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ENRICHMENT OF COMMITTED, CHONDROITIN SULFATE-EXPRESSING HUMAN NUCLEUS PULPOSUS CELLS OVER PROGENITORS UNDER ALGINATE ENCAPSULATION

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INTRODUCTION: Accumulating evidence has suggested a heterogeneous NP cell population in adult human IVDs [1, 2], 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) [1, 2], mature NP cells, and local disc progenitors [1] with various capacities in proteoglycan production other than chondrocytic NP cells. Cross-linked alginate hydrogel provides a favorable three-dimensional microenvironment for promotion of chondrogenic phenotype [3] with enhanced biosynthesis of proteoglycans (PGs) and collagen II. It has been widely used as a bioactive scaffold for engineering of nucleus pulposus (NP) tissue or maintaining NP cells in vitro [4]. However, it is still not clear how the phenotype of NP cells in terms of the various subpopulations is modulated in alginate, especially under long-term culturing. Proteoglycans, in particular those composed of chondroitin sulfate glycosaminoglycans (CS-GAG), play critical roles in IVD homeostasis and function. In this study, we aimed to first investigate the expression of proteoglycans and their glycosaminoglycan composition in alginate-cultured human NP cells derived from non-degenerated and degenerated IVD, and explored their phenotypic changes in detail based on the gene expression profile that marks the various subpopulations of the NP.

METHODS: NP tissue from patients with scoliosis (age=15±2, n=3) and degenerative disc disease (age=49±5, n=5) were obtained under the approval of IRB. Cells were isolated by sequential enzyme digestion, and monolayers expanded until alginate encapsulation at passage 3. GAGs in alginate-cultured NP cells were extracted and subjected to chondroitinase digestion. Compositional analysis of CS in digests was accomplished by fluorophore-assisted carbohydrate electrophoresis (FACE). Expression of marker genes for chondrocytic and fibroblastic cells, and disc NP cells were evaluated by RT-PCR. Expression of Tie 2, CD24 and KRT19 for NP cells in alginate culture were investigated by immunofluorescence, and positivity was further calculated based on the number of expressing cells.

RESULTS:

1. Expression pattern of CS-GAG

To examine the composition of the deposited chondroitin sulfate (CS), the internal disaccharides were evaluated by carbohydrate electrophoresis (Fig. 1). It is shown of a dynamic expression pattern of CS disaccharides in ND-NPC, where a high level of ?Di4S (band e) was observed with identification of ?Di0S (band a) by 14 days and ?Di2S (band f) by 28 days (Fig. 1A). Also demonstrated were another two bands (band c & d) representing unknown substrates. In contrast, grade III D-NPC expressed a low level of ?Di4S (band e, Fig. 1A&B). A minute quantity of ?Di0S was detectable when 10-fold higher amount of sample was loaded (data not shown).

Quantitative analysis indicated a trend of increase in α -Di4S over the 28 days of culture (Fig. 1C). Grade IV D-NPC exhibited an even lower level of disaccharides expression.

