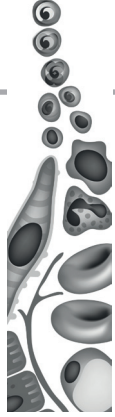

Understanding the native nucleus pulposus cell phenotype has important implications for intervertebral disc regeneration strategies

Low back pain is a leading cause of morbidity in developed societies and is strongly linked to degeneration of the intervertebral disc. The central nucleus pulposus (NP) region is most severely affected during disc degeneration and, consequently, is a focus for novel cell-based regenerative strategies. However, in order to develop such techniques, it is essential to first understand the biology and phenotype of the NP cells intended for repair. Microarray studies have highlighted novel NP markers that will allow a more accurate identification of cells for implantation, and along with other studies, have also revealed the potential importance of a developmental or immature NP cell phenotype in disseminating the optimal cell type for use. Additionally, the degenerative intervertebral disc is a harsh native environment and the effects of this on cells intended for implantation have yet to be fully elucidated; this is crucial for clinical translation of tissue engineered cell-based therapies.

KEYWORDS: degeneration ■ intervertebral disc ■ low back pain ■ notochord ■ nucleus pulposus ■ phenotype ■ regenerative medicine ■ tissue engineering

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Low back pain (LBP) is directly related to the lumbar region of the human spine and is estimated to affect 60–80% of individuals in developed societies at some point in their lives [1,2]. In the UK, LBP poses a huge economic burden, affecting over 17 million people, with some UK£12 billion in costs arising annually through treatment, lost work days and social benefit payments [3]. The UK is not alone in its economic loss through this condition, with annual costs totaling over US$85 billion in the USA [4]. Similarly, chronic neck pain associated with the cervical spine is one of the most commonly reported musculoskeletal disorders, with a lifetime prevalence in western Europe of approximately 70% [5]. Diseases of the lower back and cervical spine are multifactorial, thus complicating our understanding of their precise etiologies; however, it has been demonstrated that up to 40% of individuals with LBP also display features of intervertebral disc (IVD) degeneration, and a direct correlation has been shown to exist between pain and the severity of the tissue degeneration [6]. Furthermore, IVD degeneration is also thought to underlie degenerative spine conditions including spondylolisthesis, lumbar canal stenosis and degenerative lumbar scoliosis, all of which present neurological manifestations [7,8]. Additionally, disc degenerative changes in the cervical spine in cases of chronic neck pain have also been reported [9], further supporting the hypothesis that damage to this specialized tissue is involved in the pathogenesis of neck and back pain at various disc levels.

The IVD

The 24 articulating vertebrae of the human spine are separated by IVDs, which function not only as a supporting structure for the upper and lower extremities, but are also able to withstand load applied to the spine through movement. The IVD itself is best described as comprising three distinct regions: the cartilaginous end plates (CEPs), the annulus fibrosus (AF) and the nucleus pulposus (NP). CEPs are comprised of thin layers of hyaline cartilage whose function is threefold: to act as a physical barrier, thereby confining both the NP and AF to their anatomical boundaries; to act as a semipermeable membrane across which nutrient and fluid exchange can occur by diffusion between the NP, AF and vertebral bodies; and to absorb some of the load-induced pressure, thereby preventing pressure atrophy in the vertebral column. The AF is comprised primarily of type I collagen fibers, organized into 10–25 concentric lamellae with an alternating orientation between adjacent lamellae. The collagen fibers within these lamellae are more densely packed in the posterior region, preventing deformation of the disc when load is applied. In the cervical IVD, the AF is crescentric in form and does not demonstrate the alternating fiber orientation seen in the lumbar disc. Elastin fibers located

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between lamellae help restore disc shape after movement and also bind the individual lamellae together. The NP is the hydrated central region of the IVD and is extremely gelatinous due to the high levels of proteoglycans (PGs), particularly aggrecan, whose positive charge means water is drawn into the tissue, thereby increasing the osmotic potential [10]. Aggrecan is a large PG that aggregates with hyaluronic acid, and contains both chondroitin and keratin sulfates [11]. Additionally, other smaller PGs can be found primarily within the NP, including decorin, biglycan and lumican, the relative amounts of which have been shown to alter with disc degeneration [12], while the larger aggregate versican is localized predominantly within the AF [13]. The fluid-like nature of the disc permits deformation under load and the PG content of the disc maintains hydrostatic pressure within the tissue. A loose structure to the NP is provided by irregularly arranged type II collagen and elastin fibers. Type II collagen is the major collagen isoform present within the NP, although type IX collagen has also been localized within the healthy NP, inner AF and CEP, while type III and VI collagen increase with disc degeneration in all regions of the tissue [14]. The adult human NP is relatively hypocellular, with decreases in cell density occurring with skeletal maturity, resulting in a cell density of approximately 5000 cells/mm^3 in adulthood [15]. Extracellular matrix (ECM) therefore comprises the majority of this tissue, the biochemistry of which is carefully regulated by its resident cell population [16]. Morphologically, cells within the NP region are altered during childhood, transforming from larger, vacuolated clusters of cells noted in immature NP to smaller, rounded, chondrocyte-like cells found in adult discs [15].

