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Variations in gene and protein expression in human nucleus pulposus in comparison with annulus fibrosus and cartilage cells: potential associations with aging and degeneration

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Summary

Objective: Regardless of recent progress in the elucidation of intervertebral disc (IVD) degeneration, the basic molecular characteristics that define a healthy human IVD are largely unknown. Although work in different animal species revealed distinct molecules that might be used as characteristic markers for IVD or specifically nucleus pulposus (NP) cells, the validity of these markers for characterization of human IVD cells remains unknown.

Design: Eleven potential marker molecules were characterized with respect to their occurrence in human IVD cells. Gene expression levels of NP were compared with annulus fibrosus (AF) and articular cartilage (AC) cells, and potential correlations with aging were assessed.

Results: Higher mRNA levels of cytokeratin-19 (KRT19) and of neural cell adhesion molecule-1 were noted in NP compared to AF and AC cells. Compared to NP cytokeratin-18 expression was lower in AC, and alpha-2-macroglobulin and desmocollin-2 lower in AF. Cartilage oligomeric matrix protein (COMP) and glypican-3 expression was higher in AF, while COMP, matrix gla protein (MGP) and pleiotrophin expression was higher in AC cells. Furthermore, an age-related decrease in KRT19 and increase in MGP expression were observed in NP cells. The age-dependent expression pattern of KRT19 was confirmed by immunohistochemistry, showing the most prominent KRT19 immunoreaction in the notochordal-like cells in juvenile NP, whereas MGP immunoreactivity was not restricted to NP cells and was found in all age groups.

Conclusions: The gene expression of KRT19 has the potential to characterize human NP cells, whereas MGP cannot serve as a characteristic marker. KRT19 protein expression was only detected in NP cells of donors younger than 54 years.

Key words: Intervertebral disc cells, Phenotype expression, Nucleus pulposus, Annulus fibrosus, Articular cartilage, Human, Aging.

Introduction

Degeneration of the intervertebral disc (IVD) and related spinal disorders are leading causes of morbidity, resulting in substantial pain, disability and increased health care costs[1]. The IVD comprises the highly hydrated nucleus pulposus (NP), the surrounding multilaminar annulus fibrosus (AF) and the cartilaginous endplates. Pathophysiological evidence indicates that IVD degeneration starts in the NP, where the concentration of proteoglycans and the synthesis of type II collagen decrease[2,3]. At the same time denaturation of type II collagen fibers and synthesis of type I collagen occurs[3]. As a consequence the NP loses its osmotic properties and becomes fibrotic; the disc loses its ability to transmit intervertebral forces and further degenerative processes may occur.

Regeneration of NP tissue in the early stages of degeneration may slow down or even reverse the degenerative processes and might possibly restore part of the degenerated disc. Thus, regenerative medicine and biological therapies hold great promise. In particular the therapeutic implications of stem cells have been highly anticipated by both the clinical and scientific communities[4,5]. The challenge in characterizing cellular degeneration and ultimately accomplishing cellular regeneration begins with the identification of the molecular phenotype of the cells that constitute the NP. The NP includes small cells commonly referred to as "chondrocyte-like", since they have a similar rounded morphology and synthesize similar extracellular matrix macromolecules as articular chondrocytes. Currently, no reliable markers exist to distinguish NP cells from the chondrocytes from hyaline cartilage. A cell population with the properties of articular cartilage (AC) would fail to restore the necessary function of the IVD because the requisite fluid properties unique to the IVD would not be recreated. While the ratio of proteoglycan to collagen shows a certain potential to separate disc cells, recent research has focused on the clarification of their molecular phenotype[6]. In a recent study, rat NP cells were compared with cells from the AF and AC tissues by means of large scale microarray gene expression screening. Subsequent quantitative gene expression and immunohistochemical analyses identified distinct molecules, namely glypican-3 (GPC3) and cytokeratin-19

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(KRT19), as promising candidates for NP cell characterization[7]. Similar studies in the beagle dog revealed additional potential NP marker molecules, including alpha-2-macroglobulin (A2M), cytokeratin-18 (KRT18), desmocollin-2 (DSC2), and neural cell adhesion molecule-1 (NCAM1)[8].

However, considerable developmental, anatomical, and biochemical differences among species are likely to affect the phenotypical characteristics of the disc cells[9]. In particular the presence of notochordal cells, which are regarded to be remnants of the embryonic notochord, in the NP is the cause of substantial inter-species variation. Mice, rats, rabbits, and non-chondrodystrophoid dogs retain a predominantly notochordal NP until adulthood and often throughout life, whereas bovine, ovine and chondrodystrophoid dogs closer resemble humans in that the number of notochordal cells rapidly decreases after birth[10]. Moreover, differences in tissue size, oxygen and nutrient supply, and biomechanical requirements are also likely to affect the molecular features of the cells in the disc. As a consequence, observations from animal discs will not necessarily apply to human discs. Nevertheless, animal studies are indispensable for screening purposes, since they allow investigation of normal healthy tissues with larger sample size and smaller inter-individual variability. Due to the limited availability of healthy and viable human IVD tissue, comprehensive screening is difficult in human individuals. The aim of this study therefore was to evaluate the presence and distribution of the molecules that were found to be differently expressed in disc and cartilage cell populations in various animal species in human disc cells. To account for potential variations related to aging and degeneration, cell and tissue samples from individuals of different age groups and disc degeneration grades were examined.

