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Enrichment of committed human nucleus pulposus cells expressing chondroitin sulfate proteoglycans under alginate encapsulation

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SUMMARY

Objective: Intervertebral disc (IVD) degeneration is associated with a malfunction of the nucleus pulposus (NP). Alginate culturing provides a favorable microenvironment for the phenotypic maintenance of chondrocyte-like NP cells. However, NP cells are recently evidenced to present heterogeneous populations, including progenitors, fibroblastic cells and primitive NP cells. The aim of this study is to profile the phenotypic changes of distinct human NP cells populations and describe the dynamic expression of chondroitin sulfate glycosaminoglycans (CS-GAGs) in extended alginate encapsulation.

Method: Non-degenerated (ND-NPC) and degenerated (D-NPC) NP cells were expanded in monolayers, and subject to 28-day culture in alginate after serial passaging. CS-GAG compositional expression in monolayer-/alginate-cultured NP cells was evaluated by carbohydrate electrophoresis. Cellular phenotypic changes were assessed by immunologic detection and gene expression analysis.

Results: Relative to D-NPC, ND-NPC displayed remarkably higher expression levels of chondroitin-4-sulfate GAGs over the 28-day culture. Compared with monolayer culture, ND-NPC showed increased NP marker expression of *KRT18*, *KRT19*, and *CDH2*, as well as chondrocyte markers *SOX9* and *MIA* in alginate culture. In contrast, expression of fibroblastic marker *COL1A1*, *COL3A1*, and *FN1* were reduced. Interestingly, ND-NPC showed a loss of Tie2+ but gain in KRT19+/CD24+ population during alginate culture. In contrast, D-NPC showed more consistent expression levels of NP surface markers during culture.

Conclusion: We demonstrate for the first time that extended alginate culture selectively enriches the committed NP cells and favors chondroitin-4-sulfate proteoglycan production. These findings suggest its validity as a model to investigate IVD cell function.

Introduction

Cross-linked alginate hydrogel has been widely used as a bioactive scaffold. It provides a favorable three-dimensional microenvironment for chondrocyte culturing to prevent chondrocyte dedifferentiation and promote matrix production[1,2]. In addition, when applied to cartilage engineering, alginate was shown to not only promote the matrix production and chondrocyte proliferation[1,3], but also stimulate tissue formation with histological and biomechanical features resembling native hyaline cartilage[3,4], such as the arrangement of cells and collagen fibers and compressive properties. The intervertebral discs (IVD) are the major fibrocartilaginous joint structures of the spine and its degeneration is associated with low back pain[5]. Mature nucleus pulposus (NP) cells in human IVD are widely regarded as chondrocyte-like cells. Both of NP cells and articular chondrocytes are highly active in matrix proteins production. Therefore there is

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a growing body of literature that applies alginate encapsulation in IVD cell culturing[6–10] and IVD cell engineering based on mesenchymal stem/stromal cells[11]. In contrast to cells from annulus fibrosus (AF)[12], NP cells are reported to be relatively insensitive to alginate encapsulation in terms of matrix gene expression, including aggrecan and collagen II and I, although a comparatively higher aggrecan mRNA expression after re-encapsulation in alginate could be observed[10]. Moreover, the reestablishment of a matrix within pericellular and intercellular compartments in alginate-NP cell culture was demonstrated[9,13,14]. Compared to other three-dimensional scaffolds such as fibrin[8] and chitosan-gelatin[6], alginate was suggested to provide a microenvironment supportive of not only cell proliferation and function (such as matrix production), but also a SOX9-expressing chondrogenic phenotype[9].

As one of the predominant matrix components, proteoglycans, especially those composed of chondroitin sulfate glycosaminoglycans (CS-GAGs), play critical roles in IVD homeostasis. It is reported that the genetic variant of CHST3, an enzyme that catalyze sulfation of proteoglycan, is coupled with IDD[15]. In alginate culture, non-chondrodystrophoid NP cells[16] and non-degenerated human NP cells[17] produce GAG mainly composed of chondroitin sulfate with predominantly 6-O-sulfation (61%). Besides being an important structural component that gives IVD the necessary mechanical functions, CS-GAGs may regulate cell proliferation and migration via binding with growth factors, such as fibroblast growth factors[18]. Therefore, understanding the differential expression pattern of CS-GAG in non-degenerated and degenerated human NP cells may not be only relevant to NP cell function but also can provide insights into growth factor signaling.

Although there is extensive applied research implicating the capacity of alginate in favoring proteoglycan production in NP cells, these studies have not adequately examined the phenomenon in detail, and it has not been clearly addressed how the phenotype of NP cells is modulated in alginate, especially under long-term culturing. To date however, the rationale behind the phenotype maintenance of alginate in culturing NP cells was based upon the sustained expression of *SOX9*[8]. Accumulating evidence has suggested a heterogeneous NP cell population in adult human IVDs[19–22], possibly a result of cells being at different differentiation states. The heterogeneous cell populations include the primitive NP cells (also regarded as notochordal NP cells)[21–23] and local disc progenitors[19,20] with various capacities in proteoglycan production. Therefore, the overall NP cell behavior or activity is likely to be contributed to by the response of each of the subpopulations to the microenvironment or stimuli. A better understanding of the response of human NP cells in alginate is therefore a prerequisite for accurate interpretation of data derived from relevant *in vitro* assays. This is also of paramount importance to the design of future *in vitro* studies of therapeutics or molecular agents that aim to intervene IVD degeneration via the targeting of human NP cells.

We here investigated the capacity of human NP cells to produce proteoglycan and their phenotypes with respect to the various subpopulations in alginate. We explored the expression of proteoglycans and their GAG composition in human NP cells derived from non-degenerated (ND-NPC) and degenerated (D-NPC) IVD, under extended alginate encapsulation. With this system, we also conducted a phenotype study based on the gene expression profile that marks the various subpopulations of the NP. Our findings indicate that three-dimensional culturing in alginate promotes proteoglycan production in ND- and D-NPC with differential expression patterns of chondroitin sulfates, possibly via selection of a committed NP phenotype over progenitor cell phenotype.

Materials and methods

Patient samples

Under the approval of Institutional Review Board with patient consent, degenerated lumbar disc tissues were collected from symptomatic patients undergoing disc excision and spinal fusion surgery at the Queen Mary Hospital of Hong Kong and First Affiliated Hospital of Guangzhou. Non-degenerated lumbar disc samples were collected from adolescent scoliosis patients undergoing deformity correction surgery (Table I). The NP was retrieved and processed within 1 h after the surgery. Cells from each individual IVD tissue were regarded as an independent biological sample.

Isolation and culture of human disc cells

Unless otherwise stated, all reagents were supplied by Sigma–Aldrich (US). Cells were extracted from the NP tissue by sequential enzyme digestion[24]. Briefly, after 1–2 h digestion in 0.25% (w/v) pronase (Roche Diagnostics, US), NP tissues were further subjected to 600 U/mL collagenase II (Worthington Biochemical Co, US) digestion at 37°C. Tissue debris was removed using a cell strainer (70 μm, BD Falcon, US), and cells were washed with sterile PBS twice. The primary cells were expanded in high glucose DMEM, supplemented with 10% fetal calf serum (FCS, Biosera, FR), 4.5 mg/ml L-glutamine, 1% Penicillin/Streptomycin, 0.4% Fungizone and 1 ug/ml gentamicin, with media refreshed every 2 days at 37 °C at 5% CO_2. NP cells at passage 3 were subjected to encapsulation in 1.2% alginate hydrogel and cultured in a seeding density of 2×10^6 cells/mL (approximately 4×10^4 cells in each bead) as previously described[10]. Alginate beads were cultured in complete DMEM with additional 50 ug/mL ascorbic acid for up to 28 days, and collected for further tests. The composite was subjected to LIVE/DEAD® cell viability assays (Life Technologies, HK) to test the biocompatibility of alginate with the NP cells after 14 days of culture.

