymerase chain reaction (RT-
PCR). Total RNA was extracted with TRIzol (Invitrogen, Beijing, China) according to the manufacturer’s instructions. Then, 1–2 µg total RNA was reverse transcribed into cDNA using an A3500 reverse transcription system (Promega, WI, USA). PCR in a total volume of 25 µl consisted of 5 µl 10× Taq buffer, 0.5 µl dNTP (10 mmol/L), 0.5 µl of each primer (20 µmol/l), 2 µl cDNA, 0.5 µl Taq polymerase (Toyobo, Japan), and ddH₂O. PCR conditions were at 94°C for 5 minutes, 30 cycles at 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1 minute, and then 72°C for 10 minutes. Primers based on rabbit gene sequences were designed and synthesized by integrated DNA technologies (Table 1). PCR products were electrophoresed in 1.5% agarose gel with ethidium bromide and visualized by a Bio-Imaging System (Bio-Rad).

Statistical analysis
Data are presented as mean±standard deviation. Comparisons between groups were analyzed by one-way analysis of variance (ANOVA). Statistical analysis was performed with SPSS 16.0 (SPSS Inc., Chicago, USA). The mean difference was considered significant when a P value less than 0.05.

RESULTS

Histomorphology of C/A gel scaffold
A gross view of the C/A gel scaffold resembled a pink translucent jelly (Figure 1A). Sections of the C/A gel scaffold were observed by light microscopy. The C/A gel scaffold was a porous material in which most of the pores appeared nearly round with different sizes and a uniform distribution (Figure 1B). Scanning electron microscopy revealed that the C/A gel scaffold surface was rough and slightly granular and had large pores ranging from 50 to 200 µm (Figure 1C). The porosity of the C/A gel scaffold was 80.57%.

Culture and characterization of ADSCs
Partial adherence of rabbit ADSCs was observed at 24 hours after primary culture. After 4 days of culture, ADSCs reached 90% confluence and exhibited short spindle and small polygonal shapes under an inverted phase contrast microscope (Figure 2A). Passage 2 ADSCs grew faster than the primary cells and reached 90% confluence in 3 days.

Flow cytometric analysis showed that 95.2% of rabbit ADSCs expressed CD90, a specific marker of stem cells. However, CD45 expression, which is a lymphohematopoietic marker, was very low (Figure

Table 1. RT-PCR primers

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>Forward: 5′-GTCGCTCGGCGAGTGTG-3′</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GGAAAGGCAAGTCAGGGT-3′</td>
<td></td>
</tr>
<tr>
<td>Col II</td>
<td>Forward: 5′-TCCCAGAACTACCTACCA-3′</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-CATCCTGAGCAGCTATAG-3′</td>
<td></td>
</tr>
<tr>
<td>Aggrecan</td>
<td>Forward: 5′-GCTGCTAGGAAAGACAGATG-3′</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-CTACCTGTCATCCTTCGT-3′</td>
<td></td>
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</tbody>
</table>

Figure 1. A: Gross view of the C/A gel scaffold resembling a pink translucent jelly. B: Under a light microscope, rounded, variably sized, and uniformly distributed pores were found in the C/A gel scaffold. C: Scanning electron microscopy revealed that the pore size of C/A gel scaffold ranged from 50 to 200 µm.

Figure 2. A: Primary ADSCs exhibited short spindle and small polygonal shapes under an inverted phase contrast microscope (Original magnification ×200). B: Flow cytometric analysis of passage 2 rabbit ADSCs. CD90 was expressed at a very high level (left panel), while CD45 was expressed at a very low level (right panel).
These results were in line with the phenotypic characteristics of ADSCs.

**Co-culture of ADSCs and the C/A gel scaffold**

There were some morphological changes of induced ADSCs, such as contracting and becoming a rounded shape with no obvious edges and a significant increase of light transmittance in the C/A gel scaffold. These changes indicated that ADSCs were NP-like cells (Figure 3A). However, the growth of passage 2 ADSCs was slower and 90% confluence was reached within 6 days. BrdU immunofluorescence and DAPI staining indicated that most ADSCs survived and proliferated in the C/A gel scaffold on Day 21 (Figure 3B). Proteoglycans in the co-cultures were stained with safranin O, particularly in normoxia-induced and hypoxia-induced groups on Day 21 (Figure 3C and D). Scanning electron microscopy showed that the cells grew well in the C/A gel scaffold. Cell adhesions were found in the C/A gel scaffold. Microvilli were observed clearly under the scanning electron microscope (Figure 4).

