

Cartilage regeneration in SCID mice using a highly organized three-dimensional alginate scaffold

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ABSTRACT

Tissue engineering for cartilage regeneration provides an alternative to surgery for degenerative osteoarthritis. Recently, a highly organized three-dimensional (3D) alginate scaffold was prepared using a microfluidic device; this scaffold is effective for chondrocyte culture *in vitro*. The performance of this scaffold was further demonstrated; an alginate scaffold seeded with porcine chondrocytes was implanted in the dorsal subcutaneous site of SCID mice. The recipients were sacrificed at 2, 4, and 6 weeks after transplantation. The grafted implants retrieved from the subcutaneous site were analyzed with histologic examinations. Real-time PCR was used to identify the gene expression patterns of the chondrocytes. The hematoxylin and eosin staining showed that the chondrocytes survived normally in SCID mice; cartilage-like structures were formed after 4 weeks implantation. Immunohistochemical staining revealed cells secreted type II collagen, produced glycosaminoglycans (proved by alcian blue stain), and maintained the expression of S-100. On the other hand, the cells were negative for type I and type X collagen staining. PCR showed that the mRNA expressions of aggrecan and type II collagen were up-regulated at weeks two and four, while type I and type X collagen were down-regulated during the study period. In summary, this highly organized 3D alginate scaffold provided a suitable environment and maintained functional phenotypes for chondrocytes in this animal study.

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1. Introduction

Articular cartilage is a connective tissue that exists in synovial joints, but lacks a blood supply for nutrition and transportation of metabolites. The absence of blood vessels also limits self-healing and remodeling for minor chondral injury, which subsequently progresses to degenerative osteoarthritis [1]. Many treatment

procedures have been proposed for cartilage injuries, such as chondral injury and osteochondritis dissecans. Pridie's procedure (subchondral drilling), abrasion arthroplasty, and microfracture are all bone marrow stimulating methods used to recruit mesenchymal stem cells from subchondral bone for cartilage regeneration [2]. The main shortcoming of these approaches is that the defect is repaired by fibrocartilage, which has less ability to absorb shock and decrease shear force around the synovial joint [3]. Other approaches, such as mosaicplasty and autogenous chondrocyte transplantation, which require harvesting a great deal of autologous tissues or cells, have also been proposed to repair chondral defects [4]. However, the problem of most concern with these approaches is donor site morbidity. Furthermore, non-homogeneous distribution of chondrocytes and loss of chondrocytes from the sealed edge of the periosteum are possible pitfalls of autogenous chondrocytes transplantation [5,6]. Allograft

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is another treatment choice, although poor integration between the edge of the chondral defect and the donor graft, with a high failure rate is reported and difficult to overcome [7].

Due to the above limitations, a tissue engineering strategy for cartilage regeneration was proposed as a new modality for chondral injury repair. This cell-based therapy relies on a three-dimensional (3D) porous scaffold. Many fabricating methods such as freeze drying, electrospinning, phase separation, gas foaming, solvent casting and particulate leaching have been developed to produce a 3D scaffold [8–11]. However, the pore size diameter may not be homogeneous and the closed-porous structure is generated in scaffolds [12]. These factors result in impediments and are factors that interfere with cell seeding and growing. A scaffold with a highly ordered and uniform spatial structure may resolve these problems. Aside from the fact that photolithographic patterning and layering, direct writing, and two-photon stereolithography are used to fabricate organized 3D scaffolds, the expensive equipment and robotic control limit its wide application [13–15].

In a previous study, a highly organized 3D alginate scaffold was prepared with a 2-channel fluid jacket microfluidic device [16]. This alginate scaffold has appropriate mechanical strength, high porosity, and was non-cytotoxic to chondrocytes. *In vitro* analyses showed that chondrocytes proliferated well and maintained a functional phenotype in this scaffold. Increases in type II collagen and GAG production were also observed. This scaffold, which was prepared using a reliable and economic method, was substantiated to be adequate material for cartilage tissue engineering [17]. The performance of this alginate scaffold was further investigated via an experimental animal model to examine the feasibility of cartilage regeneration. The alginate scaffold seeded with porcine chondrocytes was implanted into a subcutaneous site in the back of mice. The implants were retrieved to perform histological examination and immunohistochemical (IHC) staining to identify the morphology and compositions of the extracellular matrix (ECM) of the tissue engineering cartilage construct at pre-determined intervals. Real-time PCR was used to identify the gene expression patterns of retrieved chondrocytes. The purpose of this study was to evaluate the performance of the highly organized 3D alginate scaffold for cartilage tissue engineering in this animal study.