Measurement of GAG production

Glycosaminoglycan (GAG) content was quantified by the 1,9-dimethylmethylene blue dye (DMMB)-binding assay modified for alginate culture system. Briefly, 100 μL products from papain digestion were incubated with 1 mL DMMB reagent solution (21 mg DMMB in 1 L dH_2O containing 1.72 g sodium hydroxide and 0.5% ethanol, pH adjusted to 1.5 by formic acid) for 30 min at room

Table I
Demographic data. A total of 13 subjects were recruited with an age between 13 and 71 (average = 36.4). Grade of disc degeneration was determined by MRI according to Schneiderman's Scale. F, female; M, male; IS, idiopathic scoliosis; DDD, degenerative disc disease; ND, not determined

Sample	Sex	Donor's age (years)	Level	Disease	Grade
1	F	13	L2-3	IS	ND
2	F	13	L2-3	IS	ND
3	M	15	L2-3	IS	ND
4	F	15	L2-3	IS	ND
5	F	16	L1-2	IS	ND
6	M	28	L5-S1	DDD	IV
7	M	40	L3-L4	DDD	III
8	F	42	L4-L5	DDD	IV
9	F	47	L3-L4	DDD	V
10	F	56	L4-L5	DDD	III
11	M	58	L3-L4	DDD	V
12	M	59	L4-L5	DDD	IV
13	M	71	L4-L5	DDD	IV

temperature. The DMMB-GAG complex precipitates were dissolved in dissociation buffer (38.1 g Guanidine hydrochloride, 0.68 g sodium acetate trihydrate in 100 mL dH_2O containing 10% propan-1-ol). Absorbance readings at a wavelength of 650 nm were obtained with a spectrophotometer (Varioskan Flash, Thermo Scientific). The DNA content of the samples was measured using Hoechst 33258 method for data normalization.

Analysis of GAG composition

Composition of GAGs was investigated by fluorophore-assisted carbohydrate electrophoresis (FACE)[25]. Briefly, samples in dissociation solution were subjected to ethanol precipitation, and pellets were dried in a vacuum concentrator (SC 210A SpeedVac Plus, Sigma, US). Following the digestion with chondroitinase ABC (100 mU/mL, from *Proteus vulgaris*, Seikagaku, JP), samples were lyophilized prior to derivatization. Digested products were conferred to fluorescent tagging by incubating with 2-aminoacridone (dAMAC) at 37°C overnight in the dark. Samples were fractionated in 15% polyacrylamide gels and visualized under illumination of UV light. Gel images were captured by ChemiDoc MP imager (Bio-Red, US) and analyzed using the gel densitometry function of Image J (Version 1.46r, National Institutes of Health, US).

Histology and immunostaining

The NP cell-alginate beads were harvested after 7, 14 and 28 days of culture in the medium and processed for paraffin sections as previously described[24]. Briefly, the beads were fixed in 4% paraformaldehyde, 0.1 M cacodylate buffer, pH 7.4, containing 10 mM $CaCl_2$ overnight at 4°C, and then washed in cacodylate buffer with 50 mM $BaCl_2$. Following routine dehydration procedures, beads were embedded in paraffin and sectioned at 5 μm thickness. Cell distribution and morphology were examined by Gill's hematoxylin and eosin staining (H&E). For immunohistochemistry, sections were incubated for 50 min at 37°C with 0.8% hyaluronidase for antigen retrieval. Non-specific background was blocked with 2% BSA for 30 min, followed by incubation overnight at 4°C with polyclonal rabbit antibody against collagen II or aggrecan with dilution of 1:20 and 1:50 respectively (Abcam, US). Incubation with alkaline phosphatase-conjugated goat anti-rabbit antibody (1:200; Abcam, US) was accomplished at room temperature for 45 min followed by color development in liquid permanent red (DAKO). The sections were counterstained by Mayer's Hematoxylin and then mounted with aqueous mounting agent (Merck Millipore) for light microscopy.

In immunofluorescence staining, primary antibody of monoclonal goat anti-KRT19 (1:50, Santa Cruz Biotechnology, US), mouse anti-CD24 (1:100, Abcam, US) or mouse anti-Tie2 (1:100, Santa Cruz Biotechnology, US) in 2% BSA was implicated to detect KRT19+, CD24+ or Tie2+ cells, respectively. Sections were then incubated with Alexa Fluor 488-conjugated goat anti-rabbit or donkey anti-mouse (Life Technologies, HK) in 1:100. Slides were mounted using Vectashield mounting medium with DAPI (Vector Labs, US) and visualized at 488 nm for antibody signal and 360 nm for DAPI signal using the Nikon Eclipse 80i imaging system.

Gene expression analysis

Total RNA was isolated from the monolayer culture or the alginate beads at 7, 14 and 28 days using TRIzol reagent (Invitrogen) and PureLink RNA Mini Kit (Invitrogen). Reverse transcription was performed with a PrimeScript RT Master Mix (TaKaRa) according to the manufacturer's instructions. Standard TaqMan gene expression assays were performed with StepOnePlus Real-Time PCR System (Applied Biosystems). Genes investigated included *ACAN* (Hs00153936_m1), *CD24* (Hs03044178_g1), *CDH2* (Hs00983056_m1), *COL1A1* (Hs00164004_m1), *COL3A1* (Hs00943809_m1), *FN1* (Hs01549976_m1), *HSP47* (Hs00241844_m1), *KRT18* (Hs01941416_g1), *KRT19* (Hs00761767_s1), *MIA* (Hs00197954_m1), *SOX9* (Hs00165814_m1), and *TEK* (Hs00945146_m1). *GAPDH* (Hs03929097_g1) was used as the endogenous control. Relative expression levels were determined by the comparative ΔΔCT method.

Statistical evaluation

For all investigations, assuming data in Gaussian distribution, *P* values were calculated via unpaired *t* test by GraphPad Prism version 5.0 (GraphPad Software Inc.). A P value <0.05 was considered statistically significant.

Results

Viability, morphology and ECM deposition of NP cells in alginate

Both human ND- and D-NPC showed a high survival rate in alginate by 14 days of culture [Fig. 1(A)]. ND-NPC exhibited relatively higher cell proliferation rate in alginate culture (Supplementary Fig. S1). Both ND- and D-NPC attained a round morphology in the alginate [Fig. 1(B)]. Formation of lacunae, as indicated by the transparent pericellular space [arrow in Fig. 1(B)], was observed with a higher frequency in the center area. To depict the cell function in matrix production, we examined the expression of aggrecan and collagen II, the two main ECM components in IVD, and showed that both were expressed in the inter-territorial and territorial matrix of ND-NPC culture [Fig. 2(A) and (B)], whereas the expression was more confined to territorial matrix in D-NPC culture. Consistent with the *in vitro* findings, such differential expression patterns were also observed in the IVD tissue samples *in situ*, where their expressions were largely restricted to the pericellular and territorial matrix domain in the degenerated NP samples when compared to non-degenerated samples [Fig. 2(C)]. By examining the overall GAG production, we found an increasing trend of proteoglycan deposition during the extended culture, and ND-NPC showed a significantly higher rate than D-NPC over 28 days of culturing [Fig. 2(D)].