Materials and methods

ISOLATION OF NP, AF, AND AC CELLS

The study was approved by the medical ethical committee of the University Medical Center (UMC) Utrecht and the scientific committee from the Department of Pathology of the UMC Utrecht. Eleven patients with no known history of IVD disease were included in the study. Samples were obtained within an average of 17.5 h after death of the patient (range 6.25–23.0 h). The age of the individuals ranged from 22 to 81 years (average 46 ± 20 years; median 43 years), and the average degree of disc degeneration, assessed according to the Thompson score[11], was 2.2 ± 1.0 (median 2) (Table I). IVD tissue was harvested from segments between L1 and L5 and was separated into NP and AF tissue. To exclude any contamination by AF tissue, only the innermost part of the disc was harvested to be assigned to NP tissue, whereas the transition zone, including part of the inner AF, was entirely excluded from analysis. This is of particular importance for aged discs, where it can be difficult to clearly distinguish NP and AF tissues. AC was harvested from the patellae of the same patients. Chondrocytes were extracted from full thickness cartilage which implies mixed populations of superficial, middle and deep zone cells. Gene expression data thus represent an average cellular expression of target mRNA.

Tissue was cut into small pieces and cells were enzymatically isolated using sequential pronase (Roche) and type II collagenase (Worthington Biochemical) digestion with DNase II (Sigma) added to prevent cell clumping. AC and AF were treated with 0.2% pronase/0.004% DNase for 1 h, then with 200 U/mL collagenase/0.004% DNase over night. NP was treated with 0.2% pronase/0.004% DNase for 1 h, then with 100 U/mL collagenase/0.004% DNase for 8 h, stirring at 37°C in humidified atmosphere. After enzymatic isolation cell suspensions were filtered through a 70 μm cell strainer, washed twice with Dulbecco's Modified Eagles Medium, and lysed in TRI Reagent (Molecular Research Center, Cincinnati, OH). Samples were stored at −80°C until RNA isolation.

Table I
Patients included for gene expression analysis of NP, AF, and AC cells. IVD tissue was harvested from discs between L1 and L5 and separated in NP and AF; AC was harvested from the patella joint surface. Cartilage quality was macroscopically assessed and was without detectable changes for patients 1–9. Slight degenerative changes were detected in patient 10, and signs of osteoarthritis in patient 11

Patient number	Age	Gender	Thompson grade
1	22	M	1
2	25	M	1
3	25	M	1
4	32	M	1–2
5	40	M	2
6	43	M	2
7	46	M	2–3
8	56	M	3
9	61	F	3
10	72	M	4
11	81	F	3

RNA EXTRACTION AND REAL TIME RT-PCR

RNA was isolated using a modified TriSpin method[7,12]. Briefly, bromochloro-propane (Sigma) was added to the lysate, phases were separated, and ethanol (Merck) added to the aqueous phase. Total RNA was extracted using the SV Total RNA Isolation System (Promega), which includes an on-column DNAse digestion, and eluted in 100 μl of RNase-free water. TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) were used for cDNA synthesis. PCR was performed with an SDS 7500 real time PCR instrument using TaqMan Gene Expression Master Mix (all from Applied Biosystems) and standard thermal conditions (10 min 95°C for polymerase activation, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s). Primer-probe systems, purchased as Gene Expression Assays, were from Applied Biosystems. Genes that were previously found to be differentially expressed in NP compared to AF and/or AC cells in the rat and/or chondrodystrophoid dog were chosen for analysis (Table II)[7,8,13]. Expression of target genes was normalized to the 18S ribosomal RNA as the endogenous control. Relative mRNA levels were calculated according to the 2^(delta Ct) method and presented as log(2) transformed values[14,15].

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For histological analysis human IVD tissue was obtained as part of a standard postmortem procedure, in which a section of the lumbar and thoracic spine is removed for diagnostic purposes. IVD samples were stored in the UMC Utrecht Biobank of the Department of Pathology. Collection and analysis of the IVDs was approved by the medical ethical committee of the UMC Utrecht and the scientific committee of the Department of Pathology of the UMC Utrecht. Samples were obtained within a mean of 17.7 h after death of the patient, 95% within 24 h after death. Between death and tissue collection the deceased patients were kept at the mortuary at 4°C. From all patients the IVD between the fourth and fifth lumbar vertebra (spinal motion segment L4–L5), including the adjacent endplates, was obtained. The grade of degeneration was scored by three individual observers using the classification of Thompson *et al.*[11]. After individual scoring the values were averaged; outliers, i.e., differences of more than 1 Thompson grade, were re-evaluated by the three observers at a consensus meeting.

