A phenotypic comparison of intervertebral disc and articular cartilage cells in the rat

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Abstract The basic molecular characteristics of intervertebral disc cells are still poorly defined. This study compared the phenotypes of nucleus pulposus (NP), annulus fibrosus (AF) and articular cartilage (AC) cells using rat coccygeal discs and AC from both young and aged animals and a combination of microarray, real-time RT-PCR and immunohistochemistry. Microarray analysis identified 63 genes with at least a fivefold difference in fluorescence intensity between the NP and AF cells and 41 genes with a fivefold or greater difference comparing NP cells and articular chondrocytes. In young rats, the relative mRNA levels, assessed by real-time RT-PCR, of annexin A3, glypican 3 (*gpc3*), keratin 19 (*k19*) and pleiotrophin (*ptn*) were significantly higher in NP compared to AF and AC samples. Furthermore, vimentin (*vim*) mRNA was higher in NP versus AC, and expression levels of cartilage oligomeric matrix protein (*comp*) and matrix gla protein (*mgp*) were lower in NP versus AC. Higher NP levels of *comp* and *mgp* mRNA and higher AF levels of *gpc3*, *k19*, *mgp* and *ptn* mRNA were found in aged compared to young tissue. However, the large differences between NP

and AC expression of *gpc3* and *k19* were obvious even in the aged animals. Furthermore, the differences in expression levels of *gpc3* and *k19* were also evident at the protein level, with intense immunostaining for both proteins in NP and non-existent immunoreaction in AF and AC. Future studies using different species are required to evaluate whether the expression of these molecules can be used to characterize NP cells and distinguish them from other chondrocyte-like cells.

Keywords Nucleus pulposus · Articular cartilage · Phenotype expression · Glypican 3 · Keratin 19

Introduction

Low-back pain affects up to 80% of adults, with annual estimated direct and indirect costs upwards of \$90 billion in the United States alone [25]. Although the aetiology of chronic back pain remains unknown and is likely to be multi-factorial, intervertebral disc degeneration appears to be a leading cause for chronic axial low-back pain [26, 40].

A healthy intervertebral disc consists of three distinct regions the central nucleus pulposus (NP), the outer annulus fibrosus (AF) and the inferior and superior cartilage endplates. The NP is a highly hydrated gel-like matrix composed of negatively charged aggregating proteoglycans, randomly organized collagen fibres and radially oriented elastin fibres [5, 9, 18, 32, 50]. The surrounding AF consists of a series of concentric lamellae of predominantly type-I collagen fibres [19, 28]. Throughout growth and skeletal maturation, the boundary between annulus and nucleus becomes less obvious, with the nucleus generally becoming more fibrotic and less gel-like with age [4, 27, 30]. The most significant biochemical change to

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occur at the onset of disc degeneration is a loss of proteoglycan [1, 27], and a subsequent decrease in the load-bearing capability of the disc—as the proteoglycan content decreases, the osmotic pressure of the disc falls and the disc is less able to maintain hydration under load [1, 27].

The cells of the AF, particularly in the outer region, appear “fibroblast-like”, with an elongated and thin morphology aligned parallel to the collagen fibres and primary synthesis of type-I collagen. By comparison, the cells of the mature human NP are commonly characterized as “chondrocyte-like”—spherical cells synthesizing primarily type-II collagen and aggrecan. This traditional characterization of AF cells as “fibroblast-like” and NP cells as “chondrocyte-like”, however, appears to be an over-simplification. It has recently been suggested that cells of the AF could be chondrocytic at a different stage of differentiation than cartilage cells [35]. From the tissue-level perspective, the structure of the proteoglycans differs [6] and the proteoglycan : collagen ratio is higher in NP compared to articular cartilage (AC) [31]. Functionally, such differences are manifested as distinctly different mechanical properties—whereas cartilage behaves largely like a viscoelastic solid, NP can behave both as a fluid and as a viscoelastic solid under different loading conditions [17].

As an added complication, in young individuals and in adults of certain species there is a second population of cells in the NP, the so-called “notochordal cells”, presumed remnants of the embryonic tissue that guided formation of the spine and the NP [47, 48]. Based on the concurrent timing of age-related changes and the disappearance of notochordal cells in humans [48] and the prevalence of disc degeneration in chondrodystrophic canines (no notochordal cells in adult animals) versus non-chondrodystrophic canines (notochordal cells present in adults), a possible connection between loss of notochordal cells and disc degeneration has been surmised.

One of the fundamental issues in understanding disc degeneration and regeneration is to comprehend the cellular biology of the intervertebral disc. Despite the high prevalence of degenerative disc disease (DDD) and the debilitating consequences, the answer to such a basic question as to what defines a disc cell remains surprisingly puzzling. Furthermore, if a stem cell-based tissue engineering approach is envisaged for NP regeneration, it is crucial to know whether stem cell derived “chondrocyte-like” cells are more similar to AC or NP cells.

Thus, the current study was performed to identify a set of genes that could potentially be used to characterize a NP cell. Since—as stated above—the phenotype expression of NP cells and articular chondrocytes are very similar and essentially the same markers, such as type-II collagen, aggrecan, or Sox-9 are used to identify these two cell types, we focused on genes that may distinguish NP from AC

cells. We used rat coccygeal discs and AC from both young and aged animals and a combination of microarray, real-time RT-PCR and immunohistochemistry techniques to compare NP, AF and AC cells.

