GENETIC MURINE MODELS OF SPINAL DEVELOPMENT AND DEGENERATION PROVIDE VALUABLE INSIGHTS INTO INTERVERTEBRAL DISC PATHOBIOLOGY

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Abstract

Disc degeneration and associated back and neck pain elicits a substantial burden on healthcare systems and the individuals affected, necessitating the development of novel therapeutic strategies. This goal can only be achieved by a better understanding of intervertebral disc development, homeostasis and pathogenesis. A number of genetic and in-bred murine models are reviewed to underscore the importance of the mouse as an animal model of choice for the assessment of intervertebral disc pathobiology. Appraisals of the differences between mouse and human musculoskeletal systems and proteoglycan structures are also included. A number of important target pathways and molecules have been identified, many of which are worthy of further examination, requiring that the activity of these be confirmed in large animal models and assessed in the context of therapeutic intervention.

Keywords: Murine model, intervertebral disc, nucleus pulposus, annulus fibrosus, spine.

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List of Abbreviations

ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AF	annulus fibrosus
Akr1b1	aldo-keto reductase family 1 member B
ALPL	alkaline phosphatase
ANK	ankyrin
AP-1	activator protein 1
Aqp2	aquaporin-2
ARP2/3	actin-related proteins 2/3
ATM	ataxia-telangiectasia mutated kinase
B3gat3	beta-1,3-glucuronyltransferase 3
BACH-1	BTB and CNC homology-1
BGN	biglycan
BMP	bone morphogenic protein
BNIP3	BLC2 interacting protein 3
CA3	carbonic anhydrase 3
Can	aggrecan
Ccl2	monocyte chemoattractant protein-1, aka MCP-1
CD44	cluster of differentiation 44
CDK2	cyclin-dependent kinase 2
CEP	cartilaginous endplates
Chm1	chondromodulin I
COL1A1	collagen type I alpha chain
COL2A1	collagen type II alpha chain
COX-2	cyclooxygenase-2
CS	chondroitin sulphate

CTGF	connective tissue growth factor
E2F	elongation factor 2
ENPP1	ectonucleotide pyrophosphatase/ phosphodiesterase 1
ENT1	equilibrative nucleoside transporter 1
ERCC1	excision repair 1 endonuclease non-catalytic subunit
ERK	extracellular signal-regulated kinase
FasL	Fas ligand
FGF	fibroblast growth factor
FGFRs	fibroblast growth factor receptors
FLNB	filamin B
FMOD	fibromodulin
FOXA1/2	forkhead box A1/A2
FOXO1/3/4	forkhead box O1/O3/O4
GAG	glycosaminoglycan
Gal	D-galactose
GalNAc	N-acetylgalactosamine
GDF5	growth differentiation factor 5
GlcA	glucuronic acid
GlcNAc	β1-3 D-N-acetylglucosamine
GlcUA	glucuronic acid
HA	hyaluronic acid
HAS2	hyaluronan synthase 2
HIF-1/2α	hypoxia-inducible factor 1alpha/2alpha
HO-1	haem oxygenase-1
HOX	homeobox
HS	heparan sulphate
Hspg2	perlecan
IGD	interglobular domain
IGF-1	insulin-like growth factor 1
IGFR	insulin-like growth factor receptor
IL-1rn	interleukin 1 receptor antagonist
IL-1α/β	interleukin 1 alpha/beta
IL-6	interleukin-6
IVD	intervertebral disc
JNK1/2	Jun N-terminal kinase 1/2
KS	keratan sulphate
LAMSHF	Lamb-Shaffer syndrome
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LP	link protein
LRP6	low-density lipoprotein receptor-related protein 6
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MCT4	monocarboxylate transporter 4
MKX	Mohawk
MMP	matrix metalloproteinase
MT1-MMP	membrane type 1-matrix metalloproteinase, aka MMP14
mTOR	mechanistic target of rapamycin
NF-1	neurofibromatosis type 1
NFAT5	nuclear factor of activated T-cells 5
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOTO	homeobox protein notochord
NP	nucleus pulposus
OREBP	osmotic response element binding protein
p16	cyclin-dependent kinase inhibitor 2A
PAX1/9	paired box 1/9
PHLPP1	PH domain leucine-rich repeat protein phosphatase
PRG4	lubricin
Pt	Pintail
PTCH1	Patched 1
QTL	quantitative trait locus
RAS	rat sarcoma
RB1	retinoblastoma transcriptional corepressor 1
RHAMM	receptor for HA-mediated motility
SCX	scleraxis
Sd	Danforths short tail
SHH	Sonic Hedgehog
Skt	Sickle tail
Slc6A6	sodium- and chloride-dependent taurine transporter
SLRP	small leucine-rich proteoglycan
SMAD3	mothers against decapentaplegic homologue 3
Smo	smoothened
SOX5/6/9	Sry-related HMG box 5/6/9
SPARC	secreted protein acidic and rich in cysteine
T	brachyury
Tc	truncate
Tgfb2	TGF-β type II receptor
TGF-β	transforming growth factor beta
TNF-α	tumour necrosis factor alpha
TNMD	tenomodulin
TonEBP	tonicity enhancer binding protein
TSC	tuberous sclerosis complex
VEGF	vascular endothelial growth factor
WNT	wingless/integrated

Introduction

Insight into disc pathobiology through the murine model

The murine model is an invaluable experimental tool for the investigation of several degenerative human conditions. Studies of a number of genetically modified mice have contributed immensely to our understanding of spinal development and homeostasis (Table 1-4). Indeed, manipulations of the mouse genome through mis-expression, knocking-out, knocking-in, or the introduction of mutations into genes, as well as inbred mouse models, have transformed our understanding of spinal development and the complex functional properties of the IVD. In many cases, such mutants are also genetic models of relevant human degenerative disorders characterised by, for example, reduced bone mass (osteopenia, osteoporosis) or abnormalities in endochondral bone formation affecting development

and growth of the skeleton (chondrodysplasias). The value of the murine model is further underscored by the high conservation of skeletal development between mice and humans, despite differences in skeletal size and time required for reaching skeletal maturity. From a regenerative perspective, understanding the regulatory molecules that are operative during such developmental processes provides information applicable to therapeutic tissue repair strategies (Tessier and Risbud, 2020). Moreover, the development of quantitative histopathological scoring schemes for murine IVDs have further increased the utility of the murine model for pathobiological evaluations. It is within this context that we have summarised numerous studies, whose insightful conclusions relied heavily upon the use of the mouse as an experimental model.

Intervertebral disc structure and comparative anatomy

The spinal column was evolutionarily preceded by the notochord, a rod-like structure that provided protection to the underlying neural tube and mechanical support to the chordate organism. In higher vertebrates, which possess the advantage of distinct spinal joints (syndesmosis/symphysis) consisting of mineralised vertebral bodies joined by IVDs, the notochord exits as merely a transient embryonic structure. While transient, it is crucial for mechanical support and morphogenic signalling during development. Its fate is to give rise to the NP, a tissue found at the centre of the IVD, distinguished for its gel-like appearance on gross examination, high proteoglycan content, and load-bearing properties. The uniqueness of the NP is further exemplified by its microenvironmental conditions (*i.e.* hyperosmolarity, hypoxia and loading), which have guided the focus of research efforts in the quest of delineating the molecular mechanisms that underly NP cell survival. Such salient discoveries, facilitated by the mouse model, will be discussed in this review. The circumferential disc tissue that surrounds the NP is called the AF. Under the microscope, the collagenous lamellae so characteristic of the AF are conspicuously prominent and show a graded transition between the inner-most and outer-most regions. AF cells, or annulocytes, also differ morphologically by regional location along the AF (Bruehlmann *et al.*, 2002). Together, the NP and AF make up the bulk of disc tissue. Also important to disc anatomy are the thin layers of hyaline cartilage that form the superior and inferior boundaries, called the CEPs. It is their presence that prevents the disc from applying its biomechanical forces directly against the vertebral bone. The CEPs, due to their porous nature, permit diffusion and exchange of nutrients and metabolites between the vascular beds in subchondral vertebral bone and avascular compartments of the disc. This is a process facilitated by the diurnal loading and unloading of the spinal motion segments. The disc, along with the adjoining vertebrae, forms a complex polyaxial, diarthrodial joint that allows for wide ranges of 3-dimensional movement (Shapiro *et al.*, 2012).