2. Materials and methods

2.1. Fabrication of the alginate scaffold

The preparation of the 3D highly organized alginate scaffold was described in a previous study [17]. Briefly, a microfluidic device equipped with a micropipette (inner diameter: 45 μm , and outer diameter: 95 μm) was used. 1.5% alginate (A2158, Sigma–Aldrich, St. Louis, MO, USA) with 1% Pluronic[®] F127 surfactant (P6866, Invitrogen, UK) was prepared. The alginate solution was dropped by the syringe pump (PHD 22/2000, Harvard Apparatus, USA) under a controlled flow rate (300 $\mu\text{l}/\text{min}$) and gas pressure (6psi). The nitrogen gas was pumped through the inner channel and the aqueous alginate solution with surfactant was pumped through the outer channel, respectively. The droplets were injected into a 2% calcium chloride solution (C2661, Sigma–Aldrich, St. Louis, MO, USA) and gelled as microspheres. After collection for 2 h, an alginate scaffold was obtained. The scaffold was then put in the vacuum system overnight to remove air bubbles and synthesize the interconnecting pores (Fig. 1). Finally, the alginate scaffold was immersed in 5% penicillin-gentamycin-streptomycin (P4083, Sigma–Aldrich, St. Louis, MO, USA) 1 h for disinfection.

2.2. Chondrocyte harvest and culture

Porcine chondrocyte was used in this study and cartilage was harvested from the porcine hind leg which was disinfected initially with alcohol and then betadine after scrubbing. medial parapatellar arthrotomy was performed and the knee joint was fully exposed. The cartilage was harvested from the femoral condyle, tibial plateau and patella as thin slices. The slices of cartilage were washed with sterilized phosphate buffered saline (PBS) and then immersed in 5% penicillin-streptomycin-neomycin for 15 min. After treatment, the tissues were washed with PBS and digested in 2% collagenase (C0130, Sigma–Aldrich, St. Louis, MO, USA) for 12 h. The digested cartilage was collected and centrifuged to obtain a cell pellet. The cell pellet was then re-suspended in Dulbecco's modified eagle's medium (DMEM) (SH30003.01, Hyclone, Logan, UT) and seeded to a 10 cm culture dish at the cell density of 5×10^5 . Chondrocytes were cultured in DMEM, supplemented with 50 $\mu\text{g}/\text{ml}$ L-ascorbic acid (A5960, Sigma–Aldrich, St. Louis, MO, USA), 10% fetal bovine serum (100–106, Gemini Bio-Products, USA), and 1% antibiotic in an incubator set at 5% CO_2 , 37 $^\circ\text{C}$. Cells were passaged after 70–80% confluent formed. The medium was changed every 2–3 days.

2.3. Cell seeding and cells/scaffold construct culturing

Alginate scaffold in the dimension of 4 mm in diameter and 4 mm in length was used. Chondrocytes (passage 3–5) were detached by trypsin-EDTA (15400, Gibco, USA), suspended in DMEM, and seeded into the alginate scaffold using a 24 gauge needle. Each scaffold contained 6×10^6 cells. Cells/scaffold constructs were first placed in a culture plate for 1 h for cell adhesion, and then medium was added. The cells/scaffold constructs were cultured in an incubator for another 3 days. Optical microscope was used to examine the cell distribution within the alginate scaffold before implantation.

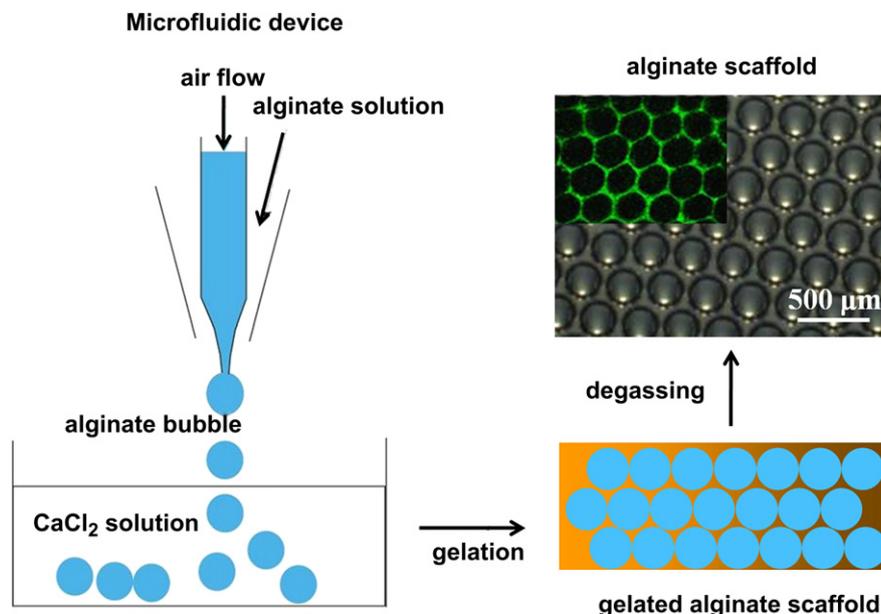


Fig. 1. Illustration of the procedure of alginate scaffold preparation.

2.4. Surgical implantation

Totally twenty-one CB-17/lcr-severe combined immunodeficient mice (SCID, male, 6 weeks old, weight: 19.3 ± 1.4 g) obtained from National Taiwan University Hospital Laboratory Animal Center were used as recipients. Animals were maintained in accordance with the guideline for the care and use of laboratory animals. Experimental protocols and surgical procedures were approved by the Institutional Animal Care and Use Committee. Mice were fed with Purina Laboratory Chow and tap water was given *ad libitum*; they were housed in a temperature-, humidity-, and light-controlled environment. Animals were marked to permit individual identification, and kept in their cages for 14 days prior to the beginning of experiments to allow for acclimatization to the laboratory conditions.

