Identification of novel nucleus pulposus markers

INTERSPECIES VARIATIONS AND IMPLICATIONS FOR CELL-BASED THERAPIES FOR INTERVERTEBRAL DISC DEGENERATION

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Mesenchymal stem-cell based therapies have been proposed as novel treatments for intervertebral disc degeneration, a prevalent and disabling condition associated with back pain. The development of these treatment strategies, however, has been hindered by the incomplete understanding of the human nucleus pulposus phenotype and by an inaccurate interpretation and translation of animal to human research. This review summarises recent work characterising the nucleus pulposus phenotype in different animal models and in humans and integrates their findings with the anatomical and physiological differences between these species. Understanding this phenotype is paramount to guarantee that implanted cells restore the native functions of the intervertebral disc.

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Introduction

Back pain is an almost universal symptom, with its prevalence by lifetime, month and time-point being 84%,1 23.2% and 11.9%,2 respectively. The economic impact of managing this condition and compensating for its associated losses and disabilities accounts for £12 billion annually in the United Kingdom3 and $85.9 billion in the USA.4

Although mechanical loading has been frequently been implicated in the pathogenesis of back pain,5–7 it is now known that genetic predisposition may account for a significant proportion of back pain associated clinical conditions.1,8,9 Significantly, MRI has shown that around 40% of patients with back pain have associated degeneration of the intervertebral disc (IVD),8,10 with a causal relationship between both having been identified.11–13 IVD degeneration, as described by Kirkaldy-Willis et al14 in their seminal work in 1978, triggers a cascade of events that lead to most of the degenerative spinal conditions treated in current clinical practice (disc bulging and herniation, spinal stenosis without spondylolisthesis, spinal stenosis with degenerative spondylolisthesis and degenerative scoliosis).14,15 Pain arising from disc degeneration may be caused by inflammatory responses triggered by degenerated disc tissue herniating into the spinal canal,16 nerve ingrowth into the IVD itself following annular and endplate ruptures13,17 or due to altered spine biomechanics.18

The IVD is responsible for shock absorption and mobility of the spinal unit.19,20 It is composed of a central nucleus pulposus (NP) and a peripheral annulus fibrosus (AF), and is separated from the vertebral bodies by two cartilaginous endplates. The NP forms the gelatinous inner core of the IVD. It comprises large quantities of the proteoglycan aggrecan within an irregular mesh of type II collagen fibres. The AF is subdivided into outer AF, which is formed by distinct lamellae, composed of type I collagen fibres oriented obliquely between each lamella,21,22 and a less fibrous and less organised inner AF, characterised by a transition to type II collagen and increased proteoglycan content.23 This architecture enables the AF to constrain the hydrostatic pressures generated within the NP upon compression, facilitating mobility between the spinal segments.24–27 The endplates are hyaline cartilage structures that form the interface between the IVD and the upper and lower platforms of the vertebral bodies. They protect the NP and AF inferiorly and superiorly and allow diffusion of oxygen and nutrients to the NP. Their structure is formed by a network of type II collagen fibrils and proteoglycans. Adjacent to the vertebral body the endplate is richer in collagen, whereas adjacent to the NP it is richer in proteoglycans.28
autologous chondrocyte implantation and matrix-induced results obtained in the treatment of osteoarthritis using a healthier extracellular matrix. The promising clinical importance of phenotyping the NP

autologous chondrocyte implantation, in which autologous articular chondrocytes are harvested from non-damaged AC areas, expanded ex vivo and re-implanted (with or without a scaffold) into damaged osteo-arthritic regions of joints, have led researchers to attempt a similar strategy in the IVD. With this aim, dog IVD cells expanded ex vivo and re-implanted into its discs were shown to integrate and produce a cartilaginous extra-cellular matrix; these results have led to the initiation of the Euro Disc Randomized Trial, an ongoing human clinical trial. Implantation of autologous NP cells harvested from degenerate discs may not be ideal, however, as degenerate NP cells have an altered phenotype, with increased expression of senescence markers, increased expression of matrix catabolic enzymes and decreased expression of matrix components factors that would impair the ability of these cells to produce a healthy matrix.