IVD degeneration

Degeneration of the IVD causes both gross morphological and molecular alterations. The degenerative process is thought to be initiated within the NP [17] and the most profound effects of this pathology are noted within this region. With progressing degeneration, the previously distinct boundary between the NP and AF becomes more ambiguous, with dehydration and fibrosis of the NP becoming apparent. This loss of hydrostatic pressure in the NP causes a decrease in size, leading to decompression and an increase in load distribution to the AF. The lamellar organization within the AF becomes more irregular and radial fissures originating in the NP extend into the annulus. Fissures also develop in the CEP, which becomes calcified and sclerotic, thereby preventing normal diffusion of nutrients into and waste products out of the disc. Other visible changes include nerve ingrowth into the disc [18], which has been linked to the induction of nociceptive pain as well as neovascularization [19], cell senescence [20,21], increased incidence of cell death [22] and cellular proliferation and cluster formation [23].

It has previously been postulated that IVD degeneration is a cell-driven process, with cells producing factors that result in a loss of ECM, leading to the gross morphological changes described. In nondegenerate discs, there is a fine balance between matrix production and breakdown, but with progressing disease status, the phenotype favors disc catabolism. Key factors involved in this process are the matrix-degrading enzymes, including matrix metalloproteinases and ADAMTS, whose expression is increased in the degenerative IVD [24–26]. Abnormal expression levels of inflammatory cytokines, such as IL-1, TNF-α and IL-6, have also been linked to disc degeneration, where they are thought to alter matrix synthesis, induce IVD cell senescence, stimulate apoptosis, increase expression of matrix-degrading enzymes and promote neural and vascular ingrowth [27–29]. In response to a cytokine challenge, cells of the NP begin to synthesize atypical matrix molecules, with a switch from type II to type I collagen leading to fibrosis of the nucleus, and decreased PG expression, resulting in dehydration through loss of osmotic pressure [27,30]. Other cellular processes are also disrupted in the NP during IVD degeneration. Increased incidence of cell death via apoptosis has been observed in degenerate discs [22], although evidence suggests that overall cell numbers are actually unchanged. This may be due to proliferation of NP cells and formation of cell clusters, which is a feature of ongoing IVD degeneration [31] and is thought to be an attempt to repair the tissue damage. Moreover, although a decrease in IVD cell density is noted during infancy, increasing age beyond childhood and progressing severity of degeneration does not significantly alter cell numbers in all regions of the disc [15]. However, it has been noted that NP cells found in clusters may, in fact, have a differential gene expression profile compared with the singular NP cells [32]. While there may be cellular proliferation in the degenerate NP, cells within degenerate disc tissue also demonstrate features of cellular senescence. Upregulated expression of senescence-associated

markers has been shown, as well as decreased telomere length with progressing age and loss of replicative potential [20,21]. Importantly, these senescent disc cells have been shown to adopt a more catabolic phenotype and are thought to play a role in the pathogenesis of disc degeneration [21]. Thus, the evidence suggests that the process of disc degeneration is complex and likely to be driven by changes in the function and biology of native NP disc cells.

Current treatments for LBP

Currently, the major obstacle for back pain sufferers is the inadequacy of treatments offered to them. Most methods are largely conservative and are administered as a means of symptomatic relief rather than treating the underlying pathology, including physical therapy and the prescription of pain-reducing medicines. In more severe cases, it is possible to perform spinal surgery in the form of discectomy, spinal fusion or total disc replacement, but these interventions often result in reduced mobility or increase the likelihood of degenerative changes at adjacent disc levels requiring further surgeries [33], and are therefore not entirely optimal.

Biological & cell-based therapies

Several other approaches aimed at treating disc degeneration have been tested, including the injection of biologically active substances, such as growth factors that promote tissue anabolism, but as they require frequent administration, they are not considered ideal when attempting to reduce morbidity [34–36]. In order to fully restore IVD structure and function, it would be optimal to replace lost or damaged disc cells with healthy cells from either autologous or allogeneic sources. Although autologous NP cells would overcome the issues of immune rejection, their limited number, availability and unpredictable behavior postimplantation pose problems, as does the injury caused to the site of harvest, which may result in degeneration at donor disc levels. Furthermore, cells from degenerate discs have been shown to possess a more catabolic phenotype [24]; therefore, reimplantation of these cells may result in advancement of disc degeneration rather than normal tissue formation. Allogeneic NP cells are also of limited use, as the risk of immune rejection and consequent long-term reliance on immunosuppressive drugs outweighs any benefits that may ensue. Therefore, alternative cell sources have been investigated, including the use of adult mesenchymal stem cells (MSCs). Unlike native disc cells, these cells can be isolated from a number of tissues, and are able to undergo differentiation to an NP-like phenotype and produce an appropriate matrix [37] that, when paired with a hydrogel or artificial scaffold to mimic the 3D environment of the NP, seems to be an optimal regenerative strategy [38,39]. Indeed, several *in vivo* animal studies have demonstrated histological and biochemical improvements upon implantation of MSCs into experimentally induced degenerate discs [38,40], while a small study in humans demonstrated that implantation of MSCs resulted in an improvement in clinical symptoms, although no increase in disc height at 1 year follow-up was noted [41]. However, it remains unclear whether such effects are a direct consequence of MSC differentiation or whether stimulation of the native disc cells by implanted MSCs underlies this. Importantly, however, while tissue engineering applications offer significant potential for regeneration of the disc, it appears that the optimal phenotype of cells to be used for implantation is unclear, and therefore, when considering the transplantation of stem cells for tissue repair, this must be determined initially to maximize tissue repair/regeneration.