The operation was done under general anesthesia via intraperitoneum Ketamine injection. After adequate skin preparation and sterilization, two cells/scaffold constructs were placed subcutaneously in the left side of the back of one mouse ($n = 5$ for each time point) by using a straight-line incision in the right side of the back, with a pocket to the contralateral side. The incision was then closed with absorbable and non-absorbable sutures in turn. The control group ($n = 2$ for each time point) was mice that had received a blank scaffold without cell seeding. Cephalosporin (4 mg/kg) was injected in the mice to prevent infection post-operatively.

2.5. Histologic examination

Mice were sacrificed at 2, 4, and 6 weeks after surgery. The implants were removed with covered surrounding tissues from the subcutaneous site and fixed in 10% formalin of neutral buffer. The samples were then dehydrated in a graded series of ethanol and embedded in paraffin wax. Consecutive sections were cut from the paraffin blocks into 5 μ m slide; sections were deparaffinized and stained with hematoxylin and eosin (H&E, 3008-1&3204-2, Muto, Japan) to assess the general construct morphology under an optical microscope (BX51, Olympus, Japan). Glycosaminoglycan (GAG) is a major component of ECM of hyaline cartilage. Alcian blue forms complexes with anionic glycoconjugates such as GAGs. Therefore, alcian blue staining (Muto, Japan) was used to evaluate the production of GAGs of the chondrocytes/scaffold construct.

2.6. Immunohistochemistry

For the IHC staining, the expressions of S-100, type I, type II, and type X collagen were analyzed. The sections were first immersed in a methanol solution with 3% H₂O₂ for 10 min to quench the endogenous peroxidase activity, and then pre-incubated with serum blocking solution for 20 min to block the non-specific binding. The sections were labeled with streptavidin-biotin (85-8943, Histostain-Plus, Invitrogen, CA, USA) following incubated with anti-S-100 antibody (Novocastrol Laboratories Ltd, Newcastle upon Tyne, UK) at room temperature for 2 h. Other sections were incubated with anti-type I collagen antibody (CSI 008-01-02, Thermo, IL, USA), anti-type II collagen antibody (PAB13494, Abnova, Taipei, Taiwan), and anti-type X collagen antibody (ab49945, Abcam, Cambridge, UK) for overnight. The presence of the antigen was indicated by a brown color with the 3, 3'-diaminobenzidine (00-2014, DAB kit, Invitrogen, CA, USA). The sections were further counter-stained with hematoxylin. Negative controls were processed identically, except that the primary antibodies were replaced with IgG.

2.7. Real-time PCR analysis of gene expression

After implantation for pre-determined intervals, the harvested cells/scaffold construct was lysed in TRIzol (11596-026, Invitrogen, CA, USA). Total RNA was extracted following the manufacturer's instructions and the RNA yield was quantified using an ultra-violet/visible/near-infrared (UV/VIS/NIR) spectrophotometer (DU 7500, Beckman, USA). The cDNA was synthesized from RNA using Superscript II RT (18064-014, Invitrogen) with a RT-PCR machine (PTC-200, MJ Research, USA). The cDNA was stored at -80 °C until further analyses.

Aggrecan, type I collagen, type II collagen, and type X collagen were chosen as target genes to analyze the gene expression pattern of engrafted chondrocytes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous housekeeping gene (primer sequences are listed in Table 1). ABI PRISM 7900 sequence detection system with software 1.9.1 was used for PCR. A single reaction included 4 μ l of cDNA (50 ng), 4 μ l of 1 μ M sense and antisense primer solution, 12.5 μ l of PCR Mix (4304437, X TaqMan® Universal PCR Master Mix, ABI, USA), and supplemented with distilled water to the final volume of 25 μ l. PCR reaction started with an initial enzyme activation step at 50 °C for 2 min, samples were then denatured at 95 °C for 10 min. Subsequently, the samples were cycled 40 times in 2 stages, including a denaturation step at 95 °C for 15 s and a following annealing/extension step at 60 °C for 1 min. The gene expression pattern of engrafted cells/scaffold construct was compared with cells/scaffold construct cultured *in vitro*, and the relative expression of each target gene was examined using the $2^{-\Delta\Delta Ct}$ method.

Table 1

The primer designed for the real-time PCR.

Gene	Gene bank number	Primer sequence	Size (basepair)
Type I collagen	AF201723	Sense CAGAACGGCCTCAGGTACCA	101
		Antisense CAGATCACGTCATCGCACAAAC	
Type II collagen	AF201724	Sense GAGAGGTCTCTGGCAAAG	118
		Antisense AAGTCCCTGGAGCCAGAT	
Type X collagen	AF222861	Sense CAGGTACCAGAGGTCCCATC	117
		Antisense CATTGAGGCCCTTAGTTGCT	
Aggrecan	AF201722	Sense CGAAACATCACCCAGGGT	107
		Antisense GCAAATGTAAAGGGCTCCTC	
GAPDH	AF017079	Sense GTCATCCATGACAACCTCCG	103
		Antisense GCCACAGTTCCTCCAGAGG	

GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

2.8. Statistical analysis

Statistical analyses for real-time PCR were analyzed by ANOVA. Difference was considered significant when the *p*-value was less than 0.05.