**Comparison of proteoglycan levels produced by ADSCs in each group**

The data differences between the mean of each group were tested by one-way ANOVA ($F=57.46$, $P<0.05$). There were no significant changes in the proteoglycan levels of control group ADSCs during culture. ADSCs in the hypoxia-induced group ($P<0.05$) and normoxia-induced groups were indicated to produce more proteoglycans compared to the control group (Figure 3C and D).

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**Figure 3.**

- **A:** There were some morphological changes of induced ADSCs in the hypoxia-induced group, such as contracting and becoming a rounded shape with no obvious edges, which resembled NP cells (Original magnification ×200).
- **B:** BrdU immunofluorescence. A large number of green fluorescent nuclei demonstrated the ADSCs survived and proliferated in the C/A gel scaffold on Day 21 (original magnification ×200). The same view with DAPI staining revealed blue fluorescent nuclei (original magnification ×200).
- **C:** Proteoglycans were stained with safranin O. Staining was weakly positive in the extracellular matrix of the normoxia-induced group on Day 21 (original magnification ×200).
- **D:** The amount of staining was obviously higher in the extracellular matrix of the hypoxia-induced group on Day 21 (original magnification ×200).

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**Figure 4.**

- **A:** ADSCs grew well and showed normal adhesion in the C/A gel scaffold.
- **B:** Cell adhesion in the C/A gel scaffold.
- **C:** High magnification revealed clear microvilli.
group \((P<0.05)\) produced significantly more proteoglycans than those in the control group. The proteoglycan level in the hypoxia-induced group was higher than that in the normoxia-induced group \((P<0.05, \text{Table 2})\).

Comparison of type II collagen levels produced by ADSCs in each group
The data differences between the mean of each group were tested by one-way ANOVA \((F=19.38, P<0.05)\). There were no significant changes in the Type II collagen levels in control group ADSCs during culturing. Compared with the control group, ADSCs in the hypoxia-induced group \((P<0.05)\) and normoxia-induced group \((P<0.05)\) produced significantly more Type II collagen. The level of Type II collagen in the hypoxia-induced group was significantly higher than that in the normoxia-induced group on Day 14 \((P<0.05, \text{Table 3})\).

RT-PCR analysis of ADSC and C/A gel co-cultures
The mRNA expression differences of HIF-1α, aggrecan, and Type II collagen between the mean value of each group were tested by one-way ANOVA \((P<0.05)\). On Day 21, compared with the control group, HIF-1α, aggrecan, and Type II collagen mRNA expression was higher in the hypoxia-induced group \((P<0.05)\) and the normoxia-induced group \((P<0.05)\). Significant differences were also found between hypoxia-induced and normoxia-induced groups (Table 4). mRNA expression of HIF-1α, aggrecan, and Type II collagen in the hypoxia-induced group was significantly higher than that in the normoxia-induced group \((P<0.05)\). The amplification and dissolution curves of aggrecan, Type II collagen, and HIF-1α genes in RT-PCR analyses are shown in Figure 5.

**Table 2.** Comparison of proteoglycan levels produced by ADSCs in each group

<table>
<thead>
<tr>
<th>Groups</th>
<th>7 Days</th>
<th>14 Days</th>
<th>21 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.17±2.47</td>
<td>11.36±0.96</td>
<td>9.15±1.54</td>
</tr>
<tr>
<td>Normoxia induced</td>
<td>21.41±2.10</td>
<td>46.73±4.14*</td>
<td>59.12±6.34*</td>
</tr>
<tr>
<td>Hypoxia induced</td>
<td>18.69±1.56</td>
<td>63.82±3.22*</td>
<td>93.78±5.31*</td>
</tr>
</tbody>
</table>

*\(P<0.05\), compared with the control group; †\(P<0.05\), compared with the normoxia-induced group.

**Table 3.** Comparison of Type II collagen levels produced by ADSCs in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>7 Days</th>
<th>14 Days</th>
<th>21 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.27±0.17</td>
<td>1.22±0.25</td>
<td>1.08±0.44</td>
</tr>
<tr>
<td>Normoxia induced</td>
<td>2.51±0.34*</td>
<td>5.49±0.59*</td>
<td>6.94±1.63*</td>
</tr>
<tr>
<td>Hypoxia induced</td>
<td>2.20±0.61*</td>
<td>9.52±0.41*</td>
<td>8.46±0.89*</td>
</tr>
</tbody>
</table>

†\(P<0.05\), compared with the control group; †\(P<0.05\), compared with the normoxia-induced group.