Elucidation of the NP cell phenotype

It is imperative for the success of novel cell-based therapies that any implanted cells or cells seeded onto biomaterials or scaffolds must have the correct phenotype so that they function correctly *in situ*. Classically, cells of the adult NP were considered chondrocyte-like in that their morphology is small and round, and that they express the chondrocyte markers Sox-9, type II collagen and aggrecan [30]. Thus, given the lack of more specific phenotypic markers, it was assumed that for the purposes of regenerating the NP, differentiation of MSCs to cells with a chondrocyte-like phenotype would be sufficient, and that these cells would produce a matrix that would function adequately in the IVD environment. However, this view was challenged when it was shown that chondrocytes and NP cells produce a distinctly different ECM in terms of the ratio of PG to collagen [42]. Additionally, transplantation of autologous chondrocytes (isolated from articular cartilage) into the IVD of the same rabbits resulted in the formation of hyaline-like cartilage [43]. Given this, and the known differences between chondrocyte and NP ECM, differentiating MSCs to a chondrocyte-like phenotype is deemed to be insufficient to ensure regeneration of an appropriate functional

tissue, thus prompting detailed phenotypic investigation of NP cells specifically, in order to ensure differentiation to the correct phenotype.

Several microarray studies have been undertaken by a number of groups with the view to elucidating unique markers of NP cells [37,44–47]. One of the key advantages of gene profiling by cDNA microarray analysis is that expression levels of thousands of genes can be determined simultaneously, allowing for the identification of a panel of marker genes from a single experiment. Initially, the phenotypic analyses were performed in small animal models, including the rat [45] and dog [44], although the canine arrays compared NP and AF tissue, with differential levels of phenotypic markers in articular chondrocytes (ACs) and NP cells being identified by reverse transcription PCR rather than microarray investigation. Unique phenotypic markers of NP cells distinguishing them from ACs and, in some cases, AF cells were identified, which supported previous evidence in a rabbit model that NP and AC cells are dissimilar in terms of gene expression [48], even though they are identical in terms of morphology. Further gene expression analyses of rabbit NP, AF and AC cells also highlighted vital markers for distinguishing the cell types [49]. Key NP and AC markers identified by microarray analysis are summarized in Table 1. Interestingly, the top differentially expressed genes between NP and ACs differed in each animal model, suggesting that interspecies variation would make isolating definitive markers of adult human NP cells problematic. Additionally, the NP of rabbit, rodent and some canine species is known to be populated by morphologically distinct notochordal cells (NCs) – a feature of IVD development that, in humans, disappears during infancy – and therefore may not be best suited for characterization, as such cells may have a distinct gene profile as well as appearance [50–52].

As the ultimate goal of these studies was to provide evidence of the NP-specific phenotype so that those trialing novel cell-based regenerative strategies are equipped with confirmation of the optimal NP profile, it was essential that microarrays were performed in animal models more akin to that of the human IVD. Bovine discs provide the only animal model in which disc structure, load and environment is similar to that observed in the human spine [53,54]. Additionally, unlike the discs of rats, mice, rabbits, pigs and some canine breeds, the mature bovine coccygeal discs are considered to be non-notochordal in terms of the cells residing within the tissue; a feature most like the mature human IVD. Initial studies by our group highlighted several differentially expressed genes between NP, AF and AC cells (see Table 1) [46], although many of the identified genes were not consistent with those highlighted in previous microarray and phenotyping studies using alternative animals, intimating that interspecies differences were responsible. With such variations noted between the phenotypes of NP cells obtained from different animal models, it was crucial that such comparisons be performed utilizing human samples, in order to ascertain the accurate human NP and AC phenotype. Our group undertook a microarray study using adult human IVD and articular cartilage samples, and a panel of differentially expressed genes were identified [37]. Interestingly, evidence from the human microarray supported findings of the previous bovine model work, in that analysis of the array data determined several of the same genes as NP-specific markers, including *FOXF1*, *KRT18* and AC-specific markers such as *IBSP*, as well as additional differentially expressed genes (e.g., *CAXII* and *PAX1*) that were subsequently confirmed by quantitative reverse transcription PCR to be expressed by bovine NP cells (Figure 1). In addition to corroborating the conclusions of the bovine investigation, the comparable findings in the human paper further validate the use of bovine discs in the study of the IVD. A subsequent phenotypic study of the human NP by Power *et al.* confirmed the expression of a number of marker genes from our previous microarray study, but focused on the identification of cell-surface markers uniquely expressed in the NP compared with AC or AF cells, which may enable the isolation of these cells from directly extracted human tissue [47]. Importantly, their study also identified *CAXII* as a key NP-specific cell-surface marker, confirming the results of our previous human microarray study, where it was determined to be a unique marker of human NP cells compared with ACs [37]. In addition to these findings, a small number of studies have identified age-dependent variations in the expression profiles of NP cells. *CAXII* expression was found to be negatively correlated with both age and degenerative score [47], while gene expression levels of *KRT19* (a NP marker identified in the rat and bovine microarray investigations [45,46]) have been shown to decrease significantly with age [55]. However, there is conflicting evidence regarding the expression of some of these novel markers [32,55]. For example, *KRT18* was not observed to be differentially expressed between