2. Phenotypic changes of disc NP cells in alginate

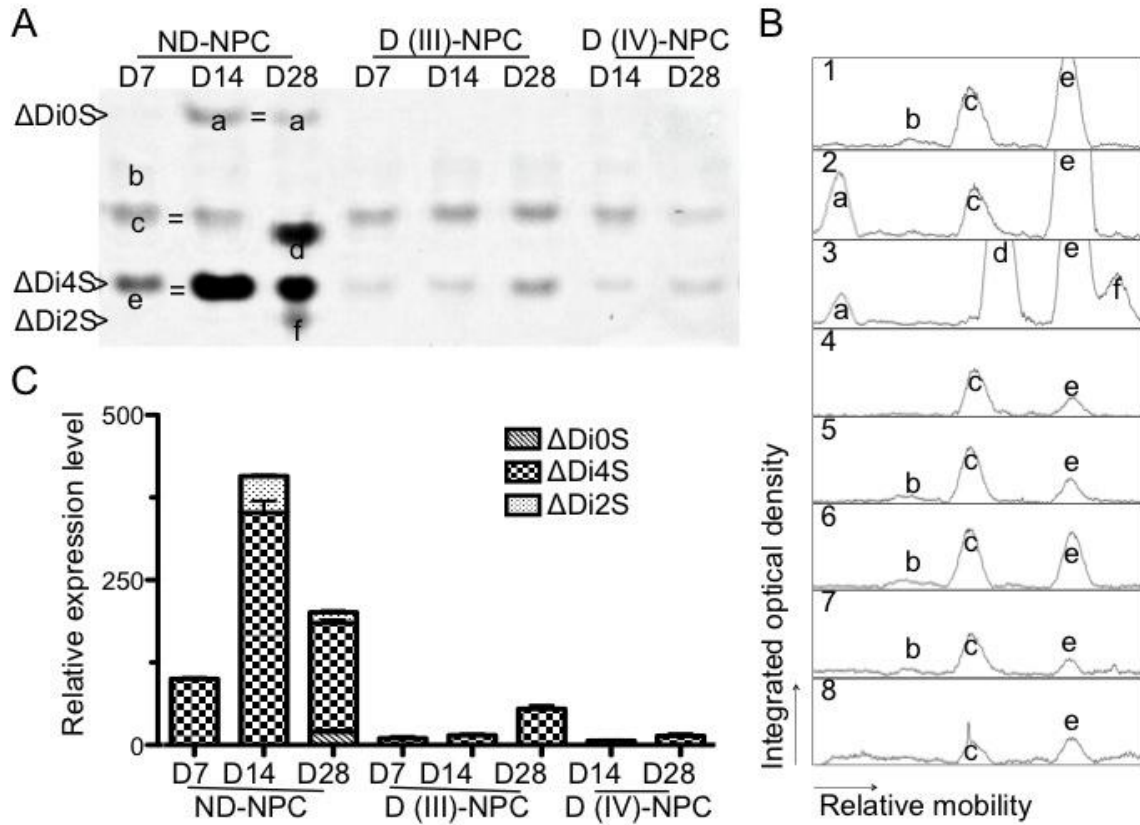
Human NP cells are thought to be heterogeneous. Expression of KRT18 (keratin 18), KRT19 (keratin 19) and CDH2 (cadherin 2/N-cadherin) has been suggested as candidate primitive NP cell markers. These genes showed reduced expression in ND-NPC in monolayers passaging but the expression was enhanced (Fig. 2A) after encapsulation in alginate. In contrast, D-NPC showed an increase of CDH2 expression in monolayers culture, and a diminished expression after long-term culture in alginate (Fig. 2B). KRT19 expression remained unchanged. The tyrosine kinase receptor Tie2 were defined as one of the crucial markers for disc progenitors that decreased markedly with age and degeneration of the IVD [1]. When cultured in alginate, ND-NPC showed lower expression of TEK2 (encoding Tie2) and the expression continued to decrease during extended culture (Fig. 2A). CD24, as a NP-specific marker in committed NP cells, showed a reduced expression when transferred from monolayer to alginate culture, and gradual recover during the extended culture in alginate (Fig. 2A). Immunodetection of KRT19+ cells (Fig. 2C and D, a) and CD24+ cells (Fig. 2C and D, c) presented a lower frequency in ND-NPC than D-NPC samples at early alginate culture (7 day). 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, Tie2 expressing cells were reduced during culture for ND-NPC, and maintained at a relatively low level in D-NPC (Fig. 2C and D, b).