A different approach would be to harvest cells from non-degenerate discs. However, the method currently used to harvest cells from healthy discs (disc needle puncture) has been shown to induce degeneration. Alternatively, Nomura et al have proposed transplanting allogenic NP tissue or cells. The use of such cells or tissue, however, would require a donor bank of healthy human samples, which would be difficult to obtain and could pose immune rejection problems.

Stem cells, particularly mesenchymal stem cells, have also been widely proposed as a source of cells in the treatment of disc degeneration. A PubMed search including the terms (‘intervertebral disc’ or ‘spinal degeneration’ or ‘disc degeneration’ or ‘degenerative disc disease’) AND (‘stem cells’ or ‘stem cell’ or ‘mesenchymal cell’ or ‘stromal cells’ or ‘MSC’) retrieved 261 papers, of which over 50% have been published in the last three years. Mesenchymal stem cells are mesoderm-derived adult stem cells, for which there is a growing body of evidence confirming that they can be differentiated to adult NP, suggesting they may be the ideal candidates for novel cell-based therapies for disc degeneration.

Assessing stem cell differentiation: the importance of phenotyping the NP

For cell-based therapies to be successful, it is fundamental that implanted cells have the correct phenotype to produce an appropriate functioning matrix in vivo. Stem cell fate or differentiation can be influenced by co-culture, growth factors and/ or biophysical conditions. However, in order to identify which differentiating factors should be used and to assess the differentiation (particularly the “end-stage” cell created), it is important to understand the NP cell phenotype and specific cell markers.

To date, most of the studies assessing differentiation of MSC to NP cells analyse differentiation and ‘end-stage’ cell phenotype using traditional chondrogenic genes, such as collagen type II alpha 1 (COL2A1), aggrecan (ACAN) and sex determining region Y (SRY)-box 9
(SOX9), markers that are known to be expressed by healthy adult human NP cells. However, while NP cells have some similarities with AC cells, these cells and the tissues in which they reside have considerable differences in terms of cell ontogeny, morphology, matrix composition and biomechanical behaviour (Table I). 68-71 and despite being important in the tissue’s function, they have not been taken into account when designing and assessing cell-based therapies for disc degeneration. This is highlighted by the study by Gorensek et al.22 in which elastic cartilage from a rabbit’s ear was transplanted into its IVD and shown to form a solid tissue resembling AC, rather than a hydrated-gelatinous tissue like the NP. More recently, the relevance of these differences to the tissue’s biology has led to a growing interest in identifying specific NP markers, characteristic of its phenotype. Several years ago the proteins hypoxia inducible factors 1 alpha and beta (HIF-1α and HIF-1β), glucose transporter 1 (GLUT-1), matrix metalloproteinase 2 (MMP-2) and vascular endothelial growth factor (VEGF) were shown to have higher expression in the rat NP in comparison with its adjacent AF and cartilage. These molecules are associated with responses to hypoxia and glucose starvation and may correspond to an adaptation of NP cells to the unique metabolic conditions NP cells have to withstand rather than marking a distinct cellular phenotype. Consequently, a more thorough characterisation of these cells was needed and, with recent advances in transcriptomic profiling, the NP phenotype has now been described in several species.

Analysing the NP phenotype: gene expression profiling

In molecular biology, gene expression profiling is the measurement of the activity of genes being expressed by a given cell in a specific moment. Extensive characterisation of these genes is permitted by the use of powerful technologies, such as microarrays, ribonucleic acid (RNA)-sequencing and chromatin immuno-precipitation sequencing. The wide availability of microarrays has provided disease-related research with valuable transcriptomic information on the interactions between cells and the environment in which they reside with this information being used to characterise disease states, predict disease progression and develop new therapies. 76-78

In the IVD field, microarrays have been used to characterise and define the NP cell phenotype, particularly by highlighting differences between the NP and similar or neighbouring tissues, such as AC, AF and endplate. Due to the difficulty in obtaining human intervertebral disc tissue, particularly non-degenerate, several of these studies have used animal models. Furthermore, for safety reasons, experimental treatments must be tested in animals and, therefore, it is important to understand if disease mechanisms differ between species and if/how they can be translated from animal research to human therapy. Phenotyping studies have thus been conducted on frequently used animal models, such as the rat,79-81 but also animals whose IVD is thought to more closely resemble the human, such as the dog82 and the bovine.36 More recently, the human NP phenotype has also been assessed. 37,83

Results from these studies have provided new insights into NP markers in different species, but also on fundamental aspects regarding the ontogeny, maturation and degeneration of NP cells. As such, these findings have important implications for the understanding of the pathogenesis and treatment of disc degeneration and will be the focus of the following sections of this review.

