The role of adipose-derived stromal cells and hydroxypropylmethylcellulose in engineering cartilage tissue in vivo

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Abstract This study demonstrated a newly developed method using adipose tissue-derived stromal cells (ADSCs) and hydroxypropylmethylcellulose (HPMC) in building injectable tissue engineered cartilage in vivo. ADSCs from rabbit subcutaneous fatty tissue were cultured in chondrogenic differentiation medium and supplemented with transforming growth factor- β 1 (TGF- β 1) and basic fibroblast growth factor (bFGF). Histological, immunohistochemistry and RT-PCR analysis confirmed that the ADSCs differentiated into chondrocytes following induction. Induced ADSCs mixed with 15 % HPMC were injected into the

subcutaneous tissue of nude mice and, after a period of 8 weeks, newly formed cartilage was observed at the site of injection. The ability of ADSCs cultured in the induction medium with TGF- β 1 and bFGF to differentiate into chondrocytes and construct new cartilage indicates that ADSCs are suitable for use as seed cells in cartilage tissue engineering. HPMC, according to its good water solubility and being able to transform from liquid to solid at body temperature, was found to be an ideal scaffold for tissue engineering.

Keywords Adipose tissue-derived stromal cells · Hydroxypropylmethylcellulose · Transforming growth factor- β 1 · Basic fibroblast growth factor · Cartilage · Differentiation

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Introduction

Injury and other types of tissue damage can result in cartilage failure, which constitutes a significant health problem. Cartilage like laryngeal and tracheal cartilage has very limited potential to spontaneously heal as it lacks blood vessels and is isolated from systemic regulation. No treatment has yet been developed to repair the defects associated with long-lasting hyaline cartilage (Ochi 2004). Tissue engineering can produce a functional cartilage substitute through the combined principles of engineering, biology and medicine, and holds significant potential for the repair or replacement of diseased or damaged tissues.

Responsive seed cells and supportive scaffold are two critical components of successful cartilage tissue engineering. In 2001, Zuk et al. (2001). Obtained adipose tissue-derived stromal cells (ADSCs) from liposuction specimens and demonstrated their differentiation into adipogenic, chondrogenic, myogenic, and osteogenic cells in the presence of specific induction factors. Alginate and ADSCs grown in chondrogenic media for 2 weeks in vitro and implanted subcutaneously in nude mice for 4 and 12 weeks have been shown to abundantly synthesize cartilage matrix molecules, including collagen type II, type VI, and chondroitin 4-sulfate in vivo and in vitro (Erickson et al. 2002). In 2004, Lendeckel et al. (2004) reported the successful use of ADSCs to repair skull defects in a 7-year old girl and ADSCs have also been used in the prevention of graft-versus-host reactions (Fang et al. 2007). Exhibiting stable growth and proliferation kinetics, human-derived ADSCs as mesenchymal stem cells have important research value (Peterson et al. 2005). The prospect of ADSCs for use in cartilage tissue engineering, therefore, warrants further investigation.

Tissue bioengineering is the process of creating functional biological prostheses by entrapping dissociated cells into synthetic biodegradable polymer substrates that act as scaffolds. These scaffolds allow the diffusion of nutrients into cells, as well as cell-to-cell contact that leads to the formation of new tissue (Vacanti 1988). Several biomaterials are available for cell immobilization, but most of these polymers lack moldable properties and can be delivered only by implantation (Freed et al. 1993). An alternative biocompatible synthetic polymer named hydroxypropylmethylcellulose (HPMC) can be delivered via injection. As a cheap and nontoxic material, HPMC has been studied and used in food, pharmaceutical, and biomedical applications. Its properties include high water retention, high film forming ability, high solution viscosity, and, more importantly, high rates of biocompatibility (Picker 2003). In ophthalmic surgery, HPMC is used as ophthalmic viscosurgical device during cataract surgery (Maltese et al. 2006). For non-invasive surgery and bone repair, HPMC has also been used as a matrix to develop an injectable bone substitute, associated with biphasic calcium phosphate (Weiss et al. 1999). The aim of the present work was to determine whether HPMC could be a suitable

scaffold for engineering substitute cartilage in vivo using ADSCs as seed cells.