Materials and methods

Microarray analysis

A total of 16 Wistar rats (14–16-weeks old) were used for tissue harvest for the microarray analysis. AC was harvested from the femoral heads, trochlear grooves, femoral condyles and tibial plateaus; AF and NP were harvested from coccygeal discs (8–10 discs/rat). Cells were enzymatically isolated from their respective tissues using sequential pronase (Roche, Basel, Switzerland) and type 2 collagenase (Worthington Biochemical, Lakewood, NJ) digestion with DNase II (Sigma, St. Louis, MO) to prevent cell clumping (AC: 0.2% pronase, 1 h and 0.1% collagenase, overnight; AF: 0.2% pronase + 0.004% DNase, 1 h and 0.1% collagenase + 0.004% DNase, overnight; NP: 0.2% pronase + 0.004% DNase, 1 h and 0.04% collagenase + 0.004% DNase, 8 h). After enzymatic isolation, cells were lysed in TRI Reagent supplemented with 0.5% polyacryl carrier (both Molecular Research Center, Cincinnati, OH), and RNA was cleaned using a modified TRIspin method [37]. Briefly, 1-bromo-3-chloro-propane (0.1 ml/1.0 ml of TRI; Sigma) was added to the cell lysate, and the RNA in the resulting aqueous layer was cleaned by column purification with the GenElute Mammalian Total RNA Kit (Sigma) according to the manufacturer’s specifications. RNA was eluted in 50 µl of RNase-free water and treated with DNase I (Sigma) to remove genomic DNA. RNA was precipitated (7 mM NaCl and 70% EtOH, 3 h at –80°C) and dissolved at a concentration of 1 µg/µl.

RNA was sent to MWG, Ebersberg, Germany, for Hybridization Plus Service. This service included T7 amplification of the RNA, Cy3 or Cy5 fluorescent labelling of the RNA, co-hybridization (NP/AF and NP/AC) to the MWG Rat 10K array, array imaging, and analysis. A total of four microarrays were prepared: NP/AF and NP/AC co-hybridizations, each with dye-swap to exclude dye bias. The relative expression of each gene in NP versus AF and NP versus AC was expressed as a ratio of fluorescence intensities.

Real-time RT-PCR

The expression levels of seven genes [annexin A3 (*anx3*), cartilage oligomeric matrix protein (*comp*), glypican 3 (*gpc3*), keratin 19 (*k19*), matrix gla protein precursor

(*mgp*), pleiotrophin heparin binding factor (*ptn*) and vimentin (*vim*)] were further evaluated using real-time RT-PCR. In addition to confirming the findings of the microarray analysis for the isolated rat cells, the effects of enzymatic cell isolation on gene expression was evaluated by comparing expression profiles in RNA extracted from isolated cells to RNA extracted directly from tissue. Furthermore, real-time PCR was used to compare the levels of these genes in young (same age as cell source; $n = 3$ isolations separate tissue pools) and old rat tissue (~ 2 -years old; $n = 6$ animals). For RNA extraction from tissues, tissue was first powderized under liquid nitrogen prior to the addition of the TRI Reagent, and RNA was isolated as described above.

Reverse transcription of total RNA to cDNA was performed using the TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the primers and probes listed in Table 1 and TaqMan Universal PCR MasterMix (Applied Biosystems) under standard thermal conditions (10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C; AB 7500, Applied Biosystems). Human 18S rRNA (Applied Biosystems) was used as the endogenous control and data were analysed using the Relative Quantification Method with C_t values from isolated AC cells as the arbitrarily chosen experimental calibrator (ABI Prism User's Bulletin, Applied Biosystems).

Immunohistochemistry

Coccygeal discs and femoral condyles were dissected from 16-week-old rats, fixed in 4% paraformaldehyde, decalcified, dehydrated, frozen in OCT compound and cryosectioned. Sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase and then washed with phosphate-buffered saline (PBS). Then they were blocked with 2% normal serum for 30 min at room temperature and incubated with the primary antibody at 4°C for 18–24 h. Mouse monoclonal anti-cytokeratin 19 antibody (Progen Biotechnik GmbH, Heidelberg, Germany) and rabbit anti-*gpc3* antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were applied at concentrations of 12.5 and 8 µg/ml, respectively, in 1% BSA/PBS. Negative controls consisted of respective isotype matched irrelevant antibodies (rabbit or mouse IgG). Sections were washed and then treated with biotinylated anti-mouse or anti-rabbit IgG (Vector Laboratories, Burlingame, CA), respectively, for 45 min at room temperature. Slides were then processed using the Vectastain ABC Kit (Vector Laboratories), developed with 3,3'-diaminobenzidine (DAB) substrate (Vector Laboratories) and counterstained with hematoxylin.

Table 1 GenBank reference of the genes evaluated, and oligonucleotide primers and probes used for real-time RT-PCR