The expression and localization of KRT19 and matrix gla protein (MGP) in IVD tissue was evaluated in 41 human individuals aged between 3 and 86 years (average 47 ± 25 years; median 51 years, Table III). Sagittal slices of the motion segments were fixed in formalin, decalcified with Kristensen's solution (50% formic acid and 68 g/L sodium formiate) in a microwave at

Table II
Gene expression assays used for real time PCR (from Applied Biosystems)

Gene	Assay code
A2M	Hs_00163474_m1
CD24	Hs_00273561_s1
COMP	Hs_00164359_m1
DSC2	Hs_00245200_m1
GPC3	Hs_00170471_m1
KRT18	Hs_01920599_gH
KRT19	Hs_00761767_s1
MGP	Hs_00179899_m1
NCAM1	Hs_00169851_m1
PTN	Hs_00383235_m1
VIM	Hs_00185584_m1

Table III
Immunohistochemical results obtained from IVD sections from the UMC Utrecht Biobank of the Dept. of Pathology. Only discs between the fourth and fifth human lumbar vertebra (L4–L5) were assessed. Cells were classified according to their topographical position within the disc tissue

Patient number	Age	Gender	Thompson grade	KRT19	MGP				
				NP	NP	IAF	OAF	EP	AOA
1	3	F	1	++	+++	−	−	−	++
2	3	F	1	+++	N/A	N/A	N/A	N/A	N/A
3	6	F	1	−	+++	++	++	−	N/A
4	14	M	1	+++	+++	++	+++	−	+++
5	14	F	1	++	+++	+++	+++	−	N/A
6	14	F	1	−	−	−	++	−	N/A
7	17	M	2	+++	+++	−	−	−	+++
8	18	M	1	+	−	−	−	N/A	N/A
9	19	F	1	−	+++	++	++	−	+++
10	21	M	1	++	+++	+	++	−	+++
11	22	F	1	+	++	−	N/A	−	N/A
12	25	M	1	−	++	−	−	−	++
13	35	F	2	+++	++	+++	+++	−	+++
14	35	M	N/A	−	+++	++	++	−	+++
15	36	M	2	−	++	+	+	−	N/A
16	38	M	2	−	+	−	−	−	+
17	41	M	2	(+)	+++	+	+++	−	+++
18	44	M	2	−	−	−	−	−	−
19	47	F	3	−	++	−	−	−	−
20	51	F	2	−	(+)	−	+	−	+++
21	51	F	2	(+)	++	−	−	−	+
22	54	F	4	(+)	+++	−	+++	−	N/A
23	57	M	4	−	++	++	+++	−	N/A
24	59	M	4	−	+++	++	N/A	−	N/A
25	60	M	5	−	+++	++	++	−	+++
26	62	F	2	−	−	−	−	−	+++
27	62	M	5	−	+++	+++	+++	−	+++
28	63	M	3	−	++	+	−	−	N/A
29	67	F	5	−	+	N/A	N/A	N/A	N/A
30	68	F	5	−	N/A	N/A	N/A	+	++
31	70	M	4	−	++	−	−	−	++
32	71	M	3	−	++	−	−	N/A	NA
33	72	M	N/A	− (*)	(+)	−	−	−	NA
34	72	M	5	−	+++	−	+++	+	+++
35	73	F	3	−	+++	+++	−	−	N/A
36	74	F	3	−	++	−	−	−	+++
37	76	M	3	−	++	+++	−	−	+++
38	76	F	5	−	+	(+)	−	−	+++
39	80	F	5	− (*)	+++	++	N/A	N/A	+++
40	82	F	4	−	++	−	−	−	N/A
41	86	F	4	−	+	−	−	−	N/A

Grading scheme: (+) = 1–2 positive cells; + = 3–4 positive cells; ++ = 5–10 positive cells; +++ = >10 positive cells per field of view. For analysis a Zeiss Axioplan2 microscope equipped with a 20× objective (Neofluar) and a 10× ocular was used. IAF: Inner annulus fibrosus; OAF: Outer annulus fibrosus; EP: Cartilaginous endplate; AOA: Attachment of outer annulus fibrosus; N/A: not available. KRT19 positive cells were only detected in the NP and in 2 cases of severe degenerative disc changes in the EP (*).

150 W and 50°C for 6 h, dehydrated in graded ethanol series, and embedded in paraffin[16]. Sections were deparaffinized, treated with 3% hydrogen peroxide in methanol for 30 min and then with heated (95°C) citrate buffer (10 mM sodium citrate, 0.05% Tween20, pH 6.0) for 20 min for antigen retrieval. Then they were blocked with 5% normal horse serum for 1 h, and were incubated with mouse monoclonal anti-KRT19 antibody (clone A53-B/A2; cat. no. EXB-11-120, Exbio, Praha, CZ) or mouse monoclonal anti-MGP antibody (clone 52.1C5D; cat. no. ALX-804-512, Alexis Biochemicals, Lausen, CH) at a concentration of 5 μg/ml over night at 4°C. Negative control sections were incubated without primary antibody. Biotinylated secondary anti-mouse antibody (dilution 1:200; Vectastain ABC-kit *Elite*, cat. no. PK-6102, Vector Laboratories, Burlingame, USA) was applied, followed by ABC complex, and chromogen development using diaminobenzidine (DAB Kit, cat. no. SK-4100, Vector Laboratories, Burlingame, USA). Sections were counterstained with Mayer's haematoxylin.

