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OSMOSENSING, OSMOSIGNALLING AND INFLAMMATION: HOW INTERVERTEBRAL DISC CELLS RESPOND TO ALTERED OSMOLARITY

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Abstract

Intervertebral disc (IVD) cells are naturally exposed to high osmolarity and complex mechanical loading, which drive microenvironmental osmotic changes. Age- and degeneration-induced degradation of the IVD's extracellular matrix causes osmotic imbalance, which, together with an altered function of cellular receptors and signalling pathways, instigates local osmotic stress. Cellular responses to osmotic stress include osmoadaptation and activation of pro-inflammatory pathways. This review summarises the current knowledge on how IVD cells sense local osmotic changes and translate these signals into physiological or pathophysiological responses, with a focus on inflammation. Furthermore, it discusses the expression and function of putative membrane osmosensors (*e.g.* solute carrier transporters, transient receptor potential channels, aquaporins and acid-sensing ion channels) and osmosignalling mediators [*e.g.* tonicity response-element-binding protein/nuclear factor of activated T-cells 5 (TonEBP/NFAT5), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)] in healthy and degenerated IVDs. Finally, an overview of the potential therapeutic targets for modifying osmosensing and osmosignalling in degenerated IVDs is provided.

Keywords: Intervertebral disc degeneration, degenerative disc disease, osmolarity, hyper-osmolarity, hypo-osmolarity, osmotic, inflammatory, transient receptor potential channel, aquaporin, tonicity-responsive enhancer binding protein.

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List of abbreviations

4αPDD	4α-phorbol-12,13-didecanoate
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AF	annulus fibrosus
AQPs	aquaporins
ASICs	acid sensing ion channels
ATM	ataxia telangiectasia-mutated
BMP-2	bone morphogenetic protein 2
Cas	CRISPR-associated
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
CS	chondroitin sulphate
dCas	deactivated Cas
DDD	degenerative disc disease
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
GAG	glycosaminoglycan
IL	interleukin
IVD	intervertebral disc
JNK	c-Jun NH_2-terminal kinase
KRAB	Krüppel-associated box

KS	keratan sulphate
MAPKs	mitogen-activated protein kinases
MMPs	matrix metalloproteinases
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-like receptor protein 3
NOD	nucleotide-binding domain
NP	nucleus pulposus
OREBP	osmotic response-element-binding protein
PGE2	prostaglandin E2
PGs	proteoglycans
PKC	protein kinase C
ROS	reactive oxygen species
RVD	regulatory volume decrease
RVI	regulatory volume increase
SLC	solute carrier
TFG-β	transforming growth factor-β
TNF-α	tumour necrosis factor-α
TonEBP/NFAT5	tonicity response element-binding protein/nuclear factor of activated T-cells 5
TRP	transient receptor potential
TRPV	TRP vanilloid
TZ	transition zone

Introduction

The IVD is a mechanically loaded tissue with early signs of degeneration that are associated with a loss of PGs and, thus, changes in the osmotic environment (Roughley *et al.*, 2002; Urban and McMullin, 1988). The IVD contains a small population of cells embedded in an ECM that is predominantly composed of water (60-99 %) (Cassinelli *et al.*, 2001; Oegema, 1993). The mechanical properties of the IVD are determined by its biochemical structure, with the highly hydrated NP in the middle, surrounded by the AF. The AF is rich in collagen type I towards the outer rim, where it provides greater strength, whereas the inner part is composed of fibrocartilage that steadily fuses into a TZ with the NP (Urban and Roberts, 2003). The NP, an immune-privileged structure, consists of sparsely distributed cells surrounded by a gelatinous network that is primarily composed of collagen type II and PGs (Takada *et al.*, 2002), showing the IVD's similarity to cartilage. With aging, the IVD undergoes degenerative changes, which are associated with tissue weakening, dehydration and loss of ECM components (Luoma *et al.*, 2000; Maher *et al.*, 2017). Associated changes in tissue hydration and, hence, in the IVD's osmotic environment influence the IVD's mechanical properties (Wuertz *et al.*, 2007), possibly leading to lower-back pain, activity limitation, disability (Walker, 2000) and, consequently, a high economic burden on the society (Wieser *et al.*, 2011). The goals of this review were (1) to summarise the current knowledge on how IVD cells sense and respond to osmotic changes, (2) to provide an outlook on possible future research on disc hydration and osmolarity and (3) to highlight potential therapies related to IVD osmosignalling.

Osmoregulation

General concept of osmoregulation

The capacity to maintain an osmotic balance (= osmoregulation) and control the cell volume is important for preserving cell function. The volume of a cell depends on the water movements across its membrane, driven by osmotic gradients that develop from differences in the chemical concentrations of the intra- and extra-cellular fluids under normal physiological conditions (Lodish *et al.*, 2000). Solutions with higher solvent concentration tend to have a lower water content and *vice versa*. Hence, water will move across the membrane from the solution with the lower solute (or higher water) content to the one with the higher solute (or lower water) content – a phenomenon that is defined as osmosis (Lodish *et al.*, 2000). Osmolarity (or osmotic concentration) is the concentration of solutes in a solution and it is expressed in osmol/L, whereas osmolality is expressed in osmol/kg (or Osm/kg); both terms are often used interchangeably (Baltz, 2012). A decrease or increase in the extracellular osmolarity will result in cell swelling (= inflow of water) or cell shrinkage (= outflow of water), respectively (Hoffmann *et al.*, 2009). To resist cell swelling, the osmotic pressure – defined as a minimal hydrostatic pressure necessary to stop water from diffusing across two barriers – has to be developed (Lodish *et al.*, 2000).

Osmoregulation in the IVD

PGs are crucial for maintaining hydration and osmotic pressure in the IVD, with aggrecan being the primary type (Urban and Roberts, 2003). Aggrecans are composed of three globular domains (G1, G2, G3) and attached GAG side chains (Sivan *et al.*, 2014). The primary types of GAGs found in the IVD are CS and KS. The ionic balance of the IVDs extracellular matrix is regulated by the negatively charged GAGs (Johnson *et al.*, 2014). The sulphated GAGs of the aggrecans create a high negative charge, contributing to the aggrecan's ability to electrostatically bind water. On the tissue level, this translates into the generation of an osmotic pressure in the IVD, causing the NP to ingest water; it also contributes to the high swelling pressure and load-bearing ability of the IVD (Erwin and Hood, 2014; Urban and Maroudas, 1981; Urban *et al.*, 1979; Urban and Roberts, 2003) and the resistance to high compressive loads experienced, for example, during weight lifting or forward bending activities (Shirazi-Adl, 2006). If the applied loading exceeds the osmotic pressure, water is diffused and the osmotic pressure increases, while water is absorbed during IVD unloading, resulting in an osmotic equilibrium (McMillan *et al.*, 1996; Urban, 1993). In the lack of external loads, the hydrostatic

pressure is maintained due to the structure of the AF and endplates that constrain the swelling of the NP (Newell *et al.*, 2017). *In vivo* studies demonstrate that the NP's water content decreases by around 15 % under loading conditions (human lumbar IVDs, unloaded *vs.* loaded, 1500 N, 6 h) (McMillan *et al.*, 1996). However, in contrast to static or high-impact loading, daily physiological loading, such as dynamic loading or loading at moderate speed [*e.g.* jogging (Belavy *et al.*, 2017)], are beneficial for the IVD's hydration and, therefore, the tissue's health (Belavy *et al.*, 2016).

