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IVD Development: Nucleus pulposus development and sclerotome specification

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Abstract

Purpose of review—Intervertebral discs (IVD) are derived from embryonic notochord and sclerotome. The nucleus pulposus is derived from notochord while other connective tissues of the spine are derived from sclerotome. This manuscript will review the past 5 years of research into IVD development.

Recent findings—Over the past several years, advances in understanding the step-wise process that govern development of the nucleus pulposus and the annulus fibrosus have been made. Generation of tissues from induced or embryonic stem cells into nucleus pulposus and paraxial mesoderm derived tissues has been accomplished *in vitro* using pathways identified in normal development. A balance between BMP and TGF- β signaling as well as transcription factors including Pax1/Pax9, Mxk and Nkx3.2 appear to be very important for cell fate decisions generating tissues of the IVD.

Summary—Understanding how the IVD develops will provide the foundation for future repair, regeneration, and tissue engineering strategies for IVD disease.

Keywords

axial skeleton development; sclerotome; notochord; annulus fibrosus; nucleus pulposus

Introduction

Intervertebral discs (IVDs) are derived embryonically from structures called the notochord and sclerotome [1–3]. The nucleus pulposus (NP), the central core of the IVD, is derived from the notochord while the annulus fibrosus (AF), the fibrous tissue that provides the structural characteristics to the IVD, is derived from the sclerotome [4–6] (Figure 1).

The notochord is a transient, rod-like mesodermal structure that forms from ectodermal cells during the third week of development in human embryos [7]. In mice and humans, the

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Compliance with Ethical Standards

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Conflict of Interest

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notochord will eventually develop into the NP of the IVD. In vertebrates, the notochord arises from the dorsal organizer during gastrulation around E6.0 in mouse [8]. Initially, the dorsal organizer must transition into the chordamesoderm [8]. The chordamesoderm is the direct antecedent of the mature notochord. It is morphologically and molecularly distinct from other mesoderm. Cell migration and movement forces the mediolateral intercalation and convergence of cells towards the midline of the embryo (convergent extension) resulting in elongation of the notochord between E8.5 and E10.5 in mice [8]. At this stage, the chordamesoderm is viewed as an elongated stack of cells. Subsequently, chordamesodermal cells acquire a thick extracellular sheath, called the notochordal sheath, and the cells become vacuolated [8]. Osmotic pressure acts against the sheath to give the notochord its finalized rod-like appearance [8]. A recent study investigated the importance of mechanical forces during notochord morphogenesis. This study established that mechanical forces from the amniotic cavity are required for the process of convergent extension [9]*. Mechanical forces during embryogenesis are also important for the proper elongation of the embryo and locomotion in some invertebrate and vertebrate species [10]. It is possible that mechanics-based mechanisms such as this assist later in the formation of the NP specifically during the exclusion of the notochord from the vertebral body and segmentation of the notochord at E15.5 days in the mouse. Recent evidence suggests that the notochord and notochordal cells will directly develop into the NP.