Pattern of chondroitin sulfate expression

To examine the composition of the deposited GAGs, the internal disaccharides from chondroitin sulfate proteoglycans were evaluated by carbohydrate electrophoresis (Fig. 3). Quantitative assessment for different disaccharides species was achieved by using a calibration curve (Supplementary Fig. S2). ND-NPC showed a dynamic expression pattern of chondroitin sulfate disaccharides, where a high level of ΔDi4S (band e) was observed with identification of ΔDi0S (band a) by 14 days and ΔDi2S (band f) by 28 days of culture [Fig. 3(A)]. Also demonstrated were another two bands (band c & d) representing unknown substrates. In contrast, grade III D-NPC expressed a low level of chondroitin sulfate predominantly composed of ΔDi4S [band e, Fig. 3(A) and (B)]. A minute quantity of ΔDi0S (8%, band a, Fig. S3) was detectable when 10-fold higher amount of sample was loaded. Quantitative analysis indicated a trend of increase in ΔDi4S over the 28 days of culture [Fig. 3(C)]. Grade IV D-NPC exhibited an even lower level of disaccharides expression.

Effect of extended alginate culturing on NP cell phenotype

Human NP cell culture is thought to be heterogeneous. We postulated that the dynamic CS-GAG expression patterns are attributed to varied response of the NP cell subpopulations to

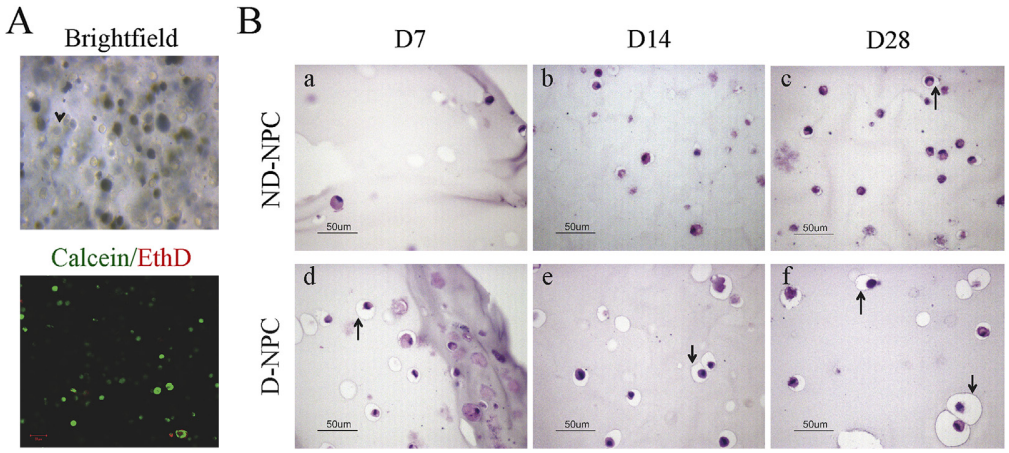

Fig. 1. Viability and morphology of NP cells cultured in alginate. (A) Assessment of biocompatibility of alginate with NP cells cultured for 28 days by LIVE/DEAD assays. Calcein AM (green) and ethidium dimer (EthD) (red) reflects the viable and non-viable NP cells, respectively. (B) Morphological assessment of non-degenerated (ND-NPC, a, b and c) and degenerated NP cells (D-NPC) in alginate cultured for 7 (D7), 14 (D14) and 28 days (D28) by H&E staining. Lacunae formation surrounding the cells was shown by arrow.

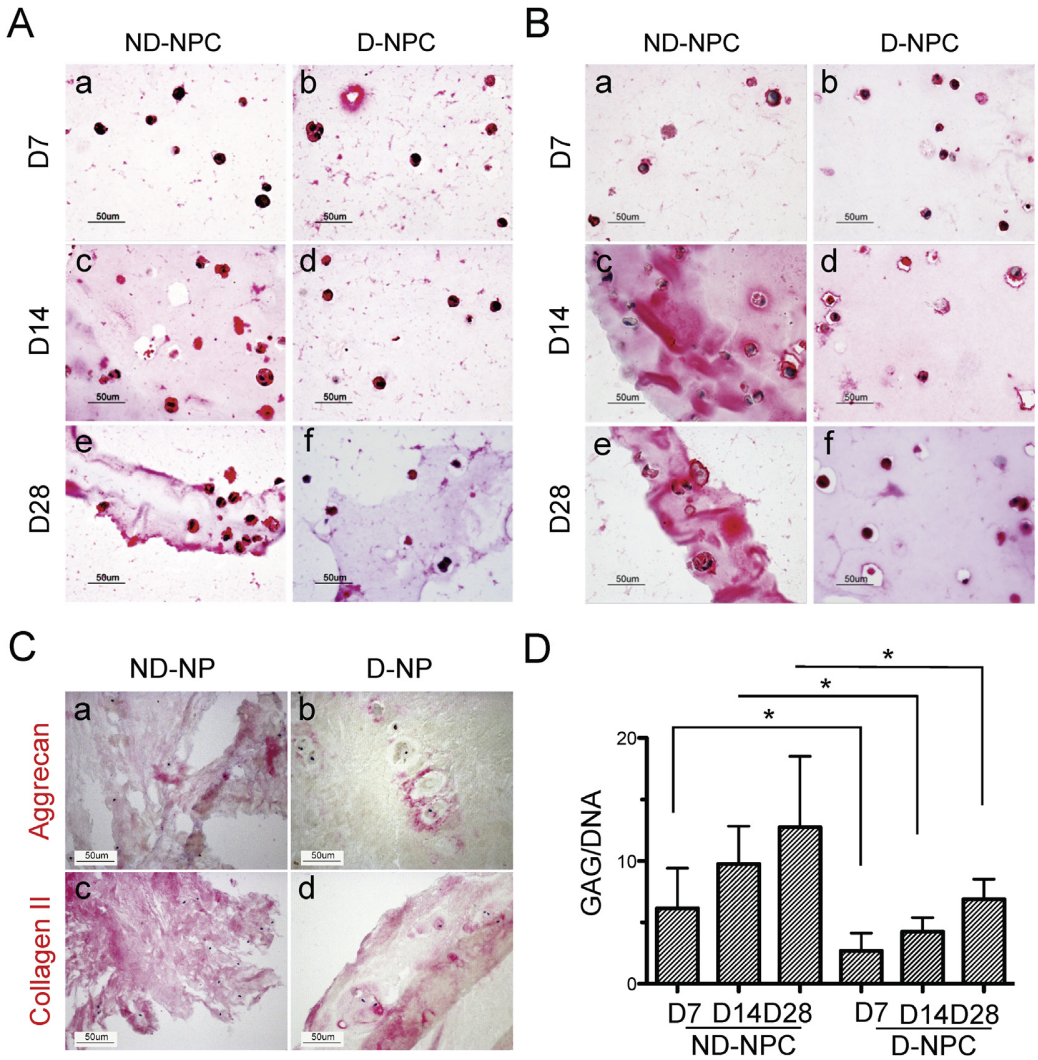

Fig. 2. Extracellular matrix deposition of NP cells in alginate and tissue. Aggrecan (A) and collagen II (B) accumulation was examined by immunohistochemistry in the alginate encapsulated NP cells cultured for 7 (D7), 14 (D14) and 28 days (D28). (C) Immunodetection of aggrecan and collagen II in NP samples from non-degenerated (ND-NP) and degenerated (D-NP) human discs. (D) Quantification of glycosaminoglycan (GAG) in alginate containing ND-/D-NPC cultivated for up to 28 days (D28). DNA content was used for normalization. Graph represents mean ± 95% CI of three pooled independent experiments with $n = 5$ discs; $^{*}P \leq 0.05$; two tailed-unpaired t test.