Materials and methods

Isolation and phenotype analysis of ADSCs

Experiments were performed according to animal experimental ethics committee guidelines. ADSCs were obtained from subcutaneous fatty tissue of adult New Zealand white rabbits. The samples of fatty tissue were washed with a series of phosphate buffered saline (PBS), digested with 0.075 % collagenase type II (Sigma, Saint Quentin Fallavier, France) in a 37 °C water bath shaker at 160 rpm for 45 min, and then filtered through a 100- μ m mesh filter to remove debris. The suspension was transferred into 50 ml tubes, then centrifuged at 3,000 rpm for 10 min to obtain the useful cells. After centrifugation, the solution was washed extensively with red blood cell lysis buffer (0.38 % NH_4Cl 0.05 mmol, Na_2EDTA 0.1 %, K_2HCO_3 pH 7.3) to remove the red blood cells, filtered through a 150- μ m mesh filter to remove cellular debris, and centrifuged again at 1,800 rpm for 10 min. Following centrifugation, the supernatant was discarded and the resultant cellular fraction was resuspended and cultured in normal medium containing Dulbecco's modified Eagle's medium (DMEM; Sigma), fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen). The cells were incubated at 37 °C in a humidified atmosphere containing 5 % carbon dioxide and 95 % air.

ADSCs were then immunophenotyped by fluorescence-activated cell sorting (FACS). Cells were detached with trypsin–EDTA (Invitrogen), washed in PBS, and immediately stained with mouse monoclonal antibodies against rabbit CD49d and CD106 conjugated with phycoerythrin (PE) (Biolegend, San Diego, CA, USA), CD14, CD31 and CD105 conjugated with fluorescein isothiocyanate (FITC), CD34, CD45 and CD56 conjugated with PE (Immunotech Beckman Coulter, Paris, France). ADSCs were washed twice with ice-cold PBS supplemented with 1 % bovine serum albumin (BSA) (Invitrogen), followed by incubation with saturating concentrations of the appropriate antibodies for 15 min at room temperature. Thereafter, cells were washed twice in ice-cold

Table 1 Primer sequences for RT-PCR analysis

Gene	Primer	Size (bp)
Aggrecan	F: 5'-GTA CCC CAT CCA CAC ACC-3'	136
	F: 5'-GAC CTC ACC CTC CAT CTC-3'	
Type II collagen	F: 5'-GAC CCC ATG CAG TAC ATG CG-3'	370
	R: 5'-AGC CGC CAT TGA TGG TCT CC-3'	
CDMP-1-1	F: 5'-GCT GTC CGA TGC TGA CA-3'	127
	F: 5'-CCT CTG CTT CCT GAC CAC-3'	
GAPDH	F: 5'-TCA CCA TCT TCC AGG AGC GA-3'	299
	R: 5'-CAC AAT GCC GAAGTG GTC GT-3'	

PBS, 1 % BSA and fixed with 1 % paraformaldehyde in PBS. Samples were analyzed using a FACSCalibur (Becton–Dickinson, Franklin Lakes, NJ, USA) and CellQuest analysis software.

Multipotent cell differentiation assay

To test the adipogenic capacity of ADSCs, cells were seeded at a density of 1×10^4 cells/cm² in 6-well plates (Nunc, Thermo Scientific, Roskilde, DK) and cultured with a serum-free-adipogenic medium including DMEM/F12 (Gibco, Invitrogen), transferrin (Sigma Aldrich) (10 µg/ml), biotin (Sigma Aldrich) (33 µM), insulin (Sigma Aldrich) (0.5 µM), triiodo-thyronine (Sigma Aldrich) (0.2 nM), IBMX (Sigma Aldrich) (0.5 mM), hydrocortisone (Sigma Aldrich) (0.1 µM) and pantothenate (Sigma Aldrich) (17 µM). After 14 days of culture, adipogenesis of ADSCs was assessed by Oil Red O staining for the lipid droplet detection. ADSCs were washed with ice-cold PBS for 5 min and fixed in 10 % formalin for 15 min at room temperature. The formalin was then discarded and the preparation was rinsed with 60 % isopropanol for 2 min, then 0.35 % Oil Red O (Sigma Aldrich) solution in 60 % isopropanol was added for a 10 min incubation. Stained cells were extensively washed with deionized water to remove the nonspecific staining.

For the in vitro osteogenic differentiation, ADSCs were seeded at a density of 5×10^5 cells/cm² in 6-well plates and cultured in the presence of osteogenic medium including DMEM/F12 (Gibco, Invitrogen), dexamethasone (Sigma Aldrich) (100 nM), L-ascorbic acid 2-phosphate (Sigma Aldrich) (100 mM), β-glycerophosphate (Sigma Aldrich) (10 mM) and penicilline-streptomycin (10,000 U/ml–10,000 µg/ml) (Gibco, Invitrogen) (0.5 %), and the medium was changed every 2–3 days.

Osteogenesis was evaluated by the deposition of calcified matrix that was detected using Alizarin Red staining. ADSCs were washed with cold PBS and stained with 2 % Alizarin Red S (Sigma Aldrich) solution for 2 min. Stained samples were extensively washed with deionized water to remove nonspecific staining.