The murine IVD shares the structural characteristics described above with that of humans. An important difference to highlight, nonetheless, is that notochord-like NP cells persist longer in the murine model than in humans. The murine and human IVD are embryonically derived from the aggregation of the notochord and perinotochordal mesenchyme during vertebral column development. Notochordal cells produce a matrix rich in proteoglycan, important for generating central swelling pressure in the developing IVD and essential for normal NP formation (Adams *et al.*, 1977). In humans, within 4 to 10 years of age after birth, the notochordal cells of the disc disappear or undergo significant morphological changes, whereas they persist throughout adulthood in the healthy murine system (Trout *et al.*, 1982). Human NP cells acquire a round, chondrocytic appearance as they age, becoming increasingly dispersed throughout the matrix. By contrast, the adult vacuolated NP cells of the healthy mouse disc assemble into a central band. For reference to the histomorphology of human disc tissue, please consult the studies cited here (Boos *et al.*, 2002; Rutges *et al.*, 2013). Notochordal NP cells are large and vacuolated, often forming groups and clusters and described as a physaliforous cell type (Pandiar and Thammaiah, 2018). They are relatively quiescent with regards to proteoglycan metabolism, but have a marked influence on the metabolism of proteoglycans within the NP (Aguiar *et al.*, 1999). A series of mouse mutants with defective notochordal development such as the Brachyury curtailed (Stott *et al.*, 1993), Truncate (*tc*) (Theiler, 1959), Pintail (*Pt*) (Hollander and Strong, 1951), and Sickle tail (*Skt*) (Semba *et al.*, 2006) allow for testing of the relationship between disc health and notochordal cell defects. These mutants show acceleration in age onset-IVD degeneration correlated with the loss of notochordal cells from the NP. In severe cases, the NP actually does not develop (Table 1). Accordingly, vacuolated notochord-like NP cells are typically associated with a healthy IVD.

There are other differences between humans and the murine system that should be taken into account. Unlike humans, for one, the quadrupedal mouse does not ambulate vertically. Quadrupedalism subjects the horizontally oriented spine to greater amounts of axial compression stress, which results in higher trabecular bone density when compared to humans. Nevertheless, both murine and human spines are primarily loaded along the cephalic-caudal axis, and the lumbar discs of both species are loaded by ground-reactionary and endogenous forces (Smit, 2002). The lumbar vertebrae and discs, too, are the largest in both species. When geometry is normalised, the average compressive and torsional stiffness of the murine disc is quite similar to that measured in humans (Elliott and Sarver, 2004). Another obvious difference is that the mouse has an additional

vertebra at the thoracic and lumbar spinal levels, one vertebra less at the sacral level, and up to 28 coccygeal/tail vertebrae. The tail vertebrae and discs have morphological features that differ from the lumbar segments likely due to differences in weight-bearing loads (Elliott and Sarver, 2004). In addition, there are important species-dependent differences in the structure of disc proteoglycans, which will be addressed in the following section.

Consideration of differences between murine and human proteoglycans

High proteoglycan content, most notably aggrecan, is a hallmark feature of the healthy IVD, as it provides the tissue with robust load-bearing properties. Structurally, the aggrecan core protein comprises 3 globular domains: G1 and G2, positioned at the N-terminal end, and G3, positioned at the C-terminal end. The G1 and G2 domains are separated by an

Table 1. Mouse spinal models assessing the effects of mutations in genes to morphogens, regulatory signalling molecules, and transcription factors.

Model	Phenotype/insight	References
Pintail (*Pt*) and truncate (*tc*) mouse models	Premature truncation of notochord /development of the vertebral column, loss of notochordal cells. Accelerated age onset disc degeneration. Total absence of NP at all levels in the *Sd* knockout mouse.	Hollander and Strong, 1951 Theiler, 1959
Skt Sickle tail mouse	*Skt* is a gene linked to the Danforth *Sd* short tail locus, required for correct IVD development. Size of NP in WT mouse is normal until birth, after birth the *Skt* NP does not expand and is dislocated to the periphery, resulting in a kinky tail phenotype in the adult.	Semba *et al*., 2006
FOXA2 knockout	Defective node and notochord formation; secondary impairment of dorsal-ventral neural tube patterning.	Ang and Rossant, 1994
Foxa1$^{-/-}$;*Foxa2*$^{c/c}$; *Shh*creER^{T2} double mutant	Defective notochord sheath, deformed NP, cell death, and reduced SHH signalling; secondary impairment of dorsal-ventral neural tube patterning.	Maier *et al*., 2013
T deficiency	Homozygous null: lack notochord, abnormal somites, allantois defect, lethal. Haploinsufficiency: short-tailed phenotype.	Stott *et al*., 1993
NOTO deficiency	Disruption of the caudal notochord. The autosomal recessive mutation in the mouse is known as Truncate (tc).	Ben Abdelkhalek *et al*., 2004
TonEBP deficiency	Homozygous null: delayed spinal development and expression of notochord markers. Haploinsufficiency: Accelerated age-dependent degeneration, matrix fibrosis, alterations to the actin cytoskeleton, and decreased expression of proinflammatory genes.	Tessier *et al*., 2019, 2019b
MitoQC reporter	NP cells possess numerous tubular and hypoxia-responsive mitochondrial networks that undergo increased mitophagy with ageing.	Madhu *et al*., 2020
MCT4 knockout	Disc degeneration with increased aggrecan degradation, MMP13, and type X collagen levels.	Silagi *et al*., 2020
*Foxa2*cre; *Hif-1α*$^{fl/fl}$ *Shh*cre; *Hif-1α*$^{fl/fl}$	Notochordal cell loss and severely prominent fibrosis of the disc by 1 month of age.	Wu *et al*., 2013 Merceron *et al*., 2014
Tnmd$^{-/-}$ *Tnmd*$^{-/-}$;*Chm1*$^{-/-}$	Increased angiogenesis and macrophage invasion of the outer AF; accelerated disc degeneration including hypertrophic cells residing in the NP compartment.	Lin *et al*., 2020
SOX5/6 double knockout	Defective notochord sheath formation, apoptosis of notochord cells, and spine lacking NP.	Smits and Lefebvre, 2003
SOX9 knockout	Notochord disintegration and disc degeneration.	Barrionuevo *et al*., 2006
AcanCre^{ERT2}*Sox9*$^{fl/fl}$	Severe degeneration of all disc compartments. Matrix remodelling, cell death, and compartment-specific transcriptomic changes.	Henry *et al*., 2012 Tsingas *et al*., 2020
PAX-1/9 double knockout	Vertebral column and disc defects; sclerotomes fail to undergo chondrogenesis.	Peters *et al*., 1999
MKX knockout	AF degeneration.	Nakamichi *et al*., 2016
BACH-1 knockout	Protective effects on annular puncture.	Ohta *et al*., 2012
*Col2a1*Cre;*Foxo1*$^{fl/fl}$; *Foxo3*$^{fl/f}$; *Foxo4*$^{fl/fl}$	Progressive increase in NP cellularity. Marked degeneration by 6 months. Kyphosis.	Alvarez-Garcia *et al*., 2018
AcanCre^{ERT2};*Foxo1*$^{fl/fl}$; *Foxo3*$^{fl/f}$; *Foxo4*$^{fl/fl}$	Marked disc degeneration by 12 months of age.	Alvarez-Garcia *et al*., 2018
Hoxd-3 knockout & Hoxb-4 knockout	Transformation of atlas and axis of the craniocervical joint, deletion of dens and superior facet joints, axis shows atlas like characteristics.	Condie and Capecchi, 1993) Ramfrez-Solis *et al*., 1993
Hox-10 knockout & Hox-11 knockout	Lumbar vertebrae do not develop in Hox-10 knockouts, sacral vertebrae do not develop in Hox-11 knockouts; associated downline abnormalities in IVD development.	Wellik and Capecchi, 2003
*Shh*GFPcre;*Smo*$^{fl/fl}$	Defective notochord sheath formation, axial patterning, and IVDs.	Choi and Harfe, 2011
Wnt/β-catenin knockout reporter (TOPGAL)	Deterioration of the GP and AF when β-catenin is overexpressed in both *Col1a1* and *Col2a1*-expressing cells; accelerated subchondral bone formation when β-catenin is deleted in *Col2a1*-expressing cells.	Dahia *et al*., 2009 Kondo *et al*., 2011
*Noto*Cre;*Ccn2*$^{fl/fl}$	Age-associated degeneration of IVDs, decreased levels of aggrecan and type II collagen, and increased levels of type I collagen within the NP.	Bedore *et al*., 2013
*Col2a*Cre;*Tgfbr2*$^{fl/fl}$	Mutant embryos with irregularities in the size and shape of vertebrae. The intervertebral discs were either completely lost or reduced in size due to AF defects.	Baffi *et al*., 2004, 2006
*Acan*Cre^{ERT2};*Tgfbr2*$^{fl/fl}$	Progressive degenerative phenotype with calcifications in the disc space by 12 months of age.	Alkhatib *et al*., 2018
SMAD-3 knockout	Spinal malformations and kyphosis, changes in CEP structure, and decreased IVD proteoglycan and collagen content.	Li *et al*., 2009
IGF1R haploinsufficiency	Accelerated disc degeneration.	Li *et al*., 2013
GDF-5 knockout	Reduced proteoglycan content.	Li *et al*., 2004
*Col2a1*Cre; *Nf1*$^{fl/fl}$	Progressive scoliosis and kyphosis with IVD defects.	Wang *et al*., 2011
TSC1 knockout	Congenital spinal defects.	Yang *et al*., 2017
*Col2a1*Cre; *Jnk1*$^{fl/fl}$/*Jnk2*$^{-/-}$	Severe, early onset scoliosis with disc fusions.	Ulici *et al*., 2019
PHLPP1 knockout	Preserves cellularity and matrix homeostasis in an injury model.	Zhang *et al*., 2019
Ercc1 deficiency	Advanced age-related degenerative changes in the vertebral bodies and intervertebral discs, including reduced proteoglycan content, increased apoptosis, and increased p16.	Vo *et al*., 2010
TNFα overexpression (Tg197 and hTNFαTg)	Compromised vertebral bone parameters, greater propensity for herniations at the EP/AF junction, evidence of AF defects, expanded NP cell band.	Gorth *et al*., 2018, 2020
IL-1α/β double knockout	Evidence of AF degeneration.	Gorth *et al*., 2019
*Acan*CreER^{T2}; *p16*^{Ink4a}	Decreased senescence markers with age-dependent degenerative changes.	Novais *et al*., 2019
IL-1RN knockout	Spinal abnormalities.	Phillips *et al*., 2013