For a normal osmotic function in the IVD, it is essential that the aggrecan content, charge and size remain as large as possible. However, aggrecans are enzymatically cleaved by proteinases such as MMPs and aggrecanases, whose expression increases in degenerated IVDs (Le Maitre *et al.*, 2004; Molinos *et al.*, 2015; Sztrolovics *et al.*, 1997). Age-related or degeneration-induced loss of aggrecan causes a drop in osmotic pressure, reducing the IVD's ability to respond to mechanical loads. In a healthy state, the extracellular osmolarity can vary from ~ 430 (iso-osmotic) to ~ 496 mOsm/L (hyper-osmotic) (Ishihara *et al.*, 1997; van Dijk *et al.*, 2011) – values which are in a physiological range for IVD cells but would be considered high for most mammalian cells (Appelboom *et al.*, 1956; Brocker *et al.*, 2012). The IVD's osmolarity can decrease to around 300 mOsm/L (hypo-osmotic) in degenerated IVDs (Wuertz *et al.*, 2007) due to a loss of PGs and, thus, IVD hydration and occurrence of fibrosis; however, the same osmolarity would be considered physiological for cells of other tissues (Hooper *et al.*, 2015). In this review, the terms: hyper-, hypo- and iso-osmotic are used in the context of the IVD tissue. Although an altered osmotic environment is rather a hallmark of IVD degeneration than its primary cause, the reduced tissue osmolarity can activate and/or interplay with pro-inflammatory factors and catabolic responses and, hence, promote IVD inflammation and DDD (see 'Cell responses to osmotic changes: target genes and signalling pathways').

Osmolarity-related cell volume changes

At the cellular level, a change in intra- or extra-cellular osmolarity causes mammalian cell volume regulation by the solubility-diffusion water transport across the cell membrane through several water, ion and molecule transport pathways, such as pores, ion channels and membrane carriers (see 'Membrane proteins as potential osmosensors in the IVD') (Dawson, 1988; Reuss, 2012). The increased cell volume induces a prompt activation of the RVD, a mechanism that acts to recover the cell volume homeostasis. In contrast, hyper-osmolarity causes cell shrinkage, to which a cell responds by activating the RVI mechanism (McManus *et al.*, 1995). However, very limited information on the cell volume regulation in the IVD exists. In bovine NP cells, a decrease in extra-cellular osmolarity (from 430 to 230 mOsm/L) increases the cell volume by up to 20 %, leading to a reduced PG synthesis rate (Ishihara *et al.*, 1997). Changes in the IVD cell volume may involve depolarisation and reorganisation of the actin cytoskeleton and initiation of calcium transits from the intra-cellular stores (Pritchard *et al.*, 2002). Interestingly, the response to the hyper-osmotically-induced volume changes may be zone-dependent (AF *vs.* NP) due to the differences in the mechanical composition of these cells. In comparison with AF cells, NP cells were found to be stiffer and more viscous due to differences in the cytoskeletal arrangement (Guilak *et al.*, 1999). Additionally, vacuoles (or vesicles), which can be found in the notochordal cells within the NP tissue (canine), contain a low-osmolarity solution that is released into the cytoplasm under a condition of hyper-osmotic stress and helps to restore the osmotic balance (Hunter *et al.*, 2007). However, this mechanism has not been confirmed in other notochordal-free species and, therefore, cannot be generalised.

Membrane proteins as potential osmosensors in the IVD

Cells sense osmotic stress through membrane proteins such as carriers (SLC) or channels (TRP and AQPs) (Table 1**a-c**, Table 2, Fig. 1), which are responsible for transporting molecules (*e.g.* ions, sugars *etc.*) across the cell membrane. Carriers physically bind to a specific solute and change their own conformation to release the solute on the other side of the cell membrane; channels form a pore, which can open to allow a specific molecule to pass by diffusion or osmosis (Kulbacka *et al.*, 2017).

The SLC is a group of membrane transport proteins that includes over 400 transporters in humans. The SLC transporters play an important role in homeostasis by transporting soluble molecules (such as nutrients) across the lipid membranes (Perland and Fredriksson, 2017) and are involved in *i.e.* glucose transport (SLC5) (Hediger *et al.*, 2004), pH regulation (SLC16) (Jones and Morris, 2016), hormone and/or drug uptake (SLC21A12) (Alam *et al.*, 2016; Williams, 2013; Zair *et al.*, 2008). As shown in human NP cells, an increase in osmolarity (450 mOsm/L) upregulates the expression of the solute carriers SLC21A12 (= SLCO) and SLC5A3 but downregulates the expression of SLC16A6 (Boyd *et al.*, 2005). Although they have a putative role in regulatory volume mechanisms in other tissue types (Arroyo *et al.*, 2013), their exact role in the IVD is largely unknown.

The TRP channels, a superfamily of cation-selective transmembrane receptors, have recently emerged as potential contributors to IVD degeneration and discogenic pain (Alfredo Franco-Obregón, 2017; Krupkova *et al.*, 2017; Sadowska *et al.*, 2017; Walter *et al.*, 2016). TRP channels are multimodal ion channels regulated by a diverse range of stimuli, including mechanical and osmotic stress (Krupkova *et al.*, 2017; Numata *et al.*, 2011). Previous studies examining

Table 1a. Targets regulated by hyper-osmotic treatment. ↑ = upregulation, ↓ = downregulation. Only selected targets are discussed in the text.

Target gene (symbol)	Expression level change	Function	Model	Reference
Guanylate binding protein 1 (GBP1)	Gene ↓	Cell-cell interaction/ adhesion	Human IVD tissue	Boyd *et al.*, 2005
Kelch motif protein (KIAA1309)	Gene ↓	Cell-cell interaction/ adhesion	Human IVD tissue	Boyd *et al.*, 2005
Small inducible cytokine A2 (SCYA2)	Gene ↓	Cell-cell interaction/ adhesion	Human IVD tissue	Boyd *et al.*, 2005
Vascular cell adhesion molecule 1 (VCAM1)	Gene ↓	Cell-cell interaction/ adhesion	Human IVD tissue	Boyd *et al.*, 2005
Cyclin-dependent kinase inhibitor 1 ($p21^{WAF1}$)	Protein ↑	Cell-cycle, arrest of G0/ G1	Bovine NP cells	Mavrogonatou and Kletsas, 2009
Phospho- p38 MAPK	Protein ↑	Cell-cycle, arrest of G0/ G1	Bovine NP cells	Mavrogonatou and Kletsas, 2009
Phospho- p53 MAPK	Protein ↑	Cell-cycle, arrest of G0/ G1	Bovine NP cells	Mavrogonatou and Kletsas, 2009
Aryl hydrocarbon receptor translocator-like (ARNTL)	Gene ↑	Cell-cycle/apoptosis	Human IVD tissue	Boyd *et al.*, 2005
CDC28 protein kinase 2 (CKS2)	Gene ↑	Cell-cycle/apoptosis	Human IVD tissue	Boyd *et al.*, 2005
Growth arrest specific 1 (GAS1)	Gene ↑	Cell-cycle/apoptosis	Human IVD tissue	Boyd *et al.*, 2005
Mucosa-associated lymphoid tissue translocation gene (MALT1)	Gene ↑	Cell-cycle/apoptosis	Human IVD tissue	Boyd *et al.*, 2005
ATPase Na^+/K^+ transporting subunit alpha 1 (ATP1A1)	Gene ↑ Protein ↑	Cell-cycle/DNA synthesis	Bovine IVD	Mavrogonatou *et al.*, 2015
Caspase 8 (CASP8)	Gene ↓	Cell-cycle/apoptosis	Human IVD tissue	Boyd *et al.*, 2005
Rho-related BTB domain containing 1 (RHOBTB1)	Gene ↓	Cell-cycle/apoptosis	Human IVD tissue	Boyd *et al.*, 2005
TNF-induced protein (GG2-1)	Gene ↓	Cell-cycle/apoptosis	Human IVD tissue	Boyd *et al.*, 2005
AQP-3	Protein ↑	Cell membrane protein	Mouse NP cells	Palacio-Mancheno *et al.*, 2017
TRPV4	Protein ↓	Cell membrane protein	Mouse NP cells	Palacio-Mancheno *et al.*, 2017