STATISTICAL ANALYSIS

Differences in relative gene expression levels between paired NP and AF and paired NP and AC were assessed by the Wilcoxon Signed Ranks test. Correlations between relative gene expression and age or disc degeneration grade were determined using the Pearson correlation analysis. $P < 0.05$ was considered as significant.

Results

GENE EXPRESSION

In both the NP vs AF and the NP vs AC comparisons, pronounced gene expression differences were observed for KRT19. Levels of KRT19 mRNA were constantly higher in the NP than in both AF and AC cells, although the extent of up-regulation varied between individuals. NCAM1 expression was also increased in NP compared to AF and AC cells, while the expression of A2M and DSC2 was higher in NP than in AF cells and KRT18 expression was higher in NP than in AC cells. On the other hand, mRNA

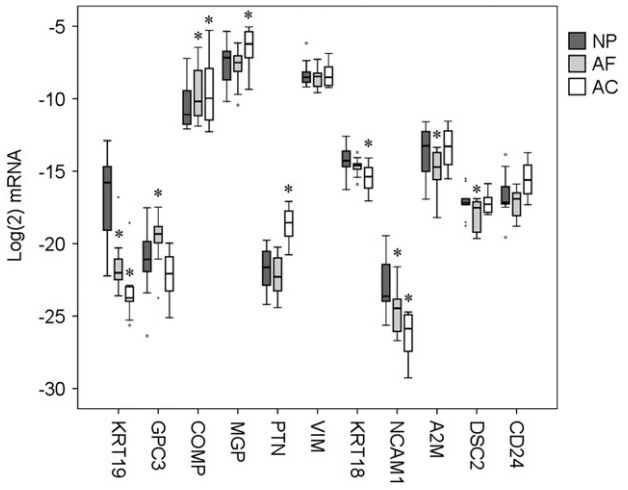

Fig. 1. Relative mRNA expression in human NP, AF, and AC cells. Expression levels were normalized to 18S rRNA as the endogenous control. *$P < 0.05$ in pair-wise comparison of NP with corresponding AF or AC; N = 9–11.

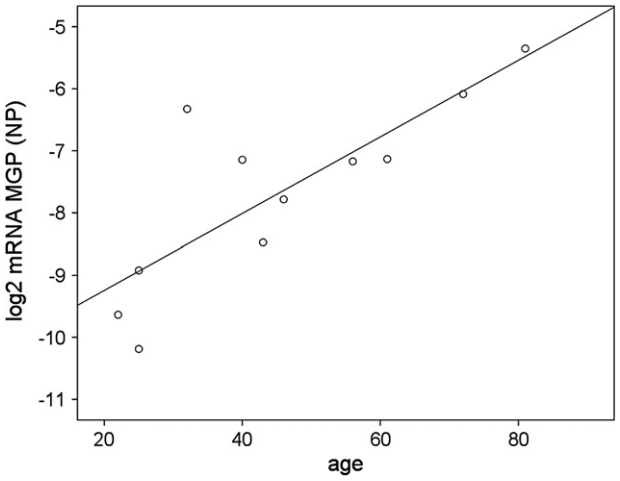

Fig. 3. Relative mRNA expression for MGP in NP cells of 11 individuals between 22 and 81 years of age. Gene expression was normalized to the 18S ribosomal RNA. An increase in the MGP expression level with increasing age is noted.

expression levels of GPC3 and cartilage oligomeric matrix protein (COMP) were higher in the AF compared to the NP, whereas COMP, MGP, and pleiotrophin (PTN) were expressed more highly in the AC than in the NP cells. No differences in vimentin (VIM) and Cluster of Differentiation 24 antigen (CD24) expression were noted between NP and AF or AC cells (Fig. 1).

While the KRT19 expression of AC and AF cells did not change throughout age groups, its expression in the NP cells showed a decrease with age ($P = 0.032$; Fig. 2). However, in spite of this decrease, KRT19 was still more highly expressed in the NP compared to the AF and AC cells even in older individuals. A correlation between age and gene expression in NP cells was also found for MGP mRNA, which increased with increasing age ($P = 0.003$; Fig. 3). In the AF cells, increasing levels of PTN mRNA were noted with aging ($P = 0.023$; data not shown). The expression of MGP in NP cells was also positively correlated with the degree of degeneration ($P = 0.007$). The relation between KRT19 level in the NP and degeneration grade was found to be almost significant ($P = 0.061$). No association between age or degeneration grade and the level of expression was detected with any of the other genes analysed. However, in agreement with previous reports, there was a strong relationship between age and degeneration grade of the disc ($P < 0.001$).