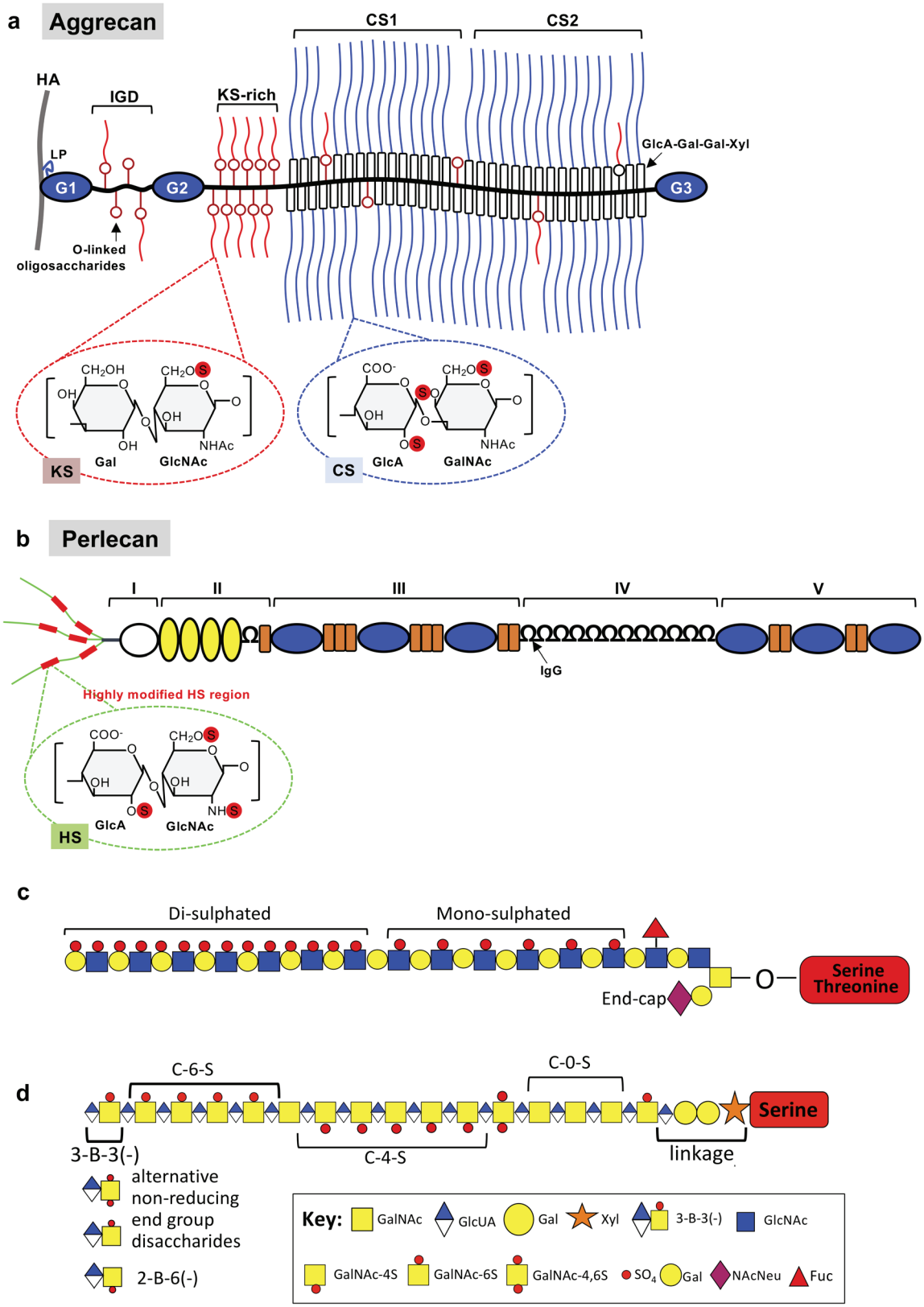

Fig. 1. Schematic depictions of the structural organisation of aggrecan, perlecan, and their KS-II and CS chains. These schematics should be viewed as generic depictions based on reported structural data. The depictions shown are for human (**a**) aggrecan and (**b**) perlecan; murine aggrecan has a truncated KS rich region and domain IV of murine perlecan also has a 40 kDa truncation (Farach-Carson *et al.*, 2014). (**b**) N- and O-sulphation positions of an HS disaccharide of perlecan in a highly modified region of the HS chain are also shown. Note: while perlecan is referred to as an HS-proteoglycan, intervertebral disc cells synthesise a hybrid form where at least one of the HS chains is replaced by a CS chain. (**c**) Typical regions of mono-, di- and non-sulphated regions of KS-II are shown along with possible fucosylation and neuraminic acid regions (Caterson and Melrose, 2018). (**d**) The CS disaccharides of aggrecan are heterogenous with regard to their sulphate distributions.

IGD and the G2 and G3 domains are separated by an extended region. The extended region contains a KS attachment domain and 2 CS attachment domains (CS-1, CS-2). During its synthesis, several O-linked oligosaccharides on the aggrecan core protein are extended to form KS chains, while CS is attached by a tetrasaccharide linkage region of GlcA-Gal-Gal-Xyl. Several aggrecan molecules attach to HA by their G1 domains and LP, forming a supramolecule that holds a huge density of electro-negative charges (Fig. 1**a**,**c**,**d**). These charges attract water and ions into the disc compartment through the Gibbs-Donnan effect, creating a hyperosmotic niche within which the resident cells must survive (Silagi *et al.*, 2018b).

Unlike human aggrecan, the murine aggrecan core protein is truncated and largely devoid of a KS-rich region. Complete sequencing of the murine core protein (Walcz *et al.*, 1994; Watanabe *et al.*, 1995) shows that it does not contain the consensus sequences for attachment of KS as found in human aggrecan core protein (E-(E,K)-P-F-P-S or E-E-P-(S,F)-P-S) (Antonsson *et al.*, 1989; Doege *et al.*, 1991). Core protein sequencing data thus explains the reduced KS content of rodent aggrecan compared to other species (Stevens *et al.*, 1984; Venn and Mason, 1985). While the KS-attachment region is shorter in murine aggrecan, some KS in the IGD of mouse aggrecan is thought to be present (Fosang *et al.*, 2008). It should be noted that species-specific differences have been demonstrated in the amino acid sequences of the aggrecan IGD (Flannery *et al.*, 1998). These differences can have consequences on the susceptibility of this region to proteolysis by ADAMTS-4/5 and MMPs (Lark *et al.*, 1995; Little *et al.*, 1999). Yet still, the mouse aggrecan core protein (222-259 kDa) shares 72.5 % homology with human aggrecan, and the G1, G2, and G3 domains are homologous (Walcz *et al.*, 1994; Watanabe *et al.*, 1995). In addition, the spatio-temporal distribution of aggrecan in murine disc and cartilages is similar to that observed in humans, suggesting that murine aggrecan has similar weight-bearing roles in joint tissues despite the aforementioned subtle differences in structural organisation.

Some differences are also evident in the GAG substitution pattern of human and murine perlecan, the HS proteoglycan found in disc and cartilage (Knox *et al.*, 2005; Melrose *et al.*, 2003; Melrose *et al.*, 2006). Perlecan has 5 domains, labelled I-V, where domains I and V may be substituted with HS or CS (Fig. 1**b**). Domain I, a domain unique to perlecan, is the main region of GAG substitution and contains a cluster of 3 potential attachment sites, while domain V has additional GAG attachment sites (Costell *et al.*, 1997; Tapanadechopone *et al.*, 1999). Furthermore, domain IV of mouse perlecan contains less IgG repeats than human perlecan. Consequently, its core protein is ~ 40 kDa smaller than the human counterpart (Kallunki and Tryggvason, 1992; Noonan *et al.*, 1991). Mouse perlecan also contains an RGD cell attachment sequence in domain III, which is absent in human perlecan (Chakravarti *et al.*, 1995), although a cell adhesion site in domain IV has been identified in human perlecan (Farach-Carson *et al.*, 2008) indicating that the overall functionality of the molecule is preserved between the species.