several tissue types, such as kidney (Birder *et al.*, 2002; Vriens *et al.*, 2004), CNS (Liedtke and Friedman, 2003), smooth muscles (Muraki *et al.*, 2003) and others (Ueda *et al.*, 2011; Yang *et al.*, 2012), point towards the TRPV subfamily (especially TRPV4) as a potential cellular osmo- and volume-sensor involved in the RVD mechanism (Becker *et al.*, 2005; Hdud *et al.*, 2014; Liedtke *et al.*, 2000; Pan *et al.*, 2008). Interestingly, Becker *et al.* (2005) demonstrate that TRPV4 play a key role in the cell-volume regulation by transiently transfecting CHO cells with TRPV4: CHO cell volume decreases after hypo-osmotic (200 mOsm/L)

Table 1b. Targets regulated by hyper-osmotic treatment. ↑ = upregulation, ↓ = downregulation. Only selected targets are discussed in the text.

Target gene (symbol)	Expression level change	Function	Model	Reference
MMP-2	Gene ↑	Enzyme-matrix turnover	Bovine NP cells (3D)	Neidlinger-Wilke *et al.*, 2012
ADAMTS1	Gene ↑	Enzyme-matrix turnover	Human IVD tissue	Boyd *et al.*, 2005
Brain-derived neurotrophic factor (BDNF)	Gene ↑	Growth factor	Human IVD tissue	Boyd *et al.*, 2005
Muskelin (MKLN1)	Gene ↑	Growth factor	Human IVD tissue	Boyd *et al.*, 2005
Zinc finger protein 238 (ANF238)	Gene ↑	Growth factor	Human IVD tissue	Boyd *et al.*, 2005
IL-6	Gene ↓	Growth factors/ cytokines	Human IVD tissue	Boyd *et al.*, 2005
Norrie disease protein/ Norrin (NDP)	Gene ↓	Growth factors/ cytokines	Human IVD tissue	Boyd *et al.*, 2005
Homologous to mouse potassium-gated channel, Isk-related subfamily (KCNE4)	Gene ↑	Ion transport	Human IVD tissue	Boyd *et al.*, 2005
Solute carrier family 16 (monocarboxylic acid transporter) (SLC16A6)	Gene ↓	Ion transport	Human IVD tissue	Boyd *et al.*, 2005
Solute carrier family 21 member 12 (SLC21A12)	Gene ↑	Ion transport	Human IVD tissue	Boyd *et al.*, 2005
Solute carrier family 4 member 11 (SLC4A11)	Gene ↑ Protein ↑	Ion transport	Bovine NP cells	Mavrogonatou *et al.*, 2015
Solute carrier family 5 member 3 (SLC5A3, SMIT1)	Gene ↑	Ion transport	Human IVD tissue	Boyd *et al.*, 2005
Solute carrier family 5 member 3 (SLC5A3, SMIT1)	Gene ↑ Protein ↑	Ion transport	Bovine NP cells	Mavrogonatou *et al.*, 2015
Aggrecan (ACAN)	Gene ↑	IVD's ECM components	Bovine NP cells (3D), human NP and AF cells (3D)	Neidlinger-Wilke *et al.*, 2012; Wuertz *et al.*, 2007
Aggrecan (ACAN)	Gene ↓ Protein ↓	IVD's ECM components	Porcine NP in organ culture	Li *et al.*, 2016
Biglycan (BGN)	Gene ↑	IVD's ECM components	Porcine TZ cells	Chen *et al.*, 2002
Biglycan (BGN)	Gene ↓	IVD's ECM components	Porcine NP cells	Chen *et al.*, 2002

Table 1c. Targets regulated by hyper-osmotic treatment. ↑ = upregulation, ↓ = downregulation. Only selected targets are discussed in the text.

Target gene (symbol)	Expression level change	Function	Model	Reference
Collagen-1 (COL1A1)	Gene ↓	IVD's ECM components	Human NP, AF cells (3D)	Wuertz *et al.*, 2007
Collagen-2 (COL2A1)	Gene ↑	IVD's ECM components	Bovine AF cells (3D)	Wuertz *et al.*, 2007
Collagen-2 (COL2A1)	Gene ↓ Protein ↓	IVD's ECM components	Porcine NP in organ culture	Li *et al.*, 2016
Decorin (DCN)	Gene ↑	IVD's ECM components	Porcine TZ cells	Chen *et al.*, 2002
Decorin (DCN)	Gene ↓	IVD's ECM components	Porcine NP cells	Chen *et al.*, 2002
Lumican (LUM)	Gene ↓	IVD's ECM components	Porcine NP cells	Chen *et al.*, 2002
Ephrin-B2 (EFNB2)	Gene ↑	Signal transduction/ transcription	Human IVD tissue	Boyd *et al.*, 2005
Musculoapo-neurotic fibrosarcoma oncogene (MAF)	Gene ↑	Signal transduction/ transcription	Human IVD tissue	Boyd *et al.*, 2005
Nuclear receptor coactivator 3 (NCOA3)	Gene ↑	Signal transduction/ transcription	Human IVD tissue	Boyd *et al.*, 2005
Oncogene TC21 (RRAS2/TC21)	Gene ↑	Signal transduction/ transcription	Human IVD tissue	Boyd *et al.*, 2005
SOX9	Gene ↓ Protein ↓	Transcription factor	Porcine NP in organ culture	Li *et al.*, 2016

treatment (RVD response after swelling), in contrast to untransfected CHO control cells. Hence, Becker *et al.* (2005) and others (Arniges *et al.*, 2004; Phan *et al.*, 2009) suggest that cell swelling caused by hypo-osmotic treatment leads to the generation of tension on the cell membrane and, thus, activation [= opening of a channel pore (Liu and Montell, 2015)] of the TRPV4 channel, which mediates the influx of extra-cellular Ca^{2+} that initiates a signalling cascades, causing an RVD response. TRP channels, including TRPV4, are expressed in the human, bovine and mouse IVD (Palacio-Mancheno *et al.*, 2017; Sadowska *et al.*, 2017; Walter *et al.*, 2016). In the human IVD, hypo-osmotic conditions (200-334 mOsm/L) induce an up-regulation of TRPV4 (on the protein level), leading to an activated calcium influx (Walter *et al.*, 2016). In contrast, hyper-osmolarity (530 mOsm/L), combined with cyclic loading (10 min on and off for 1.5 h/d), significantly downregulates TRPV4 expression (mouse NP cells) (Palacio-Mancheno *et al.*, 2017). Moreover, the expression and/or activity of TRPV4 is modulated by the inflammation (upregulated cytokines IL-6 and IL-1β) and correlates with pro-inflammatory cytokines in the IVD and cartilage (Clark *et al.*, 2010; Phan *et al.*, 2009; Sadowska *et al.*, 2017; Walter *et al.*, 2016). Therefore, Walter *et al.* (2016) suggest that alternations in TRPV4-mediated sensation of osmotic changes (also known as osmosensing) could aid the progression of disc degeneration. Additionally, both TRPV4-mediated osmotic and inflammatory signals may be regulated through the p38/MAPK and ERK1/2 pathways (Chen *et al.*, 2013; Hdud *et al.*, 2014; Qu *et al.*, 2016).