For the in vitro chondrogenic differentiation of ADSCs, cells were seeded at a density of 5×10^5 cells/cm² in 6-well plates and cultured with cytokine-supplemented medium containing 10 ml/L insulin transferrin-sodium selenite medium supplement (ITS; Sigma), 10 µg/L TGF-β1 (Sigma), 25 µg/L bFGF (Sigma), 1×10^{-7} M dexamethasone (Sigma), 10 % FBS (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). All cells were incubated at 37 °C in a humidified atmosphere containing 5 % carbon dioxide and 95 % air. The culture medium was renewed every 2–3 days. When the cells were grown to 90 % confluence and then detached from the cell culture flask using trypsin/EDTA (Sigma). Cell viability and proliferation was evaluated by MTT assay according to the instructions of the manufacturer (Sigma Aldrich). Briefly, ADSCs were seeded at a density of 2×10^5 cells and incubated either in the presence of normal or chondrogenic medium. Cell viability was detected on each day at 37 °C and 5 % CO₂, each experimental point was performed in quintuplicate. For all subsequent experiments the ADSCs were used at passage 6.

For immunocytochemistry analysis, passage 6 ADSCs at a density of 1×10^4 cells/cm² were placed into an 18 mm × 18 mm glass slide containing 2 ml of chondrogenic medium. The cell slides were incubated for 10 min with newly diluted 3 % H₂O₂ solution to inactivate internal peroxidases, followed by incubating with 2 % diluted goat serum for 20 min. Slides

were then incubated with the primary monoclonal mouse antibody against rabbit type II collagen (ZSGB Bio, Beijing, China) at 1:100 in PBS for 1 h at room temperature. Incubation with a biotin-labeled anti-mouse antibody (ZSGB Bio) for 30 min was followed by 30 min of incubation with horseradish peroxidase-conjugated streptavidin. Antibody binding was visualized with diaminobenzidine (DAB) (ZSGB Bio) and the nuclei were counterstained with hematoxylin.

For real-time PCR analysis, total RNA from the samples was extracted using the TRIZOL isolation system (Invitrogen). Reverse transcription (RT) was performed using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). The real-time PCR was performed with the Master Mix SYBR green kit (TaKaRa, Shiga, Japan) according to manufacturer's instructions. Amplification conditions were: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 45 s and extension at 72 °C for 45 s. The products were analyzed on 1 % agarose gel and visualized using ethidium bromide staining. All primer sequences can be found in Table 1. GAPDH was used as a reference. Relative abundance of RNA were calculated from the cycle threshold (Ct) using the formula $2^{-\Delta\Delta C_t}$ and expressed as arbitrary units.

Preparation of HPMC hydrogel and seeding cells

HPMC powder was sterilized by Co⁶⁰ irradiation, then the appropriate amount of HPMC (15 % w/v) was mixed with the culture medium and stirred to dissolve. The final product was a viscous liquid at PH 7.4. Before transplantation, the passage 6 ADSCs after chondrogenic differentiation (seeding cells, 5×10^7 /ml) were mixed with the above HPMC hydrogel gently in 6-well plates and incubated at 37 °C and 5 % CO₂ for 12 h. 5-Bromodeoxyuridine (BrDU, 5 µmol/L) (Sigma), using to label mitotic cells in ADSCs, was incubated with the passage 6 ADSCs after chondrogenic differentiation at 37 °C and 5 % CO₂ for 48 h. Then the labeled cells were added into the prepared HPMC and co-incubated at 37 °C and 5 % CO₂ for 12 h.

In vivo assays

Sixteen BALB/C nude mice, aged 6–8 weeks, were purchased from the Animal Center of the Fourth

Military Medical University. Four injection mixtures were selected: mixture 1 contained passage 6 ADSCs induced by TGF-β1, bFGF and HPMC (15 % w/v, MW, Sigma); mixture 2 contained only passage 6 ADSCs induced by TGF-β1 and bFGF; mixture 3 contained ADSCs cultured in normal medium and HPMC (15 % w/v, MW); and mixture 4 contained only HPMC (15 % w/v, MW). The nude mice were anesthetized with methoxyflurane, and injected with one of the four mixtures at 64 sites in total (4 sites per mouse). The injection sites were distributed symmetrically along the dorsal midline and about 1 centimetre near the middle line of the mouse back. At each site, 0.3 ml mixture was injected into the dorsal subcutaneous tissue. Specimens were harvested at 8 weeks following implantation. Specimens were fixed for 18–24 h in 10 % buffered formalin, routinely processed, and embedded in.