Gene (Abbr.)	Accession number	fw primer (5'–3')	rv primer (5'–3')	Probe (5'–3')	Normalized fluorescence	
					AC/NP	AF/NP
Keratin 19 (k19)	X81449.1	cca gga ccg acc tgg aga t	act aat ttc ctc ctc gtg gtt ctt c	cct gaa gga gga gct ggc cta cct ga	15.48	10.94
Glypican 3 (<i>gpc3</i>)	NM_012774.1	ggc cct gag cca gtg gtt	tft acc ctt ggg cac aga cat	caa act gaa gca cat taa cca gct cct gag a	12.93	10.87
Pleiotrophin heparin binding factor (<i>ptn</i>)	NM_017066.1	ctc aga gat gta aga tcc ctt gca	caa gcc tgg aac tgg tat ttg c	ctg gaa gaa gca gtt tgg agc tga g	9.88	10.80
Vimentin (<i>vim</i>)	NM_031140.1	gca ccc tgc agt cat tca ga	gca agg att cca ctt tac gtt ca	agg atg ttg aca atg cgt ctc tgg cac g	7.87	2.97
Annexin A3 (<i>anx3</i>)	NM_012823.1	gtg gaa gag acg aaa gcc tga a	atc cgt gcc cca ttt ttt ct	tgg cca aaa aag atg ccc aga ccc	5.90	5.68
Cartilage oligomeric matrix protein (<i>comp</i>)	NM_012834.1	gtt tcc cgg acg aga agc tt	atc ctc ctg ccc tga att gg	agc gcc agt gcc gca agg ac	0.11	0.11
Matrix gla protein precursor (<i>mgp</i>)	NM_012862.1	gct ccc tet gcc cat cct	ttc cat gct ttc gtg aga ttc ata	tgg cgg tgg cag ccc tgt g	0.06	0.24

Statistical analysis

Statistical significance for RT-PCR data was determined using Kruskal–Wallis non-parametric analysis with Mann–Whitney *U* post-hoc testing. Significance was set at $P < 0.05$.

Results

Microarray

To identify genes that could be used to uniquely distinguish NP cells from AF cells and articular chondrocytes, a comparative microarray analysis of 10,368 genes was performed. NP/AF and NP/AC comparisons were made by co-hybridizing the RNA from the two pairings onto separate arrays. Since the purpose of this study was to identify genes that could be used to characterize a given cell type, genes that had normalized fluorescence intensities <5 for both cell types on an array were excluded from further analysis. Furthermore, since we were looking for genes that could be used to distinguish between the different cell types, we further narrowed our search to genes with relative intensity differences of at least five, rather than the more commonly used factor of two.

From the NP/AC co-hybridization, 19 genes were identified that had a fluorescent intensity ratio of at least five, and 22 genes with a ratio <0.2 (i.e. AC/NP ratio >5) (Table 2). Three of these genes had ratios of ten or higher and four had ratios of 0.1 or lower. Interestingly, among the list of genes showing substantially higher expression in articular chondrocytes compared to NP cells was *procol2a1* (NP/AC = 0.14), the gene coding for the $\alpha 1$ chain of type-II collagen, which is the predominant collagen in the NP.

From the NP/AF co-hybridization arrays, 27 genes were identified with fluorescence ratios of at least five, and 36 genes with ratios below 0.2 (Table 3). Three of these genes had ratios of ten or higher and ten genes had ratios of 0.1 or lower.

Keratin 19 and *gpc3* had ratios >10 in both the NP/AC and NP/AF comparisons (Table 4). *ptn* also showed large differences between NP/AF and NP/AC (11.1- and 9.9-fold, respectively). *vim* also showed a large difference between NP and AC cells (NP/AC = 7.7), but not between NP and AF cells (NP/AF = 2.9). These four genes were chosen for further analysis by real-time RT-PCR as potential “markers” for NP cells. Additionally, to obtain a better understanding of the relative sensitivities between microarray analysis and RT-PCR, *anx3* was chosen for evaluation by RT-PCR; the ratios of both NP/AC and NP/AF were at least five, but still had a relatively low intensity in the NP (normalized fluorescence intensity = 5.4).

In an effort to also identify genes that could potentially be used to determine that a cell is *not* a NP cell, two genes which were highly expressed in articular chondrocytes and had AC/NP ratios near ten were also analysed by RT-PCR—*comp* and *mgp* (9.3- and 15.8-fold lower intensity in NP versus AC, respectively).

Real-time RT-PCR

The differences in *anx3*, *comp*, *gpc3*, *k19*, *mgp*, *ptn* and *vim* expression were confirmed by real-time RT-PCR using RNA extracted from isolated cells in the same manner as for the microarray hybridization (Fig. 1; cells). Relative mRNA levels of *anx3*, *gpc3*, *k19* and *ptn* were significantly higher in NP samples compared to AF and AC samples ($P < 0.05$). Additionally, *vim* mRNA levels were higher in NP samples compared to AC, and *comp* and *mgp* levels were lower in NP compared to AC ($P < 0.05$). It should be noted that the magnitude of difference in gene expression levels as measured by RT-PCR was much greater than shown by microarray fluorescence intensities. For example, whereas there was a 13–14-fold difference in intensities for *k19* and *gpc3* (NP versus AC) on the microarrays, there was a 100–1,000-fold difference by RT-PCR.

The RT-PCR data also confirmed that there were no significant differences in the relative gene expression measured from RNA extracted from tissue compared to RNA extracted from isolated cells (Fig. 1). There were, however, significant differences in gene expression among young and aged rats (Fig. 2). Compared to levels measured in young tissue, aged tissue had higher NP levels of *comp* and *mgp* mRNA, and higher AF levels of *gpc3*, *k19*, *mgp* and *ptn* mRNA. The elevated *comp* expression of aged NP samples was similar to the level measured for AC (young rat tissue), but the elevated *mgp* expression in the aged NP remained nearly tenfold lower than in AC. The age-related changes in AF expression of *gpc3*, *k19* and *ptn* resulted in statistically similar expression levels of these genes in the NP and AF for aged rats.

Immunohistochemistry

Immunohistochemical analysis was performed to identify and localize the protein expression of *k19* and *gpc3*, whose mRNA expression levels were strikingly different between NP and AC cells in both young and aged rats.