While results obtained from murine IVDs should be interpreted with these points in mind and with regard to genetic background, the ease with which the mouse can be genetically manipulated and the abundance of knowledge the murine model has offered strongly outweighs the differences described above.

Murine model systems

Transcription factors

The formation of the notochord during embryonic development requires the coordinated action of many transcription factors (Tessier and Risbud, 2020). Among these, FOXA2 (Ang and Rossant, 1994), T (Stott *et al.*, 1993), and NOTO (Ben Abdelkhalek *et al.*, 2004) are instrumental as their absence results in failure of notochord formation and NP development. The loss of a notochord in *FoxA2* null mice is lethal, precluding the study of FOXA1 and FOXA2 and their roles in IVD formation. Nevertheless, by using a conditional *FoxA2* allele in conjunction with a tamoxifen inducible Cre allele (*Shh*CreER^{T2}), *FoxA2* was removed from the notochord of E7.5 mice, which were also null for *FoxA1*. These double mutant animals had defects in the notochord sheath, severely deformed NP, increased cell death in the tail, decreased SHH signalling, and resultant disruption of dorsal-ventral patterning of the neural tube. Notably, embryos that were *FoxA1* null or that had *FoxA2* conditional deletion alone did not show abnormalities in the IVDs, demonstrating functional redundancy (Maier *et al.*, 2013).

Once the embryonic spine has successfully developed, the maturing notochordal-NP cells produce a proteoglycan-rich matrix that creates a hyperosmotic niche for resident cells. Seminal studies conducted by Tsai *et al.* revealed that the osmoadaptive transcription factor, tonicity enhancer binding protein (TonEBP aka NFAT5 or OREBP), is required for survival of NP cells under these hyperosmotic conditions (Tsai *et al.*, 2006). TonEBP modulates intracellular concentrations of organic osmolytes and the hyperosmotic environment by controlling the transcription of osmosensitive and matrix-related genes such as *Slc6A6*, *Akr1b1*, *Aqp2*, *Acan*, *B3gat3*, and *Col2a1* (Gajghate *et al.*, 2009; Johnson *et al.*, 2014). TonEBP has also been shown to regulate pro-inflammatory genes including *COX-2* (Choi *et al.*, 2018a), *IL-6*, and *Ccl2* (Johnson *et al.*, 2016). The understanding of TonEBP in disc homeostasis has been elaborated upon by recent studies of TonEBP-deficient mice. While complete ablation of TonEBP is perinatally lethal with most embryos dying around E18.5, embryonic investigations have revealed that TonEBP loss causes developmental delay of the spinal column and aberrant levels of the notochord markers SHH, T, CA3, and vimentin. Interestingly, this study

raised an interesting possibility that SHH receptor PTCH1 is a transcriptional target of TonEBP (Tessier *et al.*, 2019). Subsequent studies of adult TonEBP haploinsufficient mice showed accelerated age-related disc degeneration and matrix fibrosis, with greater propensity for annular and endplate herniations. Moreover, TonEBP haploinsufficient mice displayed disc compartment-specific effects evidenced by increased expression of actin cytoskeleton-related genes in AF cells and reduced expression of immune and pro-inflammatory genes in NP cells (Tessier *et al.*, 2020b).

An important determinant of the NP niche is its avascular and hypoxic nature. This notion of limited oxygen availability has led to the general belief that NP cells primarily produce energy *via* glycolysis with little dependence on mitochondrial metabolism. Remarkably, however, recent investigations of mitoQC reporter mice have conclusively shown that NP cells possess mitochondrial networks consisting of numerous tubular mitochondria which undergo increased mitophagy with aging, emphasising the importance of the mouse model as a key investigational tool. Furthermore, mitochondrial fragmentation, morphology, and mitophagic flux was shown to be mediated by HIF-1α and mitochondrial receptor BNIP3 (Madhu *et al.*, 2020).

Among its numerous functions, HIF-1α regulates NP cell metabolism under hypoxic conditions by coordinating interactions between glycolysis and the TCA cycle (Madhu *et al.*, 2020). HIF-1α also contributes to the transmembrane balance of high intracellular H^+ and lactate concentrations by mediating expression of MCT4 (Silagi *et al.*, 2020) and the bicarbonate recycling enzymes carbonic anhydrase 9 and 12 (Silagi *et al.*, 2018a). MCT4 null mice show classical signs of disc degeneration with increased aggrecan degradation, elevated MMP13, and collagen X levels (Silagi *et al.*, 2020). As one would thus expect, analyses of the IVDs of HIF-1α conditional knockout mice in either SHH or FOXA2 expressing cells has demonstrated early-onset postnatal degenerative features with notochordal cell loss and severely prominent fibrosis (Merceron *et al.*, 2014; Wu *et al.*, 2013). HIF-1α also regulates galectin-3, a highly expressed lectin in the IVD. While its *in vivo* role in this tissue is poorly understood, *in vitro* studies suggest that galectin-3 promotes NP cell survival under conditions of oxygen deprivation. In NP cells, suppression of galectin-3 activity promotes the expression of FasL, an inducer of apoptosis, suggesting that this HIF-1α-regulated lectin plays a role in NP cell survival (Zeng *et al.*, 2007). HIF-1α and HIF-2α have also been shown to negatively regulate the expression of ANK and thereby control local levels of pyrophosphate, an important inhibitor of tissue mineralisation (Skubutyte *et al.*, 2010). Therefore, the hypoxic environment and the consequent role of HIF transcription factors is critical to the NP cell identify and function. The importance of low oxygen tension is further accentuated by recent investigations of TNMD-null mice. TNMD is an anti-angiogenic factor with robust AF expression, especially by the outer annulocytes. Its loss ($Tnmd^{-/-}$), as well as a double knockout with its homologue, chondromodulin I ($Tnmd^{-/-}$;$Chm1^{-/-}$), results in vascular invasion of the AF tissue, macrophage infiltration, and accelerated disc degeneration with hypertrophic cells. The double knockouts show greater progressive changes including ectopic bone formation (Lin *et al.*, 2020).

Central regulators of spinal development and skeletogenesis include a number of SOX transcription factors. SOX9 and SOX5/6 have been shown to cooperate *via* super-enhancers to drive chondrogenesis (Liu and Eronique Lefebvre, 2015); hence, these three SOX proteins are often referred to as the SOX trio. The importance of the SOX family members is made apparent by the various human skeletal diseases caused by their heterozygous mutations. Mutation of a copy of *Sox9* or disturbance to its regulation causes campomelic dysplasia (Wunderle *et al.*, 1998); haploinsufficiency of *Sox5* causes LAMSHF, a neurodevelopmental disorder associated with variable skeletal abnormalities (Lamb *et al.*, 2012). While SOX5/6 are functionally redundant, their dual ablation prevents formation of the notochord sheath, evidenced by notochordal and peri-notochordal downregulation of collagen II, versican, and aggrecan. These aberrations are also met by subsequent apoptosis of notochord cells and culminate in formation of a severely defective spine lacking NP (Smits and Lefebvre, 2003). Similarly, mouse embryos null for *Sox9* show notochord disintegration as early as E9.5 (Barrionuevo *et al.*, 2006) and deletion of *Sox9* in the disc (AcanCre^{ERT2}*Sox9*$^{fl/fl}$) results in degeneration (Henry *et al.*, 2012). Early degeneration of the CEP is the first conspicuous change seen in AcanCre^{ERT2}*Sox9*$^{fl/fl}$ mice, followed by degeneration of all disc compartments and severe disc collapse involving apoptosis and progressive loss of disc cells. Importantly, transcriptomic profiling indicated that SOX9 has distinct compartment-specific functions (Tsingas *et al.*, 2020). The SOX trio also works with paired box transcription factors PAX1 and PAX9, which are essential for AF development (Peters *et al.*, 1999; Sivakamasundari *et al.*, 2017). Despite the key importance of the *Sox* genes in disc homeostasis and skeletogenesis, the precise roles that many of these transcription factors play in the adult disc remain elusive, representing a necessary avenue for future investigations.

In addition to PAX1/9 and the SOX trio, among others, the MKX and SCX transcription factors are key to AF development. Lineage tracing studies have shown that an AF multipotent progenitor population co-expressing SOX9 and SCX contribute to the formation of the inner and outer AF (Sugimoto *et al.*, 2012). Furthermore, lineage studies have revealed that SCX-expressing cells drive AF regeneration after annular puncture of neonatal discs. This finding offers an interesting direction for future disc regeneration therapies, highlighting the importance of genetic

lineage tracing studies in mice (Torre *et al.*, 2018; Torre *et al.*, 2019). By a similar token, complete knockout of *Mkx* results in smaller collagen fibril diameter in the outer AF and early onset disc degeneration. Transplantation of MKX-overexpressing MSCs into the injured AF of a tail-loop mouse model was shown to promote AF regeneration with abundant collagen fibril formation (Nakamichi *et al.*, 2016). It is worth noting here that mice deficient in the transcription factor BACH-1 are less impacted by annular puncture. BACH-1 deficiency results in increased levels of HO-1, which protects disc cells from oxidative stress (Ohta *et al.*, 2012).