3. Results

3.1. Macroscopic observation

Fig. 2a shows a mouse that received a chondrocyte/scaffold construct. At day 0, the scaffold was well located in the mouse's back and easily movable by palpation (Fig. 2b). The graft gradually hardened and became well fixed subcutaneously in the implant area (Fig. 2c). After autopsy, the transplanted scaffold was exposed and the soft tissue adhering around the scaffold was removed (week 2, Fig. 2d). At week 4, the color of the scaffold turned white (Fig. 2e). At week 6, the scaffold became whiter and harder, like a block of cartilage (Fig. 2f).

3.2. Histologic examinations of engrafted chondrocyte/scaffold construct

Fig. 3a and b showed a chondrocyte/scaffold construct observed using an optical microscope before implantation. Incorporation of chondrocytes into the alginate scaffold resulted in the formation of lacunas in the alginate scaffold (Fig. 3c and d). The cell number increased at week 4 (Fig. 3e and f), and the cells almost filled the scaffold at week 6 (Fig. 3g and h). The chondrocytes maintained their morphology with a round shape throughout the culture period. No elongation or deformation of the cells was found, which indicated no transformation of the fibroblast-like cells. Secretion of ECM by chondrocytes was observed in week 2 (Fig. 3d), and the production of ECM increased at week 4 (Fig. 3f). Lacuna formation was seen in the matrix surrounding the chondrocytes (Fig. 3h), the morphology of these lacunas being similar to that of natural cartilage. Histologic examination of the empty scaffold harvested from control group showed mild cells infiltration at week 6.

The lacuna structure with round-shaped chondrocytes was observed clearly and an increasing production of ECM was confirmed by positive staining with bluish hue (bluish hue, alcian blue staining, Fig. 4a). This critical structure revealed that cartilage-like matrix was generated. The alcian blue staining of the matrix in the chondrocyte-seeding scaffold was enhanced at 6 weeks (Fig. 4c) post-implantation in the SCID mice, compared to that at 2 and 4 weeks (Fig. 4b), which indicated an increase in GAGs production from the chondrocytes. A graft retrieved from control group at week 6 underwent alcian blue staining was shown in Fig. 4d.

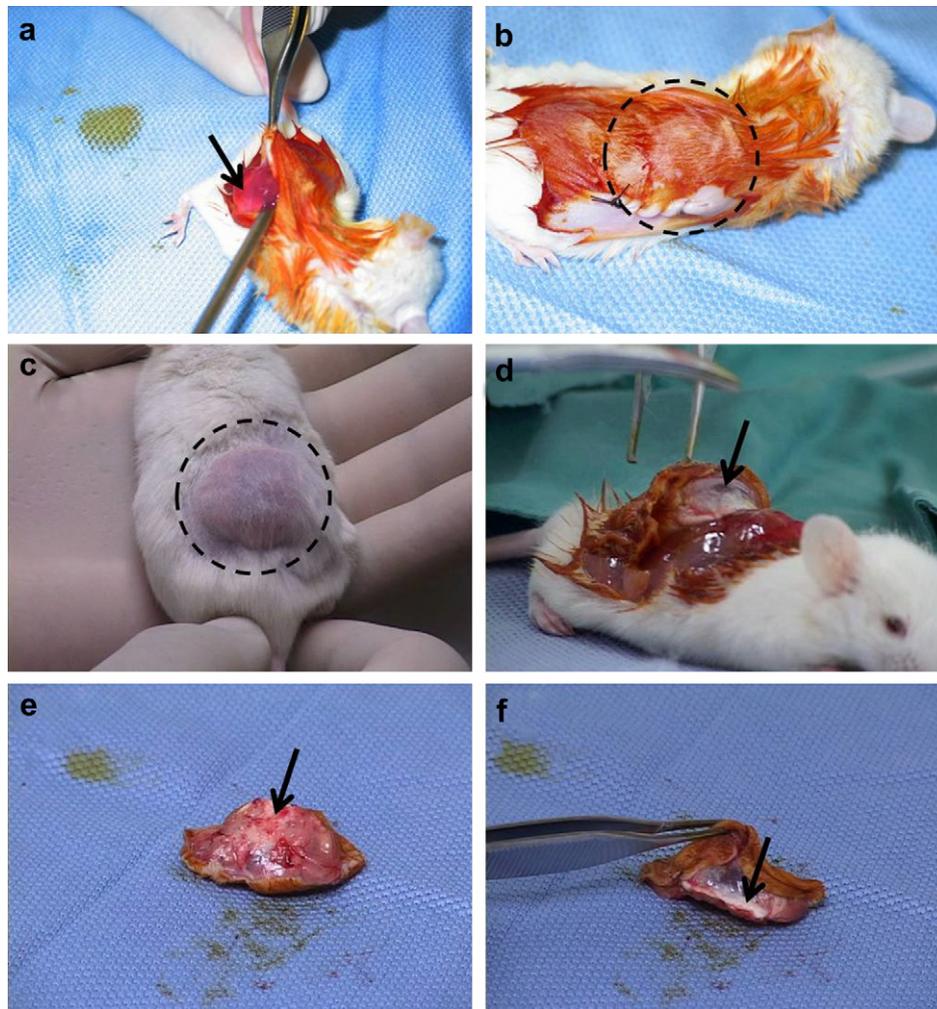


Fig. 2. (a) A mouse received the chondrocyte/scaffold construct. (b) At day 0, the scaffold was located over the mouse back and easily movable by palpation. (c) The graft became harder and fixed well under the implant area subcutaneously gradually (d) After autopsy, the transplanted scaffold was exposed at week 2. (e) At week 4, the color of scaffold turned white. (f) At week 6, the scaffold became whiter and harder that similar to a native cartilage.