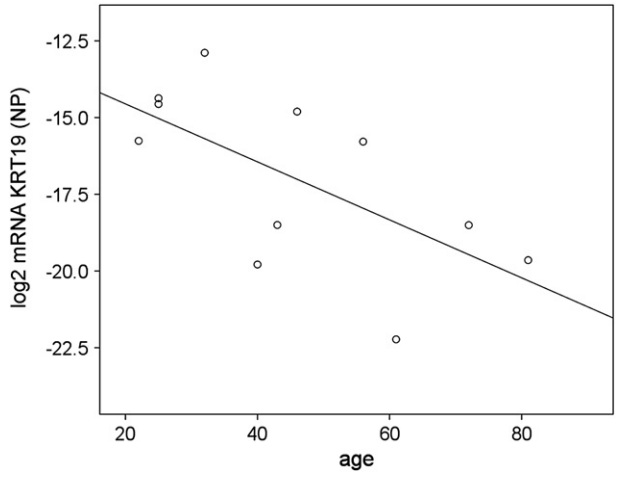

Fig. 2. Relative mRNA expression for KRT19 in NP cells of 11 individuals between 22 and 81 years of age. Gene expression was normalized to the 18S ribosomal RNA. A decrease in the KRT19 expression level with increasing age is noted.

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KRT19 was chosen for immunohistochemical analysis, since this molecule showed most pronounced differences between NP and AF or AC with respect to mRNA expression, whereas MGP was selected for analysis at the protein level because of its apparent age- and degeneration-dependent increase in the NP cells, which are of main interest in this study.

In the NP of juvenile discs (<5 years of age), clusters of large cells with a notochordal morphology were identified. These cells were positive for KRT19 [Fig. 4(A)], while neither cells within the AF nor cells of the cartilaginous endplate revealed any positive labelling [Fig. 4(B), Table III]. In the NP of young discs with Thompson score 1 but without apparent existence of notochordal cells (age range 6–25 years), KRT19 positive cells were observed in 60% ($n = 6/10$) of the individuals. Labelling was located predominantly intracellular and was limited to a small number of cells that had a somewhat chondrocytic appearance with no morphological evidence of a notochordal cell phenotype [Fig. 4(C, D)]. On the other hand, the majority of healthy adult discs did not reveal any immunoreactivity for KRT19 [Fig. 4(E)]. In two degenerate discs however (patients with sepsis), positive cells were detected adjacent to fissures associated with degenerative changes of the cartilaginous endplate [Fig. 4(G)]. These cells exhibited a characteristic chondrocyte-like morphology, were located at the border between cartilaginous endplate and NP and were not regarded as NP cells.

The number of MGP positive cells in general was higher than the number of KRT19 positive cells, but non labelled

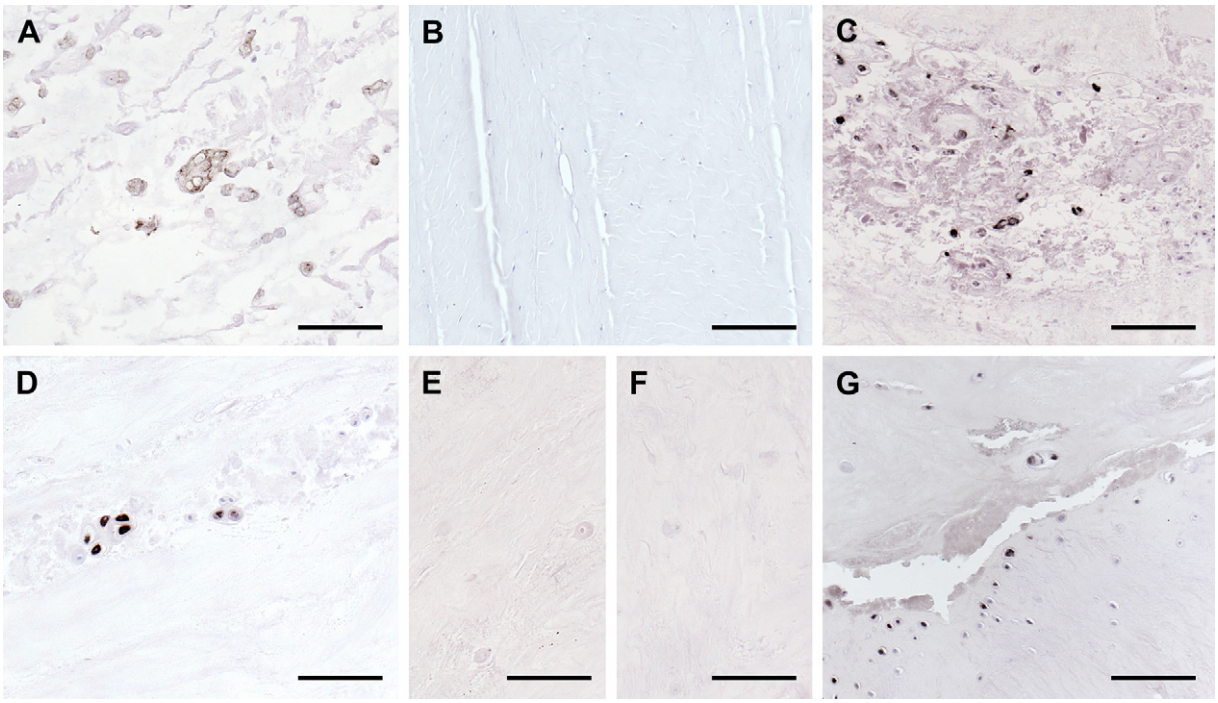

Fig. 4. Immunolabelling characteristics for KRT19. (A) Cells within the NP of a 3-year-old human female label positive for KRT19. (B) No KRT19 positive cells are detected in the inner annulus of a 21-year-old male individual. (C) A group of KRT19 positive cells in the NP of a 14-year-old male. (D) Small group of KRT19 positive cells from the NP of a 21-year-old male (same individual as in B). (E) NP of a 47-year-old female with Thompson grade 3. No positive labelling can be detected. (F) Control from same individual as in E. (G) 72-year-old male donor with disc degeneration grade 4. The image shows the edge of the NP region. The cartilaginous endplate is at the bottom of the image. Several KRT19 positive cells with a chondrocyte-like morphology can be detected. All scale bars = 100 μm.