Table 1. A summary of the most highly differentially expressed nucleus pulposus marker genes as identified by microarray in rat, canine, bovine and human models.

Species investigated	Top differentially expressed genes determined	Gene abbreviation	Confirmed in other animal models?	Gene or protein expression data?	Ref.
Rat	Cytokeratin-19	*KRT19*	Bovine, human	Gene and protein	[45,46]
	Annexin A3	*ANXA3*	–	Gene	[45]
	Glypican 3	*GPC3*	–	Gene and protein	[45]
	Pleiotrophin	*PTN*	–	Gene	[45]
	Vimentin	*VIM*	Human	Gene	[45]
Canine	Cytokeratin-18	*KRT18*	Bovine, human	Gene and protein	[44,46]
	α2-macroglobulin	*A2M*	Human	Gene and protein	[44]
	Neural cell adhesion molecule-1	*NCAM1/CD56*	Bovine	Gene and protein	[44,46]
	Desmocollin-2	*DSC2*	Human	Gene and protein	[44]
Bovine	Cytokeratin-8	*KRT8*	Human	Gene	[46]
	Synaptosomal-associated protein 25	*SNAP25*	–	Gene	[46]
	N-cadherin	*CDH2*	Human	Gene	[46]
	Forkhead box protein F1	*FOXF1*	Human	Gene	[46]
	Brain acid-soluble protein 1	*BASP1*	Human	Gene	[46]
Human	β-globin	*HBB*	Bovine	Gene	[46]
	Ovostatin 2	*OVOS2*	Bovine	Gene	[46]
	Paired box protein 1	*PAX1*	Bovine	Gene	[46]
	Carbonic anhydrase XII	*CAXII*	Bovine	Gene and protein	[46,47]
	C-type lectin domain family 2 member B	*CLEC2B*	–	Gene	[47]
	γ-sarcoglycan	*SGCG*	–	Gene	[47]
	Tyrosine protein kinase receptor 3	*TYRO3*	–	Gene	[47]

the NP and AF of human degenerate disc samples [55], although work from our own laboratory has shown this not to be the case, with expression in human fetal NCs and a proportion of adult NP cells. Additionally, we found no change in expression with age or degeneration (FIGURE 2). Similarly, while gene expression of *KRT8* has been shown in both bovine and human NP cells, studies to date assessing protein expression have shown its detection in a subset of bovine NP cells [32].

Such studies highlight some of the limitations of microarray analyses. One of the major limitations of the microarray data previously published is the small sample cohorts used for experimentation. Although validation of these results in a larger cohort is unlikely to challenge the findings presented, it is essential to assess expression levels in a number of samples encompassing a wide range of ages and levels of degeneration, in order to accurately represent the NP phenotype at all stages of development, maturity and degeneration. Additionally, much research regarding the NP phenotype has focused on gene expression analyses, and there are limited studies regarding the expression of these genes at the protein level. In order to fully understand the expression profiles of adult human NP cells, it is important to localize expression of these proteins to identify cells with positive expression in order to ascertain any patterns between marker expression that may exist. Importantly, if subpopulations of cells within the NP displaying differential phenotypic profiles are then identified, their role within the adult tissue should be elucidated.