Recent investigations by Alvarez-Garcia *et al.* revealed that all compartments of the IVD express FOXO1, FOXO3, and FOXO4. Downregulation of FOXO levels, notably FOXO1 and FOXO3, was observed during aging, preceding major degenerative changes seen on histology (Alvarez-Garcia *et al.*, 2017). Simultaneous knocking out of these three FOXO isoforms using *Col2a1*Cre resulted in a progressive increase in NP cellularity, due to increased proliferation, and an associated increase in disc height. By 4 and 6 months, these discs began to show marked features of degeneration and a severe kyphosis. Interestingly, analysis of *Acan*Cre^{ERT2} driven triple knockout at skeletal maturity showed degenerative changes more conspicuous in the NP than the AF, while analysis of single gene knockouts showed that FOXO1 and FOXO3 are the dominant members, playing a role in mediating autophagy and antioxidant defences in NP tissues (Alvarez-Garcia *et al.*, 2018).

Development of the axial skeleton relies on the tight spatio-temporal control of HOX transcription factors. The *Hox* genes that encode these factors differentially control the proliferation rates of the mesenchymal condensations that give rise to the vertebral cartilages. For instance, mutations in the homeobox containing gene *Hoxb-4* (Hox 2.6) causes overt changes in the axial skeleton that are characterised by a transformation of the second cervical vertebra from axis to atlas (Ramfrez-Solis *et al.*, 1993). Similarly, *Hoxd-3* gene (Hox-4.1) disruption causes anterior transformations of the atlas and axis and a drastically remodelled craniocervical joint (Condie and Capecchi, 1993). The *Hox10* and *Hox11* genes are also involved in the global patterning of the axial and appendicular skeleton. Lumbar vertebrae fail to develop with loss of *Hox10*, while loss of *Hox11* results in formation of lumbar vertebrae in the place of sacral vertebrae (Wellik and Capecchi, 2003).

Cell signalling pathways

An intricate balance of morphogenic signals is required for proper embryonic development of the IVD. A centrally important morphogen in this process is notochord-secreted SHH, which not only patterns surrounding embryonic structures such as the neural tube and AF anlagen, but is also required for maintenance of the notochord itself and subsequent formation of the NP. Hedgehog proteins interact with the 4,6-disulphated non-reducing terminal glycans of CS chains in aggrecan and the HS chains of perlecan and this may localise SHH in tissues, aid in the formation of SHH gradients, and regulate SHH cell signalling (Bandari *et al.*, 2015; Cortes *et al.*, 2009; Ortmann *et al.*, 2015).

The necessity of SHH for disc development was demonstrated by Choi and Harfe who removed SHH signalling from *Shh*-expressing cells, including the notochord and floorplate, using a floxed mouse allele of the SHH receptor *Smo* and *Shh*GFPcre (Choi and Harfe, 2011). Conditional ablation of *Smo* during early embryogenesis resulted in defects in IVDs and vertebrae formation; however, removal of *Smo* once the notochord sheath had formed did not alter disc development, highlighting the importance of the notochord sheath during spinal patterning. The role of SHH in IVD development and postnatal homeostasis has been investigated and reviewed by Dahia and colleagues (Rajesh and Dahia, 2018).

The function of WNT/β-catenin signalling in the regulation of IVD development and degeneration has been examined in WNT/β-catenin reporter (TOPGAL) mice (Dahia *et al.*, 2009; Kondo *et al.*, 2011). It was noted that during embryonic stages, WNT signalling was active in the CEPs and AF but very low in the NP. Postnatally, however, WNT signalling by NP cells was found to increase. Overexpression of β-catenin in both *Col1a1* and *Col2a1*-expressing disc cells resulted in AF disorganisation, hyperplasia, and osteophyte formation in adult mice (Kondo *et al.*, 2011; Wang *et al.*, 2012). Deletion of β-catenin in *Col2a1*-expressing cells in 5-day-old mice resulted in accelerated maturation of subchondral bony endplate by 9 weeks of age. A later study using TOPGAL mice showed that WNT signalling in the NP declines with aging in both lumbar and caudal discs (Holguin *et al.*, 2014). Of note, WNT signalling positively regulates SHH, which activates the expression of differentiation factors and cell proliferation in NP cells, but undergoes a decline with IVD maturation (Winkler *et al.*, 2014). WNT and SHH are poorly soluble proteins and in order to establish gradients of these morphogenetic components in tissues, perlecan may have a transporter role. Indeed, WNT binds to the LDL receptor-like domains of perlecan domain II (Matsuo and Kimura-Yoshida, 2014).

Growth factors stimulate matrix production and NP cell proliferation. CTGF, aka CCN2, serves key functions in this regard. CCN2 binds to LDL receptor LRP6, which is homologous to domain II of perlecan (Segarini *et al.*, 2001), as well as integrins $\alpha5\beta1$ and $\alpha v\beta5$ (Tran *et al.*, 2014). CCN2 modulates BMP, TGF-β, and WNT signalling to coordinate chondrogenesis and angiogenesis during skeletal development (Abreu *et al.*, 2002; Mercurio *et al.*, 2004), and more recently in the prevention of degenerative disc disease (Matta *et al.*, 2017). Deletion of the *Ccn2* gene in notochord-derived cells results in age-associated degeneration of IVDs, decreased levels

of aggrecan and collagen II, and increased levels of collagen I within the NP (Bedore *et al.*, 2013; Tran *et al.*, 2013). The ability of CCN2 to regulate the composition of the IVD suggests that it may be a clinical target for the treatment of IVD degeneration.

The TGF-β superfamily are secreted proteins that play crucial roles in the determination of the notochord lineage and in skeletal development, growth, and cell differentiation. The importance of these signalling proteins in IVD development was well-demonstrated by the constitutive conditional knockout of the TGF-β type II receptor (*Col2a*Cre;*Tgfbr2*$^{fl/fl}$). These mutant embryos showed vertebrae deformities and IVDs that were either completely lost or reduced due to AF defects (Baffi *et al.*, 2004; Baffi *et al.*, 2006). Subsequent microarray analyses of the embryonic *Tgfbr2*-null discs revealed that the expression profiles were similar to that of vertebrae profiles (Sohn *et al.*, 2010), and identified the transcription factor, avian erythroblastosis virus E-26 oncogene homologue (ERG), as a player in AF differentiation. Accordingly, the function of TGF-β in disc development is thought to involve the induction of AF differentiation from the sclerotome while inhibiting chondrocyte differentiation of the presumptive IVD. More recently, *Acan*Cre^{ERT2};*Tgfbr2*$^{fl/fl}$ mice were generated to study the role of *Tgfbr2* in adult tissue. Deletion of *Tgfbr2* at 2 weeks-of-age resulted in progressive degeneration underscored by disorganisation of AF lamellae with thinning of AF collagen bundles, delayed bony endplate formation, and dystrophic calcification of the disc by 12 months of age (Alkhatib *et al.*, 2018). These studies highlight that TGF-β also plays a clear role in IVD homeostasis postnatally.

TGF-β signals through SMAD3 and AP-1, positively regulating CCN2 expression in the NP and may represent a limited reparative response in IVD degeneration (Tran *et al.*, 2010). *Smad3* null mice are smaller in size, show spinal malformations and kyphosis, exhibit alterations in CEP structure, and show decreased IVD proteoglycan and collagen content (Li *et al.*, 2009). TGF-β/SMAD3 signalling in NP cells regulates the expression of β-1,3-glucuronosyltransferase-1 (GlcAT-1), an important regulator of GAG synthesis, which may partly explain the reduced GAG levels in SMAD3 knockout mice (Wu *et al.*, 2012). It may be concluded that CCN2 and TGF-β1 are actively secreted by notochordal cells and that these direct resident disc cell populations in the maintenance of IVD homeostasis.

Additional growth factors that have a key role to play in disc homeostasis include IGF-1, FGF, and GDF5. IGF-1 and its receptor (insulin-like growth factor-1 receptor, IGF1R) have regulatory roles over extracellular matrix (ECM) synthesis and crucial roles in the maintenance of IVD homeostasis. IGF-1 and TGF-β1 both regulate MMP-2 production by NP cells (Pattison *et al.*, 2001). The IVDs of IGF1R$^{+/-}$ mice display greater histopathological scores of degeneration, reduced collagen II and proteoglycan levels, and elevated MMP13 (Li *et al.*, 2013). Aberrations to FGF-FGFR signalling are responsible for a diverse group of skeletal disorders including those that afflict the axial skeleton (Table 3). Polymorphism in GDF5 has been associated with disc degeneration in humans (Huang *et al.*, 2018); accordingly, IVDs in GDF5 knockout mice show decreased proteoglycan content and loss of water binding, evident from reduced T2-weighted signal intensity of lumbar levels (Li *et al.*, 2004).