The rat nucleus pulposus. The rat is a frequently used model for human diseases and has been extensively used to understand disc degeneration.84-87 At birth, the rat NP is populated by large vacuolated notochordal cells and this population of cells is reported to be maintained during the first 12 months of life, after which it starts to be replaced by a smaller population of chondrocyte-like cells.88

Fujita et al.79 have compared the transcriptome of the rat NP (eight-week-old) with that of two avascular tissues (AF and tendon), five mesenchymal tissues (skeletal muscle, skin, blood, bone and bone marrow) and two neurogenic tissues (spinal cord and brain). This comparison, however, failed to include the AC, which, as aforementioned, is the tissue that more closely resembles the NP. The authors identified cluster differentiation 24 (CD24), a cell adhesion glycoprotein expressed at the surface of B-lymphocytes and differentiating neuroblasts, as a cell surface marker with significantly higher expression in the NP than in the other tissues analysed.

**Table I.** Main differences between articular cartilage and nucleus pulposus

| Characteristics          | Articular cartilage | Nucleus pulposus |
|--------------------------|----------------------|------------------|
| Cell ontogeny (34,35)    | Lateral plate mesoderm | Notochord (axial mesoderm) |
| Cell morphology          | Small and round      | Variable between species. In humans, they are large and vacuolated at birth, becoming small and round with maturaion/degeneration |
| Extracellular matrix proteoglycans (68) | Large aggregates (hyaluronic acid and central filaments), multiple monomers and large non-aggregated monomers | Short non-aggregated proteoglycan monomers and clusters of monomers without apparent central filaments |
| Aggrecan/type II collagen ratio (70) | 2/1                  | 27/1             |
| Collagen network (70)    | Rigid                | Loose            |
| Biomechanical behaviour (71) | Viscoelastic solid in response to shear transient and dynamic deformation | Fluid under transient and of a viscoelastic solid under dynamic deformation |
| Mechanical loads experienced (71) | Compressive loading | Compressive and shear loading |

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Additionally, CD24 immunopositivity was shown to be present in six out of seven human chordomas (a notochord-derived bone tumour found in the spine) but not in chondrosarcoma samples.79

In a more recent study, Tang et al81 compared the NP and AF gene expression and again identified CD24, but also brain abundant membrane attached signal protein (BASP1), N-Cadherin (N-Cad), neuropilin (NRP-1), CD155 and CD221 as specific rat NP markers. Interestingly, CD24 has also been shown to be expressed by NP cells from six- to 17-year-old patients undergoing scoliosis surgery, having been proposed as an immature human NP-marker.59

In another rat phenotyping study, Lee et al80 identified annexin A3 (ANXA3), glypican 3 (GPC3), keratin 19 (KRT19) and pleiotrophin (PTN) as highly differentially expressed NP markers genes (compared with the AF and AC), with KRT19 and GPC3 differential expression being confirmed at the protein level. CD24, which had been proposed by Fujita et al79 as a NP-specific cell surface marker was also found to be highly differentially expressed in the NP, but failed to meet the criteria of at least five-fold differential expression used in this study. GPC3, KRT19 and PTN are all involved in tissue development and differentiation, with KRT19 in particular being expressed by the human embryonic notochord90 and chordoma.91 Interestingly, KRT19 expression in the rat NP is not restricted to the notochordal-cell-rich NP and has also been found in adult (two-year-old) rat NP, where large vacuolated notochordal cells were reported to be absent.80

Stressing the importance of distinguishing the NP from the AC, this study found that the gene procolIIa1, which codes for COL2A1, the predominant collagen type in NP and AC, although being highly expressed in both tissues, had five times higher expression in the AC.80 supporting the more rigid nature of the AC compared with the more gelatinous NP.