In general, the NP of young rat discs appeared inhomogeneous with a central area consisting of single cells including large vacuoles and of acellular regions, whereas the peripheral part contained groups of condensed cells and an extended extracellular matrix. Since the acellular

Table 2 Genes with NP/AC fluorescence intensity rates of >5 or <0.2 in the NP/AC co-hybridization arrays

Gene	Accession number	Normalized fluorescence		
		AC	NP	NP/AC
<i>Keratin 19</i>	<u><i>X81449.1</i></u>	5.10	79.71	15.48
<i>Avian sarcoma virus 17 v-jun oncogene homologue</i>	<u><i>NM_021835.1</i></u>	2.84	41.10	14.11
<i>Glypican 3</i>	<u><i>NM_012774.1</i></u>	2.64	34.58	12.93
Pleiotrophin heparin binding factor	<u><i>NM_017066.1</i></u>	2.60	27.94	9.88
Vimentin	<u><i>NM_031140.1</i></u>	31.02	263.39	7.87
Serum-inducible kinase	<u><i>NM_031821.1</i></u>	0.78	5.79	7.39
Sialoprotein osteopontin	<u><i>NM_012881.1</i></u>	14.00	104.39	7.00
Lysosomal trafficking regulator	<u><i>NM_053518.1</i></u>	2.77	18.70	6.87
Ige binding protein	<u><i>NM_031832.1</i></u>	63.85	431.35	6.73
Glutathione s-transferase	<u><i>J03752.1</i></u>	1.36	10.62	6.61
Annexin a3	<u><i>NM_012823.1</i></u>	0.94	5.43	5.90
Carbonic anhydrase iii ec 4.2.1.1	<u><i>NM_019292.1</i></u>	30.74	196.36	5.85
c-fos protein aa 1-380	<u><i>X06769.1</i></u>	27.42	158.33	5.61
Striated muscle alpha tropomyosin aa 81-284	<u><i>NM_057208.1</i></u>	0.88	5.47	5.54
Class i beta-tubulin	<u><i>AB011679.1</i></u>	8.30	44.32	5.28
Precursor of alpha-1-protease inhibitor	<u><i>NM_022519.1</i></u>	3.26	18.84	5.23
Cytokeratin 8 polypeptide	<u><i>M63482.1</i></u>	15.78	83.64	5.21
Basigin ox47 antigen	<u><i>NM_012783.1</i></u>	6.18	31.13	5.12
Hypothetical protein smap31—homo sapiens	<u><i>RATTUS01096</i></u>	6.41	33.03	5.07
Testosterone-repressed prostate message 2	<u><i>NM_053021.1</i></u>	62.80	12.91	0.19
Arachidonate 5-lipoxygenase activating protein	<u><i>NM_017260.1</i></u>	14.15	4.14	0.18
Collagen alpha 1 type x col10a1	<u><i>AJ131848.1</i></u>	8.48	2.02	0.17
Early quiescence protein-1	<u><i>RATTUS00790</i></u>	5.79	1.00	0.17
Nuclear protein 1	<u><i>NM_053611.1</i></u>	67.14	10.66	0.16
Chondroadherin	<u><i>NM_019164.1</i></u>	46.23	7.93	0.16
Connective tissue growth factor	<u><i>NM_022266.1</i></u>	286.93	44.82	0.16
<i>o</i> -acetyltransferase milk fat globule membrane protein	<u><i>NM_012811.1</i></u>	79.71	12.32	0.16
Procollagen ii alpha 1 col2a1	<u><i>NM_012929.1</i></u>	819.25	134.84	0.14
Scrapie responsive protein 1	<u><i>NM_033499.1</i></u>	12.38	1.69	0.14
3- <i>o</i> -sulphotransferase	<u><i>AF177430.1</i></u>	11.03	1.41	0.13
Protease nexin	<u><i>M17784.1</i></u>	33.30	4.24	0.13
Collagen alpha 2 type v	<u><i>AJ224880.1</i></u>	5.54	0.67	0.12
Integrin-binding sialoprotein bone sialoprotein ii	<u><i>NM_012587.1</i></u>	13.06	1.50	0.11
smhs1 protein	<u><i>NM_080399.1</i></u>	20.81	3.03	0.11
Organic cation transporter	<u><i>NM_022270.1</i></u>	7.86	0.81	0.11
Cartilage oligomeric matrix protein	<u><i>NM_012834.1</i></u>	269.93	29.00	0.11
Myocilin	<u><i>NM_030865.1</i></u>	13.03	1.41	0.10
<i>Zinc finger protein 6</i>	<u><i>U78117.1</i></u>	31.55	3.36	0.08
<i>Matrix gla protein precursor</i>	<u><i>NM_012862.1</i></u>	252.20	15.95	0.06
<i>Megakaryocyte stimulating factor precursor</i>	<u><i>RATTUS02946</i></u>	52.08	3.05	0.05
<i>60 kDa secreted protein transin aa 1-475</i>	<u><i>X02601.1</i></u>	64.63	3.21	0.05

Italicized text indicate a ratio of >10. Genes analyzed by RT-PCR are marked in bold-face type

regions and the vacuoles did not exhibit any immunoreactivity, the central part generally appeared less stained than the peripheral areas.