Importantly, one of the most interesting findings of these studies was the identification of expression of a number of notochordal markers by NP cells. *KRT8*, *KRT18* and *KRT19* and *T* are genes known to be expressed in the developing notochord [56–59], and were shown to be more highly expressed in mature NP cells compared with AC and AF cells in a number of the animal models tested [37,44–46]. In addition to this, the separation of cells by size using serial filtration allowed for analysis of bovine NP cells and NCs individually and highlighted

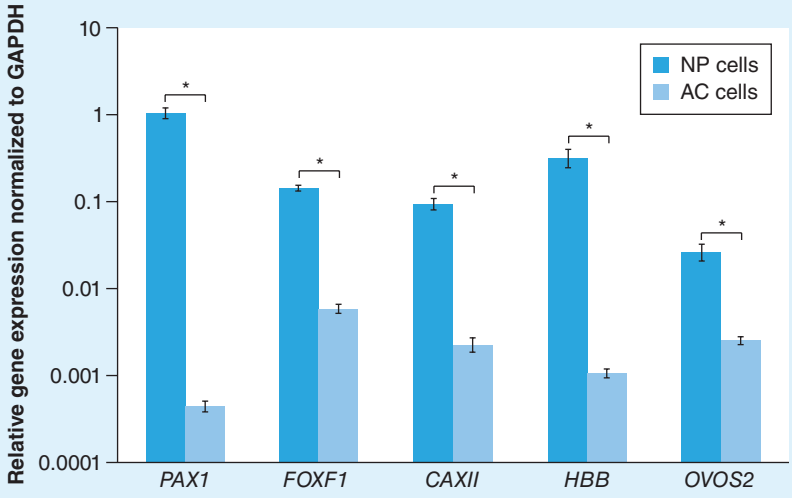

Figure 1. Expression of phenotypic markers identified in the human microarray investigation by bovine cells. *PAX1*, *FOXF1*, *CAXII*, *HBB* and *OVOS2* were also differentially expressed between bovine NP (n = 5) and AC cells (n= 5). *$p < 0.05$.
AC: Articular chondrocyte; NP: Nucleus pulposus.

overlapping expression profiles between the two cell types [46]. Thus, the identification of similar gene expression profiles between NCs and the mature NP suggests a common ontogeny, the full elucidation of which may further benefit regenerative strategies.

Importance of elucidating NP cell ontogeny

In order to effectively target IVD degeneration, it is essential to fully understand the origins of the native IVD cells intended for regeneration. In 1953, it was first proposed that the embryonic NP is notochordally derived, while the AF and CEP are mesenchymal in origin [60]. More recently, it was hypothesized that the marked differences in organization and structure in the NP and AF/CEP may be attributed to the differences in developmental origins [61]. During early development, the notochord lies between paraxial sclerotome-containing somites. Condensation of sclerotome cells around the notochord occurs [62,63], forming layers of condensed and less condensed cells that go on to form the AF/CEP and vertebrae, respectively [64]. Following this, notochordal condensation occurs, with the notochord contracting from within the vertebrae and expanding within the intervertebral space, eventually going on to form the NP [63–66]. The condensed sclerotomal cells simultaneously transform into fibroblastic-like cells, and begin to alter their alignment so that matrix can be deposited between them to form the familiar lamellar structure of the AF [67]. However, what remains unclear is whether the cells of the adult human NP are derived from NCs or from cells infiltrating from the surrounding AF, which is mesenchymally derived.

Recently, a wealth of data has been published supporting the theory that adult NP cells are notochordally derived. The rationale for NC loss and their replacement by chondrocyte-like NP cells with skeletal maturity in some species, including humans, is unclear. As observed in humans, the immature murine NP contains NCs that undergo a transformation to NP-like cells with the onset of disc degeneration [68]. Importantly, lineage-tracing studies have increased our understanding of NP development, and the use of mouse models has identified a common ontogeny between all cells of the mature mouse NP, which contains notochordal and NP cells, as well as cells from the embryonic notochord [50,69–71]. Evidence of NC marker expression, such as that of *KRT8*, *KRT18* and *KRT19*, *T* and *LGALS3* in cells of the adult human NP, also supports this hypothesis [32,37,44–46,57,72,73].

Contradictory to this, it is also hypothesized that the NP is, in fact, synthesized by the mesenchyme surrounding it during development. It is suggested that this process is driven by NCs, which then undergo a form of cell death [62]. Evidence supporting this theory is limited; however, the identification of a progenitor cell niche expressing markers that are characteristic of bone marrow-derived MSCs in the adult human IVD was thought to corroborate this [74,75]. However, such conclusions may be flawed, in that similar patterns of MSC marker expression were also noted in cells extracted from the AF of the same tissues and detection of these in the NP may be due to infiltration of cells from the inner AF to the NP, which is hypothesized to coincide with the degenerative process and is considered to be an endogenous attempt at repairing tissue damage [76]. Alternatively, it is possible that contamination of NP tissue with annular material during separation of the regions *in vitro* would result in such observations [74].