Despite being a commonly used animal model for disc degeneration, findings from studies in the rat may not be directly translatable to human research, as there are considerable cellular and microenvironmental differences between the IVD in both species. Being small quadrupeds, the load applied to the rat spine is substantially smaller than the load applied to the human spine.92 Additionally, as the human IVD cross-sectional area is significantly larger than the rat’s, and since nutrient intake and waste product removal from the NP are made through diffusion from the endplate,29 it is possible that the human NP is subjected to much more stressful conditions (e.g. glucose starvation, hypoxia and acidic pH) than the rat NP. Most importantly, rats retain morphologically distinct notochordal cells during at least half of their lifespan, whereas such cells disappear from the human NP soon after birth. These differences may have a considerable impact on the NP phenotype and on the translation of these findings to humans.

The canine nucleus pulposus. Another animal frequently used to study disc degeneration, particularly in NP phenotyping studies, is the dog93-96 Dogs are larger quadrupeds and have a larger IVD than rats. Additionally, similar to humans, certain breeds of dogs – such as the dachshund, the beagle, the bulldog and the bassett hound (together named chondrodystrophoid dogs) – are reported to lose their notochordal cells soon after birth; these species then undergo spontaneous disc degeneration.93,97 These aspects make these dog species potentially more suitable as a model for human disc degeneration.

Sakai et al82 performed comparative microarray analysis between the NP and AF from 16- to 18-month-old beagles and further analysed the identified molecules at the gene and protein level in NP, AF and AC from the same animals. Among the identified markers, α-2-macroglobulin (A2M), keratin-18 (KRT18) and neural cell adhesion molecule (NCAM1) were identified as highly expressed in the NP compared to the AF and AC and desmocolin-2 (DSC2) compared with the AF. The high differential expression of A2M in the aggrecan-rich dog NP may account for its biological function – A2M has been shown to be a potent inhibitor of the aggrecanases ADAMTS-4 and -5,98 two aggrecanases implicated in NP matrix degradation.46,99 NCAM1 (CD56) and DSC2 are two cell surface markers involved in cell-cell adhesion and interaction but their specific function in the dog NP remains to be explained. Like the rat NP marker KRT19, KRT18 has also been identified in human notochordal cells during development90 and also in the human NP.100,101

Of relevance is also the fact that this study identified significant differences between the dog and the rat NP phenotype. From the previously reported rat NP markers (ANXA3, GPC3, KRT19, PTN and CD24) only GPC3 was detected in this study and, contrary to the rat, it showed higher expression in the AF than in the NP. These differences in gene expression between species are possibly a reflection of the differences in the different cellular and biochemical environment in the NP of these two species. The bovine nucleus pulposus. The finding that there are significant variations between the NP phenotype of different species triggered the search for the NP phenotype in other species, which would more closely resemble the human NP. Bovine caudal discs have been widely used as a model to study the human IVD.51,65,102,103 The IVD and the endplate of both species have been reported to have similar diameters and thicknesses and, due to the cow’s musculature, to be submitted to similar biomechanical forces.92 In terms of cellular population, the adult bovine NP predominantly contains a large population of small chondrocyte-like cells, although some authors have described a coexistent small population of large vacuolated notochordal cells in animals aged < 30 months.19,36

A comparative microarray analysis was performed on bovine NP, AF and AC36 which identified and further
validated KRT8, KRT18, KRT19, N-Cad, synaptosomal-associated protein 25 (SNAP25) and sclerostin domain containing 1 (SOSTDC1) as specific NP markers; BASP1, KRT8, KRT18, tenomodulin (TNMD), TNF alpha induced protein 6 (TINFAP6), N-cad, forkhead box F1 (FOXF1), forkhead box F2 (FOXF2), aquaporin 1 (AQP1), SOSTDC1 and SNAP25 as specific IVD (NP and AF) markers; and integrin binding sialoprotein (IBSP) and fibulin 1 (FBLN1) as negative NP markers. Confirming the interspecies variations that had been suggested in the canine array, this study failed to identify any of the rat NP markers GPC3, ANXA3, VIM, COMP, PTN and MGP or canine NP markers A2M, ANXA4, DSC2, NCAM1 as bovine NP markers.

In an attempt to address the transcriptional profile of markers related to the unique IVD microenvironment, Minogue et al. investigated the expression of previously identified rat microenvironment-related NP markers (MMP-2, HIF1A, GLUT-1 and VEGF) in bovine tissues, having failed, however, to identify significant differences between their gene expression in the bovine NP and AC. Although these differences may again be related to significant interspecies variations, it is of important note that these markers were originally identified by changes in protein expression and, therefore, the lack of differences in the bovine transcriptome may also be due to post-translational regulation or protein degradation, altering protein half-life, which would not be reflected at the mRNA level.