The sclerotome is derived from the somites, structures that pattern and segment the embryo (Reviewed in [5, 11, 12]). This process begins with dynamic reorganization of the embryo and the formation of a structure called the primitive streak [13, 14]. The primitive streak marks the beginning of gastrulation (E6.0 in mouse), which establishes the three germ layers of the embryo [15, 14]. The germ layers are ectoderm, endoderm and mesoderm, with the somites and sclerotome being derived specifically from the paraxial mesoderm, the tissue immediately adjacent to the neural tube. The rod-shaped paraxial mesoderm forms the somites beginning at day E8.0, mouse. Somitogenesis occurs in an anterior to posterior fashion along the embryo axis, where the spherical structures bud off from the paraxial mesoderm in a fashion that is highly regulated temporally and spatially [16, 17]. The last somite in the tail forms around E15, mouse. The accepted model for somite formation is the “Clock and Wavefront Model” where Notch and Wnt signaling acts as the “clock” that provides the temporal, permissive signal for somitogenesis, and FGF signaling acts as the “Wave”. The wave is responsible for the anterior to posterior formation of somites, due to FGF signaling providing the directionality and spatial regulation needed to stimulate the epithelial to mesenchymal transition of the paraxial mesodermal cells, thus pinching off of a newly formed somite [17, 16, 18]. The outer layer of the somites consist of epithelial cells while the inner cells called the somitocoel, which can contribute to the AF of the IVD, remain mesenchymal [19, 20]. Almost as soon as it is formed, the somite differentiates into separate tissues: the dermamyotome, which further differentiates into the dermatome and the myotome, and the sclerotome. The dermatome forms the epidermis of the back, the myotome forms the skeletal muscle, and the sclerotome forms the vertebrae, IVD, cartilaginous end plates and other connective tissues of the spine [21, 17]. Dermamyotome differentiation is stimulated in the dorso-lateral region of the somite by Wnt, Noggin, and Bmp from the roof plate and epidermis and the inhibition of Shh. Sclerotome differentiation

is stimulated in the ventro-medial region of the somite by Shh signaling from the floor plate and notochord, which induces expression of the sclerotomal markers Pax 1, Pax 9 and Mfh1 [17, 22–25]. The AF is subsequently divided into two compartments, the inner AF (iAF) and outer AF (oAF). iAF is a fibrocartilage exhibiting characteristics of both fibrous tissues and cartilage. oAF is more fibrous and resembles ligaments in many ways. The AF is highly organized and exhibits a lamellar structure [26].

This manuscript will review the past 5 years of research into the development of the IVD. Specifically, what has been learned about the formation of the NP from the notochord and how the sclerotome becomes specified to form vertebral body (VB) or the AF.

Development of the nucleus pulposus

The notochord sheath

The notochord sheath is a thick extracellular matrix that surrounds the vacuolated cells of the notochord. Mutations in genes encoding proteins that compromise the notochordal sheath have been demonstrated to affect the development of the NP. Sox5/6 are required for the formation of the notochordal sheath and subsequent survival of the notochord cells and development of the NP [27]. Deletion of *Smo* from Sonic Hedgehog-expressing cells resulted in complete absence of the notochordal sheath, and the NP subsequently did not expand into the IVD region, indicating the importance of the notochord sheath in full development of the NP [28].

Identification of NP cells

Discerning the notochord from the NP is a difficult task. MRI and X-ray techniques do not offer sufficient resolution to distinguish notochord from developing NP in mice. Using high resolution contrast-enhanced micro-computed tomography (microCT), Bhalla et al have attempted to distinguish these two tissues in mice [29]*. Phosphomolybdic acid (PMA), which targets collagen residues, was used to track changes in the notochord as it developed into the NP. PMA-stained functional units revealed increased attenuation of the notochord tissues across 1 day, 10 day, 3 month, 6 month, and 1 year timepoints. Surface area to volume ratio (S.a/V.) provided a measure for the morphology of the notochord. High S.a/V. indicated more surface than volume suggesting a more diffuse structure. Low S.a/V. suggested a more block-like structure. At earlier timepoints (1 day) the S.a/V. ratio was high, however, at 10 days the ratio decreased dramatically indicating that the notochord had condensed. With aging, the notochord increased in volume but maintained its S.a/V. ratio, suggesting that the NP develops as the structure enlarges but maintains its morphology.