AQPs are small transmembrane channel proteins responsible for water transport and are of relevance in osmoregulation. The presence of AQPs is confirmed in the healthy human NP (Richardson *et al.*, 2008), with decreased expression during degeneration as an adaptive mechanism demonstrated in various species: rat NP and AF (Tas *et al.*, 2012), rabbit NP (Wang and Zhu, 2011), human NP (Hoffman *et al.*, 2017; Johnson *et al.*, 2015). These findings are in line

with the observed downregulation of AQP-1 under reduced osmolarity [rabbit NP (Wang and Zhu, 2011)] and the increased expression of AQP-3 under hyper-osmotic conditions [mouse NP (Palacio-Mancheno *et al.*, 2017)] and consequent NP maturation (but without changes in AQP-1). Furthermore, TRPV4 and AQP-4 interact and form a channel complex (in astrocytes), which might constitute an important link in the cell volume homeostasis by integrating water transport and calcium signalling (at least in the CNS) (Benfenati *et al.*, 2011; Jo *et al.*, 2015).

Additionally, AQPs might play a role in inflammation in the IVD and cartilage (Haneda *et al.*, 2018; Takeuchi *et al.*, 2018; Xie *et al.*, 2016). In human chondrocytes, AQP-1 co-localises (on the protein level, as shown by immunofluorescence) with the catabolic factor ADAMTS-4 (an aggrecan-degrading enzyme involved in IVD and cartilage degeneration) and a downregulation of AQP-1 decreases the expression of ADAMTS-4 (Haneda *et al.*, 2018; Sun *et al.*, 2015). Additionally, a knockdown of AQP-9 (human chondrocytes) decreases the mRNA levels of other catabolic factors (Takeuchi *et al.*, 2018). On the other hand, an overexpression of AQP-3 (human degenerated NP cells) decreases the expression of ADAMTS 4 and 5 and suppresses the Wnt/β-catenin signalling (Xie *et al.*, 2016), which is involved in IVD cell senescence (Hiyama *et al.*, 2010b; Hiyama *et al.*, 2011; Wang *et al.*, 2012). These findings suggest a protective role of AQP-3 against disc degeneration (Xie *et al.*, 2016). Yet, in corneal epithelial cells, hyper-osmotic treatment (450, 500 and 550 mOsm/L) induces the upregulation of AQP-5 (*via* JNKs pathway), leading to an upregulation of IL-1β, IL-6, IL-8, TNF-α and caspase-1 (Ren *et al.*, 2017), with similar findings in cartilage (Cai *et al.*, 2017) and IVD (Hoffman *et al.*, 2017; Snuggs *et al.*, 2017).

Cell responses to osmotic changes: target genes and signalling pathways

Cellular responses to changes in the osmotic environment are facilitated through multiple signalling mediators, including TonEBP/NFAT5, also known as OREBP, or signal-transduction pathways, such as p38/MAPK, ERK and JNK pathways (Aramburu and Lopez-Rodriguez, 2009; Dong *et al.*, 2014; Hdud *et al.*, 2014; Li *et al.*, 2016; Ren *et al.*, 2017; Sheikh-Hamad and Gustin, 2004). These mediators do not only play a role in the cell volume regulatory mechanisms, but also initiate changes in other cellular processes, such as cell survival, matrix turnover and inflammation (Table 1**a**-**c**, Fig. 1). Hence, the osmotic challenge and accompanying altered gene expression can contribute to the development and/or progression of the inflammatory responses in the IVD. However, the inflammation in the IVD is much more complex than how it is presented in the context of the IVD's osmotic environment (present review) and is reviewed by Molinos *et al.* (2015). Briefly, inflammation can occur within the IVD, despite the IVD being immune-privileged (in a healthy state), as NP cells produce pro-inflammatory cytokines, while macrophages infiltrating the damaged IVD can further exacerbate the inflammation (Molinos *et al.*, 2015).

One of the most prominent signal transduction pathways that facilitates mammalian cell responses to numerous extracellular signals is the MAPK family. Three major members of the MAPK family are ERK, JNK and p38, which can be activated by multiple stimuli, such as growth factors (Hiyama *et al.*, 2010a; Uchiyama *et al.*, 2007), inflammatory cytokines (Klawitter *et al.*, 2012) and osmotic stress (Li *et al.*, 2017). Activation of each of these pathways controls several cellular functions, including cell cycle progression (ERK), cell proliferation and survival (JNK), cell growth, cell differentiation, cell death, inflammation and matrix catabolism (p38) (Johnson and Lapadat, 2002; Studer *et al.*, 2007; Yang *et al.*, 2016). In this review, each signal-transduction pathway is presented in the context of the IVD cell response to osmotic stress. However, these signalling pathways are also activated by different stimuli and can induce a range of cell responses not necessarily addressed within this review (Wong, 2009). In the IVD, a hyper-osmotic treatment (500 and 600 mOsm/L) participates in the activation of the p38 pathway in bovine and rabbit NP cells (Dong *et al.*, 2014; Mavrogonatou and Kletsas, 2009) and results in the ATM-mediated phosphorylation of p53 in response to DNA damage caused by hyper-osmotic shock (Kishi *et al.*, 2001; Mavrogonatou and Kletsas, 2009). However, MAPK activation and signalling are cell-type specific, with JNK activation occurring upon hypo-osmotic stimulation in IVD cells and upon hyper-osmotic stimulation in chondrocytes (Racz *et al.*, 2007). Furthermore, conflicting activation triggers are described for the ERK pathway, with authors reporting distinctive osmotic conditions for both activation and inhibition. These differences may arise from i) using different animal models and different definition of osmolarity levels (hypo-, iso- and hyper-osmotic values), ii) adjusting the osmolarity with various agents and iii) using different culture conditions, *e.g.* supplementation with growth factors (Mavrogonatou and Kletsas, 2010). Activation of the ERK pathway under hyper-osmotic stress is observed in rabbit NP cells treated with 500-600 mOsm/L of medium adjusted with NaCl (Dong *et al.*, 2014), rat NP cells treated with 450 mOsm/L of medium (adjusting agent unknown) (Tsai *et al.*, 2007) and bovine NP cells treated with 500 mOsm/L of medium adjusted with urea (Mavrogonatou and Kletsas, 2012). In rat NP cells, ERK phosphorylation leads to the activation of TonEBP/NFAT5 (Tsai *et al.*, 2007) – an adaptation factor to high osmotic stress that protects IVD cells from undergoing apoptosis (Tsai *et al.*, 2006; Tsai *et al.*, 2007). In contrast, ERK inhibition can

Table 2. Targets regulated by hypo-osmotic treatment. ↑ = upregulation, ↓ = downregulation. Only selected targets are discussed in the text.