Interestingly, the only common genes to the bovine NP phenotype and other species were KRT18 (dog NP marker), KRT19 (rat NP marker), KRT8 (rat NP marker identified by Lee et al. but not chosen for qRT-PCR validation) and N-Cad (rat NP marker).

The human nucleus pulposus. The aforementioned transcriptional profiling studies were the first to provide a comprehensive list of NP markers for each specific species. They highlighted, however, the profound interspecies variations in gene expression, which possibly reflect differences in tissue and cell organisation, IVD and spine size and biomechanics and different local physico-chemical environment in each species. This emphasised even more the need for the identification of the human NP genetic signature.

Such a study was recently performed by Minogue et al. Using a stringent criteria of 20-fold differential expression between the human NP and AC, the authors identified and validated FOXF1, ovostatin 2 (OVOS2), haemoglobin beta chain (HBB), carbonic anhydrase XII (CA12) and paired box 1 (PAX1) as specific human NP markers (compared with AC), and growth differentiation factor 10 (GDF10), integrin binding sialoprotein (IBSP) and cytokine-like 1 (CYTL1) as specific human AC (NP-negative) markers.

PAX1 is a transcription factor expressed by the developing sclerotome and involved in the Shh-dependent mesenchymal-epithelial transition seen during the migration of sclerotomal cells towards the midline. The role of FOXF1 in the intervertebral disc has been less studied but recent findings have shown an association between deletions in the FOXF1 gene and the VACTERL association, a non-random association of birth defects which includes spinal malformations and fusion of spinal vertebrae.

The high differential expression of HBB and CA12 is possibly related to the adaptive mechanisms of the NP cells to the harsh niche in which they reside. Haemoglobin, an oxygen transport metalloprotein – usually found in erythrocytes – has recently been identified in neurons, macrophages, alveolar cells and mesangial kidney cells where it has been proposed to act by storing oxygen under hypoxic conditions; its identification in the hypoxic NP may represent a similar homeostatic mechanism. As NP cells have to survive in oxygen-deprived conditions, they rely mostly on glycolysis to produce energy and produce lactic acid as an end product; the accumulation of lactic acid in the NP leads to an acidic environment. CA12 is an enzyme that catalyses the conversion of carbon dioxide and water to bicarbonate and protons and is important in maintaining acid-base equilibrium by transporting carbon dioxide from cells residing in acidic environments, together with CA9, it has also been identified in the developing IVD. It is possible that its presence in the human NP serves to counteract the accumulation of acidic waste products in this tissue.

Again, these results demonstrate high variability between species. From the previously identified rodent NP markers, only KRT19 and GPC3 showed a similar trend in the human arrays (higher differential expression in NP compared with AC); from the identified canine markers, a similar trend was found for DSC12, KRT18 and NCAM1; from the previously identified bovine markers, FOXF1 and FOXF2 were the only genes with at least two-fold differential expression between the two human tissues. Among the negative markers previously identified, the bovine markers IBSP (80-fold differential expression between both human tissues) and FBLN1 (qRT-PCR data) were also validated as negative human NP markers.