Histological analysis

Paraffin sections (5 µm thick) were deparaffinized with toluene, rehydrated through a graded series of ethanol, and rinsed in distilled water. For the histological analysis, the sections were stained with hematoxylin and eosin (H&E), alcian blue (pH 2.6), and periodic acid-Schiff (AB-PAS) to identify acid and neutral mucins. Analysis with Masson's trichrome (MT) (Lazarous et al. 1992) and picrosirius red staining (PSR) was performed to detect the presence of interstitial collagen. For picrosirius red staining, the de-waxed paraffin sections were stained with haematoxylin for 8 min first and then stained with picro-sirius red (Sigma Aldrich) for one hour. The stained samples were washed with acidified water and dehydrated in 100 % ethanol for three times, then cleared in xylene and mounted in a resinous medium.

Statistical analysis

Experimental data were analyzed using SPSS 11.0 statistical software (SPSS Predictive Analytics, Chicago, IL, USA). Pearson's Chi square test was applied to assess statistically significant differences and correlations. Values of $p < 0.05$ were considered to indicate statistically significant differences.

Fig. 1 Characterization of ADSCs **a** Microphotographs of cultured ADSCs prior to induction.

b Microphotographs of ADSCs following induction with TGF- β 1 and bFGF. Scale bar = 50 μ m. **c** MTT analysis showed that the ADSCs cultured in cytokine-supplemented medium grew much faster than the cells cultured in normal medium (DMEM)

Table 2 Flow cytometric analysis of the mean number of ADSCs expressing hematopoietic lineage CD markers

CD antigen	Geometric mean
CD49d	58.5
CD14	9.8
CD31	13.8
CD34	3.7
CD45	4.6
CD56	12.5
CD105	11.5
CD106	4.9

Results

Microscopic and phenotype characteristics of isolated ADSCs

The ADSCs cultured in normal medium maintained a fusiform shape with oval nuclei, arranged in a swirl-like pattern (Fig. 1a), with an average population doubling time of 55 h. After the ninth passage, the cells grew more slowly and became obviously aged. As the culture time continued, ADSCs cultured in chondrogenic medium became apparently enlarged, broader in shape and with even extended pseudopodia

relative to the control group (Fig. 1a). The nuclei also enlarged and prominent nucleoli formed (Fig. 1b). MTT assay results showed that the induced cells entered the exponential growth phase at the second day, exhibited a population doubling time of 30 h. The ADSCs cultured in chondrogenic medium grew faster than the cells cultured in normal medium (Fig. 1c), and the cells in chondrogenic medium became senescent at the fifteenth passage.

The passage 6 ADSCs were immunophenotyped by fluorescence-activated cell sorting (FACS). After plastic adherence selection, the antigenic phenotype of ADSCs was carefully checked by flow cytometric analysis. The results showed that cells weakly expressed hematopoietic lineage markers CD14, CD34, CD45, CD106 and so on, but highly expressed CD49d that was not expressed in hematopoietic lineage but in ADSCs (Ranera et al. 2011) (Table 2), confirming that these cells were ADSCs.

Adipogenic and osteogenic differentiation potential of ADSCs

To study the differentiation of ADSCs toward adipogenic and osteogenic potential, we evaluated the morphological characters of ADSCs after adipogenic and osteogenic differentiation. As a result, a few lipid

Fig. 2 Adipogenic and osteogenic differentiation potential of ADSCs. **a** Lipid droplets were observed by Oil Red O staining in the ADSCs after 14 days of adipogenic differentiation.

b Alizarin Red staining results revealed calcific deposition in the ADSCs after 14 days of osteogenic differentiation. Scale bar = 100 μ m

Fig. 3 In vitro chondrogenic differentiation of ADSCs. **a** Type II collagen staining is negative in the ADSCs without chondrogenic induction, as revealed by immunohistochemical analysis. **b** Type II collagen is present in the extracellular matrices of induced ADSCs. Scale bar = 100 μ m. **c** Expression of cartilage-related genes by ADSCs with or without chondrogenic differentiation. Three genes were evaluated by real-time

PCR (AGC, Col-II, CDMP-1). Left panel agarose gel electrophoresis results; right panel statistical analysis results. Group 1 ADSCs in normal medium; Group 2 ADSCs in chondrogenic medium. The mRNA levels of the three genes were significantly upregulated in ADSCs treated with TGF- β 1 and bFGF. All results were normalized by GAPDH. *Significant difference between two groups at $P < 0.01$

droplets were observed stained with Oil Red O after 14 days of adipogenic differentiation (Fig. 2a); and Alizarin Red staining also revealed a large number of

mineralized areas/calcific deposition almost throughout the plate after 14 days of osteogenic differentiation (Fig. 2b).