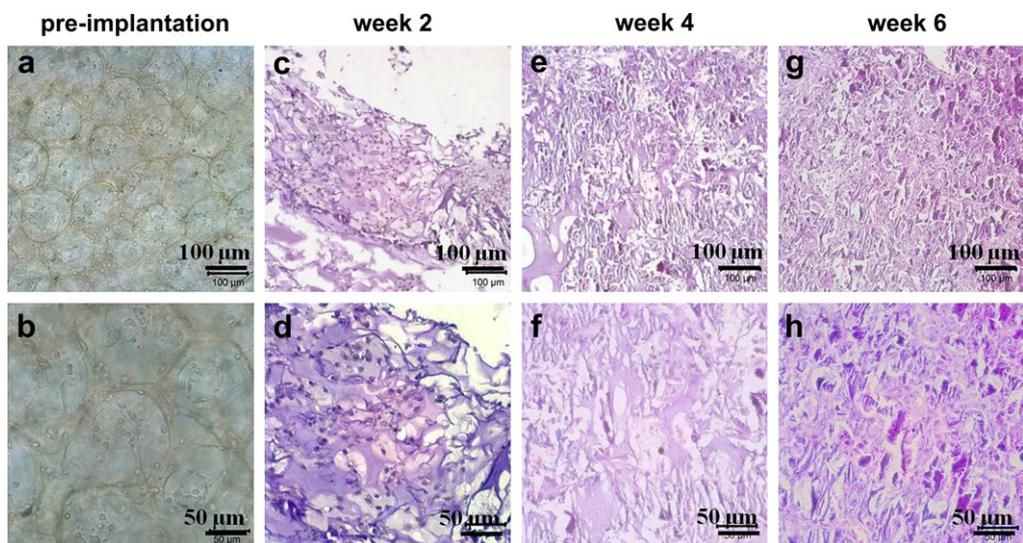


Fig. 3. (a) and (b) showed a chondrocytes/scaffold construct observed using an optical microscope before implantation. (c) and (d) showed the incorporation of chondrocytes in the alginate scaffold resulted in the formation of lacunas in the alginate scaffold. The cell number increased at week 4 ((e) and (f)) and almost fulfill with the scaffold at week 6 ((g) and (h)) ((c)–(h), H & E staining, purple represents the cell nuclear and red color represents cytoplasm).

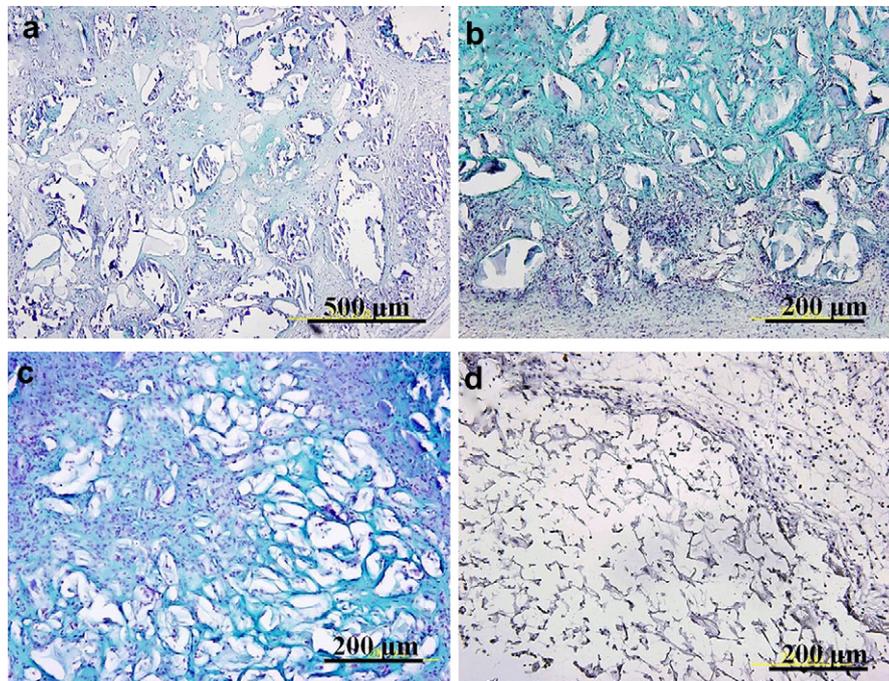


Fig. 4. (a) Alcian blue staining showed chondrocytes produced ECMs (bluish hue represents the positive staining of GAGs) at week 2, (b) week 4, and (c) week 6. (d) A graft retrieved from control group at week 6 underwent alcian blue staining.