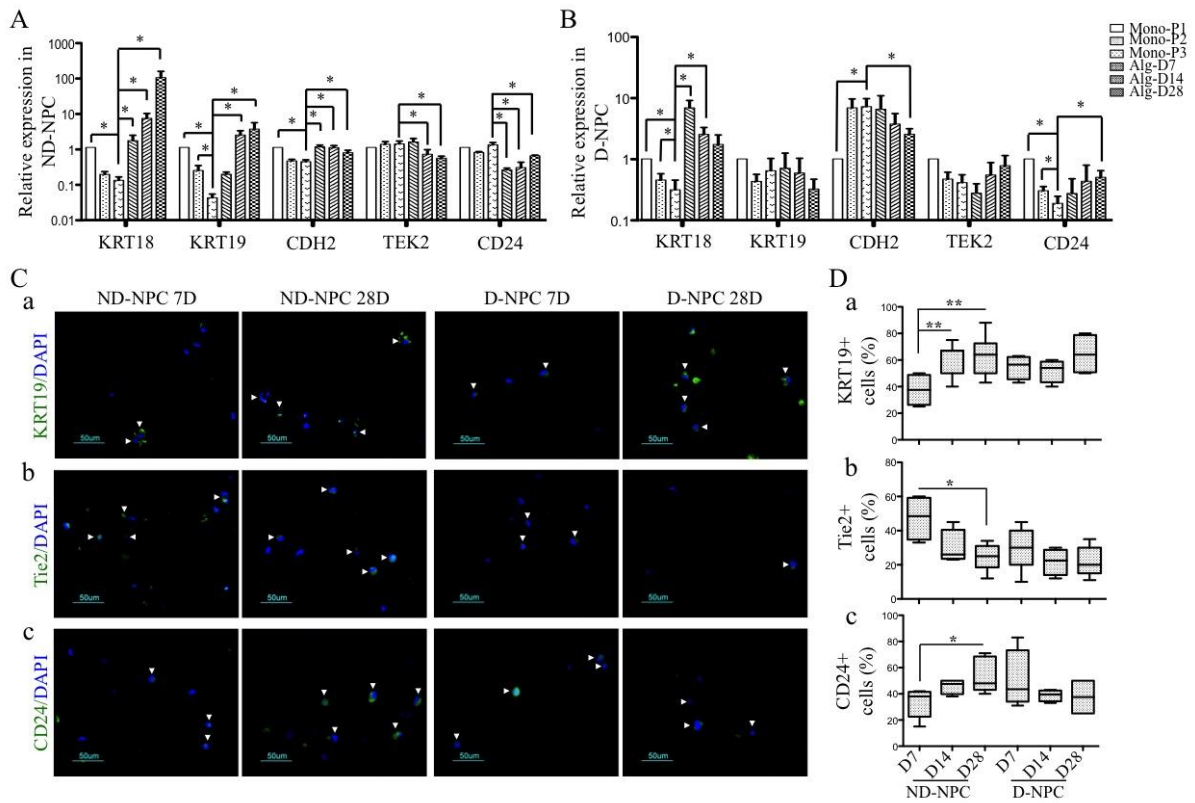
DISCUSSION: This is the first study to examine the chondroitin sulfate expression and molecular phenotype of human NP cells in terms of the sub-populations in a long-term alginate culture model. Although vacuolated notochordal cells were purported virtually lost after adolescence in humans, our staining for keratin 19 supports the existence of notochordal NP cells in human degenerative and non-degenerative IVD. The extended alginate encapsulation is evidenced to be able to enrich these primitive NP cells as well as mature chondrocyte-like cells, rather than disc progenitors or fibroblastic cells. This proposes the use of extended alginate encapsulation to preserve the native cell composition in the culture of primary human NP cells and tissue engineering for NP. Notwithstanding the reports of 6-O-sulfate occupying the GAG composite over 4-O-sulfate in tissue, our data suggest that α -Di4S, but not α -Di6S, is the predominant disaccharide synthesized in human NP cells, and that its decrease is associated with the severity of disc degeneration. Unknown disaccharide species (band b-d) identified by FACE is supposed to be generated from GAG reducing terminals or dermatan sulfate, which need more evidences.

SIGNIFICANCE: Our findings propose an impact of alginate on the activity of specific NP cell sub-populations, providing insights into NP engineering that utilizes alginate as the scaffold. This study is also of paramount importance to the design of future in vitro studies into therapeutics or molecular agents that aim to intervene IVD degeneration via the targeting of human NP cells.

Figure 1. Expression pattern of CS by NP cells in alginate culture. (A) Comparison of disaccharides expressions in ND-NPC and D-NPC with disc degeneration grade of III (D (III)-NPC) and IV (D (IV)-NPC). (B) (1-8) represent line plots of initial integrated optical density versus relative mobility from the image in Figure 1A. (C) Quantification of disaccharides composition. All values were normalized to the level of D7 in ND-NPC and represented by the percent change.

Figure 2. Phenotypic changes of NP cells in alginate culture. Gene expression of markers for disc NP cells was evaluated in ND- (A) and D- (B) NPC. (C) Immunolocalization of keratin 19, Tie2 and CD24 expressing cells in alginate beads. Cells with positive signals (green) were indicated by arrowhead. Frequency of their expressions was further calculated (D). Data are represented as mean \pm S.E.M of four to six independent experiments; * $p < 0.05$, ** $p < 0.01$ versus P3; unpaired t test.





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