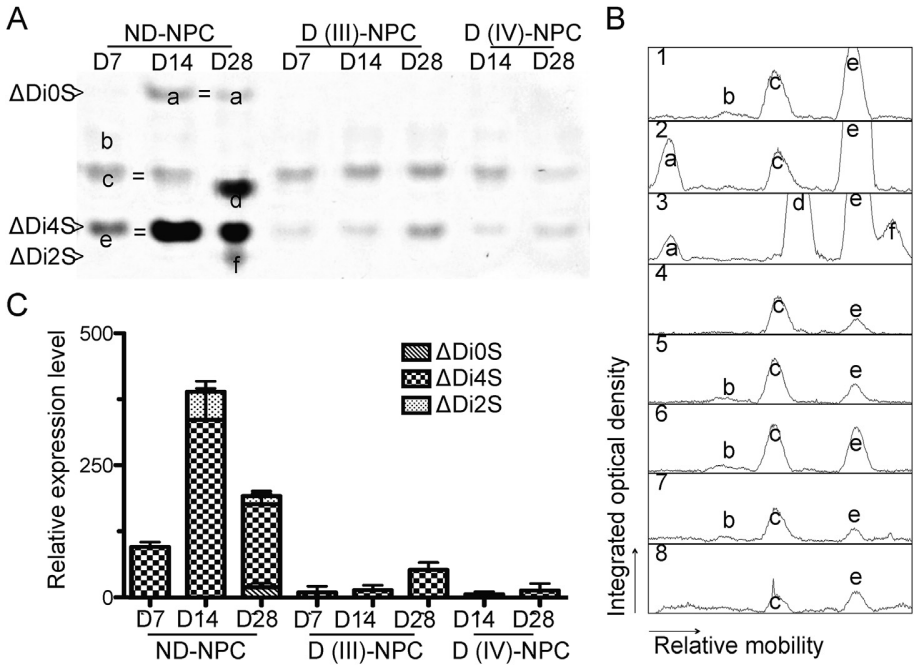

Fig. 3. Chondroitin sulfate expression pattern by NP cells in alginate. (A) Comparison of FACE profiles in samples with NP cells isolated from non-degenerated (ND-NPC) or degenerated discs with grading III (D (III)-NPC) or IV (D (IV)-NPC) and cultured in alginate for up to 28 days. Representative photograph from three independent experiments is shown. (B) (1–8) represent line plots of initial integrated optical density vs relative mobility from ND-NPC (D7, D14 and D28), D (III)-NPC (D7, D14 and D28) and D (IV)-NPC (D14 and D28) of the image in (A), respectively. Peak designations correspond to the bands in (A). (C) Quantification of disaccharides compositions via measuring the intensity of the bands in the image in (A). All values were normalized to the level of D7 in ND-NPC and represented by the percent change.

alginate. Hence the expression changes of the representative markers relevant to the subpopulations, including primitive NP cells, disc progenitors and mesenchyme-derived chondrocytes and fibroblastic cells were evaluated. Both chondrocytes and IVD cells were reported to undergo dedifferentiation in monolayer expansion, showing decreased expression of matrix genes[26]. To minimize dedifferentiation of the NP cells, we conducted the monolayer culture at a high cell density, and monitored the gene expression profile at passage 1–3 (P1–3) to assess any phenotype shift. Cells at P3 were further cultured in alginate to investigate the effect of alginate encapsulation on cell phenotype.

Expression of *KRT18* (keratin 18), *KRT19* (keratin 19) and *CDH2* (cadherin 2/N-cadherin) has been suggested as candidate primitive NP cell markers at the transcriptional and protein levels[22]. These genes showed reduced expression in ND-NPC when passaged from P1–P3 in monolayer culture but the expression was enhanced after encapsulation in alginate (relative to P3 monolayer culture) [Fig. 4(A)]. In contrast, D-NPC showed an increase of *CDH2* expression after serial passaging, and diminished expression after long-term culture in alginate [Fig. 4(B)]. *KRT19* expression remained unchanged in D-NPC. The tyrosine kinase receptor Tie2 was defined as one of the crucial markers for disc progenitor cells that decreased markedly with age and degeneration of the IVD[20]. When cultured in alginate, ND-NPC showed lower expression of *TEK2* (encoding Tie2) and the expression continued to decrease during extended culture [Fig. 4(A)]. CD24 has been reported as an NP-specific marker in rats[23], and suggested to be expressed in committed NP cells[20,23]. While *CD24* expression was reduced when transferred from monolayer to alginate culture, the expression was gradually recovered during the extended culture in alginate [Fig. 4(A)]. Similarly, D-NPC showed a trend of recovery of *CD24* expression under extended alginate encapsulation [Fig. 4(B)].

A combination of *SOX9* (sex determining region Y box 9), *MIA* (CD-RAP, melanoma inhibitory activity protein), and ACAN (aggrecan) may identify a chondrocyte phenotype[10,11,23]. A significant increase of *SOX9* (ND-NPC) and *MIA* (ND-DPC and D-NPC) expression was detected under extended alginate culture compared to monolayer culture [Fig. 5(A) and (B)]. No significant differential expression of *ACAN* was found in alginate compared to monolayer culture. The expression of fibroblastic markers *FN1* (fibronectin), *COL1A1* (collagen I, alpha 1), *COL3A1* (collagen III, alpha 1), and *HSP47* (heat shock protein 47) were up-regulated in both ND- and D-NPC under extended monolayer culturing [Fig. 6(A) and (B)]. However, their expression were down-regulated (*COL1A1* and *COL3A1*) or relatively maintained (*FN1* and *HSP47*) when both cells were under extended alginate culture. Altogether, these findings suggest that alginate encapsulation may selectively promote primitive NP cell and chondrocyte-like cell activities rather than disc progenitor or mesenchyme/fibroblast activities, and that ND- and D-NPC exhibit distinct responses to alginate.

Immunodetection of KRT19, Tie2 and CD24 expressing cells in alginate beads

To gain insight into the relative ratio of the committed, primitive NP cells and the disc progenitor cells in alginate culture, we examined the expression of their corresponding markers in the alginate beads [Fig. 7(A)] and the positive expressing cells were counted [Fig. 7(B)]. Data showed that the ND-NPC samples have a lower frequency of KRT19+ cells [Fig. 7(B, a)] and CD24+ cells [Fig. 7(B, b)] than D-NPC samples at early alginate culture (7 days). Consistent with the findings of gene expression, we found that the number of KRT19+ and CD24+ cells increased significantly with increasing time of culture. In contrast, ND-NPC showed a decrease in the number of Tie2+ cells during culture, whereas D-NPC maintained a relatively low level of Tie2+ cells [Fig. 7(B, c)].