3.3. Immunohistochemical analysis of engrafted chondrocyte/scaffold constructs

The expressions of S-100 protein and type II collagen in retrieved chondrocyte/scaffold constructs were examined by IHC staining. In general, S-100 protein is expressed in cells of ectodermal origin, such as neurogenic cells; chondrocytes, except those of mesenchymal origin, also express S-100 protein. Lack of S-100 expression indicated that the chondrocytes lose their phenotype. As shown in Fig. 5a, the chondrocytes retained their round-shape morphology and positive for S-100 protein staining (brown color, IHC for S-100). The round shape of the chondrocytes revealed that the normal phenotype of the cells was maintained; the normal phenotype is essential to production of ECM by the chondrocytes. Type I collagen is the most abundant collagen in human body and found in fibrocartilage and bone. Negative staining means the chondrocytes from porcine hyaline cartilage can keep their phenotype as shown in Fig. 5b. Type II collagen, a major component of hyaline cartilage, is produced by functional and healthy chondrocytes only. Fig. 5c shows that the retrieved implants at weeks 2, 4, and 6 were all positive for type II collagen staining (brown color, IHC for type II collagen). These results therefore revealed that the chondrocytes in the scaffold were functioning well. Type X collagen is found in hypertrophic and mineralizing cartilage. Fig. 5d shows the negative staining in the retrieved implant, which revealed the chondrocytes were not transformed into osteoblasts.

3.4. Gene expression in chondrocytes transplanted into the SCID mice

The gene expression patterns of engrafted cells/scaffold construct was compared with that of cells/scaffold construct cultured *in vitro* (Fig. 6). Real-time PCR showed that aggrecan expression was up-regulated at week 2 (1.65 ± 0.44 folds, $p < 0.01$), further increased at week 4 (1.89 ± 0.82 folds, $p < 0.05$) but decreased at week 6 (0.61 ± 0.19 folds, $p < 0.05$). On the other hand, the type I collagen mRNA expression for the engrafted

chondrocytes/scaffold was suppressed ($p < 0.01$), and the mRNA expressions were at similar levels at weeks 2, week 4, and week 6 ($p > 0.05$). The mRNA expression of type II collagen was up-regulated to 3.49 ± 0.60 folds ($p < 0.01$) at week 2 and 2.21 ± 1.00 folds ($p < 0.05$) at week 4, and then the expression decreased to 0.88 ± 0.29 ($p > 0.05$) folds at week 6. The expression of type X collagen was similar to that of type I collagen, in which the expression was highly down-regulated ($p < 0.01$). The results revealed that the cells maintained their normal phenotypes in the alginate scaffold in the SCID mice model.

4. Discussion

In contrast to expensive and complex techniques such as photolithographic patterning and layering, direct writing, and two-photon stereolithography, we developed an economic and easy method to fabricate a 3D organized scaffold using a microfluidic device [16]. This alginate scaffold maintains normal phenotypes of chondrocytes with up-regulation in aggrecan, collagen type II, and down-regulation in collagen types I and X *in vitro* [17]. In this study, the chondrogenesis of porcine chondrocytes seeding in the alginate scaffold using a mouse model. Scaffold seeding with chondrocytes was implanted in the subcutaneous portion of the back of the SCID mice. In the macroscopic view, the scaffold color had turned white with a polished surface at week 4 (Fig. 2). The cartilage-like structure became harder with less elasticity relative to the surrounding tissues at week 6. H & E staining showed chondrocytes secreted ECM at week two, and a lacuna structure similar to natural cartilage was observed at week 4 (Figs. 3 and 4). Moreover, the subcutaneous site has low oxygen partial pressure and limited nutrition supply which may detrimental to engraft. However, in this present study, H&E staining revealed the cell numbers increased gradually within the alginate scaffold. This may be attributed to the construction of the alginate scaffold, which its uniform-size pores with a highly organized stack. The pores mimic the shape and environment of the lacuna so that the chondrocytes will stay and proliferate [18].