Fig. 4 Histological analysis of ADSCs after chondrogenic differentiation. AB-PAS analysis indicated the expression of the acid mucopolysaccharide component in chondrogenic induced cells (**a** Cells without induction; **b** Cells with induction).

Histological analysis in the chondrogenic potential of ADSCs in vitro and in vivo

Immunocytochemistry results showed that the passage 6 ADSCs cultured with chondrogenic medium for 14 day were positive for type II collagen, while type II collagen expression was negative in the cells cultured with normal medium (Fig. 3a, b). By doing real time PCR, three cartilage-related genes including Aggrecan (AGC), type II collagen (Col-II) and the cartilage-derived morphogenetic protein 1 (CDMP-1-1)(Zhang et al. 2011) were tested. The results showed that the mRNA levels of all three genes were significantly upregulated in ADSCs treated with TGF- β 1 and bFGF than the cells in normal medium (Fig. 3c).

The histological analysis of the paraffin embedded tissues of the nude mice showed that ADSCs induced

Picrosirius red staining analysis indicated the presence of type II collagen in chondrogenic induced cells (**c** Cells without induction; **d** Cells with induction). Scale bar = 50 μ m

with chondrogenic medium secreted the specific cartilaginous matrices acid mucopolysaccharide by AB-PAS analysis (Fig. 4b). Furthermore, different collagen fibers were detected by picrosirius red staining under polarized light microscopy in the ADSCs cultured in chondrogenic medium (Fig. 4d). But such results could not be found in the ADSCs cultured in normal medium (Fig. 4a, c).

In vivo cartilage tissue formation

Nude mice displayed normal physical activities during the experimental period. On examination of the sacrificed mice, eight weeks after injection, chronic congestion and a small amount of capillary proliferation were observed at the injection sites. No edema or abnormal secretions were observed in the

Fig. 5 In vivo cartilage formation of induced ADSCs. **a** tissue engineered cartilage formation under subcutaneous tissue injected with ADSCs cultured in induction medium and HPMC (arrow). **b** Hematoxylin-eosin staining showed mature tissue engineered cartilage in the subcutaneous tissue (arrow). **c** AB-PAS analysis indicated expression of the acid mucopolysaccharide component

in the tissue engineered cartilage. **d** the presence of type II collagen in tissue engineered cartilage could be tested using Masson's trichrome staining analysis. **e** Picrosirius red staining analysis showed the presence of type II collagen in cartilage formations. Scale bar = 50 μ m

Fig. 6 The difference of in vivo cartilage formation using induced ADSCs with and without HPMC. **a** tissue engineered cartilage formation under subcutaneous tissue injected with ADSCs cultured in induction medium and HPMC. **b** Cartilage

formation using induced ADSCs without HPMC. The numbers of chondrocytes decreased obviously and the cells were also disarranged. Hematoxylin-eosin staining, scale bar = 100 μ m

subcutaneous tissue. 7 weeks after the injection, cartilage-like tissues were observed under the injection sites. (arrow in Fig. 5a). Hematoxylin-eosin

staining and AB-PAS sections of the neo-cartilage formation in group 1 showed chondrocytes in lacuna with a sulfated proteoglycans extracellular matrix

Table 3 Rates of cartilage formation (%)

Group	Number of nude mice	Number of injection sites/repeats	Number of formations	Rate of cartilage formation (%)
Mixture 1	4	16	13	81.25*
Mixture 2	4	16	2	12.50
Mixture 3	4	16	0	0.00
Mixture 4	4	16	0	0.00

* $P < 0.05$ (mixture 1 contained passage 6 ADSCs induced by TGF- β 1, bFGF and HPMC; mixture 2 contained only passage 6 ADSCs induced by TGF- β 1 and bFGF; mixture 3 contained ADSCs cultured in normal medium and HPMC; and mixture 4 contained only HPMC.)

Fig. 7 The contribution of the injected ADSCs in the neo-cartilage formations in vivo. **a** Immunocytochemistry analysis showed that BrDU was successfully labeled in passage 6 ADSCs after chondrogenic differentiation. Scale bar = 20 μ m.

b Immunohistochemistry for anti-BrDU showed that most of the cells in the formed cartilage were positive while the collagen fiber and muscle cells around the cartilage were negative. Scale bar = 50 μ m

(Fig. 5b, c). Figure 5d clearly showed an abundance of collagen fibers in the formed cartilage, identified by Masson's trichrome (MT) staining. In addition, using birefringence properties under polarized microscope, picrosirius red staining revealed that mature collagen fibers (red/orange) and immature collagen fibers (green/yellow) were present in the cartilage (Fig. 5e).