cells clearly constitute the largest cell fraction. However, in juvenile and young adult discs positive cells could be detected in the NP (Table III). MGP positive cells were found in distinct clusters of NP cells in young individuals [Fig. 5(A, B)]. In older more degenerated discs (grade 3 and higher) positive cells were found adjacent to clefts and cracks [Fig. 5(D)]. Furthermore, the outer AF was often immunopositive [Fig. 5(E)], with a decreasing intensity towards the inner AF [Fig. 5(F)]. Labelling was limited to the cells and to a very small portion of the pericellular matrix in their immediate vicinity. The cells and extracellular matrix of the mineralized cartilage of the endplate often demonstrated a positive reaction for MGP, especially at the tidemark [Fig. 5(G, H)]. The non-mineralized cartilage of the endplate was always negative [Fig. 5(G, H)]. The fibrocartilaginous attachment of the outer AF frequently labelled positive [Fig. 5(I)].

Discussion

Degenerative changes occurring in the IVD have been extensively described and mechanisms, including genetic variations that may cause a predisposition to IVD degeneration are being elucidated. However, the molecular profile that characterizes the normal disc, and in particular the NP cell is still unknown. Previous investigations have suggested potential markers for IVD cells and more specifically for NP cells[7,8,13,17]. The present study is a further contribution to the identification of molecules expressed in human disc cells. It was undertaken to validate potential NP marker molecules identified by large scale gene expression screening in the rat and dog for their potential use with human cells.

Looking at genes previously identified as markers for the rat NP, the expression of KRT19 could be confirmed for human cells. However, a decrease in KRT19 expression with age was noted in the NP, while the expression in AF and AC remained constant. As KRT19 has also been associated with notochordal cells and chordoma, its expression in healthy adult human NP cells may be unexpected[18,19]. Indeed, immunohistochemical analysis confirmed its presence in cells with a notochordal phenotype and further supported the suggestion of an age-dependent expression pattern. At the protein level, KRT19 was barely detectable in the NP after the third decade, although mRNA expression was still clearly measurable. Possible explanations for this finding may include differences in the detection limit between mRNA and protein expression, instability of the mRNA, short protein half-life, or inhibition at the translational level.

In contrast to KRT19, the expression pattern of the other genes that had been found to be expressed more highly in the NP than in the AC in rat specimens, including CD24, was not confirmed in human samples[7,13]. However, GPC3 and PTN expression profiles were similar to observations in beagle dogs[8]. This might be related to the comparable development of the NP with respect to cell phenotype in human individuals and chondrodystrophoid dogs, which clearly differs from the development in rats. Looking at the genes evaluated for the beagle dog, the human samples generally showed a similar expression pattern, with higher levels of KRT18, A2M, and NCAM1 in NP vs AF and/or AC. Besides KRT19, only NCAM1 was expressed more