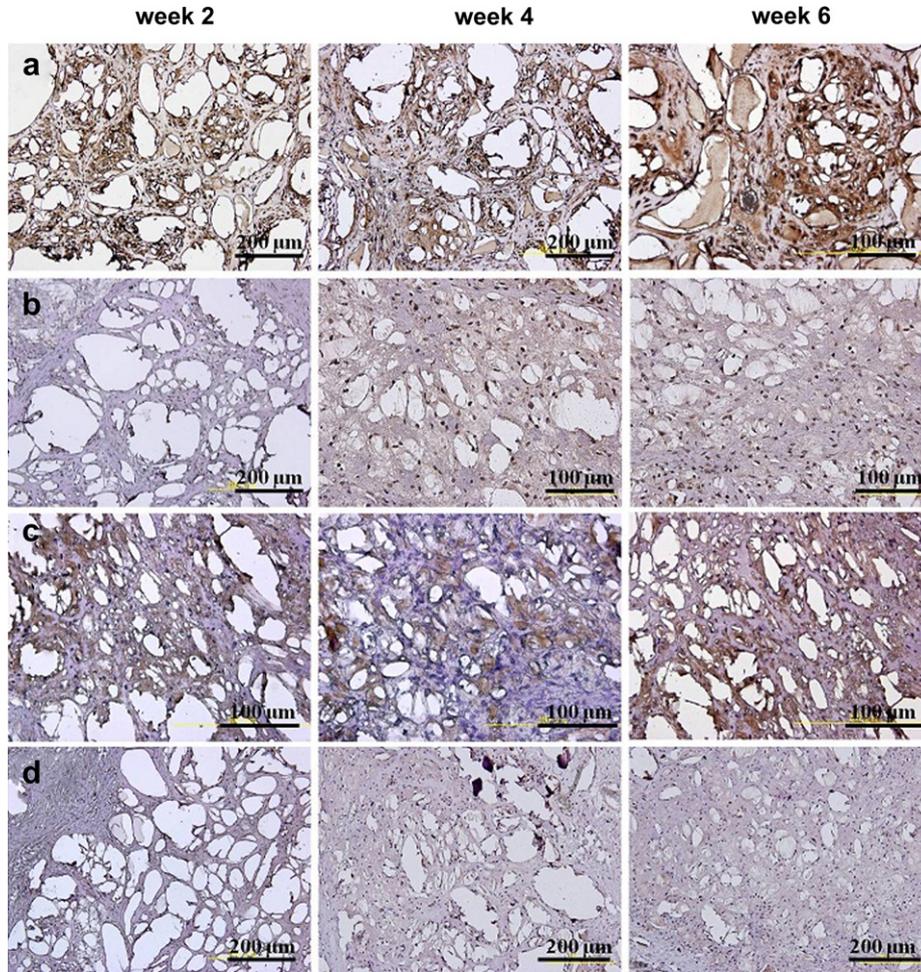


Fig. 5. IHC staining (brown color represents the positive staining of each targeting protein) for (a) S-100 protein, (b) type I collagen, (c) type II collagen, and (d) type X collagen at week 2, 4, and 6, respectively.

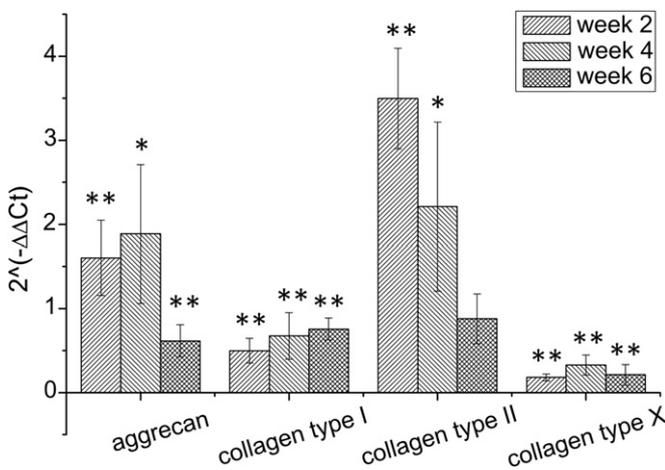


Fig. 6. Real-time PCR showed that the gene expression patterns of engrafted cells/scaffold construct relative to that of cells/scaffold construct cultured *in vitro*. The aggrecan expression was up-regulated at week 2 (1.65 ± 0.44 folds), further increased at week 4 (1.89 ± 0.82 folds) but decreased at week 6 (0.61 ± 0.19 folds). Type I collagen mRNA expression was suppressed, and the mRNA expressions were at similar levels during the study periods. Type II collagen expression was up-regulated to 3.49 ± 0.60 folds at week 2 and 2.21 ± 1.00 folds at week 4, and then the expression decreased to 0.88 ± 0.29 folds at week 6. The expression of type X collagen was similar to that of type I collagen, in which the expression was highly down-regulated.

Previous studies reported that alginate-based scaffold for cartilage tissue engineering induces foreign body giant cell reactions and immunological responses when implanted in experimental animals [19]. Several studies suggested that alginate microspheres implanted in rodents require low-dose immunosuppression [20]. On the contrary, no macrophage proliferation, giant cell infiltration or fibroblast invasion was observed surrounding or within the engrafted scaffold (Fig. 3). Compared with traditional method to prepare a alginate scaffold, the scaffold prepared by microfluidic device is constructed with fewer raw materials. The relative fewer material shall attribute to the mild immunological responses. Furthermore, alginate is composed of 1,4'-linked β -D-mannuronic acid and α -L-guluronic acid that is negatively charged, which may impede the diffusion of negatively charged nutrition and metabolized waste into and out of the alginate-based scaffold [21,22]. Therefore, the lesser amount of material used in the scaffold structure may decrease the effect of the negative charge from alginate molecules.

The chondrogenesis of the engineered cartilage is influenced by many factors. One important factor is the thickness of the scaffold. A thickness greater than 2 mm would impede the chondrogenesis, especially in the center zone [23]. The thickness of the scaffold as a limitation for cartilage regeneration is similar to the characteristics of articular cartilage. Without blood vessels circulating into the cartilage, nutrition and metabolism are dependent on diffusion with joint fluid [24]. In this study, less necrosis or liquefaction of the

engineered cartilage was noted in the center, which was more than 2 mm from the surface of the scaffold. The high porosity, thin wall and good interconnection between pores of our scaffold contributed to the good permeation of nutrition and metabolized waste.