In the sites where the mixture 1 was injected, 13/16 cartilage formations were observed. By contrast, in the sites where the mixture 2 was injected, cartilage developed much slower and only 2/16 formations were observed, and the formations were not typical mature cartilage because the chondrocytes were very less in number and arranged in disorder (Fig. 6). No cartilage was observed in the sites injected with mixture 3 and 4. (Table 3).

The contribution of the injected ADSCs in the neo-cartilage formation

BrDU can be incorporated into the newly synthesized DNA of replicating cells during the S phase of the cell cycle. In this study, BrDU labeling was analyzed by immunocytochemistry and immunohistochemistry methods using antibody specific for BrDU (Sigma). Immunocytochemistry result showed that BrDU was successfully labeled in passage 6 ADSCs after chondrogenic differentiation (Fig. 7a). In the neo-cartilage formation, immunohistochemistry results showed that most of the cells in the cartilage formation were positive for anti-BrDU, while the collagen fiber cells and the muscle cells around the cartilage formation were negative for anti-BrDU (Fig. 7b).

Discussion and conclusions

Cartilage defects are the leading cause of disability. Although artificial joints or metal inserts are widely utilized in most cases, therapies based on tissue-engineering represent a potential method in clinical treatment, owing to the application of multilineage stem cells to differentiate into bone, cartilage, heart and nerve cells, as well as their biocompatibility and long-term prognosis (Chao et al. 2007).

In recent years, ADSCs have played an increasingly important role in the clinical applications of the repair or replacement of damaged tissues. The advantages of ease of isolation, high cell yields, immunomodulation and multipotency qualify ADSCs as an ideal source of autologous stem cells for cell therapy or tissue engineering (Du et al. 2010; Rodriguez et al. 2004). Abdanipour et al. found that ADSC treated by selegiline at certain concentrations could differentiate into neuronal lineage expressing neurotrophins (Abdanipour et al. 2011). In addition, it was also reported that ADSC cultured under keratocyte-differentiation conditions expressed corneal-specific matrix components, thus ADSC can adopt a keratocyte phenotype and therefore have potential for use in corneal cell therapy (Du et al. 2010).

In the present study, ADSCs obtained from subcutaneous fatty tissue of adult New Zealand white rabbits successfully differentiated into cartilage cells in the presence of TGF- β 1 and bFGF, which then began secreting type II collagen, a three-dimensional fibrillar network essential for the tensile stiffness and strength of cartilage (Oesser and Seifert 2003). A number of growth factors including TGF- β , FGF, BMP (bone morphogenic protein) and IGF (insulin-like growth factor) along with other soluble factors, such as HA (hyaluronic acid), chondroitin sulfate, and insulin, have been examined in previous studies for their effects on cartilage tissue engineering. These factors have been investigated independently and synergistically, with outcomes dependent on cell type and culture conditions (Cuevas et al. 1988; Fortier et al. 2002; Sellers et al. 2000). Many studies have found that TGF- β can evoke important signaling pathways that induce cell differentiation during the development of various organs (Watabe and Miyazono 2009). In cartilage development, TGF- β acted mainly as a stimulator of extracellular matrix production, such as collagen type II and proteoglycan (PG), or as a

downregulator of matrix-degrading enzymes (Edwards et al. 1987; Chung and Burdick 2008). But many studies have found that TGF- β may interact in a negative feedback loop to regulate chondrogenesis and the onset of hypertrophy during endochondral bone formation, while FGFs maintain continuous growth of cells and prevent premature chondrogenic differentiation (Weiss et al. 2010). bFGF has also proven to be an important growth factor that affects the growth and differentiation of cartilage, especially in preventing chondrocytes to progress from maturation to the hypertrophic state (Grimsrud et al. 1999). The results of our study also supported that TGF- β family play a major role in cartilage development.