More recently, Power et al. used a similar strategy to identify the human NP phenotype, and particularly NP-specific genes containing transmembrane domains, in comparison with the AF and AC. The rational for this strategy was, besides adding to information about the human NP phenotype, to identify potential NP-specific cell surface markers, which could be used to target specific cells with nanoparticles and, thereby, systemically delivering potential therapies to the NP cells. However, it should be noted that for such a strategy to be accomplished, the NP would have to have sufficient blood inflow to receive those particles, and the identified markers should not be expressed by any other cell in the human body, therefore assuring that the nanoparticles would only target NP cells. Surprisingly, the array data showed high similarity between the NP and the AF transcriptome and failed to identify a differentially expressed
Keratin expression in the NP provides clues to the ontogeny of the NP cells, which has been a subject of a long lasting debate. This controversy has been clarified by recent fate-mapping studies in mice where the embryonic notochord was shown to give rise to all the cells populating the adult mice NP. However, it was still not clear why the NP of some species lacks cells with a notochordal morphology. In order to further investigate the expression of keratin and also brachyury (also known to be expressed by notochordal cells) in animals where notochordal cells are either absent or present in small numbers, we have isolated two subpopulations of bovine NP cells – large vacuolated and small chondrocyte-like (Fig. 1). Analysis of these two cell populations showed that, although these notochordal markers were highly expressed in larger cells, they were still very highly expressed in small chondrocyte-like cells, and that large cells share the expression of the chondrogenic markers (SOX9, COL2A1 and ACAN) with small NP cells (Fig. 2). This, together with the fact that keratins are expressed in the NP of all the animal species assessed to date, independently of the resident NP cell morphology, suggests that large vacuolated and small chondrocyte-like cells may all share a same notochordal lineage and that, contrary to what has been postulated, notochordal cells do not disappear but differentiate to smaller non-vacuolated cells. This supports previous findings of rabbit notochordal cells differentiating to chondrocyte-like cells as a response to injury.

Gene expression variations with degeneration

The identification of the novel NP markers has given not just a more comprehensive gene signature of the NP, but also triggered an interest as to whether this gene expression varies with ageing and disc degeneration. Using the spontaneous dog model of disc degeneration (chondrodystrophic dogs), Smolders et al recently identified a down-regulation of genes involved in Wnt signalling and caveolin-1 occurring during the transition from a notochordal-rich to a chondrocyte-like-rich NP seen during early disc degeneration in these animals. The
Notochordal cells are crucial for the regulation of the expression of NP markers such as SNAP25, KRT-8, KRT-18, and N-Cad. This regulation is important throughout degeneration, as shown by the findings of Yang et al., where the onset of degeneration was shown to coincide with differentiation of mouse notochordal to chondrocyte-like cells. Interestingly, some of the markers reflecting the notochordal ontogeny are also those that are common across species (KRT8, KRT18, KRT19, and brachyury). Conversely, other NP markers, such as HIF-1A, GLUT-1, HBB, and CA12, may thus reflect responses to the microenvironmental conditions to which the NP cell is exposed in various species.

**Future perspectives**

Transcriptional profiling of the NP has provided invaluable information that can help to establish the NP phenotype in various species. Researchers in the IVD field have now a library of specific genes that can be used to assess the NP phenotype, some of which are common to different animal species. Additionally, the bovine and human studies have reignited a debate that had been ongoing for decades concerning the fate of notochordal cells in the NP of these species. Evidence now supports the premise that these cells do not disappear but rather differentiate to chondrocyte-like cells. Interestingly, some of the markers reflecting the notochordal ontogeny are also those that are common across species (KRT8, KRT18, KRT19, and brachyury). Conversely, other NP markers, such as HIF-1A, GLUT-1, HBB, and CA12, may thus reflect responses to the microenvironmental conditions to which the NP cell is exposed in each species.

As is norm in research, every scientific advance unveils undiscovered fields that warrant future research. It is yet to be clarified why in some animals NP cells maintain a notochordal morphology throughout life, whereas in others they differentiate to morphologically distinct cells. Additionally, it is not clear how homogeneous the small chondrocyte-like population of adult NP cells is. A recent report suggests that the expression of notochordal markers is restricted to subpopulations of the adult NP and that the non-notochordal population of NP cells may have migrated from the AF to the NP in response to degenerative stimuli. If so, it can be hypothesised that the elongated fibroblastic AF cells adopt a round chondrocyte-like morphology in response to the loose collagen network and high proteoglycan content in the NP, as opposed to the more ordered, fibrous matrix found in the AF. If this holds true, it remains to be shown whether these cells display different metabolism, response to injury, catabolic and anabolic properties. The identification of specific AF markers and, hence its phenotype, would allow identification of AF-derived cells with an NP-like morphology within the NP.

In respect to the treatment of intervertebral disc degeneration, particularly a cell-based therapy, it is still not know which phenotype should be targeted (young notochordal or a more mature chondrocyte-like). Understanding this is fundamental to guarantee that implanted cells have a NP phenotype capable of producing an appropriately functioning hydrated tissue that may regenerate and restore the functions of the intervertebral disc.
A study of the

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ICMJE Conflict of Interest:
- None declared

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