Controversy has surrounded the true identification of the NP cell type. Many argue that the NP population of cells is heterogenous even in adulthood and that different cell types within the NP may be more susceptible to catabolic stimulation inducing degradation. New studies have attempted to distinguish these different subpopulations and their responses to stress that would be found in aging. Van Den Akker et al. immortalized two different NP cell clone populations which they distinguished as responders (NP-R) and non-responders (NP-nR) [30]**. Upon stimulation with chondrogenic media, NP-R cells induced Sox9 and Col2a1

expression while NP-nR cells did not. The authors of this study believe that NP-R cells could represent undifferentiated cells and NP-nR cells are differentiated cells. To further prove that cellular response is different, this group studied EGR1, an immediate early response gene (IEG). These genes are believed to provide a link between the cell micro-environment and its physiology. EGR1 is induced when NP-R and NP-nR cells were stimulated with agents such as ITS or VPA (valproic acid), which are known to induce IEGs. Baseline expression of EGR1 was low in NP-R and NP-nR cells, however, when they were challenged with IL-1 β , EGR1 expression increased significantly. EGR1 was then knocked down by siRNA in the presence of IL-1 β to test whether gene expression of genes (*COX2*, *TNF- α* , *MMP3*, and *ADAMTS4*) implicated in IVD degeneration were mediated by EGR1. NP-nR clones were more responsive to catabolic stimuli (higher catabolic gene expression) than NP-R clones. Cells in degenerate disc tissue were also positive for EGR1. Therefore, the results support the assertion that distinct subpopulations within the NP respond differently to catabolic stimuli during adulthood leading to disc degeneration.

Many notochordal and NP marker genes have been identified previously in the literature. It is not well-known whether these marker genes are continually expressed during the aging process or degenerative process. Richardson et al studied human tissues at various ages and stages of degeneration to investigate whether NP cells continue to express notochord markers [31***]. Gene expression from 60 individual tissues and immunohistochemistry from 56 individual tissues indicated that expression of NP markers FOXF1, PAX-1, KERATIN-8/18, carbonic anhydrase-12, and notochordal markers brachyury, galectin-3, and CD24 are maintained in cells of the NP irrespective of age or stage of degeneration. Furthermore, this study identified a subpopulation of NP cells that express carbonic anhydrase-12, galectin-3, and CD24. This suggested that cells with a notochordal-like phenotype could persist in the NP into adulthood.

Transition of notochord to NP cells

In the past five years, studies have been performed attempting to identify genes that are crucial for the notochord to NP transition and to identify marker genes for both cell populations. One study performed a whole transcriptome analysis of notochordal cells that were sorted from *Shh*-cre;ROSA:YFP mice at E12.5 and P0 [32***]. *Shh* is specifically expressed in the developing notochord. 5000 genes were differentially expressed in cells isolated from E12.5 versus P0 embryos. Expression of the mRNA for Sonic Hedgehog pathway elements such as *Shh* ligand, *Ptch1*, *Smo*, and *Gli1*, 2, and 3 were significantly reduced at P0 compared to E12.5 whereas TGF- β family components such as *TGF β 1* ligand and *Tgfbr2* were significantly increased. *IGF-1* ligand and *IGFBP 6* and 7 gene expression were also increased at P0 but Wnt signaling pathway components including *Wnt 1*, 3, 6, 11, 5a, 7a, and, 9b were decreased at P0 compared to E12.5. TGF β 1 protein staining was only observed in the surrounding mesenchyme at E12.5 but IGF-1 protein was observed within the notochord. Both TGF β 1 and IGF-1 were observed in NP cells at P0. As expected, expression of mRNA of several extracellular matrix components including *Acan*, *Bcan*, *Bgn*, *Dcn*, *Col1a1*, and *Col6a1* were increased at P0 in comparison to E12.5 indicating maturation of the tissue. At P0, aggrecan and COL6 protein were diffusely detected in the NP and at the outer boundary of the NP. mRNA expression for the NP marker genes, *T* gene (brachyury),

Keratins 8 and 18, and *HIF-1 α* were comparable at E12.5 and P0. These data suggest that signaling and biosynthesis of notochordal-derived cells can change depending on the developmental stage. Furthermore, it may be possible to discern notochordal cells at different stages of development during the transition to NP using these gene markers.