Much research is now focusing on the interactions between NC and mature NP cells. There is evidence to suggest that NCs may play a role in the maintenance of a healthy NP through increased synthesis of matrix PGs when NP cells and NCs are cocultured [77]. In addition to this, NCs have been demonstrated to secrete increased levels of PGs compared with mature NP cells [51,78,79]. Similarly, culture media conditioned with factors secreted by NCs have been

shown to increase PG expression in chondrocyte-like NP cells, as well as facilitating the differentiation of MSCs to an immature NP cell phenotype [80]. Interestingly, increased sensitivity of NCs to deprivation of nutrients may, in fact, precede loss of these specialized cells from the NP tissue, as the regression of NCs with maturity in the human IVD is thought to coincide with decreased vascular supply and a subsequent drop in nutrient availability [81]. Furthermore, NCs also have a decreased resistance to mechanical loading conditions and lose their notochordal phenotype compared with NP cells [82]. Finally, it appears that factors secreted by NCs are NP protective, in that they prevent NP cell death by the inhibition of key caspases in the apoptotic cascade and promote matrix anabolism through the downregulation of *MMP-3*, although it is noteworthy that *ADAMTS-4* expression was, in fact, increased in NC-conditioned media [83]. Collectively, these data suggest that NCs are predisposed to producing a healthier NP matrix. Thus, for the purposes of tissue regeneration, implantation of NC-like cells into the degenerate disc may produce a healthier matrix than that of NP-like cells under the same conditions. However, given the difficulties associated with the isolation of clinically relevant numbers of human NCs from fetal or pediatric tissue, these cells do not currently offer a viable solution for regenerative medicine applications. Differentiation of progenitor cells, such as MSCs, towards NC-like cells may offer an attractive alternative, although crucially the phenotype of human NCs is currently unknown and further studies are required to elucidate this.

The IVD niche

One of the major considerations for novel therapies is the response and tolerance of implanted cells to the physical and chemical microenvironment of the IVD. The adult IVD is generally an avascular tissue, although some vascularity is observed in the outer AF of healthy discs [84,85]. Cells in the centre of the larger lumbar NP reside up to 8 mm from the nearest blood supply [86], and oxygen and glucose levels in this central region are subsequently very low [87,88]. Nutrient and gas diffusion from blood vessels within the vertebral bodies, across the CEP, supply the NP with oxygen and glucose, while metabolic waste products are removed via the same process, and the permeability and diffusion properties of the tissue are therefore vital to the maintenance of nutritional levels in the disc [89,90]. Impaired nutrient and gaseous supply is associated with degeneration of the disc [91], as calcification of the CEP reduces permeability and prevents diffusion [92], although it is hypothesized that neovascularization in the IVD during degeneration may, in fact, result in elevated oxygen and nutrient concentrations. Nutrient deprivation in adult human NP cells negatively affects cell viability, while also reducing matrix anabolism and favoring catabolic processes, and is considered more detrimental to NP homeostasis than increased mechanical stress [93]. Hypoxia does not appear to be deleterious to the viability of NP cells [94], with increased