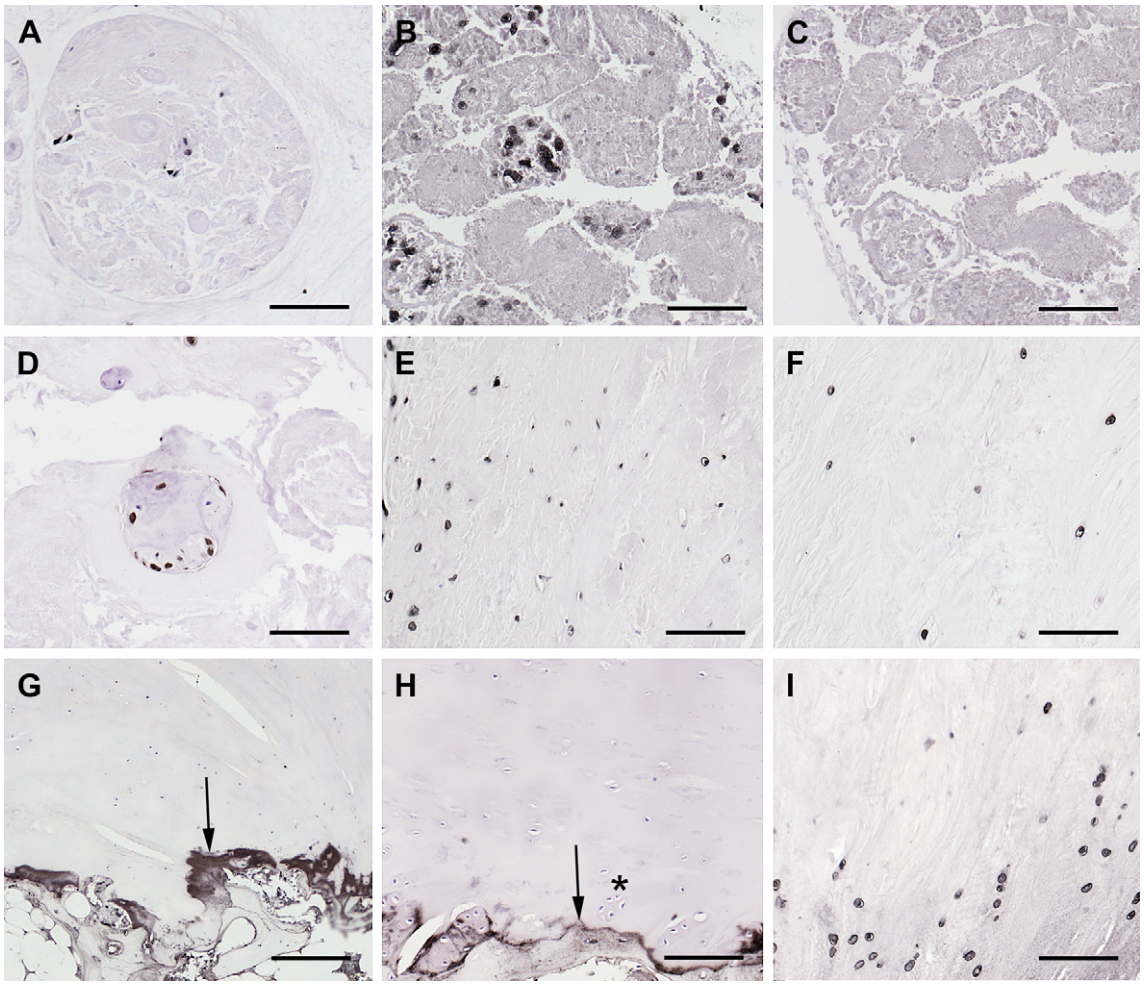

Fig. 5. Immunolabelling characteristics for MGP. (A) Cells within the NP of a 6-year-old human female label positive for MGP. (B) Group of MGP positive cells in the NP of a 17-year-old male. (C) Control from same individual and same region as shown in B. (D) Small group of MGP positive cells from the NP of a 60-year-old male individual with disc degeneration Thompson grade 5. Note that the cells are forming a cluster like structure in the neighbourhood of a fissure. (E) Outer and (F) inner annulus of a 35-year-old female with Thompson grade 2. Several positive cells can be detected. The surrounding extracellular matrix is negative. (G) Endplate cartilage from a 72-year-old male with Thompson grade 5. Note the strong positive labelling at the tidemark (arrow) and in the calcified cartilage. Cells in non-calcified cartilage are all negative. (H) Endplate cartilage from a 35-year-old female individual with Thompson grade 2. The positive labelling at the tidemark (arrow) is clearly visible. Cartilage cells form clusters (*) but are all negative. (I) Fibrocartilaginous attachment of the outer annulus of a 35-year-old female individual (same as in H). The fibrocartilage cells are arranged in characteristic rows and label positive for MGP. Scale bar in G = 200 μm, all other scale bars = 100 μm.

highly in NP than both AF and AC cells in this study. NCAM1 is an integral membrane glycoprotein that can regulate both cell–cell and cell–substrate interactions, primarily through polysialic acid[20,21]. Although it is expressed primarily in the nervous system, NCAM1 has been identified in various tissues in the adult rat[22]. In development NCAM1 plays a significant role in cell differentiation, including diverse functions in osteogenesis and chondrogenesis[23]. However, the fact that NCAM1 was expressed at a low level in all cell types analysed depreciates this molecule as a useful marker for human NP cells.

The matrix protein COMP showed consistently lower expression in NP than in AF and AC cells in all species. This differential expression of COMP in cartilage and disc adds to earlier observations of variations in the relative amounts of distinct matrix molecules in these two tissues[6]. While COMP has been identified and localized in the IVD, its relatively lower expression may reflect differences in the mechanical properties between the NP and cartilage tissues[24]. The main molecular functions of COMP include binding other matrix proteins and catalyzing polymerization of type II collagen fibrils. Furthermore, COMP is reported to prevent vascularization of cartilage and this could also be the case in the IVD tissues[25].

Commonly, work that addresses the disc cell profile leads to the conclusion that disc cells express a predominantly chondrocytic phenotype[26–28]. Investigations on mature bovine IVD cells agreed that NP cells produce more proteoglycans and less collagen than AF and cartilage cells, which is consistent with the higher hydration of the NP tissue[6,29]. In rat spinal units it was demonstrated that NP can be distinguished from adjacent tissues by the expression of proteins that are synthesized in response to restriction in oxygen and nutrient supply[17]. Additional studies in the rat revealed other potentially NP specific markers[7,13]. However, a major disadvantage of using rat NP is the presence of cells with

a notochordal phenotype. In fact several studies have used rat cells to explore the notochordal molecular phenotype in the disc[30,31]. Therefore, results from the rat, as well as mouse or rabbit have to be extrapolated with care to the human situation. Apart from the different developmental pathways of the NP cell populations the large deviations in IVD size and thus nutrition and mechanical conditions are also likely to influence the molecular characteristics of NP cells. Nevertheless, the expression profile of KRT19 demonstrates that selected genes may be valuable as markers even in different species.