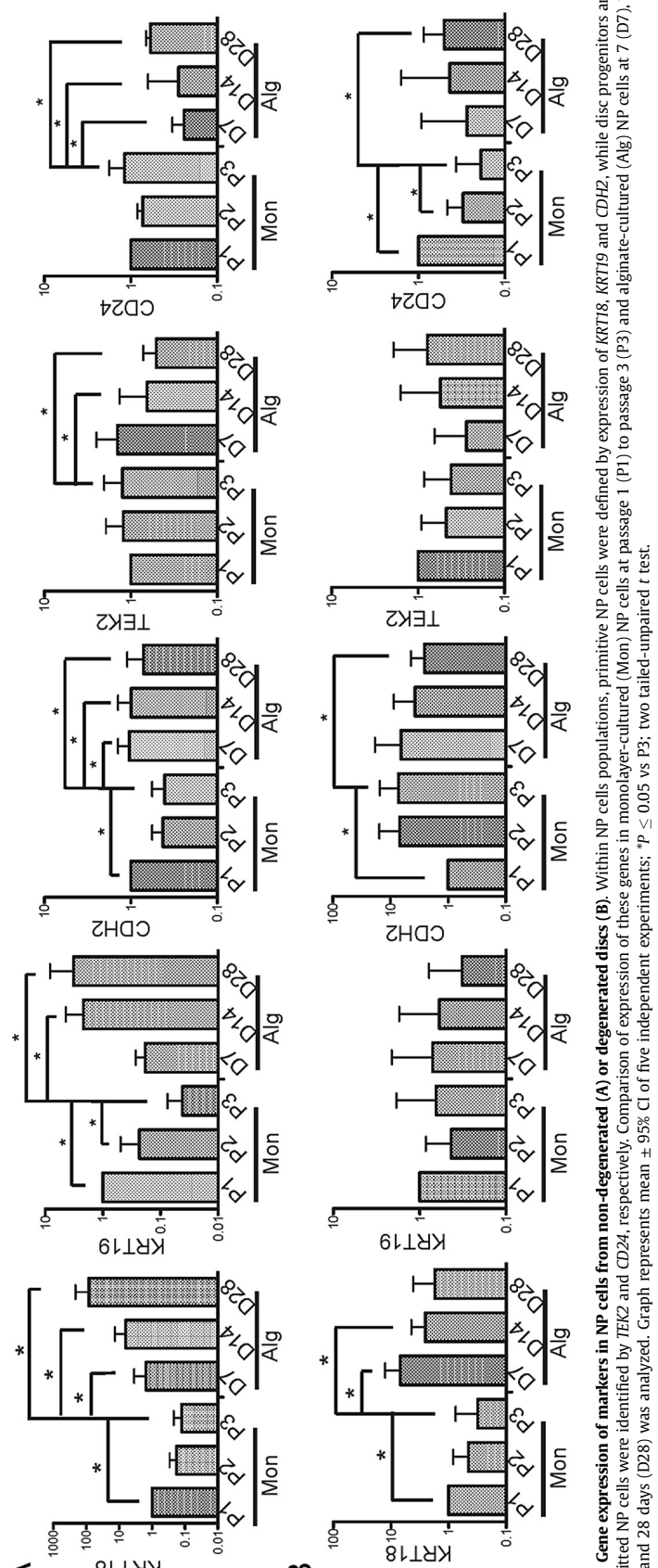

Fig. 4. Gene expression of markers in NP cells from non-degenerated (A) or degenerated discs (B). Within NP cells populations, primitive NP cells were defined by expression of *KRT18*, *KRT19* and *CDH2*, while disc progenitors and committed NP cells were identified by *TEK2* and *CD24*, respectively. Comparison of expression of these genes in monolayer-cultured (Mon) NP cells at passage 1 (P1) to passage 3 (P3) and alginate-cultured (Alg) NP cells at 7 (D7), 14 (D14) and 28 days (D28) was analyzed. Graph represents mean ± 95% CI of five independent experiments; $^{*}P \leq 0.05$ vs P3; two tailed-unpaired *t* test.

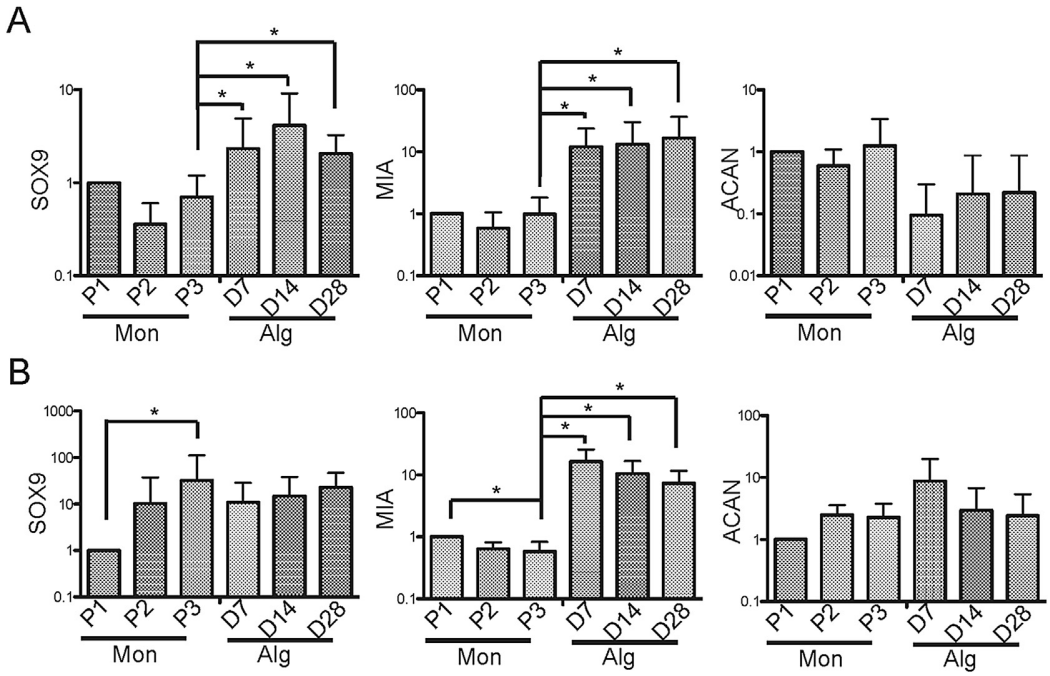

Fig. 5. Gene expression of chondrocytic markers *SOX9*, *MIA* and *ACAN* in non-degenerated (A) and degenerated (B) NP cells. Differential expression was analyzed in monolayers culture (Mon) for three passages (P1–3) or after subsequent culture in alginate (Alg) for 7 (D7), 14 (D14) and 28 days (D28). Graph represents mean ± 95% CI of five independent experiments; $^{*}P \leq 0.05$, vs P3; two tailed-unpaired *t* test.