To further characterize the genes that are essential during this notochord to NP transition, Tang et al attempted to differentiate human induced pluripotent stem cells (hiPSCs) into NP-like cells using a stepwise protocol [33***]. The study demonstrated that hiPSCs cultured initially in colonies and stimulated with BMP4/FGF2 and Wnt-3a/Activin A can express early mesodermal and notochordal marker genes. Cells were then cultured in monolayer with the addition of GDF-5 and lastly in pellet culture with TGF- β 3 and l-Proline to stimulate matrix synthesis indicative of the NP cell type. This protocol led to the formation of larger more uniform cell pellets and vacuole-like structure characteristic of NP-like cells. Immunohistochemistry staining revealed that these cells also expressed the NP markers CD24, LM- α 5 and BASP1. Furthermore, in an attempt to enrich notochordal progenitor cells, hiPSCs were transduced using lentivirus containing reporter constructs for transcription factor brachyury (*T*) promoter and GFP. Cells were sorted by flow cytometry on *T* expression based on GFP intensity. The highest GFP-expressing cells were differentiated using the established protocol and showed higher expression of the NP markers CD24, LM- α 5 and BASP1. Intense and uniform GAG expression was also observed in these pellet cultures by Safranin O staining. This study indicated that stimulation with factors identified in developmental studies can be used to guide stem cell fate to NP-like cells.

Maintenance of the NP

A recent study targeted notochordal cells using a Foxa2-cre line and deleted HIF-1 α , a transcription factor stabilized within the prospective NP [34]**. HIF-1 α is used as an adaptive response to a variety of stresses including low oxygen tension within the NP. Notochord development was not altered in this study. However, at E15.5, mutant NPs appeared smaller than control NPs and lacked the large vacuoles that are indicative of normal NPs. Furthermore, by 1 month of age, the NP had disappeared in mutants and was replaced by a fibrocartilaginous tissue that stained intensely with Safranin O. Lineage studies and TUNEL assay also revealed that NP cells did not transdifferentiate into chondrocyte-like cells but rather underwent massive cell death in mutants. Functionally, mutant NPs had a significantly reduced load damping capacity. Mutant spines had an impaired ability to absorb axial energy transmitted through them. These results identified HIF-1 α as a critical gene in the development and maintenance of the NP in mice.

Development of vertebral body and annulus fibrosus

The sclerotome is an embryonic tissue composed of pluripotent, mesenchymal stem cells. These cells will eventually form the connective tissues of the spine including the VB, the AF of the IVD, tendon and ligaments. Sclerotome is derived from somites of the paraxial mesoderm. Much research has currently been devoted to stimulating the differentiation of stem cells into paraxial mesoderm and its derivatives using growth factors and/or small

molecule inhibitors. This process of deriving tissue from undifferentiated cells by using cues from development has been coined “developmental engineering [80–83].” A recent study derived paraxial mesoderm-like cells from pluripotent embryonic stem cells (ESCs) using small molecule inhibitors *in vitro*. Formation of the paraxial mesoderm is dependent upon Wnt3a and Noggin signaling [35, 36]. By using a GSK3 inhibitor to mimic Wnt3a signaling and an inhibitor of BMP type 1 receptors to replace Noggin, the ESCs began to express Tcf15 and Meox1, which are paraxial mesoderm markers [37***]. It was noted that these cells could potentially be used as precursors for engineering or repair of connective tissues in the spine. The formation of chondrogenic mesoderm, identifiable by cell surface receptor expression of Flk-1[−]/Pdgfra⁺, from mouse ESCs was accomplished by using Activin, Wnt and VEGF to stimulate paraxial mesoderm formation and subsequent treatment with BMP4 or GDF5 to stimulate chondrocyte-like cells [39***]. Another study stimulated the differentiation of 12 human mesodermal cell lineages from induced pluripotent stem cells using extrinsic signals previously shown to be critical during mesoderm formation and differentiation [38***]. These studies highlight the current advancements in regenerative science and the potential to use these discoveries in regeneration and engineering strategies.