The detection of chondrocyte-specific type II collagen and S-100 protein in the engrafted implants indicated that the porcine cells were not replaced by mouse cells (Fig. 5). The chondrocytes maintained their round appearance without deformation/elongation to the fibroblastic phenotype. In addition, the cells/scaffold was negative for type I and type X collagen staining. The expression of type I collagen for chondrocytes may represent the trans-differentiation to fibrocartilage. Collagen type X is expressed when chondrocytes become hypertrophy. The hypertrophic chondrocytes also gradually differentiate toward osteogenesis [25]. A previous study found that calcium ion used for alginate gelation during scaffold preparation have the tendency to induce chondrocyte hypertrophic change and transform to osteogenesis [26], and that the cell differentiation to hypertrophic chondrocytes is irreversible, even with further culturing in a 3D type II collagen sponge [27]. Extracellular calcium regulates the matrix synthesis of chondrocytes, and the high calcium level induces chondrocytes to increase the expression of osteonectin, osteopontin, osteocalcin, and collagen type X [25,26]. In order to prevent chondrocyte dedifferentiate to hypertrophic chondrocyte, Chua et al. suggested that insulin-transferrin-selenium may be added as a supplement to cell culture. Although this supplement may be a considerable regimen *in vitro*, this addition may not be practicable *in vivo* [28]. On the contrary, a low calcium level decreases collagen type I expression whereas collagen type II expression remains stable [29]. Another advantage of our alginate scaffold prepared using the microfluidic is that it device requires relatively fewer calcium ions. This finding reveals less calcium in the scaffold has a lesser effect on chondrocytes changing their phenotype to osteoblasts.

The materials used for cartilage tissue engineering influence the gene expression pattern of chondrocytes dramatically [30,31]. In the present study, type II collagen mRNA level was up-regulated at weeks 2 and 4, and then gradually decreased in week 6. Aggrecan expression was highest in week 4. In combination with the findings of histologic examinations, these results reveal that the chondrocytes have secreted enough type II collagen in the first two weeks to form a 3D structure constructed by collagen fibrils, which provides the ECM architecture for proteoglycan and chondrocytes to fill. This finding resembled that of a study reported by Tilo et al., in which human articular chondrocytes showed up-regulated type II collagen at week 3 but the expression decreased thereafter [32]. Several studies have reported a slightly decrease in aggrecan for cells cultured in a 3D scaffold [33], but chondrocytes expressed up-regulation of aggrecan in this study. Aggrecan, the predominant proteoglycan, links to the collagen fibrils via the connection of hyaluronic acid. It is the functional component of cartilage and its polyanionic character attracts water, which allows the cartilage to swell. Pressure on the articular cartilage causes aggrecan to release water and form a surface of minimal friction. The release of pressure allows joint fluid to be draw back into the cartilage and supply the chondrocytes nutrients. In week 4, the chondrocyte/scaffold construct with a great deal of aggrecan secretion provided more elastic strength similar to the environment for articular cartilage.

Type II collagen and aggrecan mRNA expression were decreased greatly in week 6. The reason may be that no mechanical force existed in the back of the mice. As found in a previous study, mechanical forces across the cartilage stimulate chondrocytes to synthesize proteoglycan and collagen type II [34]. Prolonged inactivity, such as that of a bedridden patient, leads to cartilage degeneration through a reduction in ECM synthesis by the chondrocytes. Moreover, long-term culture stimulates the expression of type X collagen [35]. In this study, collagen type I and X mRNA

expression were nearly undetectable during the experimental periods. This means the chondrocytes can keep the phenotype without transforming to fibroblast, fibrocartilage or osteoblast.

One of the problems encountered in the biomaterial scaffold is that it easily degrades and becomes weak after a period implanted inside the animal [36]. This alginate scaffold has a honeycomb structure which is substantiated with high strength and enduring shear force. An animal study has shown the engrafted scaffold retained the original shape without obvious degradation or deformation when removed from the mice at 6 weeks post-implantation. Alcian blue stain formed complexes with GAGs and substantiated that much ECM was secreted by chondrocytes. GAGs were demonstrated to provide the mechanical strength of the grafts and retained a large matrix space [37]. The retained shape of the scaffold and increased strength may be due to the abundant ECM. In spite of the fact that this animal experiment, in combination with the previous *in vitro* study, revealed this alginate scaffold is a good candidate for cartilage tissue engineering, histologic examinations showed some blood vessels which do not exist in normal cartilage penetrated the scaffold. The chondrogenesis of this scaffold requires more animal study to demonstrate for partial-thickness chondral defect in articular cartilage (like porcine or rabbit knee joint defect transplantation using these scaffolds).

5. Conclusion

This animal experiment showed that chondrocytes seeded into the highly organized 3D alginate scaffold survived normally in SCID mice; cartilage-like structures were formed after 4 weeks implantation. IHC staining revealed cells secreted type II collagen, produced GAGs, and maintained the expression of S-100. On the contrary, cells were negative for type I and type X collagen staining. PCR showed that the mRNA expressions of aggrecan and type II collagen were up-regulated at week two and four, while the type I and type X collagen were down-regulated during the study period. This study reveals that the alginate scaffold can provide suitable environment and maintain functional phenotypes for chondrocytes.

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