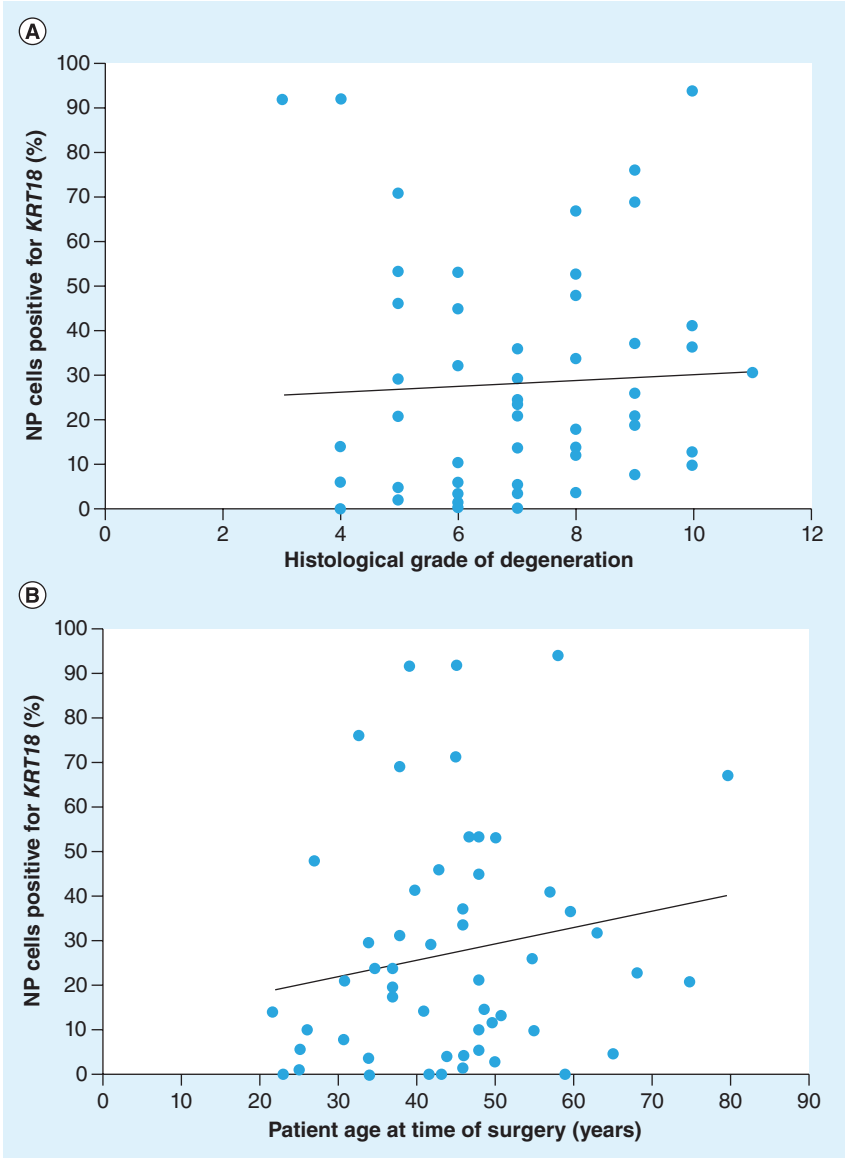

Figure 2. Analysis of *KRT18* immunopositivity. The percentage of NP cells positive for *KRT18* expression was plotted against **(A)** histological grade of degeneration and **(B)** patient age at time of surgery. No significant correlation was noted for either analysis.
NP: Nucleus pulposus.

expression of PGs and promotion of a gelatinous (as opposed to a fibrous) matrix having been reported [95]. It is thought that NP cells adapt to the hypoxic *in vivo* environment by expressing hypoxia-inducible factors [96] and exposure to a hypoxic environment may, in fact, promote cellular proliferation [97]. Decreased NP pH is characteristic of IVD degeneration and is thought to be caused by increased lactate concentration in the tissue [98], although it is hypothesized that vascular invasion in severely degenerate tissues may, in fact, increase the pH to values that are more typical of healthy NP [86]. Studies have revealed that a reduction in NP pH significantly decreases glycosaminoglycan production [99] and that expression of ASIC3 is crucial for the survival of IVD cells in the acidic disc microenvironment [100]. In addition to reduced pH, glucose and oxygen levels, cells of the NP are exposed to an escalated cytokine challenge compared with other tissues, with levels of inflammatory cytokines, such as IL-1α, IL-1β, TNF-α and IL-6, which are associated with the promotion of matrix catabolism, being increased with progressing degeneration of the disc [28,101–103]. NP cells partially adapt to this high cytokine milieu through the expression of antagonists that sequester the activated cytokines, although evidence suggests that antagonist levels are not sufficient to offset the high levels of cytokines detected in the degenerate IVD, and thus an imbalance between matrix formation and destruction persists, leading to matrix degradation [101,104].

Thus, the IVD, and particularly the central NP, is a highly specialized microenvironment, with cells of the tissue adapted to survive in a mechanically loaded, low-glucose, hypoxic and acidic niche, as well as to exposure to high levels of inflammatory cytokines. One of the important considerations for tissue engineering and regenerative medicine strategies are the effects of these *in vivo* conditions on implanted cells. Several studies have now investigated the effects of IVD-like culture conditions on MSC survival and differentiation. In hypoxia, MSC differentiation to a chondrocyte-like phenotype and cellular viability are promoted [79,105], and specifically, expression of NP-specific genes is increased in hypoxic culture in the presence of certain growth factors [105]. Similarly, an IVD-like pH of 6.8 inhibits PG expression in differentiated MSCs, with decreased cellular proliferation also having been noted [106]. Conversely, glucose deprivation has been demonstrated to stimulate the expression of matrix molecules in cultured MSCs [106]; however, combining these culture conditions is detrimental to the differentiation of MSCs, with decreased cellular proliferation and collagen and PG expression having been noted, suggesting that the beneficial effects of IVD-like low-glucose culture are not sufficient for promotion of stem cell differentiation when other environmental factors are considered [106]. Finally, it is crucial to determine MSC response to challenge by proinflammatory cytokines so that we can fully understand how these cells will react postimplantation in a degenerate IVD niche. Culture of MSCs in the presence of IL-1β significantly decreases culture pellet size, and cells produce an ECM with atypical mechanical strength and decreased expression of matrix molecules [107]. However, when the same study was performed under low-oxygen conditions, improved chondrogenic potential was noted [107]. Similarly, IL-1α and TNF-α inhibit chondrogenesis of human MSCs [108]. Inhibiting the actions of proinflammatory mediators such as IL-1 through the use of receptor antagonists or molecules designed to block downstream signaling cascades may be advantageous to IVD regenerative strategies [104], ameliorating the detrimental effects of these cytokines on potential cell sources for disc repair. As yet, however, results are inconclusive and require further investigation regarding the impact of such treatments on native disc cells.

Implications for IVD regeneration

The evidence to date would suggest that in order to provide a patient with a treatment that will regenerate tissue and restore function to the disc, it is imperative that cells implanted into the spine – be it injected or as part of an engineered scaffold – differentiate into the correct phenotype (depicted through expression of identified NP markers). In terms of ontogeny, there is evidence to suggest that cells within the adult NP are notochordally derived and that factors secreted by NCs may promote a healthy NP; therefore, cells differentiated to this phenotype may be a better end-stage cell for the purposes of tissue regeneration. Finally, the degenerate disc niche is a harsh environment that few cells are adapted to survive in, and therefore any cells to be utilized for cell-based therapies must be evaluated under these conditions, so that we are certain of their suitability to re-establish functionality *in vivo*.