Discussion

This study investigated the modulation of function and phenotype of NP cells in extended alginate encapsulation. Our data showed that alginate encapsulation could facilitate the phenotypic recovery and drive the production and accumulation of proteoglycans of characteristic chondroitin sulfate pattern in a time-dependent manner. Moreover, extended encapsulation could enrich NP cells that have primitive as well as mature chondrocyte-like phenotype. Direct comparison of ND- and D-NPC in chondroitin sulfate expression pattern and phenotypic changes was also described. Our findings provide fundamental basis to support the

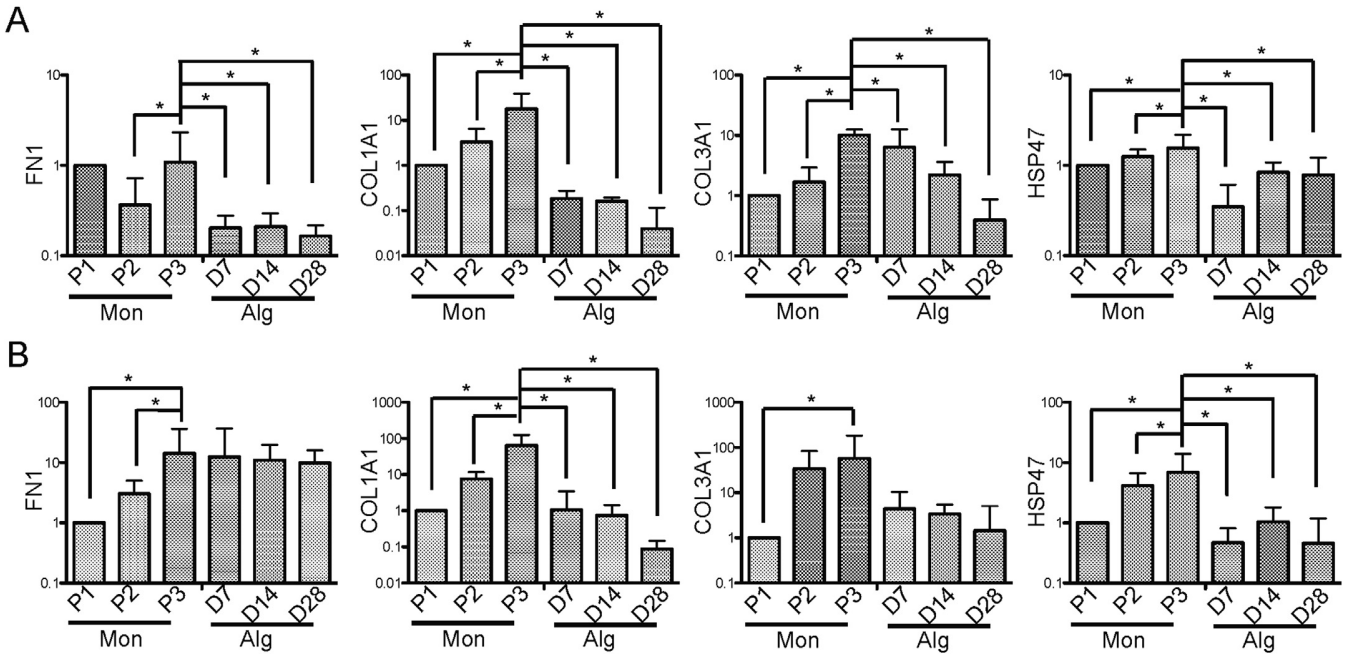

Fig. 6. Gene expression of markers for fibroblastic cells. Monolayer cultured (Mon) non-degenerated (A) and degenerated (B) NP cells from passage 1–3 or alginate-cultured cells (Alg) at passage 3 for 7 (D7), 14 (D14) and 28 days (D28) were studied for the expression of fibroblastic marker genes (*FN1*, *COL1A1*, *COL3A1* and *HSP47*). Graph represents mean ± 95% CI of five independent experiments; $^{*}P \leq 0.05$, vs P3; two tailed-unpaired *t* test.

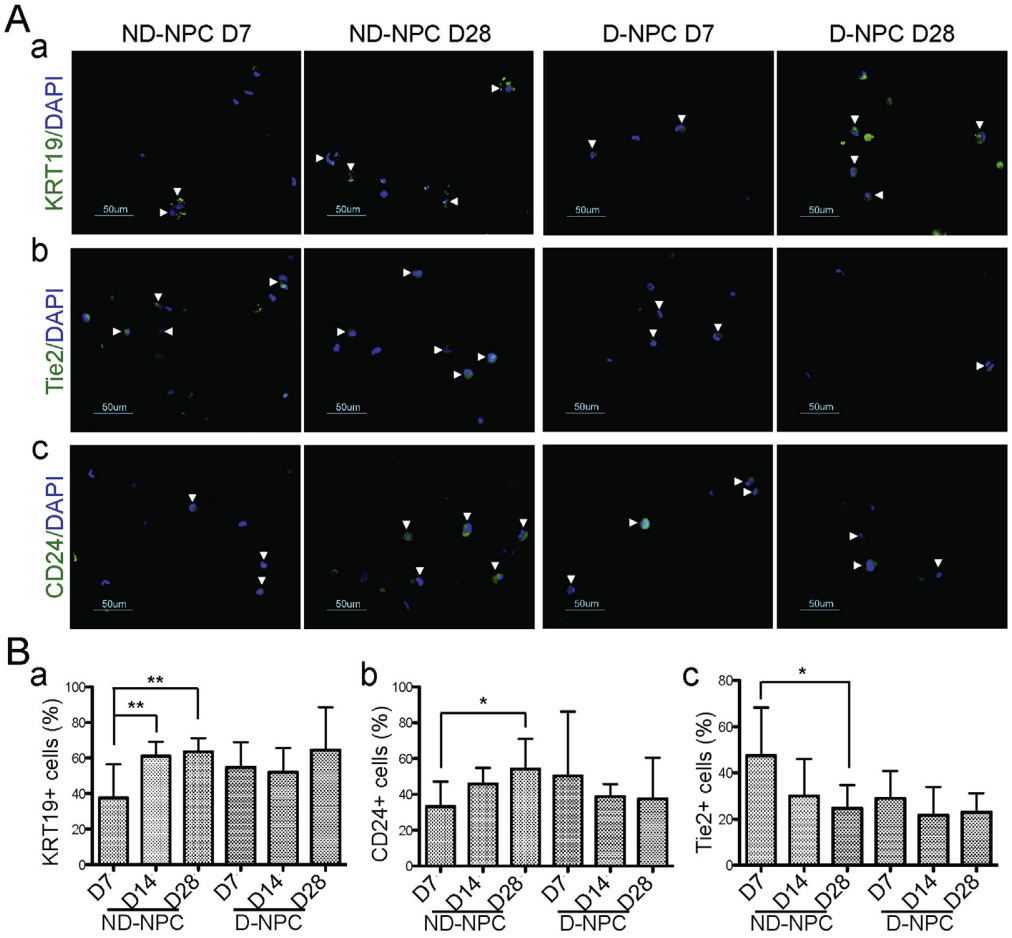

Fig. 7. Immunolocalization of KRT19, Tie2 and CD24 expressing cells in alginate beads. (A) Immunodetection of KRT19 (a), Tie2 (b) and CD24 (c) in non-degenerated (ND-NPC) and degenerated (D-NPC) NP cells cultured in alginate beads for 7 (D7) and 28 days (D28). Cells with positive signals (green) were indicated by arrowhead. Cell nuclei were represented by DAPI staining (blue). (B). Frequency of KRT19+ (a), CD24+ (b) and Tie2+ (c) cells was determined from the immunostaining data shown in Panel A. ND-NPC samples showed increased number of cells positive for KRT19 and CD24 but decreased number for Tie2 over 28 days of culture. Data are represented as mean ± 95% CI of six independent experiments; $^{*}P \leq 0.05$ vs P3; unpaired *t* test.

use of alginate as an *in vitro* cell culturing model to investigate the long-term response of NP cells to stimuli, such as cytokines and mechanical loading, and further contribute to the NP cell engineering.