Mutations in genes that cause over-stimulation of signalling pathways involved in cell growth and differentiation often yield predispositions to oncogenesis and other clinical pathologies, including skeletal abnormalities. Mutations in *Nf1*, which encodes neurofibromin, a GTPase-activating protein that downregulates the RAS pathway, causes NF1. NF1 is a disorder characterised by generalised focal bony lesions, dystrophic scoliosis, and tibial pseudoarthrosis. To investigate the aetiology of the skeletal abnormalities associated with NF1, researchers used *Col2a1*Cre;*Nf1*$^{fl/fl}$ mice to silence *Nf1* in axial and appendicular osteochondroprogenitor cells. These mice displayed progressive scoliosis, kyphosis, short stature, and IVD defects, closely recapitulating the clinical features of NF1 in human patients. Interestingly, inhibition of RAS/ERK by lovastatin mitigated the phenotype of these mice, suggesting that activation of the RAS/ERK pathway by NF1 loss-of-function is in part responsible for these spinal defects (Wang *et al.*, 2011). Similarly, tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterised by mutations in *Tsc1* or *Tsc2* that leads to hyperactivation of mTOR. Patients with TSC suffer from benign tumours known as tubers that affect several organ systems including sclerotic bone lesions. *Tsc1* null mice show congenital spinal defects marked by kyphosis and a degenerative disc phenotype (Yang *et al.*, 2017). Recent studies of PHLPP1, a phosphatase that negatively regulates Akt signalling, suggested that this axis may play a role in disc degeneration. Disc injury in *Phlpp1* null mice lead to Akt activation and cell proliferation and showed long-term positive effects on preserving cellularity and matrix homeostasis, implying that it can be explored as a therapeutic target (Zhang *et al.*, 2019). Ulici *et al.* have also recently reported the skeletal phenotype of JNK1 and JNK2 double-knockout mice (*Col2a1*Cre; *Jnk1*$^{fl/fl}$/*Jnk2*$^{-/-}$). These mice showed a severe and early onset scoliotic phenotype and vertebral and disc fusions (Ulici *et al.*, 2019).

A murine model of human progeroid syndrome was developed by genetic ablation of *Ercc1*, a gene that encodes a functional protein of the XPF-ERCC1 nuclease, ERCC1 (Niedernhofer *et al.*, 2006) and contributes to the repair of double stand breaks. Haploinsufficient *Ercc1* show advanced degenerative changes in the vertebral bodies and IVDs, including reduced proteoglycan content, increased apoptosis, and p16 positive cells, consistent with accelerated

senescence and aging (Vo *et al*., 2010). Further studies linked NF-κB-mediated signalling to the *Ercc1*$^{+/-}$ phenotype (Nasto *et al*., 2012).

Extracellular matrix and its turnover

In a polyaxial diarthrodial joint, the disc allows the spine to flex, extend, and rotate, with the NP functioning as a central fulcrum (Shapiro *et al*., 2012). This system is made possible by the relationship between the swelling forces of the NP due to its proteoglycan-rich matrix and opposing counter-forces provided by the concentric collagenous lamellae of the AF. Therefore, matrix changes and degradation often define degenerative disc pathology (Table 2).

High concentrations of aggrecan and HA are characteristic features of the IVD. Mice lacking HAS2 show prominent vertebral body defects underscored by a lack of endochondral ossification, decreased matrix deposition, and increased cartilage cellularity. Perhaps counter to what one might expect, NP cellularity was increased with copious vacuolated cells. Since HA can interact with chondrocytes through CD44, RHAMM, and other cell surface receptors, it is possible that lack of HA induced a state of proliferation and differentiation in the targeted cells of these mice (Roughley *et al*., 2011).

Chloe B6 mice have a knock-in mutation in the *Acan* gene that changes the sequence in the IGD from DIPEN↓FFG to DIPEN↓GTR for the purpose of blocking MMP-dependent cleavage of aggrecan (Little *et al*., 2005). These mice do not show major alterations in skeletal development, and their IVDs have no obvious developmental abnormalities. ADAMTS-5 is the major aggrecan degrading metalloprotease in murine articular cartilage and disc (Stanton *et al*., 2005), while ADAMTS-4 is the major aggrecanase in murine growth plates (Glasson *et al*., 2005). ADAMTS-4 knockout mice, surprisingly, do not show any defects in skeletal development, growth, or remodelling, and no alterations in the growth of their long bones (Glasson *et al*., 2004). Likewise ADAMTS-5 knockout mice have no skeletal developmental abnormalities, although deletion of ADAMTS-5 effectively prevents cartilage destruction in a murine osteoarthritis model and is shown to protect from chronic tobacco smoke-induced disc degeneration (Ngo *et al*., 2017). These findings are consistent with the view that ADAMTS-5 is largely responsible for the turnover of murine aggrecan

Table 2. Mouse models involving mutations in spine ECM molecules.

Model	Phenotype	References
HAS2 knockout	Vertebral body defects, decreased matrix deposition, and increased cartilage and NP cellularity.	Roughley *et al*., 2011
Chloe B6 mice	No obvious abnormalities.	Little *et al*., 2005
Perlecan knockout	Shortened growth plates, defective endochondral ossification leading to dwarfism, severe chondrodysplasia, dyssegmental ossification of spine. Generalised abnormalities in development of vasculature and musculoskeletal system, myotonia.	Arikawa-Hirasawa *et al*., 1999
PRG4 knockout	Increased torsional modulus and reduced transverse major diameter and height.	Teeple *et al*., 2015
BGN knockout	Premature osteoarthritis and early-onset disc degeneration involving both the NP and AF.	Furukawa *et al*., 2009
COL2A1 deficiency	Homozygous null: Severe skeletal defects and inability to pattern the notochord during disc development. Haploinsufficiency: Early skeletal defects including shorter spines, thicker and irregular vertebral endplates, and lower GAG levels in the AF.	Aszódi *et al*., 1998 Sahlman *et al*., 2001
COL9A1 knockout	Premature degeneration associated with physical impairment and degenerative changes in the disc including the CEP.	Allen *et al*., 2009 Boyd *et al*., 2008 Goldring, 2009
ADAMTS-4 knockout	No obvious skeletal phenotype.	Glasson *et al*., 2004
ADAMTS-5 knockout	Protective against cartilage destruction and chronic tobacco smoke induced disc degeneration.	Ngo *et al*., 2017
MT1-MMP knockout	Inadequate collagen turnover, dwarfism, kyphosis, accelerated age-related osteoarthritic changes evident in axial skeleton.	Holmbeck *et al*., 1999
SPARC knockout	Endplate calcification and sclerosis. Elevated cell numbers in the AF, greater incidence of AF herniations. Behavioural studies evidence pain.	Gruber *et al*., 2005 Millecamps *et al*., 2012
Thrombospondin-2 knockout	Impaired collagen fibrillogenesis, reduced levels of transglutaminase, fragile skin, and disorganisation of annular lamellae.	Gruber *et al*., 2008

Table 3. Mouse models examining aberrant FGF-FGFR signalling effects on spinal development.

Model	Mechanism	Phenotype	References
Ectopic FGF-2 expression	FGF-2 transgene	Skeletal dwarfism	Coffin *et al*., 1995
FGF-2 deficient mouse	Knockout mutation	Inhibition of bone formation/bone mass	Montero *et al*., 2000
Apert Syndrome FGF2(+/S252W) mouse	FGF-2 knock-in	Abnormalities in cartilage and bone development	Wang *et al*., 2005
Ectopic FGF-9 expression	FGF-9 transgene	Achondroplasia like dwarfism	Garofalo *et al*., 1999
FGFR3 deficient mice	Knockout mutation	Skeletal overgrowth	Colvin *et al*., 1996 Deng *et al*., 1996
FGFR3	Knock-in K644M mutation	Severe dwarfism	Iwata *et al*., 2001
FGFR3	Knock-in S365C mutation	Severe dwarfism	Chen *et al*., 1999
FGFR3	Knock-in K644E mutation	Achondroplasia like dwarfism	Li *et al*., 1999
FGFR3	Knock-in K644E mutation	Thanatophoric dysplasia like dwarfism	Iwata *et al*., 2000
FGFR3	Knock-in G380R mutation	Achondroplasia like dwarfism	Naski *et al*., 1998
FGFR3	Knock-in G369C mutation	Achondroplasia like dwarfism	Chen *et al*., 1999
FGFR2 deficient mouse	Knockout mutation	Dwarfism, abnormal spinal development	Yu *et al*., 2003
FGFR-1 deficient mouse	Knockout mutation	Osteoglophnic dysplasia abnormal long bone and skeletal development	White *et al*., 2005

in-vivo (Glasson *et al.*, 2005). ADAMTS-5-dependent aggrecanolysis predominates over ADAMTS-4 in mouse cartilaginous tissues due to an absence of highly sulphated KS chains in the murine IGD (Stanton *et al.*, 2005; Stewart *et al.*, 2006). These mutants suggest that preventing aggrecan turnover do not lead to major effects on skeletogenesis and disc health.