Resegmentation

Resegmentation is the splitting of the sclerotome along the rostral-caudal border to allow the migration and subsequent combining of the two resulting halves with the adjacent segment. This reorganization allows for the placement of the myotome adjacent to the developing vertebrae staggered at one half segment and permits the nerves projecting from the spinal cord to innervate the developing muscles [40–43]. Resegmentation is necessary for the proper formation of the IVD and vertebrae and perturbations to this process have clinical implications. Failure of the proatlax sclerotome, which will partially go on to form the C1 vertebrae, to resegment results in rare disorders observed in humans called Proatlax Segmentation Anomalies (PSA) OMIM: 109500. These disorders result in the malformation of the proatlax and thus dysplasia within the craniovertebral junction [44*, 45*, 46*]. Effects of this disorder can manifest in various ways clinically, such as cervical vertebrae fusion and ventral brain stem compression [46, 44]. Klippel-Feil Syndrome OMIM: 613702, where patients suffer from a short, truncated neck and cervical vertebrae fusion, has also been associated with a disruption in resegmentation [47].

At the start of resegmentation, the sclerotome establishes rostral-caudal polarity by the expression of rostral markers such as Mesp2 and Tbx18 and caudal markers such as Ripply 1 and 2, Uncx4.1 and Pax 1/9 [48–51]. Once polarity is established, the sclerotome migrates ventrally to surround the notochord. At this time the sclerotome splits into two separate halves along the rostral-caudal border called von Ebner’s fissure and combines with half of the adjacent sclerotome [5, 42]. As a result, in mice, the IVD is derived from the cells in the caudal half of the sclerotome near von Ebner’s fissure. However, in chicks the IVD appears to be derived from the rostral half near the fissure [52]. The newly combined halves form the VB ventrally, while some of the cells migrate dorsally to surround the neural tube and form the neural arches [6]. Resegmentation has been observed and verified in multiple vertebrate model systems, but the path that sclerotome follows to form the vertebrae after resegmentation has been brought into question. A new model was recently proposed that

suggests the sclerotome does in fact undergo resegmentation, but the migration pattern as the cells move dorsally to surround the neural tube is not linear. The resegmentation-shift model proposes that the migrating sclerotomal cells shift cranially in respect to the ventral sclerotome as the cells migrate dorsally to form the neural arches. By using lipophilic dyes to label the migrating sclerotome in the chick model, it was shown that this resegmentation-shift phenomenon happens specifically in the lumbo-sacral region of the spine, while the sclerotomal cells from the cervical and thoracic regions do not undergo this cranial shift and migrate dorsally in a linear fashion. It is believed this cranial shift is necessary to establish the slant of the neural arch pedicle specifically in the lumbo-sacral region[53]**.

Sclerotome specification

Sclerotome specification into the various tissues of the spine during development is a tightly controlled and highly dynamic process. Sclerotome cells organize to form many structures within the spine including the hyaline cartilage that will form the VB and end plate through the process of endochondral bone formation, the AF of the IVD, tendon and ligament. Improper formation of the vertebrae or IVD due to failure of adequate chondrocyte development leads to severe spinal disorders such as spinal bifida, kyphosis, scoliosis, lordosis [54]. Once the sclerotome has migrated and resegmented, the cells destined to undergo cartilage specification to form the VB proliferate, condense and differentiate. Fat4 and Dchs1, protocadherins that act[s] as a receptor-ligand pair involved in planar cell polarity, have been shown to be necessary for cell proliferation in the sclerotome and proper formation of the vertebrae. The double knockout of Fat4 and Dchs1 shows a reduced number of chondrogenic cells within the developing vertebrae and malformed vertebrae that were split along the midline [55]**.