When comparing the present study with previous studies on beagles, it also has to be noted that the human samples in this study are very heterogeneous with respect to age, while the animals all had the same age. This may explain the less pronounced differences between NP, AF, and cartilage cells in human individuals compared to the dog species, while general trends were identical in both species. Consequently, the age of the animal always has to be taken into consideration for the interpretation of results from an animal model. Moreover, although a study in the rat did not reveal major differences in gene expression pattern between RNA extracted from isolated cells and RNA extracted directly from the tissues, enzymatic cell isolation might have contributed to the reduced tissue-related differences observed in human specimens[7]. Besides, it is sometimes difficult to clearly distinguish human NP from AF tissue, especially in aged discs, which can also result in lowered gene expression differences between NP and AF cells. In view of these difficulties and the observed age-related alterations, young individuals are clearly preferred for the study of the phenotype of the healthy human NP cell.

Age-related changes have been detected regarding matrix composition, expression of matrix degrading enzymes, and other processes[3,6,32–34]. Although differences between aging and (early) degeneration were recently described in rabbits, a strong correlation exists between age and degeneration grade in human patients[3,35,36], which is confirmed in this study. Thus, it is not possible to clearly separate the influence of aging from that of degeneration mechanisms. Accordingly, Adams and Roughly defined a degenerate disc as one with structural failure combined with accelerated or advanced signs of aging[37]. This has recently been demonstrated also for the cervical spine, where in a longitudinal study no other factor except for age was related to the progression of degeneration[38]. Although cases of early disc degeneration have been described and are of particular value for specific investigation of degenerative processes, the present study evaluated individuals with "natural" disc development, where aging is accompanied with a certain degree of degeneration. This is particularly reflected in the mRNA expression of MGP in the NP, which strongly correlated with both aging and degree of degeneration. MGP is a Bone morphogenetic protein-2 (BMP-2) regulatory protein that is known as a calcification inhibitor in cartilage and in arteries[39,40]. Interestingly, MGP serum levels of community-based cohorts were also elevated with increasing age and were associated with individual risk factors for coronary heart disease[41]. It was suggested that induction of MGP expression may be a feedback mechanism to prevent mineralization of calcium deposits in the arteries[41]. Similarly, one could speculate that induction of MGP in the NP may be an attempt to prevent calcification processes that have been observed in the aging disc[42,43]. The presence of MGP in areas of mineralized cartilage in the endplate and in cells adjacent to sites of degeneration would support this hypothesis. The same is true for the expression at the fibrocartilaginous attachment of the outer AF where ectopic ossification (i.e., exophyte growth) would be prevented. Interestingly MGP expression in non-calcified AC is restricted to the superficial regions in monkeys and is barely detectable in senile human cartilage tissue[44]. In chondrocytic cells, both over-expression of MGP in maturing chondrocytes and under-expression of MGP in proliferative and hypertrophic chondrocytes may induce apoptosis[45]. As it has been reported that some cells of the IVD may differentiate towards the hypertrophic chondrocyte phenotype with age, MGP might function to prevent apoptosis in these cells[46]. Further studies are required to clarify the role of MGP expressed in the disc.

The observed rise in PTN gene expression in the AF with aging may result from a cellular attempt to restore a slowly degrading tissue. PTN functions as a growth and differentiation factor in many cell types and has been shown to induce the synthesis of matrix molecules in articular chondrocytes[47]. In cartilage, elevated PTN levels have been related to both osteoarthritis and rheumatoid arthritis[48,49]. Interestingly, an increased amount of PTN-immunopositive cells was observed in degenerated and in prolapsed disc samples and was associated with vascularization of diseased or damaged tissue[50]. Since blood vessels are mostly localized in the outer AF and rarely penetrate into deeper zones of the IVD, an increasing expression of PTN in the AF with aging would support the suggestion that PTN may function as an angiogenic factor in the degenerating IVD[50]. Age- or degeneration-associated changes might become clinically useful markers to determine the "juvenileness" or the regenerative capacity of IVD tissues sampled from discectomies or nucleotomies. More extended studies will be required to validate the potential of such markers to individually evaluate the most appropriate treatment of an IVD disorder.

In conclusion, from a selection of NP phenotype markers identified in animal studies, KRT19 and NCAM1 expression were found to be more pronounced in NP than AF and AC cells in human individuals. Whereas NCAM1 levels are relatively low even in NP cells, KRT19 may be regarded as a marker for human NP cells, being highly expressed in NP and at significantly lower levels in AF and AC cells. This observation on the subject of gene expression is at least partially reflected at the protein level, where KRT19 positive cells are almost exclusively identified in the NP of juvenile and young adult discs. This suggests that KRT19 transcripts are translated into detectable amounts of protein primarily in notochordal-like cells of juvenile NP and occasionally in young chondrocyte-like NP cells. MGP is found in a variety of human IVD tissues and thus cannot serve as a characteristic NP marker.

Conflict of interest

The authors confirm that they have no financial and personal relationships with other people or organisations that could inappropriately influence their work.

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