The formation of lacunae around the D-NPC in alginate beads is consistent with that observed in previous reports[14]. Previously, Melrose *et al.* showed that cells in the AF and transition zone (TZ) rather than NP of ovine IVD could form lacunae[14]. This implicates that AF and TZ cells might share some similarities with degenerated human NP cells in lacunae formation. Moreover, we note that D-NPC with lacunae tended to be located in the central but not the peripheral area of the alginate beads, implying that the lacunae formation may be associated with oxygen tension or nutrient diffusion.

Analogous to the expression pattern in human IVD tissues, the D-NPC accumulated less aggrecan and collagen II in the matrix compared to the ND-NPC in alginate culture. Moreover, they also have significantly lower rate of CS-GAG production. It has been previously reported that the proportion of 6-*O*-sulfate to 4-*O*-sulfate is 61 to 31 in canine NP cells[16]. However, our data suggest that ΔDi4S, but not ΔDi6S, is the predominant disaccharide in the matrix-associated CS-GAGs in human NP cells, and that its decrease is associated with the severity of disc degeneration. This may be due to different NP cell compositions between bovine and human, such as the relative abundance of primitive NP cells and chondrocyte-like cells. Alternatively, it may be related to the differences in culture duration. Indeed, our data showed that increasing time of culture can result in the generation of ΔDi0S and ΔDi2S [Fig. 3(A)], indicating dynamic CS-GAG metabolism in NP cells. Interestingly, three unknown chondroitin moieties (bands b–d) are identified with the mobility between ΔDi0S and ΔDi6S. Calabro *et al.* also detected disaccharides of similar mobility in aggrecan isolated from human cartilage, and described them as the non-reducing terminal saccharides[25]. Future characterization of these undetermined sugar moieties would require AMAC-derivatized hexosamines as references. Moreover, while this study aims to understand the GAG production by alginate-embedded human NP cells, a direct comparison of the expression of various proteoglycans, such as aggrecan, as well as the composition of intracellular and secreted GAG between monolayer and alginate culture may yield additional insights in future.

In an attempt to define the possible composition of cell subpopulations in the NP samples, we used different molecular markers to examine specific phenotypes, including disc progenitors, committed NP cells such as primitive and mature chondrocyte-like NP cells, as well as fibroblastic cells. The master chondrogenic transcription factor *SOX9* and matrix genes *ACAN* and *COL2A1* have been widely used to characterize the chondrocytic NP phenotype[10,11,23]. While NP cells in alginate culture are proposed to have a chondrocyte-like phenotype, we have noted a trend of lower

expression of *ACAN* mRNA for ND-NPC in alginate compared to monolayer culture. Previous study has indicated that NP cells under alginate re-encapsulation for 14 days did not show obvious enhancement in matrix gene expression, including aggrecan and collagen I/II[10], although aggrecan accumulation and proteoglycan synthesis could be detected[12]. In particular, primary NP cells have been suggested to exhibit lower aggrecan gene expression in alginate than in monolayer culture[10] or other three-dimensional scaffolds[6,8]. In addition, mature NP cells encapsulated in alginate were shown to have a relatively low rate of proteoglycan synthesis than AF cells[16]. Interestingly, it has been previously reported that co-culturing notochord cells with NP cells in alginate could result in lower aggrecan expression[4]. It is not clear if notochord-like NP cells were present in the ND-NPC and regulated the overall aggrecan expression. After all, our findings reflect that proteoglycan production or accumulation does not necessarily correlate with aggrecan expression at mRNA level and may rely on multi-level regulatory control.

Although it has been commonly assumed that primitive NP cells/ notochordal cells are virtually lost after adolescence in humans, recent evidence of their existence have been reported based on the expression of notochord markers keratin 8, 18 and 19[21,22]. Our staining for KRT19 supports the existence of notochordal NP cells in human NP samples. The ability of alginate to maintain both primitive NP cells and chondrocytic cells may encourage its use for culturing of NP cells derived from various species. Moreover, the expression of disc progenitor marker *TEK2* and committed NP cell marker *CD24* in ND-NPC appears insensitive to serial passaging in monolayer culture but becomes responsive when transferred to alginate culture. Consistent with these gene expression findings, we found that alginate culturing can promote the enrichment of committed NP cells in ND-NPC, as demonstrated by the increased frequency of CD24+ cells and decreased frequency of Tie2+ cells. In contrast, a low level of the Tie2+ cells in D-NPC was present, and they could not be effectively enriched in alginate. Our gene expression and immunostaining data therefore suggest that alginate encapsulation selectively promotes the activity of committed NP cells rather than disc progenitors or other cells such as fibroblasts. Further study is required to consolidate if the subpopulations were indeed differentially modulated. This can be achieved by isolating various subpopulations, such as Tie2+ and CD24+ cells, and investigating their specific response to alginate encapsulation, including cell proliferation and differentiation.

The increasing trend of *CD24* expression with time of culture in alginate appears to contradict the previous finding, which showed a decrease of *CD24* expression in alginate-cultured NP cells from rats[23]. We postulate this might be related to the difference in oxygen tension used in the culture (atmospheric vs 2% O_2). Above all, our study proposes the use of extended alginate encapsulation to preserve the native cell composition in the primary human NP cells, particularly the D-NPC, which is advantageous for investigating their *in vitro* responses to stimuli, such as cytokines and mechanical loads. Fibrosis has been suggested as a phenomenon of disc degeneration[27], implicating that resident fibroblasts may be present in the disc and activated in the degeneration process. Our findings showed that both ND- and D-NPC exhibit similar responses upon alginate encapsulation in terms of suppressed expression of fibroblastic markers. This implies that alginate culture may inhibit the activity of fibroblastic population or reverse cell de-differentiation arising from the serial monolayer culture.

Reports have shown that combined agarose and aligned electrospun poly (epsilon-caprolactone) with mesenchymal stem cells can establish a disc-like structure[28]. On the other hand, transplantation of the alginate-based disc construct can provide desirable function in small animals[29–31]. This indicates alginate may not only function in cell culturing but also support tissue engineering for total disc replacement. How alginate or alginate-based scaffolds may favor stem cell differentiation into committed NP cells to facilitate disc engineering may worth further investigations.

Conclusions

To our knowledge, this is the first study to examine the chondroitin sulfate proteoglycan expression and molecular phenotype of human NP cells in a long-term alginate culture model. Our data suggest that extended alginate encapsulation can promote CS-GAG production, possibly via selective enrichment of the committed NP cells, such as the primitive and chondrocyte-like NP cells, rather than disc progenitors. This study supports the application of alginate encapsulation to investigate the regulation of proteoglycan metabolism and other essential functions in NP cells under long-term perturbation by external agents or stimuli.

Author contributions

YS: Conception and design, data collection and analysis, drafting of article.
ML: Data collection.
LZ: Data collection.
VT: Critical revision of article.
FL: Critical revision of article.
DC: Critical revision of article.
HW: Data collection.
ZZ: Critical revision of article.
KMC: Conception and design, and final approval of manuscript, obtaining funding.
VYL: Conception and design, data analysis, drafting of article, final approval of manuscript, obtaining funding.

Conflict of interest statement

None of the authors had financial or personal relationships with any people or organizations that could inappropriately influence the bias of the presented work.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2015.02.166.