Consistent with the gene expression data, positive immunostaining for *k19* was noted in the NP of young rat discs (Fig. 3a). The staining was localized intracellularly

Table 3 Genes with NP/AF fluorescence intensity rates of >5 or <0.2 in the NP/AF co-hybridization arrays

Gene	Accession number	Normalized fluorescence		
		AF	NP	NP/AF
<i>Glypican 3</i>	<u><i>NM_012774.1</i></u>	2.35	28.91	10.94
<i>Keratin 19</i>	<u><i>X81449.1</i></u>	6.81	79.89	10.87
<i>Pleiotrophin heparin binding factor</i>	<u><i>NM_017066.1</i></u>	2.46	27.34	10.80
Avian sarcoma virus 17 v-jun oncogene homologue	<u><i>NM_021835.1</i></u>	2.15	19.40	8.76
DNA-damage-inducible transcript 1	<u><i>NM_024127.1</i></u>	2.16	18.05	8.51
Solute carrier family 12, member 2	<u><i>NM_031798.1</i></u>	2.35	19.04	8.39
Cytokeratin-18	<u><i>U67992.1</i></u>	9.04	80.81	8.17
Keratin 18	<u><i>X81448.1</i></u>	2.03	17.29	7.85
Cytokeratin 8 polypeptide	<u><i>M63482.1</i></u>	13.39	90.33	6.81
Sprague_dawley trembl1q9jme5 fragment	<i>RATTUS02626</i>	0.75	4.96	6.44
Lysosomal trafficking regulator lyst	<u><i>NM_053518.1</i></u>	2.49	16.46	6.44
Hypothetical protein smap3	<i>RATTUS01096</i>	5.18	32.75	6.39
Carbonic anhydrase iii	<u><i>NM_019292.1</i></u>	36.35	231.32	5.89
Nerve growth factor-induced protein	<u><i>NM_012551.1</i></u>	1.20	7.16	5.89
Striated muscle alpha tropomyosin aa 81-284	<u><i>NM_057208.1</i></u>	0.77	4.62	5.88
Protein tyrosine phosphatase, non-receptor type 16	<u><i>NM_053769.1</i></u>	16.84	98.14	5.75
<i>Annexin a3</i>	<u><i>NM_012823.1</i></u>	0.91	5.47	5.68
Metallothionein I	<u><i>M11794.1</i></u>	20.66	114.91	5.63
Ninjurin 2	<u><i>NM_021595.1</i></u>	0.85	4.73	5.50
Apolipoprotein e	<u><i>S76779.1</i></u>	7.39	39.92	5.43
Brain alpha-tropomyosin tmbr-1; 2; 3	<u><i>M18135.1</i></u>	0.75	4.07	5.43
Serum-inducible kinase	<u><i>NM_031821.1</i></u>	0.86	5.20	5.32
Transgelin	<u><i>NM_031549.1</i></u>	1.65	8.51	5.20
Metallothionein 2	<u><i>M11794.1</i></u>	25.26	129.84	5.16
c-fos protein aa 1-380	<u><i>X06769.1</i></u>	20.25	112.10	5.10
jun b proto-oncogene	<u><i>NM_021836.1</i></u>	5.27	26.85	5.09
Histone h4 precursor aa -1 to 102	<u><i>NM_022686.1</i></u>	0.77	3.88	5.06
nov protein	<u><i>NM_030868.1</i></u>	16.00	3.26	0.20
<i>o</i> -acetyltransferase milk fat globule membrane protein	<u><i>NM_012811.1</i></u>	47.56	9.07	0.19
Precursor cystatin c c-terminal fragment	<u><i>X16957.1</i></u>	82.32	16.63	0.19
Kelch family protein nd1-l—mus musculus	<i>RATTUS00028</i>	7.51	1.37	0.18
Lipopolysaccharide binding protein	<u><i>NM_017208.1</i></u>	21.52	3.80	0.18
Megakaryocyte stimulating factor precursor	<i>RATTUS02946</i>	7.13	1.23	0.18
4-hydroxyphenylpyruvic acid dioxygenase	<u><i>NM_017233.1</i></u>	3.90	0.67	0.18
Thrombospondin-4	<u><i>X89963.1</i></u>	5.78	1.01	0.17
Alpha-1 type i collagen colia1	<u><i>M27208.1</i></u>	435.43	75.35	0.17
Osteonectin	<u><i>NM_012656.1</i></u>	288.15	49.35	0.17
Chondroadherin	<u><i>NM_019164.1</i></u>	73.75	12.86	0.16
Protease nexin	<u><i>M17784.1</i></u>	5.05	0.78	0.16
Alcohol dehydrogenase 3	<u><i>NM_019286.1</i></u>	25.44	3.85	0.15
Fatty acid elongase 2	<u><i>AB071986.1</i></u>	8.76	1.61	0.14
Unknown	<u><i>AF093569.1</i></u>	5.36	0.76	0.14
Clone: 1300008g01—mus musculus	<i>RATTUS01267</i>	23.78	4.96	0.14
Early quiescence protein-1	<i>RATTUS00790</i>	7.17	0.98	0.14
Myocilin	<u><i>NM_030865.1</i></u>	8.48	1.19	0.13
Gelsolin protein—mus musculus	<i>RATTUS02839</i>	25.03	4.58	0.13
mhc class ib antigen rt1.m5	<u><i>AF055667.1</i></u>	16.27	2.23	0.12