Naturally occurring and engineered mutations in the gene encoding perlecan (*Hspg2*) show unequivocally that perlecan is essential for cell growth, differentiation, and the function of cartilaginous tissues (Arikawa-Hirasawa *et al.*, 1999; Arikawa-Hirasawa *et al.*, 2001). Both the core protein and the GAG chains confer perlecan's ability to modulate these processes. When substituted with HS, perlecan domain I promotes binding to laminin-1 and collagen IV. Perlecan can also interact with a number of fibrillar or cell attachment molecules in the ECM and thus plays important roles in its organisation (Melrose *et al.*, 2008). The HS side chains of domain I of perlecan act as low affinity co-receptors for growth factors, such as FGF-1, -2, -7 and -9, and a core protein receptor for FGF-7 has also been reported (Ghiselli *et al.*, 2001; Mongiat *et al.*, 2000). This complex formation is important for the correct presentation of the FGFs to FGFRs, their subsequent oligomerisation, activation to initiate cell signalling through the cytoplasmic tail of the FGFRs, and subsequent downstream effects on cell proliferation and differentiation (Chang *et al.*, 2000; Chuang *et al.*, 2010; Knox *et al.*, 2001; Knox and Whitelock, 2006). Perlecan contains multiple modules that are homologous to the LDL receptor (Murdoch *et al.*, 1992; Noonan *et al.*, 1991). When expressed on cells, domain II of perlecan binds and internalises LDL in a similar manner to LRP6 (Fuki *et al.*, 2000). The development of perlecan gene knock-out mice has demonstrated the essential role played by perlecan in cartilage development and skeletogenesis. Homozygous knockout mice, which survive to birth, display severe skeletal defects with short axial and limb bones, defects of vertebral ossification centres, cleft palate, and striking abnormalities in the growth-plates of their long-bones (Arikawa-Hirasawa *et al.*, 1999).

Null mice for the proteoglycans PRG4 and BGN have also been generated. Biomechanical studies of PRG4 knockout spines have revealed an increased torsional modulus, and disc dimension measurements suggest that null discs have a reduced transverse major diameter and height, with an NP that takes up a relatively larger area of the disc (Teeple *et al.*, 2015). BGN is a SLRP that contributes to collagen fibrillogenesis, control of the bioavailability of TGF-β activity, and cellular regulation. Consequently, BGN knockout mice display premature osteoarthritis and early-onset disc degeneration involving both the AF and NP (Furukawa *et al.*, 2009).

In addition to proteoglycans, collagens are major matrix constituents of the disc matrix. Inactivation of COL2A1 leads to premature ossification and IVD degeneration. One-month-old *Col2a1* (+/−) mice have shorter spines, thicker and irregular vertebral endplates that calcify prematurely, and lower GAG levels in the AF. By 15 months, some compensation in these spinal changes occur (Sahlman *et al.*, 2001). Homozygous transgenic mice lacking COL2A1 die at birth and show severe skeletal defects characterised by impaired endochondral ossification and an inability to pattern the notochord during disc development (Aszódi *et al.*, 1998).

Collagen IX is a nonfibrillar collagen composed of three gene products: alpha-1(IX), alpha-2(IX), and alpha-3(IX). Collagen IX molecules are localised on the surface of type II-containing fibrils and consist of two arms. These include a long arm that is crosslinked to collagen II and a short arm that projects into the perifibrillar space (Diab *et al.*, 1996). It has been proposed that collagen IX molecules are involved in the interaction of fibrils with each other or with other components of the ECM. A mouse lacking both isoforms of the alpha-1(IX) chain has been developed (*Col9a1* null). *Col9a1* null mice do not show evident abnormalities at birth, but progressively develop marked joint degeneration as is seen in osteoarthritis. *Col9a1* null mice also develop premature degeneration associated with physical impairment and degenerative changes in the disc, including the CEPs. Behavioural studies measuring reflexes, beam walking, pole climbing, wire-hanging, grip strength, sensorimotor skills, and mechanical and thermal pain sensitivity were all compromised in these knockout mice (Allen *et al.*, 2009; Boyd *et al.*, 2008; Goldring, 2009).

Interestingly, while preventing aggrecan turnover in murine tissues does not result in major phenotypes, blocking collagen turnover in some mouse models can have severe effects on skeletogenesis (Glasson *et al.*, 2004; Holmbeck *et al.*, 1999; Little *et al.*, 2005). Mouse strains deficient in MMP-1, -2, -3, -7, -8, -9, and -12 have been developed (Bian *et al.*, 2016; Foley and Kuliopulos, 2014; Van Hove *et al.*, 2016; Kato *et al.*, 2015; Robinson *et al.*, 2003; Wells *et al.*, 2003; Wilson *et al.*, 1999). While some changes in haematopoiesis and angiogenesis associated with the maturation of long bone growth plates have been reported in some of these models, little or no impairment in skeletal development has been observed. The contributions of individual enzymes and functional overlap with other MMPs is therefore important to take into consideration. In contrast, membrane type-1 MT1-MMP (aka MMP-14) knockout mice display cervical kyphosis, disc degeneration, and accelerated age-dependent osteoarthritic changes in the axial skeleton. This is apparently due to inadequate collagen turnover, which emphasises the critical role MT1-MMP plays in the conversion of MMPs from their inactive precursors into their active forms to promote collagenolysis *in-situ* (Holmbeck *et al.*, 1999). Likewise, MMP13 efficiently cleaves collagen II. MMP13 knockout mice display significant accumulation of interstitial collagen in their growth

plate cartilages and delayed endochondral ossification due to inefficient collagen turnover (Inada *et al*., 2004; Stickens *et al*., 2004). Its abundant presence in severely degenerated discs also highlights the deleterious role MMP13 can play in pathological states.

Matricellular proteins have emerged as important regulators of cell-ECM interactions and a growing body of evidence has established roles for these proteins in disc homeostasis. SPARC is a matricellular protein important in the maintenance of IVD integrity. Radiological examination of spines from 2-month-old SPARC knockout mice revealed wedging, endplate calcification, and sclerosis. At 3 months onwards, SPARC knockout mice showed elevated cell numbers in the AF and a greater incidence of AF herniations. Aged SPARC knockout mice mirrored aspects of low back pain as they displayed changes in cutaneous sensitivity to cold, heat, mechanical stimuli, and ambulation, indicating pain may be emanating from the disc (Gruber *et al*., 2005; Millecamps *et al*., 2012). Similarly, mice with disruption of the gene encoding thrombospondin-2, a multifunctional, anti-angiogenic matricellular protein, showed impaired collagen fibrillogenesis, reduced levels of the intermolecular cross-linking enzyme transglutaminase, fragile skin, and disorganisation of annular lamellae (Gruber *et al*., 2008).

Ectopic calcification is a notable feature that is known to occur in several spinal disorders. Mice lacking ENT1, a model of diffuse idiopathic skeletal hyperostosis, show reduced expression of ANK, ENPP1, and ALPL in discs, increased AF cell proliferation, and progressive ectopic calcification of fibrous connective tissues along the spine (Warraich *et al*., 2013). Analyses of AF tissue isolated from ENT1 knockout mice has further revealed that the observed increased cell proliferation is associated with an upregulation of E2F transcription factors, the cell cycle regulators RB1 and CDK2, and stimulation of the JNK/MAPK pathway (Veras *et al*., 2019). Notably, the importance of genetic background in the ectopic calcification process has been recently demonstrated by the study of LG/J inbred mice, which show an age-dependent increase in dystrophic mineralisation of discs (this mouse strain will be discussed later in the review) (Novais *et al*., 2020).