**Table 4.** mRNA expression of ADSC and C/A gel co-cultures

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control group</th>
<th>Normoxia-induced group</th>
<th>Hypoxia-induced group</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>0.38±0.21</td>
<td>1.25±0.98</td>
<td>2.96±1.35†</td>
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<tr>
<td>Coll</td>
<td>0.47±0.25</td>
<td>1.14±0.86</td>
<td>2.88±1.52†</td>
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<tr>
<td>Aggrecan</td>
<td>1.35±1.02</td>
<td>3.23±1.58</td>
<td>6.97±2.47†</td>
</tr>
</tbody>
</table>

†\(P<0.05\), compared with the control group; †\(P<0.05\), compared with the normoxia-induced group.

DISCUSSION

The mechanism of IVD degeneration is still unclear, but a change of the NP plays an important role in such degeneration. Treatment or reversal of IVD degeneration by tissue engineering has broad prospects for clinical application and an important aspect is the selection of appropriate seed cells and scaffold materials. Phenotypic changes of NP cells are found in degenerative IVDs. These changed NP cells are not the most appropriate cells for repair of degenerated IVDs. Moreover, the sources of NP cells are limited and not sufficient for IVD repair.

Many studies have reported that stem cells can be induced to differentiate into cartilage cells in vitro,\(^5\)\(^-\)\(^9\) NP and cartilage cells have similar features such as their phenotype and secretion of extracellular matrix, suggesting that stem cells may be alternative seed cells for NP cells.\(^10\) ADSCs have a multi-lineage differentiation potential. Furthermore, in vitro studies have confirmed that ADSCs differentiate toward the NP cell phenotype when co-cultured with NP cells.\(^11\) For clinical application, adipose tissue, as a rich source of stem cells, is easily harvested.\(^12\)\(^-\)\(^16\) In addition, Zhu et al.\(^17\) have proposed that ADSCs are superior to mesenchymal stem cells (MSCs).

In monolayer culture, adherent ADSCs grow as a fusiform shape and undergo contact inhibition quickly. ADSCs cultured as a monolayer are prone to senescence and spontaneous differentiation. Alternatively, 3D culture and induction systems have a similar biological environment as that of tissues in vivo.\(^17\) The extracellular matrix can provide a suitable microenvironment for stem cell growth and differentiation. Moreover, cell-matrix interactions may affect the phenotype, morphology, proliferation, and differentiation of cells.\(^18\)

In this study, ADSCs underwent morphological changes in gel scaffolds under induction conditions, such as contraction, increased translucency, and stereoscopic morphology. These morphological features are similar to those of NP cells in monolayer culture. Scanning electron microscopy showed that ADSCs adhered to the scaffold and cell surface microvilli were observed clearly, indicating that the cells grew well in the scaffold. Therefore, a 3D environment is very important for ADSC differentiation toward NP-like cells.\(^19\)\(^,\)\(^20\) Lu et al.\(^21\) found that ADSCs are more prone to differentiate toward NP-like cells in a gel scaffold. To some extent, transplantation of cells in a gel scaffold simulates the microenvironment of the NP. In addition, NP cells in a gel scaffold can stimulate ADSC differentiation to the phenotype of NP cells. Here, we created a new 3D environment with cross-linked chitosan and alginate, in which ADSCs were well dispersed to maintain their 3D shapes.

Chitosan is a natural biodegradable polysaccharide biological material that has good histocompatibility. Alginate is also a safe biodegradable polysaccharide.
material that is frequently used in tissue engineering to serve as the supporting matrix of a chitosan scaffold. In this study, we fabricated a new type of C/A gel scaffold, tested its pore properties, and evaluated the differentiation of rabbit ADSCs in C/A gels. The 3D C/A gel scaffold had a porous exterior surface. The porosity was 80.57% and the pore sizes ranged from 50 to 200 µm. However, previous studies have reported that NP cells in alginate scaffolds cannot properly produce a large amount of extracellular matrix. This effect may be observed because most proteoglycans secreted by cells do not gather sodium hyaluronate. As a result, NP cells simply diffuse in alginate gels. In our study, chitosan and alginate were stable in PBS, probably because of reverse iron electric charge as considered by Park et al. For tissue engineering, the cell activity in a scaffold is obviously important. Cell viability was evaluated by BrdU immunofluorescence in C/A gel scaffolds. A large number of green fluorescent nuclei demonstrated that the ADSCs survived and proliferated in the C/A gel scaffold. In addition, the gel scaffold used in our study can be injected or implanted with a minimally invasive technique, which is beneficial for clinical application.