Target gene (symbol)	Expression level change	Function	Model	Reference
AQP-1	Protein ↓	Cell membrane proteins	Rabbit NP cells	Wang and Zhu, 2011
TRPV4	Receptor function (tyrosine phosphorylation) ↑	Cell membrane proteins	HEK293	Vriens *et al.*, 2004; Xu *et al.*, 2003
TRPV4	Protein ↑	Cell membrane proteins	Equine chondrocytes	Hdud *et al.*, 2014
TRPV4	Protein ↑	Cell membrane proteins	Human IVD	Walter *et al.*, 2016
Large-conductance Ca^{2+}-activated K^+ (BK_{Ca})	Protein ↑	Cell membrane proteins	Equine chondrocytes	Hdud *et al.*, 2014
MMP3	Gene ↑	Enzyme-matrix turnover	Bovine NP cells (3D)	Neidlinger-Wilke *et al.*, 2012
Collagen-2 (COL2A1)	Gene ↑	IVD's ECM components	Porcine TZ cells	Chen *et al.*, 2002
Collagen-2 (COL2A1)	Gene ↓ Protein ↓	IVD's ECM components	Porcine NP in organ culture	Li *et al.*, 2016
Aggrecan (ACAN)	Gene ↑	IVD's ECM components	Porcine TZ cells	Chen *et al.*, 2002
Aggrecan (ACAN)	Gene ↓ Protein↓	IVD's ECM components	Porcine NP in organ culture	Li *et al.*, 2016
Biglycan (BGN)	Gene ↑	IVD's ECM components	Porcine TZ cells	Chen *et al.*, 2002
Decorin (DCN)	Gene ↑	IVD's ECM components	Porcine TZ cells	Chen *et al.*, 2002
Lumican (LUM)	Gene ↓	IVD's ECM components	Porcine NP cells	Chen *et al.*, 2002
Tubulin (TUB)	Gene ↓	IVD's ECM components	Porcine NP cells	Chen *et al.*, 2002
ERK1/2	Protein ↑	Signaling pathway	Equine chondrocytes	Hdud *et al.*, 2014
SOX9	Gene ↓ Protein ↓	Transcription factor	Porcine NP in organ culture	Li *et al.*, 2016

lead to the suppression of TonEBP/NFAT5 and the augmentation of cell apoptosis [NP cells in a porcine disc culture (Li *et al.*, 2017), rat NP cells (Tsai *et al.*, 2007), rabbit NP cells (Dong *et al.*, 2014)]. Activation of p38 and JNK induces cell apoptosis [rabbit NP cells (Dong *et al.*, 2014)], indicating the involvement of these pathways in the degenerative shift under hyper-osmotic conditions. This is in line with the study by Haschtmann *et al.* (2006), in which, following a hyper-osmotic treatment (485 mOsm/L), cells (rabbit IVD in organ culture) exhibit a reversible drop in viability. Moreover in the IVD, the ERK pathway controls the expression of collagen type II (Chen *et al.*, 2002; Wuertz *et al.*, 2007) and MMP-2 (Neidlinger-Wilke *et al.*, 2012), while collagen type I (Wuertz *et al.*, 2007), IL-6 (Boyd *et al.*, 2005) and MMP-3 (Neidlinger-Wilke *et al.*, 2012) are identified as targets of the p38/MAPK pathway (Jung *et al.*, 2017; Park *et al.*, 2016; Sano *et al.*, 2001). However, aggrecans are regulated by both pathways: in bovine NP cells, lactoferricin-induced upregulation of aggrecan mRNA levels are decreased when p38/MAPK and ERK pathways are

inhibited by SD203580 and PD98059, respectively (Kim *et al.*, 2012). In a study by Tsirimonaki *et al.* (2013), prolonged activation of the ERK1/2 pathway increases the mRNA expression level of aggrecan in human NP cells. On the contrary, leptin-induced p38 phosphorylation upregulates aggrecanases and downregulates aggrecan on the gene and protein level in human NP cells (Li *et al.*, 2014b). Another signalling pathway that could be involved in the IVD's osmo-adaptation, but has not been investigated thus far, is the non-canonical PKC pathway. Increased activity of the PKC pathway is involved in the regulation of aggrecan expression, matrix synthesis and cell proliferation (Arai *et al.*, 2012; Rottmar *et al.*, 2014; Tsirimonaki *et al.*, 2013) in rat and human NP cells, as well as bovine chondrocytes, and may be involved in Ca^{2+}-mediated activation of the TRPV4 channel (Fan *et al.*, 2009; Xu *et al.*, 2003), which is linked to IVD osmosensing (Walter *et al.*, 2016).

One of the key cellular osmoregulative mediators is TonEBP/NFAT5, which modulates the expression of genes induced by osmotic stress. In response to hyper-osmotic challenges and upon the activation of the ERK and p38-MAPK pathways, TonEBP/NFAT5 accumulates in the cell nucleus (Ho, 2006; Tsai *et al.*, 2007) (Fig. 1). Consequently, it induces the expression of genes that are involved in the production of organic osmolytes (to counterbalance the osmotic challenge) (Lee *et al.*, 2011b), aggrecan (Tsai *et al.*, 2006) and the pro-inflammatory cytokines IL-1β and TNF-α