Table 3 continued

Gene	Accession number	Normalized fluorescence		
		AF	NP	NP/AF
Contrapsin-like protease inhibitor related protein	<u>NM_031531.1</u>	57.44	6.84	0.12
Osteoglycin precursor	RATTUS01982	10.50	1.22	0.12
smhs1 protein	<u>NM_080399.1</u>	22.94	3.22	0.11
Testosterone-repressed prostate message 2	<u>NM_053021.1</u>	223.91	24.62	0.11
Cartilage oligomeric matrix protein	<u>NM_012834.1</u>	217.03	24.29	0.11
Ryudocan/syndecan 2	<u>NM_013082.1</u>	16.46	1.84	0.11
<i>Death effector domain-containing protein deft</i>	<u>NM_031800.1</u>	<i>11.94</i>	<i>1.18</i>	<i>0.10</i>
<i>3-o-sulphotransferase</i>	<u>AF177430.1</u>	23.24	2.25	<i>0.10</i>
<i>pro1 collagen type iii; alpha 1</i>	<u>X70369.1</u>	76.53	7.29	<i>0.09</i>
<i>Precursor truncated-cadherin cadherin-13</i>	RATTUS02522	17.15	1.48	<i>0.09</i>
<i>Dermatan sulphate proteoglycan-ii decorin</i>	<u>Z12298.1</u>	163.16	13.91	<i>0.09</i>
<i>Versican v3 isoform precursor</i>	<u>AY007691.1</u>	24.31	2.16	<i>0.08</i>
<i>Collagen alpha 2 type v</i>	<u>AJ224880.1</u>	19.23	1.33	<i>0.07</i>
<i>60 kDa secreted protein transin aa 1-475</i>	<u>X02601.1</u>	21.36	1.41	<i>0.06</i>
<i>Zinc finger protein 6</i>	<u>U78117.1</u>	34.55	2.98	<i>0.06</i>
<i>Procollagen, type i, alpha 2</i>	<u>NM_053356.1</u>	677.00	35.20	<i>0.05</i>

Italicized text indicate a ratio of >10. Genes analyzed by RT-PCR are marked in bold-face type

throughout all the NP cells. In contrast, no *k19* immunostaining was identified in AF cells. Furthermore, articular chondrocytes were also immunonegative for *k19* (Fig. 3b).

Nucleus pulposus tissue also demonstrated a strong intracellular immunoreaction for *gpc3* (Fig. 4a). *Gpc3* staining was identified in a majority of the cells throughout the NP, but was most intense towards the periphery, in particular towards the border to the AF. No immunoreactivity for *gpc3* was found in AF and AC cells (Fig. 4b).

Control sections probed with irrelevant immunoglobulins did not show any staining for neither disc nor cartilage specimens.

Discussion

No genes were identified that were clear “on/off” markers to distinguish NP cells from AC or AF cells. There were, however, a total of 63 genes with at least a fivefold difference in fluorescence intensity between the NP and AF cells compared to 41 genes with a fivefold or greater difference comparing NP cells and articular chondrocytes.

In order to increase the purity of the RNA extracted for microarray hybridization, cells were first isolated from their extracellular matrix by enzymatic digestion. It may be expected that such a process can change gene expression levels, thus it was important to confirm the findings from the microarray with real-time RT-PCR using RNA extracted both from isolated cells and directly from the

tissue. For the genes examined by RT-PCR (*annexin A3*, *comp*, *gpc3*, *k19*, *mgp*, *ptn* and *vim*), there was no significant difference in the gene expression for cell or tissue isolation. The possibility remains, however, that true differences in gene expression that would be seen with RNA extracted directly from tissue were not detected because the cell isolation procedure neutralized such differences. In addition, our results were based on one single microarray analysis. Therefore the present study cannot be regarded as comprehensive or conclusive over the phenotypical differences between the different cell types; it rather reports on the identification of a number of genes that are differently expressed in the cells/tissues analysed.

In choosing the subset of genes for RT-PCR analysis, relative levels of gene expression (very high or very low fluorescence ratios on the microarray), individual fluorescence intensities and reported functions/classifications of the genes were considered. The relative levels of individual fluorescence intensities were used to eliminate genes which were expressed at very low levels in all three cell types since the purpose was to find a set of genes that could be used to identify a given cell type. Additionally, although *procol2a1* met the criteria of at least a fivefold difference (AC > NP), it was highly expressed in both NP and AC cells; thus, while its expression is one of the criteria currently used to characterize a cell as an NP cell, it may not be used to distinguish an NP from an AC cell. Finally, it was desired to have a set of genes that could potentially lend insight into development/differentiation of the NP cell

Table 4 Genes from microarray showing at least a fivefold higher or lower intensity ratio for **BOTH** NP/AF and NP/AC comparisons