Pax1 and Pax9 are transcription factors that mark the sclerotome. Pax1/9 are first induced in the medial ventral portion of the somite by Shh from the notochord. The embryonic knock out of both Pax1 and Pax9 causes the complete loss of the VB and AF [56]. The loss of both structures could be the result of a failure in the initial formation of the sclerotome. Alternatively, the sclerotome may form but due to defects in proliferation and subsequent differentiation caused by loss of Pax1/9 the tissues do not form. Pax1/9 also regulate condensation of the sclerotome mesenchyme and the initiation of chondrogenesis [57***]. Pax 1 and Pax 9 were shown to regulate the SRY-related transcription factor Sox 5, which in conjunction with Sox 6 is necessary for the prechondrocyte aggregation and activation of early chondrocyte markers such as Col2a1 and Aggrecan. Sox 5/6 is downstream of Sox 9, and this trio of Sox proteins is well known to be necessary for chondrocyte differentiation [58, 59]. Nkx3.2, also known as Bapx1, is another early marker of sclerotome formation and is necessary to stimulate chondrogenic differentiation. Its expression is regulated by Shh and it initially has the same temporal and spatial expression pattern as Pax 1/9, however, Nkx3.2 remains expressed throughout the sclerotome and VB while Pax expression later becomes restricted to more fibrous tissues. Nkx3.2 acts as a transcriptional repressor, allowing the sclerotome to be competent to undergo chondrogenesis by responding to BMP signals, while simultaneously inhibiting anti- prechondrogenic factors in the prospective. The embryonic deletion of Nkx3.2 is perinatally lethal in mice and results in severe spinal dysplasia due to failure of the sclerotome to undergo chondrogenesis [60].

While Pax1/9 are initially expressed in the entire sclerotome, and expression is required for the initiation of chondrogenesis, expression is later enriched in the caudal region of the sclerotome, which will form the AF of the IVD. By E12.5 in mouse, expression is restricted to the developing AF and marks the boundary between the IVD and developing VB [57***]. Eventually, expression is restricted to the oAF. The restricted expression pattern to more fibrous tissues may be due to a feed-back loop in which Pax1/9 induce expression of Sox proteins and the Sox proteins in turn inhibit further expression of Pax1 [57***].

In a recent study, the transcriptome of Pax1-GFP and Pax9-GFP expressing cells sorted from embryos based on GFP fluorescence was characterized. Gene ontology analysis determined that genes involved in mesenchymal condensation, cell proliferation and collagen fibrillogenesis, which are critical processes in AF development and formation of lamellar structure, were enriched in the Pax1/9 expressing cells relative to GFP-negative controls. Cartilage development genes were down-regulated. Furthermore, when compared to lists of genes that are enriched in the developing VB or AF it was noted that vertebral enriched genes were down-regulated in Pax1/9 expressing cells. Later, after the division of the AF into iAF and oAF, gene expression patterns indicated that Pax1/9 themselves were capable of promoting iAF and oAF expression profiles; however, Pax1/9 in combination with TGF- β and BMP was primarily involved in promoting IAF. It was noted that a significant number of BMP and TGF- β regulated genes are also regulated by Pax1/9 indicating that Pax1/9 are interlinked with BMP and TGF- β signaling pathways. Smad1 and Smad2 DNA binding motifs were found near Pax9 binding elements in the regulated genes suggesting a mechanism of how Pax9, [and] TGF- β , and BMP could function together to regulate gene expression [57, 61].

BMP signaling is essential for chondrocyte specification and differentiation. BMP signaling is a potent regulator of chondrocyte differentiation upstream of Sox 5, 6 and 9 transcription factors[58, 62, 63***]. The combined embryonic knockout of BMPR1b and the conditional knockout of BMPR1a in Collagen Type 2 expressing cells in mice showed that prechondrocytic cells can condense but do not further differentiate into chondrocytes [62]. *In vitro* studies showed that BMP ligands 2, 4 and 6 all have the capacity to induce human mesenchymal stem cells derived from bone marrow stroma to form cartilage [64], and the cartilage cells induced by BMP4 overexpression express chondrocyte markers such as Col2a and Aggrecan [64]. The relationship between BMP and TGF β signaling is important for sclerotome fate decisions. The inhibition of TGF β is also essential for the formation of the vertebrae, with TGF β expression inhibiting chondrogenic differentiation and promoting fibrous tissue differentiation including differentiation of the AF, tendon and ligaments. It was recently shown that TGF β inhibits BMP-induced chondrogenesis in sclerotome grown in micromass culture [65]. TGF β induced expression of the BMP antagonist, Noggin. When Noggin was knocked down, TGF β was unable to block BMP activity as measured by phosphorylation of Smad1/5. Noggin was at least in part sufficient to induce fibrous marker genes and it cooperated with TGF- β to further induce their expression. Nevertheless, inhibition of BMP signaling alone was not sufficient to induce fibrous tissue specific gene expression suggesting that to form AF, an instructive signal is needed in addition to inhibition of BMP-mediated chondrogenesis [65].