References

1. Homicz MR, Chia SH, Schumacher BL, Masuda K, Thonar EJ, Sah RL, *et al*. Human septal chondrocyte redifferentiation in alginate, polyglycolic acid scaffold, and monolayer culture. Laryngoscope 2003;113(1):25–32.
2. Guo JF, Jourdian GW, MacCallum DK. Culture and growth characteristics of chondrocytes encapsulated in alginate beads. Connect Tissue Res 1989;19(2-4):277–97.
3. Klein TJ, Schumacher BL, Schmidt TA, Li KW, Voegtline MS, Masuda K, *et al*. Tissue engineering of stratified articular

cartilage from chondrocyte subpopulations. Osteoarthritis Cartilage 2003;11(8):595–602.
4. Wang CC, Yang KC, Lin KH, Liu HC, Lin FH. A highly organized three-dimensional alginate scaffold for cartilage tissue engineering prepared by microfluidic technology. Biomaterials 2011;32(29):7118–26.
5. Samartzis D, Karppinen J, Mok F, Fong DY, Luk KD, Cheung KM. A population-based study of juvenile disc degeneration and its association with overweight and obesity, low back pain, and diminished functional status. J Bone Joint Surg Am 2011;93(7):662–70.
6. Renani HB, Ghorbani M, Beni BH, Karimi Z, Mirhosseini M, Zarkesh H, *et al*. Determination and comparison of specifics of nucleus pulposus cells of human intervertebral disc in alginate and chitosan-gelatin scaffolds. Adv Biomed Res 2012;1:81.
7. Thonar E, An H, Masuda K. Compartmentalization of the matrix formed by nucleus pulposus and annulus fibrosus cells in alginate gel. Biochem Soc Trans 2002;30(Pt 6):874–8.
8. Yang SH, Wu CC, Shih TT, Chen PQ, Lin FH. Three-dimensional culture of human nucleus pulposus cells in fibrin clot: comparisons on cellular proliferation and matrix synthesis with cells in alginate. Artif Organs 2008;32(1):70–3.
9. Melrose J, Smith S, Ghosh P, Taylor TK. Differential expression of proteoglycan epitopes and growth characteristics of intervertebral disc cells grown in alginate bead culture. Cells Tissues Organs 2001;168(3):137–46.
10. Wang JY, Baer AE, Kraus VB, Setton LA. Intervertebral disc cells exhibit differences in gene expression in alginate and monolayer culture. Spine 2001;26(16):1747–51. discussion 1752.
11. Risbud MV, Albert TJ, Guttapalli A, Vresilovic EJ, Hillibrand AS, Vaccaro AR, *et al*. Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype in vitro: implications for cell-based transplantation therapy. Spine 2004;29(23):2627–32.
12. Gruber HE, Stasky AA, Hanley Jr EN. Characterization and phenotypic stability of human disc cells in vitro. Matrix Biol: journal of the International Society for Matrix Biology 1997;16(5):285–8.
13. Chiba K, Andersson GB, Masuda K, Thonar EJ. Metabolism of the extracellular matrix formed by intervertebral disc cells cultured in alginate. Spine 1997;22(24):2885–93.
14. Melrose J, Smith S, Ghosh P. Differential expression of proteoglycan epitopes by ovine intervertebral disc cells. J Anat 2000;197(Pt 2):189–98.
15. Song YQ, Karasugi T, Cheung KM, Chiba K, Ho DW, Miyake A, *et al*. Lumbar disc degeneration is linked to a carbohydrate sulfotransferase 3 variant. J Clin Invest 2013;123(11):4909–17.
16. Maldonado BA, Oegema Jr TR. Initial characterization of the metabolism of intervertebral disc cells encapsulated in microspheres. J Orthop Res: official publication of the Orthopaedic Research Society 1992;10(5):677–90.
17. Chelberg MK, Banks GM, Geiger DF, Oegema Jr TR. Identification of heterogeneous cell populations in normal human intervertebral disc. J Anat 1995;186(Pt 1):43–53.
18. Deepa SS, Umehara Y, Higashiyama S, Itoh N, Sugahara K. Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors. Implications as a physiological binding partner in the brain and other tissues. J Biol Chem 2002;277(46):43707–16.
19. Huang S, Leung VY, Long D, Chan D, Lu WW, Cheung KM, *et al*. Coupling of small leucine-rich proteoglycans to hypoxic survival of a progenitor cell-like subpopulation in Rhesus Macaque intervertebral disc. Biomaterials 2013;34(28):6548–58.
20. Sakai D, Nakamura Y, Nakai T, Mishima T, Kato S, Grad S, *et al*. Exhaustion of nucleus pulposus progenitor cells with ageing and degeneration of the intervertebral disc. Nat Commun 2012;3:1264.
21. Shapiro IM, Risbud MV. Transcriptional profiling of the nucleus pulposus: say yes to notochord. Arthritis Res Ther 2010;12(3):117.
22. Minogue BM, Richardson SM, Zeef LA, Freemont AJ, Hoyland JA. Transcriptional profiling of bovine intervertebral disc cells: implications for identification of normal and degenerate human intervertebral disc cell phenotypes. Arthritis Res Ther 2010;12(1):R22.
23. Rastogi A, Thakore P, Leung A, Benavides M, Machado M, Morschauser MA, *et al*. Environmental regulation of notochordal gene expression in nucleus pulposus cells. J Cell Physiol 2009;220(3):698–705.
24. Bertolo A, Ettinger L, Aebli N, Haschtmann D, Baur M, Berlemann U, *et al*. The in vitro effects of dexamethasone, insulin and triiodothyronine on degenerative human intervertebral disc cells under normoxic and hypoxic conditions. Eur Cells Mater 2011;21:221–9.
25. Calabro A, Hascall VC, Midura RJ. Adaptation of FACE methodology for microanalysis of total hyaluronan and chondroitin sulfate composition from cartilage. Glycobiology 2000;10(3):283–93.
26. Steck E, Bertram H, Abel R, Chen B, Winter A, Richter W. Induction of intervertebral disc-like cells from adult mesenchymal stem cells. Stem Cells 2005;23(3):403–11.
27. Leung VY, Aladin DM, Lv F, Tam V, Sun Y, Lau RY, *et al*. Mesenchymal stem cells reduce intervertebral disc fibrosis and facilitate repair. Stem Cells 2014;32(8):2164–77.
28. Nerurkar NL, Sen S, Huang AH, Elliott DM, Mauck RL. Engineered disc-like angle-ply structures for intervertebral disc replacement. Spine (Phila Pa 1976) 2010;35(8):867–73.
29. Bowles RD, Gebhard HH, Hartl R, Bonassar LJ. Tissue-engineered intervertebral discs produce new matrix, maintain disc height, and restore biomechanical function to the rodent spine. Proc Natl Acad Sci USA 2011;108(32):13106–11.
30. Gebhard H, Bowles R, Dyke J, Saleh T, Doty S, Bonassar L, *et al*. Total disc replacement using a tissue-engineered intervertebral disc in vivo: new animal model and initial results. Evid Based Spine Care J 2010;1(2):62–6.
31. Martin JT, Milby AH, Chiaro JA, Kim DH, Hebela NM, Smith LJ, *et al*. Translation of an engineered nanofibrous disc-like angle-ply structure for intervertebral disc replacement in a small animal model. Acta Biomater 2014;10(6):2473–81.