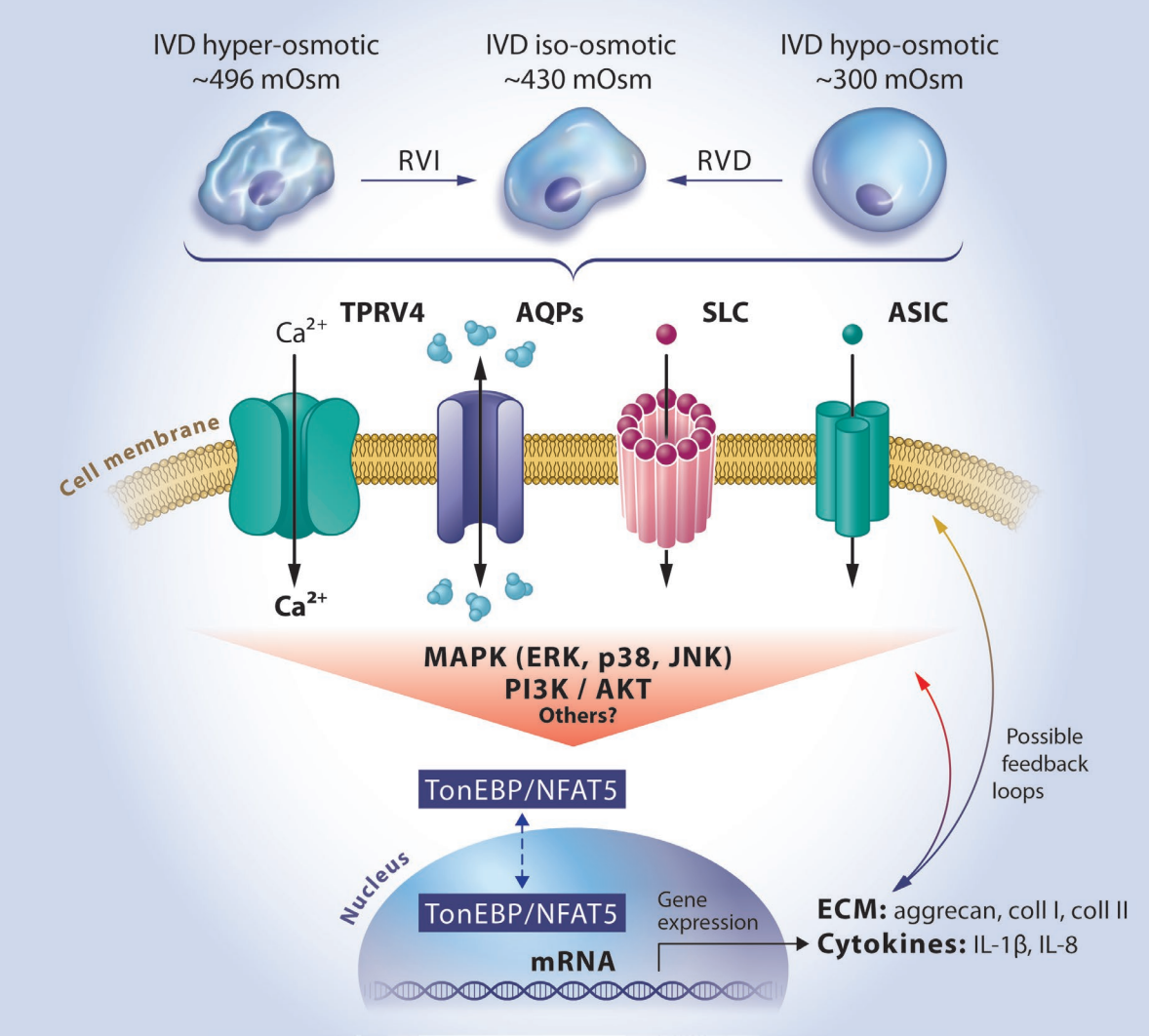

Fig. 1. Osmolarity-related changes in the IVD cells. Schematic representation of the IVD response to the osmotic stimuli. Changes in cell volume (top left: cell shrinkage under an hyper-osmotic challenge; top right: cell swelling under an hypo-osmotic challenge) trigger the activation of volume recovery mechanisms (RVI and RVD) to restore the homeostasis but can also act as stress signals, activating cell membrane receptors. TRP channels, AQPs, SLCs and ASICs are membrane proteins with a potentially crucial role in osmosensing and osmo-adaptation. Transduced signals activate the MAPKs pathway (middle). Further, osmolarity changes lead to increase in NFAT nuclear shuttling, which induces transcription of genes involved in matrix homeostasis and pro-inflammatory cytokines (bottom middle). These changes may activate a positive/negative feedback loop to the osmo-receptor and/or signalling pathways. However, the exact and complete osmosensing pathway has not been yet extensively studied in the disc.

(Johnson *et al.*, 2017; Lee *et al.*, 2008; Trama *et al.*, 2002), which have known implications in IVD degeneration (Lee *et al.*, 2011a; Risbud and Shapiro, 2014; Sadowska *et al.*, 2017). In the IVD, hyper-osmotic stress is not the only regulator of the TonEBP/NFAT5 complex. The expression and activity of TonEBP/NFAT5, which is calcium-dependent (Choi *et al.*, 2018; Hiyama *et al.*, 2009), can be also regulated by growth factors, such as BMP-2 and TFG-β (Halterman *et al.*, 2012; Hiyama *et al.*, 2010a). These multimodal activation mechanisms indicate that TonEBP/NFAT5 is critical not only for osmoregulation, but also for other cellular functions (*e.g.* cell survival, matrix synthesis, *etc.*). TonEBP/NFAT5 modulates the NF-κB pathway, which is a pivotal element in the cellular response to inflammation and stress, with downstream targets including TNF-α, IL-1β, IL-6 and IL-8 (Lopez-Rodriguez *et al.*, 2001; Roth *et al.*, 2010; Tak and Firestein, 2001). The NF-κB pathway is implicated in many chronic conditions such as osteoarthritis (Makarov, 2001), osteoporosis (Kim *et al.*, 2006) and IVD degeneration (Nasto *et al.*, 2012; Xu *et al.*, 2015; Zhongyi *et al.*, 2015). Changes in cell volume, triggered by hyper-osmotic conditions and sensed by an osmosensing channel (*e.g.* TRP), can be perceived by a cell as a stress signal. Such a signal can initiate a pro-inflammatory cascade *via* the ROS-mediated activation of the NF-κB (and/or p38/MAPK) pathway (Abolhassani *et al.*, 2008; Panahi *et al.*, 2018; Rajamaki *et al.*, 2016; Schwartz *et al.*, 2009) and activate the NLRP3 inflammasome – a protein complex, downstream targets of which are caspase-1 and IL-1β (Chen *et al.*, 2015). In various cell types, activation of the NLRP3 inflammasome during RVD mechanisms leads to the activation of the pro-inflammatory cytokine IL-1β (Compan *et al.*, 2012; Compan and Pelegrin, 2018; Ip and Medzhitov, 2015). Interestingly, a positive relationship between the expression of NLRP3, IL-1β and the IVD degeneration grade exists (NP tissue, mRNA and protein level) (Chen *et al.*, 2015; Song *et al.*, 2017). This indicates that NLRP3 may constitute a putative target between the altered osmotic environment and the inflammation and, thus, be a possible therapeutic target for the treatment of IVD degeneration (Tang *et al.*, 2018).

ASICs are proton-activated cation channels (Yoder *et al.*, 2018) that are hypothesised to be of functional importance in IVD osmoprotection and pathophysiology (Li *et al.*, 2014a; Uchiyama *et al.*, 2008; Yuan *et al.*, 2016) by facilitating the adaptation of IVD cells (rodent) to an acidic and/or hyper-osmotic environment *via* the ERK signalling pathway (Uchiyama *et al.*, 2007). The presence of ASIC is also confirmed on the mRNA and protein level in healthy and degenerated human IVD tissue (Cuesta *et al.*, 2014), with higher expression levels during IVD degeneration, possibly due to changes in pH and hydrostatic pressure (Cuesta *et al.*, 2014). The viability of rat NP cells cultured in hyper-osmotic (450 mOsm/L) medium decreases in a dose-dependent manner (from ~ 100 % to ~ 40 %) when the ASIC3 inhibitor amiloride (10-100 μM, 24 h, MTT) is added (Uchiyama *et al.*, 2007). However, the same study shows that increasing the osmolarity from 330 to 450 mOsm/L, without the ASIC3 inhibitor, does not have a significant influence on NP cells viability (Uchiyama *et al.*, 2007). In the context of the IVD and osmoregulation, an acidic pH (which is a characteristic of the degenerated IVD) down-regulates the synthesis of PGs, thereby contributing to low extracellular osmolarity (Ohshima and Urban, 1992; Wuertz *et al.*, 2009). The altered expression of ASICs can, on the one hand, indicate a causative and hence detrimental role in IVD degeneration or may, on the other hand, constitute a mechanism to better cope with the degenerative conditions (Uchiyama *et al.*, 2007).