An evolving view of inflammation in disc degeneration

An association between increased inflammation and IVD degeneration is well-described and most commonly attributed to the cytokines TNFα and IL-1β. Nevertheless, investigations of human TNFα overexpressing mice (Tg197 and hTNFαTg) and IL-1α/β double knockout (IL-1 KO) mice have led to questioning the understanding of the role inflammation plays in disc homeostasis. While polyarthritic Tg-hTNF mice show compromised vertebral bone parameters, AF defects, and a greater propensity for herniations at the CEP/AF junction, the presence of increased TNF-α in these mice did not show adverse effects on the NP compartment. Surprisingly, Tg-TNF mice showed healthy-appearing NP cells that assembled into an expanded cell band and the overall transcriptomic profile remained unaltered (Gorth *et al*., 2018; Gorth *et al*., 2020). This phenotype contradicts the existing view that chronic TNFα overexpression would cause pronounced disc degeneration, as the articular joints of these mice are severely degenerated by rheumatoid arthritis. Plausible reasons for this phenotype could be that previous studies have exclusively used herniated human disc material with infiltrated immune cells as starting material for the analysis, shaping the understanding of the function of TNFα in the disc compartment. This phenomenon, in which differential effects of inflammation are observed in discs as opposed to articular cartilage, has also been noted in TonEBP haploinsufficient mice. TonEBP-deficient NP cells downregulate pro-inflammatory molecules yet experience notable degeneration, while, on the other hand, TonEBP-deficiency is protective against immune-driven arthritis (Tessier *et al*., 2020b). The differential effects of inflammation on these joints may be credited to the fact that the inner disc is largely avascular and immune privileged, unless penetrated by herniation. Similarly, while conditional loss of p16^{Ink4a} (*Acan*CreER^{T2}; p16Ink4a$^{f/f}$) shows lower NP levels of IL-1β, IL-6, and MCP-1, these mice are not protected from age-dependent degenerative changes (Novais *et al*., 2019). Global IL-1α/β double knockout mice also show higher degenerative grades with aging than wild-type counterparts, suggesting that loss of IL-1 does not serve a protective role (Gorth *et al*., 2019). Seemingly contrary to the phenotype of these IL-1 knockout mice, mice null for the *IL-1rn* gene were reported to develop spinal abnormalities (Phillips *et al*., 2013). However, the discrepancies in these findings may be reflective of mouse strain-dependent effects (C57BL/6, IL-1KO; BALB/c, IL-1rnKO), highlighting the complexity of inflammation on disc health.

The cytoskeleton

The cytoskeleton is fundamental to cell biology as it maintains intracellular organisation and cell shape, offers mechanical support, and links the cell to extracellular components by transmembrane proteins to enable functions such as cell adhesion and migration. Although this critical component of the cell is largely understudied in IVD tissues, a few mouse models targeting the cytoskeleton have been developed. These include mice lacking ARP2/3, the branched actin nucleator, and FLNB, an actin-binding protein which forms a linking scaffold to coordinate signal transduction (Table 4).

Constitutive deletion of the *Arpc2* gene encoding a critical subunit of the ARP2/3 complex in collagen II-expressing cells (*Col2a1*-Cre; *Arpc2*$^{fl/fl}$) results in severe chondrodysplasia and spinal defects. These mice are dwarfed due to disorganised growth plates, present with kyphosis, and show dramatic changes to the IVDs, some of which are fused. On histology,

Table 4. Mouse models with mutations in cytoskeleton genes.

Model	Phenotype	References
Col2a1-Cre; *Arpc2*$^{fl/fl}$	Severe chondrodysplasia, spinal defects, dwarfism, growth plate expansion, deformation of AF and NP compartments, fused discs.	Tessier *et al.*, 2020a
Acan-CreER^{T2}; *Arpc2*$^{fl/fl}$	NP cell-band expansion and AF degeneration marked by altered matrix composition.	Tessier *et al.*, 2020a
FLNB knockout	Postnatal progressive disc degeneration, endochondral-like ossification of AF, vertebral fusions.	Zieba *et al.*, 2016

the growth plates are grossly expanded, the NP is reduced in size, and the AF is deformed and disorganised, containing rounded chondrocyte-like cells. Embryonic and postnatal lethality in these mice led to the crossing of *Arpc2*$^{f/fl}$ with the inducible *Acan*-CreER^{T2} allele to generate *Acan*-CreER^{T2}; *Arpc2*$^{f/fl}$ mice. Resultant loss of ARP2/3 at skeletal maturity was shown to induce notable AF degeneration marked by altered matrix composition. *In vitro* analyses further revealed that ARP2/3 controls TonEBP-dependent osmoadaptation and cell-ECM interactions in NP cells (Tessier *et al.*, 2020a).

Similarly, the discs of FLNB$^{-/-}$ mice show rapid and progressive degeneration during postnatal development. The AF cells of these mice undergo differentiation into the chondrogenic lineage, acquiring the signature of hypertrophic chondrocytes and upregulating TGFβ and BMP signalling. Consequently, AF tissues show endochondral-like ossification, express collagen X, and evidence increased apoptosis, leading to vertebral fusions (Zieba *et al.*, 2016). Loss-of-function of other factors known to influence the cytoskeleton such as N-cadherin and $\alpha_v\beta_6$ integrin have also been investigated in mice and are associated with altered disc cell morphology and degenerative phenotypes (Bian *et al.*, 2017; Hwang *et al.*, 2016).

Nuclear HS-proteoglycans such as perlecan have recently been identified in NP cells. These may interact with cytoskeletal components and re-organise the nucleosome and chromatin organisation which may regulate the access of transcription factors to DNA (Hayes and Melrose, 2021; Kovalszky *et al.*, 2014). Nuclear HS-proteoglycans inhibit histone deacetylases resulting in chromatin compaction affecting DNA accessibility to transcription factors. HS also inhibits DNA topoisomerase I activity, which has important roles to play in (1) the removal of DNA supercoils during transcription and DNA replication; (2) the re-annealing of DNA strands following strand breakage during re-combination and chromosomal condensation; and (3) the disentanglement of intertwined DNA strands during mitosis (Champoux, 2001; Wang, 2002). Nuclear heparanase may have roles in the regulation of nuclear HS-proteoglycans (Chen and Sanderson, 2009).

Insights from inbred mice

While genetically modified mice have offered important insights into the function of individual genes, recent investigations of inbred mouse strains (SM/J, LG/J, and C57BL/6) have provided invaluable lessons on the pathogenesis of disc degeneration. SM/J (small) and LG/J (large) mice, generated by crossing multiple inbred strains and selecting for body size, are models of choice for QTL analysis. These strains show differential cartilage healing capacities: SM/J is considered a poor healer strain whereas LG/J is considered a super healer. The SM/J is the first mouse model to show early onset and spontaneous disc degeneration without the need of overt, inflammation-causing injury such as annular puncture, tail looping, or mechanical overloading (Choi *et al.*, 2018b; Zhang *et al.*, 2018). Thus, the SM/J is proposed to more closely resemble the chronic disc degeneration seen in most humans. The disc phenotype of these mice has been characterised by matrix fibrosis, elevated markers of hypertrophic chondrocytes, increased NP cell death, decreased WNT and VEGF signalling, and dysregulated ion transport systems (Choi *et al.*, 2018b; Zhang *et al.*, 2018). An extensive characterisation and comparison of these inbred mice, as well as the commonly used C57BL/6J strain, during the aging process has been recently conducted by Novais *et al.* (2020). An important insight derived from these studies is the observation that each mouse strain possesses a unique disc degeneration phenotype, with dissimilar morphological features, transcriptomic signatures, and onset of disease. Strain-dependent differences at the gene expression and histological level following traumatic injury have also been recently demonstrated (Brent *et al.*, 2020). Ultimately, these differences underscore the notion that disc degeneration is largely influenced by genetic background and therefore, when combined with environmental and mechanical factors, presents heterogeneously across individuals as sub-phenotypes.

Conclusions

With the advances in knowledge provided by the completion of the human genome project and elucidation of the complete murine genome, the mouse represents the vehicle of choice for further examining the role of specific human gene abnormalities on spinal development and homeostasis in health and disease. While the mouse musculoskeletal system displays notable differences, including the persistence of notochordal-NP cells, quadrupedalism, additional vertebrae, and some variations in the structure of proteoglycans, the mouse model nevertheless is an attractive experimental system to answer important questions in spinal pathobiology. Due to these intrinsic differences, careful interpretation of experimental findings is indicated when attempting to make comparisons with the human musculoskeletal system. The described studies on the mouse IVD have identified many potential therapeutic targets worthy of further

examination, not only in the mouse, but also in other animal models, to ultimately identify their roles in human disc degeneration.

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Discussion with Reviewer

Reviewer 1: Which mouse model, or which combination of the described mouse models, would you recommend for studying spontaneous disc degeneration?

Authors: Spontaneity eludes to a disease process that is not initiated by intentionally made genetic or physical (*i.e.* AF puncture, tail looping) manipulations. Therefore, the selection of a mouse model that displays spontaneous disc degeneration must be limited to the inbred strains. Furthermore, the acknowledgment that disc degeneration encompasses a diversity of degenerative phenotypes precludes the use of a single model. With that held into consideration, the SM/J and LG/J inbred strains are especially useful, as they have been extensively characterised and show similar degeneration characteristics to that of human disease. SM/J mice show degeneration at a relatively early age (Choi *et al.*, 2018b), whereas LG/J mice evidence disc calcification with ageing (Novais *et al.*, 2020). In the case of SM/J mice, the early onset aspect facilitates experimental investigations when time constraints are imposed and helps to lessen the costly housing of mice for extended periods of time, as is done in aging studies.

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