Gene	Accession number	Normalized fluorescence					
		AC	NP	NP/AC	AF	NP	NP/AF
Keratin 19	<u>X81449.1</u>	5.10	79.71	15.48	6.81	79.89	10.87
Avian sarcoma virus 17 v-jun oncogene homologue	<u>NM_021835.1</u>	2.84	41.10	14.11	2.15	19.40	8.76
Glypican 3	<u>NM_012774.1</u>	2.64	34.58	12.93	2.35	28.91	10.94
Pleiotrophin heparin binding factor	<u>NM_017066.1</u>	2.60	27.94	9.88	2.46	27.34	10.80
Serum-inducible kinase	<u>NM_031821.1</u>	0.78	5.79	7.39	0.86	5.20	5.32
Lysosomal trafficking regulator	<u>NM_053518.1</u>	2.77	18.70	6.87	2.49	16.46	6.44
Annexin a3	<u>NM_012823.1</u>	0.94	5.43	5.90	0.91	5.47	5.68
Carbonic anhydrase iii	<u>NM_019292.1</u>	30.74	196.36	5.85	36.35	231.32	5.89
c-fos protein aa 1-380	<u>X06769.1</u>	27.42	158.33	5.61	20.25	112.10	5.10
Striated muscle alpha tropomyosin aa 81-284	<u>NM_057208.1</u>	0.88	5.47	5.54	0.77	4.62	5.88
Cytokeratin 8 polypeptide	<u>M63482.1</u>	15.78	83.64	5.21	13.39	90.33	6.81
Hypothetical protein smap3	RATTUS01096	6.41	33.03	5.07	5.18	32.75	6.39
Testosterone-repressed prostate message 2	<u>NM_053021.1</u>	62.80	12.91	0.19	223.91	24.62	0.11
Early quiescence protein-1	RATTUS00790	5.79	1.00	0.17	7.17	0.98	0.14
Chondroadherin	<u>NM_019164.1</u>	46.23	7.93	0.16	73.75	12.86	0.16
<i>o</i> -acetyltransferase milk fat globule membrane protein	<u>NM_012811.1</u>	79.71	12.32	0.16	47.56	9.07	0.19
3- <i>o</i> -sulphotransferase	<u>AF177430.1</u>	11.03	1.41	0.13	23.24	2.25	0.10
Protease nexin	<u>M17784.1</u>	33.30	4.24	0.13	5.05	0.78	0.16
Collagen alpha 2 type v	<u>AJ224880.1</u>	5.54	0.67	0.12	19.23	1.33	0.07
Smhs1 protein	<u>NM_080399.1</u>	20.81	3.03	0.11	22.94	3.22	0.11
Cartilage oligomeric matrix protein	<u>NM_012834.1</u>	269.93	29.00	0.11	217.03	24.29	0.11
Myocilin	<u>NM_030865.1</u>	13.03	1.41	0.10	8.48	1.19	0.13
Zinc finger protein 6	<u>U78117.1</u>	31.55	3.36	0.08	34.55	2.98	0.06
Megakaryocyte stimulating factor precursor	RATTUS02946	52.08	3.05	0.05	7.13	1.23	0.18
60 kDa secreted protein transin aa 1-475	<u>X02601.1</u>	64.63	3.21	0.05	21.36	1.41	0.06

Italicized text indicate a ratio of >10. Genes analyzed by RT-PCR are marked in bold-face type

population and/or functional differences among NP and AC or AF cells (see Table 5 for a summary of gene functions and their relevance to cartilage and disc cells).

Although it was not a primary objective of this study to identify a gene that could be used to characterize aging of

the intervertebral disc, we had anticipated substantial age-related changes in the rat NP based on reports that the cell population of the rat NP changes from a predominately notochordal cell type to a more mature (“chondrocyte-like”) cell type around 12 months of age [43]. For the

Fig. 1 Relative mRNA expression in tissue (filled circles) or isolated cells (open circles) of annulus fibrosus (AF), nucleus pulposus (NP) and articular cartilage (AC) of 14–16-week-old rats. Data were normalized to the expression levels of AC cells. No significant differences were seen between cell and tissue extracts for any genes. * $P < 0.05$ compared to AC and AF; † $P < 0.05$ compared to AC

Fig. 2 Relative mRNA expression in annulus fibrosus (AF), nucleus pulposus (NP) and articular cartilage (AC) tissue of young (14–16 weeks; circles) and old (2 years; squares) rats. Data were normalized to the expression levels of young AC cells. Shaded groupings reflect a significant difference ($P < 0.05$) between aged tissue levels of mRNA relative to young tissue

Fig. 3 Immunolocalization of keratin 19 in rat **a** coccygeal disc and **b** articular cartilage, assessed using DAB as the chromogen. **a** Intense intracellular *k19* staining was observed in the NP, which was virtually absent in the AF. **b** Articular chondrocytes were immunonegative for *k19*. Scale bars = 0.1 mm

Fig. 4 Immunolocalization of glypican 3 in rat **a** coccygeal disc and **b** articular cartilage, assessed using DAB as the chromogen. **a** Intense intracellular *gpc3* staining was observed in the NP, while AF was immunonegative for *gpc3*. **b** Articular chondrocytes showed no *gpc3* immunostaining. Scale bars = 0.1 mm

genes chosen for RT-PCR analysis in the current study, however, the only statistically significant age-related changes in the NP were with *comp* and *mgp* expression; with *comp* expression in the aged NP cells increasing to near the level measured in young rat AC.

In contrast to the few age-related changes in the NP, *gpc3*, *k19*, *mgp* and *ptn* expression changed with age in the

AF. In all cases, gene expression shifted towards levels measured in the NP in the older tissue samples. It is unclear from this study whether this shift is due to a true shift in the AF cell or due to partial inclusion of NP tissue in the AF samples. The NP/AF boundary is less distinct in the older animals and since it was anticipated that NP cells would be more similar to AC cells, tissue was harvested as to err on