A second important component in using ADSCs as seed cells for tissue engineering is obtaining an appropriate scaffold to provide a three dimensional environment required for the production of cartilaginous tissue. Numerous scaffolds have therefore been developed to repair articular cartilage like natural polymers or synthetic matrices (Vinatier et al. 2007). HPMC, a cellulose derivative polymer, is a methyl-cellulose modified with propylene glycol ether groups attached to the anhydroglucose of the cellulose (Fatimi et al. 2008). At high molecular weights and concentrations, this hydrogel forms a moldable viscous mass that is non-interactive with proteins and biologic macromolecules. Moreover, HPMC gel is able to transform from liquid to solid at body temperature. Due to these advantages, HPMC has been widely used to retard the release of materials by drug delivery devices. However, HPMC has less been reported to be used as a main scaffold material for tissue engineering. Trojani et al. (2005) evaluated the silated HPMC-based hydrogel as a scaffold for 3D culture of osteogenic cells in 2005. Vinatier et al. (2007) later assessed the ability of this hydrogel to maintain a chondrocyte-specific phenotype in vitro. In their study, they used the chondrocytes as the seed cells. Merceron et al. investigated Si-HPMC associated with autologous hADSCs might be an ideal tool in the context of cartilage damage (Merceron et al. 2011). Mathieu et al. (2012) injected Si-HPMC seeded with mesenchymal stem cells (MSC) into the myocardium of rats and successfully preserved the cardiac function and attenuated ventricular remodeling of the rats after myocardial infarction. But in our study, we just made full use of the physicochemical characteristics of HPMC in its own, because HPMC has good water

solubility and the solution is a kind of low-viscosity liquid at 23 °C but changes to be a solid gel at 37 °C. So in this study, when passage 6 ADSCs were mixed with 15 % HPMC, they were instantly injected into the the dorsal subcutaneous tissue of the nude mice. Our results also clearly demonstrated that HPMC plays a critical role as a scaffold during the formation of the cartilage. The results of the present study also demonstrated the importance for HPMC in engineering cartilage in vivo. With ADSCs cultured in induction medium and HPMC, the number of newly formed cartilages and chondrocytes in newly formed cartilage were significantly increased than that of induced ADSCs but without HPMC. In addition, the use of HPMC did not induce obvious infection or discharge in the nude mice, but further studies are also needed for longer term effects after implantation as well as cartilage repairing potentiality of HPMC.

In conclusion, our results demonstrated that following induction with TGF- β 1 and bFGF, ADSCs are capable of differentiating into chondrocytes and constructing cartilage tissue in nude mice. The fact that ADSCs have been shown to be suitable seed cells and that HPMC is an ideal scaffold for tissue engineering, could potentially serve as the experimental basis for a new treatment to repair cartilage defects in vivo.

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References

- Abdanipour A, Tiraihi T, Delshad A (2011) Trans-differentiation of the adipose tissue-derived stem cells into neuron-like cells expressing neurotrophins by selegiline. *Iran Biomed J* 15:113–121
- Chao PH, Grayson W, Vunjak-Novakovic G (2007) Engineering cartilage and bone using human mesenchymal stem cells. *J Orthop Sci* 12:398–404
- Chung C, Burdick JA (2008) Engineering cartilage tissue. *Adv Drug Deliv Rev* 60:243–262
- Cuevas P, Burgos J, Baird A (1988) Basic fibroblast growth factor (FGF) promotes cartilage repair in vivo. *Biochem Biophys Res Commun* 156:611–618
- Du Y, Roh DS, Funderburgh ML, Mann MM, Marra KG, Rubin JP, Li X, Funderburgh JL (2010) Adipose-derived stem cells differentiate to keratocytes in vitro. *Mol Vis* 16: 2680–2689
- Edwards DR, Murphy G, Reynolds JJ, Whitham SE, Docherty AJ, Angel P, Heath JK (1987) Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J* 6:1899–1904
- Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H, Guilak F (2002) Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. *Biochem Biophys Res Commun* 290:763–769
- Fang B, Song Y, Lin Q, Zhang Y, Cao Y, Zhao RC, Ma Y (2007) Human adipose tissue-derived mesenchymal stromal cells as salvage therapy for treatment of severe refractory acute graft-vs.-host disease in two children. *Pediatr Transpl* 11:814–817
- Fatimi A, Tassin JF, Quillard S, Axelos MA, Weiss P (2008) The rheological properties of silated hydroxypropylmethylcellulose tissue engineering matrices. *Biomaterials* 29:533–543
- Fortier LA, Mohammed HO, Lust G, Nixon AJ (2002) Insulin-like growth factor-I enhances cell-based repair of articular cartilage. *J Bone Joint Surg Br* 84:276–288
- Freed LE, Marquis JC, Nohria A, Emmanuel J, Mikos AG, Langer R (1993) Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers. *J Biomed Mater Res* 27:11–23
- Grimsrud CD, Romano PR, D'Souza M, Puzas JE, Reynolds PR, Rosier RN, O'Keefe RJ (1999) BMP-6 is an autocrine stimulator of chondrocyte differentiation. *J Bone Miner Res* 14:475–482
- Lazarous DF, Shou M, Unger EF (1992) Combined bromodeoxyuridine immunohistochemistry and Masson trichrome staining: facilitated detection of cell proliferation in viable vs. infarcted myocardium. *Biotech Histochem* 67:253–255
- Lendeckel S, Jodicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, Hedrick MH, Berthold L, Howaldt HP (2004) Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report. *J Craniomaxillofac Surg* 32:370–373
- Maltese A, Borzacchiello A, Mayol L, Bucolo C, Maugeri F, Nicolais L, Ambrosio L (2006) Novel polysaccharides-based viscoelastic formulations for ophthalmic surgery: rheological characterization. *Biomaterials* 27:5134–5142
- Mathieu E, Lamirault G, Toquet C, Lhomme P, Rederstorff E, Sourice S, Bateau K, Hulin P, Forest V, Weiss P, Guicheux J, Lemarchand P (2012) Intramyocardial delivery of mesenchymal stem cell-seeded hydrogel preserves cardiac function and attenuates ventricular remodeling after myocardial infarction. *PLoS ONE* 7:e51991
- Merceron C, Portron S, Masson M, Lesoeur J, Fellah BH, Gauthier O, Geffroy O, Weiss P, Guicheux J, Vinatier C (2011) The effect of two- and three-dimensional cell culture on the chondrogenic potential of human adipose-derived mesenchymal stem cells after subcutaneous transplantation with an injectable hydrogel. *Cell Transpl* 20:1575–1588
- Ochi M (2004) Clinical results of transplantation of tissue-engineered cartilage and future direction of cartilage repair—novel approach with minimally invasive procedure. *Yonsei Med J* 45 (suppl):S74–A76
- Oesser S, Seifert J (2003) Stimulation of type II collagen biosynthesis and secretion in bovine chondrocytes cultured with degraded collagen. *Cell Tissue Res* 311:393–399