Therapeutic modulation of osmosensing

Dysregulated tissue osmolarity is a hallmark of several chronic diseases, suggesting that the efficiency of the body's osmoprotective mechanisms decreases with age and/or tissue degeneration (Brocker *et al.*, 2012). Non-physiological concentrations of intra- and extra-cellular structural molecules and signalling mediators alter the cellular responses to otherwise normal stimuli, such as physiological loading. A healthy IVD could be described by its ability to effectively adapt to diurnal osmotic changes and auto-regulate itself, without damage (Sivan *et al.*, 2006). From this perspective, a deviation into extreme osmotic conditions (either hyper- or hypo-osmotic), concomitant with a decreased adaptation ability, can have detrimental consequences on the IVD. Osmotic stress can be potentially counteracted by promoting osmoadaptation through stimulation of cellular defence mechanisms (Johnson *et al.*, 2014) or by combating the consequences of a dysregulated osmosensing (van Dijk *et al.*, 2015).

A key intracellular mediator of osmoadaptation is the transcription factor TonEBP/NFAT5, which controls the expression of genes involved in the response to hyper-osmolarity (Lopez-Rodriguez *et al.*, 2004) and supports cell survival, especially in tissues regularly experiencing hyper-osmotic stress (Choi *et al.*, 2018; Gajghate *et al.*, 2009; Tsai *et al.*, 2006; Tsai *et al.*, 2007). Therefore, stimulating and promoting TonEBP/NFAT5 could have beneficial effects on the IVD homeostasis in a situation when the osmotic balance or the cell's adaptation capability are disturbed. Adaptation of rat NP cells to hyper-osmolarity is mediated *via* ERK- and p38-induced activation of TonEBP/NFAT5 (Tsai *et al.*, 2007), which regulates water balance through expression of several target genes [*e.g.* AQPs (Gajghate *et al.*, 2009)]. In rat NP cells, dominant negative NFAT5 significantly reduces cell viability and activated caspase 3,

suggesting a pro-survival role of TonEBP/NFAT5 in hyper-osmotic conditions (Tsai *et al.*, 2006). The osmoprotective activity of TonEBP/NFAT5 could be therapeutically enhanced; however, TonEBP/NFAT5 also participates in pro-inflammatory responses in the IVD (Johnson *et al.*, 2017). Therefore, the desired TonEBP/NFAT5-modulating compounds should ideally reduce its potential chronic pro-inflammatory effect, while enhancing its osmoprotective activity. TonEBP/NFAT5 inhibitors that selectively suppress the expression of pro-inflammatory genes without hampering TonEBP/NFAT5-induced osmoadaptation are developed and tested in a model of chronic arthritis, representing the first steps in this direction (Han *et al.*, 2017). The molecular signals directing TonEBP/NFAT5 towards osmoadaptation and/or inflammation in IVD cells are currently unknown. For example, in macrophages, the putative sensors that discriminate between pro-inflammatory and osmoprotective effects of TonEBP/NFAT5 are the ROS (Kim *et al.*, 2013). Among other functions, TonEBP/NFAT5 positively regulates synthesis and transport of osmolytes, gene expression of AQPs and synthesis of extracellular matrix components, all of which could be possibly therapeutically enhanced.

Organic osmolytes are solutes (*e.g.* sugars, polyols, amino acids) that protect biomolecules from the damage caused by changing osmotic pressure and dehydration and, thereby, provide cytoprotection and anti-inflammatory effects (Rabbani and Choi, 2018). Natural osmolytes participate in regenerating native protein forms from unfolded states, restoring proper protein functions and, thus, possibly preventing disease development (Alfieri *et al.*, 2002). Physiological concentrations of osmolytes in the IVD and their effects in and outside IVD cells have not yet been thoroughly investigated (Mavrogonatou and Kletsas, 2012). Synthesis and uptake of osmolytes in the IVD can be possibly regulated by TonEBP/NFAT5-COX-2-PGE2 signalling, as an osmoprotective role is demonstrated for this pathway (Favale *et al.*, 2009; Kim *et al.*, 2009). In renal cells subjected to hyper-osmolarity, COX-2 is involved in the accumulation of osmolytes (Moeckel *et al.*, 2003) and COX-2 inhibition reduces cell viability (Neuhofer *et al.*, 2004). Interestingly, a recent study testing TonEBP/NFAT5 in mouse hyper-osmotic IVD organ cultures shows that TonEBP/NFAT5 also provides cytoprotective effects in the IVD by inducing COX-2 (Choi *et al.*, 2018). In view of these findings, currently used COX-2-targeting drugs could impair IVD osmoadaptation mechanisms (Bonner *et al.*, 2009) and further contribute to the pathophysiology of DDD. Therapeutic enhancement of osmolyte function by a COX-2-unrelated mechanism could potentially increase resistance of the IVD to osmotic stress.

Dysfunction or aberrant expression of various AQPs is likely implicated in the pathogenesis of IVD degeneration. Inducing the expression and/or activity of certain AQPs can promote an exchange of fluids in the NP, possibly reducing the progression of DDD. Both water permeability and ionic conductance of AQPs can be positively regulated by PKC (Zhang *et al.*, 2007) and cyclic nucleotides (Lorenz *et al.*, 2003). However, these molecules control numerous cellular processes and their therapeutic modulation might produce detrimental off-target effects (Cooke *et al.*, 2017). Specific upregulation of AQP gene expression could be achieved by CRISPR gene editing, *e.g.* using dCas fused with VP64 domains targeted to AQP gene enhancers (Konermann *et al.*, 2015).

As a membrane receptor, TRPV4 could potentially be regulated by specific agonists or antagonists, to prevent an age-related loss of ECM and reduce inflammation in the IVD (Walter *et al.*, 2016). However, involvement of TRPV4 in these processes is rather complex and a tight balance in the expression/regulation of TRPV4 is crucial in the maintenance of the musculoskeletal health. As an example, blocking TRPV4 with the antagonist GSK205 reduces chondrocyte responses to hypo-osmotic stress, including RVD and production of PGE2 (porcine cells) (Phan *et al.*, 2009), while activating TRPV4 with 4αPDD inhibits the production of the pro-inflammatory mediator nitric oxide in rat chondrocytes (Hu *et al.*, 2013). The importance of a balance in TRPV4 expression/function is also shown in mouse models, where loss of TRPV4 leads to a progressive osteoarthritic joint degeneration (Clark *et al.*, 2010), while gain of function causes various skeletal dysplasias (Mah *et al.*, 2016; Weinstein *et al.*, 2014). Therefore, therapeutic TRPV4 agonists or antagonists should be specific [*e.g.* 4α-PDD or GSK2193874, respectively (McNulty *et al.*, 2015)], used locally (*e.g.* injections) and only once the benefits of modulating TRPV4 have been clearly demonstrated. Stable overexpression or knock-out of TRPV4 could be delivered into the IVD, *e.g.* in genetically engineered therapeutic cells.

Other therapeutic approaches could include augmentation of extracellular matrix, *e.g.* by up-regulating glucuronosyltransferase 1, a key TonEBP/NFAT5-dependent regulator of glycosaminoglycan synthesis (Hiyama *et al.*, 2009), or by implanting biomaterials. Osmoprotective moieties, such as chondroitin sulphate, can be incorporated into injectable hydrogels to increase hydration of the synthetic matrix in tissue engineering applications (Chen *et al.*, 2016; Farnsworth *et al.*, 2014).