Table 5 Genes chosen for validation using real-time RT-PCR

Abbr.	Alternate name	Description/function	References
k19	Cytokeratin-19	Intermediate filament typically co-expressed with <i>vim</i> ; expression reported in epidermal stem cells, notochord cells, various tissues during embryonic development	[44, 49]
gpc3		Cell-surface heparin sulphate proteoglycan; inhibits cell proliferation and regulates cell survival; modulates activity of several growth factors; expression reported in selective embryonic mesoderm and human fetal cartilage	[29, 34, 42, 51]
ptn	Osteoblast stimulating factor-1	Heparin-binding multi-functional protein; can up-regulate proteoglycan synthesis and osteoblast proliferation; expression reported in mesoderm and neuroectoderm during embryonic development, and fetal, juvenile, and osteoarthritic cartilage	[36, 45, 46]
vim		Intermediate filament typically co-expressed with <i>k19</i> ; expression reported in AF cells, large (“notochordal”) and small (“chondrocytic”) cells of the NP, and notochord cells; expression also documented in cartilage	[3, 7, 21, 22]
anx3		Ca ²⁺ and phospholipid binding protein; influences membrane organization and traffic; regulates Ca ²⁺ currents across membranes and/or intracellular Ca ²⁺ concentrations	[12]
comp		Extracellular matrix glycoprotein that can cross-link type-II collagen; found abundantly in articular cartilage; also identified in NP and AF	[8, 20, 39]
mgp		Vitamin-K dependent protein found in bone and cartilage; expression up-regulated in endochondral bone formation, pathological calcification of cartilage, and immature and periosteal hypertrophic chondrocytes during fracture healing	[2, 15, 16, 23, 24, 33]

the side of including NP tissue with AF rather than including AF tissue with NP.

Immunohistochemical studies basically confirmed the gene expression data at the protein level. The strong *gpc3* reaction of the NP cells, which was absent in AF and AC, suggests that *gpc3* expression may be an explicit feature of NP cells. High levels of *gpc3* have been found in most tissues during mammalian development [34], whereas in the adult, *gpc3* expression is strongly limited [42]. Supporting our observation, Pellegrini et al. reported that the *gpc3* labelling of developing mouse vertebrae remained, after ossification, only in the NP [34]. *Gpc3* is a highly versatile proteoglycan that has been shown to regulate various growth factors, depending on the tissue, the developmental stage, and its membrane-bound or secreted form [10]. Since *gpc3* has also been shown to induce apoptosis in certain cell-types [13], one could speculate that this proteoglycan may contribute to the change in cell populations within the NP. On the other hand, *gpc3* mRNA was still highly expressed in the NP of aged rats, suggesting that NP might be one of the rare tissues that retain *gpc3* expression.

The large differences in *k19* mRNA levels between NP and AC cells were also reflected by the *k19* immunoreactivity of the respective tissues. *K19* has previously been associated with notochord and chordoma [14, 44]. Thus, this protein might be expected to be present primarily in notochordal cells of the NP. Nevertheless, the majority of the NP cells stained *k19* positive. This is, however, consistent with the finding that *k19* mRNA was expressed at high levels also in NP of old rats that are expected to be devoid of notochordal cells. Notably, several of the molecules found to be highly expressed in NP, namely *k19*, *gpc* and *ptn*, play an important role in tissue development and differentiation. This reflects, in addition to the variable occurrence of notochordal cells also on a molecular basis, that in certain species the NP may continue to express developmental molecules after birth and possibly throughout life.

Recently, Risbud et al., suggested that hypoxia inducible factor 1 α (HIF-1 α) could be used to distinguish NP cells and articular chondrocytes [38]. NP cells uniquely expressed functionally active HIF-1 α protein under normoxic conditions and only modestly increased their HIF-1 α

transcriptional activity in a low-oxygen environment. In contrast, AF cells, chondrocytes and osteoblasts showed low-level expression of HIF-1 α under normoxia, with significant protein induction at hypoxia. Confirming reports that HIF-1 α mRNA is consistently expressed in most cell types and at best modestly induced by hypoxia ([41]; Risbud MV, personal communication) there were no significant differences in HIF-1 α gene expression levels between the cell types analysed by microarray. Thus, while HIF-1 α protein expression can be considered as specific for NP cells, its mRNA expression is irrelevant as a phenotypic NP marker.

A recently published study, which also employed microarray analysis of RNA extracted from rat coccygeal discs, identified CD24 as a potential marker for NP cells [11]. In the current microarray, the NP : AC intensity ratio for CD24 was 2.3, supporting their findings that CD24 is more highly expressed in the NP compared to AC, but not meeting our stricter criteria of an intensity ratio >5 to warrant further investigation. It is possible, however, that enzymatic isolation of the cells from their extracellular matrix affects the expression of cell surface proteins, such as CD24. Due to the potential utility of using a cell surface marker such as CD24 for cell sorting and/or immunolocalization, CD24 remains an interesting candidate and warrants further investigation.

Conclusions

The present study evaluated a set of genes that are distinctly higher expressed in rat NP compared to AC cells. In view of a stem cell-based tissue engineering approach for NP regeneration, expression of these molecules could become instrumental in monitoring and, eventually, triggering stem cell differentiation towards IVD cells. However, future studies using different animal models in which notochordal cells are not present after birth are necessary to confirm whether the expression of *gpc3* and *k19* can be used to distinguish a mature NP cell from an articular chondrocyte, especially with respect to the human situation. In particular, it will be essential to evaluate whether these genes can generally be attributed to NP cells or whether they depend on the presence of notochordal cells. In addition to the limitation of the rat model due to the ambiguity of the notochordal cells, the functions of the rat coccygeal disc in comparison to the functions of the human IVD need to be considered in the interpretation of the findings of this study. Nonetheless, the rat was an appropriate model for this study due to the need to have an abundant supply of healthy disc tissue, the commercial availability of a rat microarray and the common use of the rat model to study the etiology of disc diseases.

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