- Peterson B, Zhang J, Iglesias R, Kabo M, Hedrick M, Benhaim P, Lieberman JR (2005) Healing of critically sized femoral defects, using genetically modified mesenchymal stem cells from human adipose tissue. *Tissue Eng* 11:120–129
- Picker KM (2003) The 3-D model: comparison of parameters obtained from and by simulating different tableting machines. *AAPS PharmSciTech* 4:E35
- Ranera B, Lyahyai J, Romero A, Vazquez FJ, Remacha AR, Bernal ML, Zaragoza P, Rodellar C, Martin-Burriel I (2011) Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue. *Vet Immunol Immunopathol* 144:147–154
- Rodriguez AM, Elabd C, Delteil F, Astier J, Vernochet C, Saint-Marc P, Guesnet J, Guezennec A, Amri EZ, Dani C, Ailhaud G (2004) Adipocyte differentiation of multipotent cells established from human adipose tissue. *Biochem Biophys Res Commun* 315:255–263
- Sellers RS, Zhang R, Glasson SS, Kim HD, Peluso D, D'Augusta DA, Beckwith K, Morris EA (2000) Repair of articular cartilage defects one year after treatment with recombinant human bone morphogenetic protein-2 (rhBMP-2). *J Bone Joint Surg Am* 82:151–160
- Trojani C, Weiss P, Michiels JF, Vinatier C, Guicheux J, Daculsi G, Gaudray P, Carle GF, Rochet N (2005) Three-dimensional culture and differentiation of human osteogenic cells in an injectable hydroxypropylmethylcellulose hydrogel. *Biomaterials* 26:5509–5517
- Vacanti JP (1988) Beyond transplantation. Third annual Samuel Jason Mixter lecture. *Arch Surg* 123:545–549
- Vinatier C, Magne D, Moreau A, Gauthier O, Malard O, Vignes-Colombeix C, Daculsi G, Weiss P, Guicheux J (2007) Engineering cartilage with human nasal chondrocytes and a silanized hydroxypropyl methylcellulose hydrogel. *J Biomed Mater Res A* 80:66–74
- Watabe T, Miyazono K (2009) Roles of TGF-beta family signaling in stem cell renewal and differentiation. *Cell Res* 19:103–115
- Weiss P, Gauthier O, Bouler JM, Grimandi G, Daculsi G (1999) Injectable bone substitute using a hydrophilic polymer. *Bone* 25(2 Suppl):67S–70S
- Weiss S, Hennig T, Bock R, Steck E, Richter W (2010) Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells. *J Cell Physiol* 223:84–93
- Zhang B, Yang S, Sun Z, Zhang Y, Xia T, Xu W, Ye S (2011) Human mesenchymal stem cells induced by growth differentiation factor 5: an improved self-assembly tissue engineering method for cartilage repair. *Tissue Eng Part C Methods* 17:1189–1199
- Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 7:211–228