Conclusion and outlook

Changes in the IVD hydration and osmolarity ranging from ~ 430 (iso-osmotic) to ~ 496 mOsm/L (hyper-osmotic) can be observed during daily life activities. From this perspective, the osmotic environment in the IVD is unusual, as the osmotic range which is physiological for the IVD would be considered high

for other tissues (*e.g.* blood plasma with osmolarity of ~ 300 mOsm/L). However, a reduction in tissue osmolarity to ~ 300 mOsm/L (hypo-osmotic) is a consequence of a cascade of degenerative changes and a hallmark of IVD degeneration. In this review, an overview of the existing studies on IVD osmolarity, its potential intersection with IVD inflammation and how this knowledge could be translated into treatment strategies is presented. Increasing scientific evidence points towards a crucial role of ion channels (such as TRP channels) in the regulatory volume control mechanisms in the IVD, as well as in cartilage – a tissue with similar characteristics to the NP tissue. Simultaneously, AQPs are an emerging target involved not only in osmosensing, but also in IVD degeneration and inflammation. Importantly, TonEBP/NFAT5 (co-activated by calcium and ERK/p38 signalling) facilitates the IVD adaptation to fluctuations of its osmotic environment. Changes in water and ion concentrations affect the homeostasis of the IVD, as indicated by dysregulation of ECM synthesis under hypo- and hyper-osmotic conditions, but many details on the underlying mechanisms are still unknown. Several questions remain to be answered, such as:

- What is the underlying mechanism of the RVD and RVI response in NP and AF cells?
- What is the mechanistic function of cell membrane carriers and ion channels in IVD osmosensing and osmoadaptation?
- Do the cell membrane carriers and ion channels interact in the IVD?
- Can the activation of PKC and NF-κB pathways be osmolarity-induced?
- What is the role of these pathways in osmoadaptation and/or osmolarity-induced inflammation?

A broader understanding of how IVD cells react to altered osmolarity, *e.g.* in relation to PG synthesis, cell survival/apoptosis or inflammation, is crucial when aiming to advance the current concepts of IVD pathophysiology. Once these mechanisms are better understood and possible targets are identified, suitable therapeutics that successfully and specifically modulate osmoadaptation can be developed. This new class of anti-inflammatory and regenerative therapeutics may target the osmoprotective transcription factor TonEBP/NFAT5 or osmo-sensing membrane proteins such as TRPV4 or AQPs. Gene editing techniques (*e.g.* CRISPR/Cas) can be used to modulate the expression/activity of osmosensing-associated genes in locally delivered autologous therapeutic cells. For example, genes regulating the activity of osmosensors or synthesis and transport of osmolytes can be activated by dCas fused with VP64 domains or switched off by Cas-mediated knock-out/knock-down. Anti-inflammatory and regenerative therapeutics may be combined with gene editing techniques, with the overall aim of maintaining proper function of the cellular osmoadaptation sensors and the ECM and to ensure efficient transport of water and solutes through loaded IVD tissue.

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Discussion with Reviewers

Jivko Stoyanov: A change of disc osmolarity from the normal high value of 450 mOsm/L to a 'mean' of ~ 300 mOsm/L is a consequence of a cascade of degenerative changes which lead to the decrease in extracellular proteoglycan content and water-retaining capacity of the disc. How would a therapy related to osmolarity regulation or osmosensing target such a specific mechanism which is at the root of the problem?
Authors: Indeed, targeting osmoregulation or osmosensing in IVD cells would not recuperate the degenerative changes occurring in the IVD and the consequent drop of osmolarity. These approaches are rather intended to let IVD cells cope better with the arising hypo-osmotic microenvironment, thus preventing/minimising induction of detrimental cellular responses that can further accelerate the degenerative cascade.

Junxuan Ma: NP cells are naturally living in a harsh microenvironment with a high osmolarity, which is different from other tissues. How is the response to osmolarity different between NP and other cells?
Authors: NP cells are stiffer and more viscous in comparison to AF cells and many other cell types due to differences in the cytoskeletal arrangement, consequently leading to different responses to an altered osmotic environment (Pritchard *et al.*, 2002).

Junxuan Ma: A decrease in cell volume due to the hyper-osmotic stress is mentioned by the authors. What is the subsequent cellular response, *e.g.* which pathways are activated by the cell volume changes, leading to apoptosis or inflammation?
Authors: Hyper-osmotic stress (*i.e.* increase in the extracellular osmolality) leads to cell shrinkage as water moves out of the cell, generating Ca^{2+} transients (Pritchard *et al.*, 2002) and subsequent activation of MAPKs (p38, ERK and JNK) (Dong *et al.*, 2014). These Ca^{2+} transients, together with the MAPKs, activate TonEBP/NFAT5, which plays a key role in IVD cell osmoadaptation. In addition, TonEBP/NFAT5 can also induce inflammation, *e.g.* through its crosstalk with the NF-κB pathway (Johnson *et al.*, 2017). As such, activation of the same signal transduction pathway can lead to distinctive responses, depending on the cellular microenvironment. Also, hyper-osmotically-activated p38/MAPK can lead to DNA damage and trigger apoptosis by activating G2 and G1 cell cycle check points (Dong *et al.*, 2014; Mavrogonatou and Kletsas, 2009). Conflicting results are reported concerning hyper-osmotic stress and activation of the ERK pathway (see manuscript), but these differences may be due to heterogeneous experimental set-ups, such as culture conditions or animal model used.

Junxuan Ma: Inflammation causes further damage to the tissue, but also triggers repair. For the treatment, the authors mentioned the inhibition of inflammation while leaving osmoadaptive mechanism to function. Yet, what if there is a positive aspect of the inflammation being present?
Authors: Indeed, inflammation can have positive effects, for example during tissue injury or infection. However, in other instances, inflammation can also be viewed as a malfunction of a tissue and, as such, can be tissue-/cell-/ECM-induced (*e.g.* through signals released by stressed IVD cells). In the context of this review, the word inflammation is used with the meaning of 'chronic pro-inflammatory response', which is characterised by prolonged, increased expression/presence of pro-inflammatory mediators (*e.g.* TNF-α, IL-1β, IL-8) and catabolic enzymes (*e.g.* MMPs) and, at the same time, reduced expression/presence of inflammation antagonists (*e.g.* IL1RA, TNFRA).

Reviewer: Please provide more details of how CRISPR in osmosensing molecules may be used in IVD degeneration.
Authors: Gene editing techniques (*e.g.* CRISPR) can be used to modulate the expression/activity of osmosensing-associated genes in locally-delivered autologous therapeutic cells (Krupkova *et al.*, 2018, additional reference). To date, CRISPR is used to generate inflammation-resistant chondrocytes and IVD cells. As an example, stable CRISPR/Cas9 knockout of the surface receptor IL1R1 results in inflammation-resistant cell population with superior properties over non-edited therapeutic cells (Karlsen *et al.*, 2016, additional reference). Moreover, two surface receptors (TNFR1, IL1R1) are successfully targeted by epigenome editing in human primary IVD cells, suggesting the feasibility of this approach (N.L. Farhang, 2018, additional reference). Therefore, osmosensing receptors (such as TRPV4) and genes regulating their activity, synthesis and transport of osmolytes can be potentially targeted by CRISPR. CRISPR-based methods include dCas fused to VP64 domains for gene activation (Perez-Pinera *et al.*, 2013,

additional reference), dCas fused with KRAB for gene silencing (Gilbert *et al.*, 2013, additional reference) or Cas-mediated DNA cuts for gene knock-out (Jinek *et al.*, 2013, additional reference).

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