Future perspective

MSC-based therapies for the repair of the degenerate IVD offer an exciting future

possibility. However, while elucidation of the NP phenotype offers a tool to assess appropriate MSC differentiation, a number of obstacles lie between the current status in the field and the translation of a novel regenerative therapy into the clinic. The first major challenge lies in the identification of the stage of degeneration at which cell-based therapies would be most efficacious; this is most likely earlier than current surgical interventions, when cell changes occur before loss of tissue integrity. Such identification relies on the need for improved *in vivo* imaging techniques to identify earlier stages of degeneration, or the identification of appropriate biomarkers of early disc degeneration. At present, we rely on MRI methodologies to identify disc degeneration, but such images are not able to distinguish between the distinct grades of degeneration (where aberrant cell biology is apparent), and thus there is a need for more detailed systems to be developed.

Alongside this, the optimal source of cells is yet to be elucidated. While MSCs, particularly adipose-derived MSCs, appear to be the most favorable cell source for regeneration of the IVD [37], emerging evidence regarding the potential of NCs for such purposes further complicates the search for cells to be used in implantation [77,80,83]. However, the human NC phenotype is currently unknown, and thus it is crucial that we now determine unambiguously the NP cell and NC phenotype. Only once this is proven will it be possible to decide which of the two distinct cell types are most suitable for regeneration.

Assuming MSCs are to be used, it remains to be determined whether these cells should be implanted directly and appropriate differentiation allowed to occur *in vivo*, or whether pre-differentiation *in vitro* is required to ensure correct differentiation of cells, in which case a protocol for differentiation must be established. If an *in vivo* approach is to be taken, a noninvasive method for testing the appropriate differentiation of cells

Executive summary

Elucidation of the nucleus pulposus cell phenotype

- Cells of the adult human nucleus pulposus (NP) were previously considered to be chondrocyte-like in terms of their morphology and phenotype, but it was revealed that NP and articular chondrocyte cells secrete a distinctly different extracellular matrix, thus highlighting the need for the elucidation of NP-specific marker genes.
- Microarray studies in a variety of animal models identified a panel of genes that are differentially expressed between NP and articular chondrocyte/annulus fibrosus cells.
- Analysis of these marker genes in a large cohort will enable the assessment of expression levels across a wide range of developmental stages, ages and degenerative scores.
- Localization of protein expression in a large cohort of human samples may identify cell subpopulations within the adult NP with different profiles and functions.

Importance of elucidating NP cell ontogeny

- It has been demonstrated that cells of the embryonic NP are notochordal in origin.
- However, as morphologically distinct notochordal cells are lost from the human NP with aging, what remains unclear is whether cells of the adult human NP are also notochordally derived, or whether they originate from the mesenchymally derived annulus fibrosus and infiltrate the NP.
- Recent evidence from lineage tracing and gene expression studies would support the hypothesis that adult NP cells are indeed notochordal in origin.
- Data are emerging that suggests that notochordal cells are predisposed to produce a more healthy extracellular matrix than cells of the adult human NP, which may indicate that these cells are a better source of cells for regeneration.
- However, such strategies are currently limited by difficulties in isolating sufficient cell numbers for treatment and that the human notochordal cell phenotype is not known.

The intervertebral disc niche

- The chemical microenvironment of the degenerate intervertebral disc (IVD) will no doubt influence any implanted cells or scaffolds used for regeneration of the tissue.
- Thus, it is imperative to assess the impact of a harsh *in vivo* environment prior to translation of any novel methods from the laboratory to the clinic.

Implications for IVD regeneration

- Identification of the correct and optimal phenotype for cells to be implanted for IVD regenerative strategies is crucial.
- Differentiation of cells to a notochordal-like phenotype may be more optimal for cells intended for regeneration, but the phenotype of human notochordal cells is currently unclear.
- Once the most suitable cells for implantation have been identified, these must be evaluated under environmental conditions akin to those of the degenerate IVD, or through preclinical and clinical studies.

is needed, given the potential damage caused by biopsying patients postimplantation.

The use of animal models to test these potential therapies also presents difficulties, given their incompatibility with the human model of IVD degeneration and interspecies variation relating to cell phenotype. However, improved bioreactor-based *ex vivo* human models offer the potential to circumvent these issues and would allow rapid testing of cell-based therapies to elucidate optimal cell concentrations and the need for novel biomaterials. They would also offer a system that would allow testing of therapies under conditions mimicking the harsh physiochemical environment of the degenerate IVD, such as reduced pH, oxygen and nutrient concentration, abnormal mechanical load and the presence of catabolic cytokines.

Thus, while the path for any novel IVD regenerative therapy requires the elucidation of all of the factors discussed here, advances in the field over recent years suggest that the concept is a viable long-term alternative to current clinical interventions.

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