Proteoglycans, particularly the large aggregating proteoglycan aggrecan, play a major mechanical role in load-bearing IVD tissue. Aggrecan tends to imbibe water, inflate the collagen network, and maintain tissue shape because of the high osmotic pressure resulting from its sulfated glycosaminoglycans. A decrease of proteoglycan concentration is one of the first changes in IVD degeneration. Type II collagen is the main element responsible for the important function of the NP. The amount of Type II collagen in the NP decreases during IVD degeneration, but there is an increase of Type I collagen. Thus, one of the important indexes of successful repair by tissue engineering is that the regenerated IVD can produce functional proteoglycans and collagen. Our results showed that ADSCs in hypoxia-induced and normoxia-induced groups produced and concentrated more proteoglycans and Type II collagen than those in the control group. We also confirmed aggrecan and collagen gene expression by RT-PCR. During culture, proteoglycan and Type II collagen production did not decline. We also confirmed that the C/A gel is a suitable and biocompatible substrate for ADSC differentiation. These results revealed that ADSCs may secrete the same functional extracellular matrix as that

Figure 5. A: Amplification curve of the aggrecan gene. B: Dissolution curve of the aggrecan gene. C: Amplification curve of the Type II collagen gene. D: Dissolution curve of the type II collagen gene. E: Amplification curve of the HIF-1α gene. F: Dissolution curve of the HIF-1α gene.
of NP cells under induction conditions. However, further analyses should be performed to determine whether the amount of extracellular matrix secreted by ADSCs after induction is similar to that secreted by NP cells.

The normal adult human IVD is almost completely avascular, and resident cells must survive in a hypoxic environment. Therefore, we attempted to simulate the environment of normal NP cells in a low oxygen state. Under hypoxic conditions, we found that ADSCs produced more proteoglycans. Risbud et al.\(^1\) found that hypoxia and TGF-β1 induce MSC differentiation toward a phenotype consistent with that of NP cells. Pharmacological inhibition of ERK1/2 and p38 activity results in a decrease of Sox-9, aggrecan, and Type II collagen mRNA levels, suggesting that low oxygen and growth factors may promote MSC differentiation toward NP-like cells and increase extracellular matrix production through MAPK signaling pathways. The mechanism of ADSC differentiation toward NP cells under a hypoxic condition is very complex and should be explored in detail. In our study, we also examined the gene expression of HIF-1α, aggrecan, and Type II collagen by RT-PCR. Compared with control and normoxia-induced groups, the expression of these genes was higher in the hypoxia-induced group. HIF-1α may play a role in ADSC differentiation toward NP cells under hypoxic conditions. However, our results are not sufficient for clarification of the mechanism. Further studies to elucidate the mechanism should include examination of other genes such as matrix metalloproteinase (MMP)-2, type XI collagen, decorin, biglycan, fibromodulin, and lumican in a hypoxic culture system.

Growth factors of the BMP family and other factors have been widely used to induce MSCs to differentiate into cartilage or cartilage-like cells.\(^2\) BMP-7 and TGF-β1 significantly increase the proteoglycan production of NP-like cells \textit{in vitro}.\(^2\) In this study, proteoglycan production of ADSCs under TGF-β1 and BMP-7 induction was significantly higher than that in the control group, indicating that these growth factors may promote ADSC differentiation into NP cells and increase extracellular matrix secretion. However, the detailed mechanism is not very clear.

HIF-1α, glucose transporter-1, MMP-2, glypican 3, and keratin 19 have been proposed as NP cell markers.\(^3\) Notably, in contrast to HIF-1β, HIF-1α is expressed only in the NP and is absent in both the cartilage end plate and annulus.\(^4\) Therefore, HIF-1α can be considered as a phenotypic character of NP cells, indicating that ADSCs had differentiated toward an NP-like phenotype in our study. However, the exact phenotype and origin of NP cells is still unclear. Currently, the NP cells that differentiated from many kinds of stem cells can only be defined as "like" or "similar." IVD regeneration is not just anatomical, but more importantly a functional restoration. The NP and annulus must be repaired histologically and mechanically. If transplanted cells and scaffold constructs can generate the same functional extracellular matrix as those in the normal NP, it does not appear to be important for transplanted cells to differentiate into NP cells.

REFERENCES


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