Characterization of Filamin B knock out mice (Flnb^{-/-}) demonstrates how the balance of TGF β and BMP signaling is important to maintain cell fate and maintenance of postnatal AF. This study showed that Flnb^{-/-} mice demonstrate spondylodysplastic synostosis, characterized by vertebral fusion [66]. The AF cells showed disrupted morphology exhibiting hyaline chondrocyte phenotype. These cells overtook NP space. There was disrupted collagen deposition and Col2 expression in the oAF cartilage metaplasia on this normally fibrous tissue. These cells also expressed Col10, a marker of hypertrophic chondrocytes indicating inappropriate endochondral bone formation in the oAF. The results together suggest that FLNB is important to maintain cell type in the post-natal IVD. Furthermore, both canonical and non-canonical TGF β signaling was increased in Flnb^{-/-} mice as measured by increased phosphoSmad2/3 and activated ERK. TGF β target genes CTGF and P21 were increased. Likewise, both canonical and non-canonical BMP signaling was increased as measured by nuclear localization of Smad1/5, activation of P38, and increased BMP target gene, Msx2 [67]**. In contrast, a similar phenotype is observed in [observed in] mice with disrupted Tgfbr2 signaling, whether through dominant-negative interference of TGF- β signaling or deletion of Tgfbr2 in Acan-Cre expressing cells [68]. Nevertheless, together the results suggest that a balance of TGF β and BMP signaling is important for maintenance of postnatal AF.

Scleraxis (Scx) is a transcription factor that is strongly associated with tendon differentiation and function. Scx⁺ cells populate a subcompartment of the sclerotome, termed syndetome, that is adjacent to the myotome. It has been shown that the lineage of Scx⁺ cells can be traced into the AF. However, Scx does not appear to be required for formation of the AF since IVD form in Scx^{-/-} mice [69, 70]. When Sox9, a transcription factor associated with chondrogenesis, is deleted in Scx⁺ cells, mice demonstrate increased formation of oAF at the expense of iAF [71***, 72***]. The results again indicate an important role for Sox proteins in the division between iAF and oAF. The results also suggest that although Scx is not required for development of the AF, the Scx⁺ population of cells contributes to its formation. Another tendon associated transcription factor, Mohawk (Mkx), is involved in AF development [73***]. A recent study showed that Mkx is expressed in the oAF in embryonic and adult stages in mice. Mkx has a role in collagen fiber formation in oAF by affecting expression of collagens and small leucine rich proteoglycans (SLRPs). These proteins play an essential role in lamellar structure in oAF [74, 75]. This study also showed that Mkx could promote regeneration of oAF when expressed in mesenchymal cells from degenerated IVD. This result implies that Mkx is a critical regulator of cell fate in mesenchymal cells [73].

Recent work suggests Heparan sulphate proteoglycans (HSPG) may be important in the formation of lamellar structure in the AF. To generate lamellar structure, AF initially forms an intracellular network of stress fibers resulting in elongation of the AF cells and controlled deposition of extracellular matrix (ECM) by focal adhesion complex into the lamellar organization [76, 77]. A recent study showed that Syndecan-4 protein is localized to the lamellar structures in the oAF of rats [78]*. Syndecan 4 is a co-receptor with integrins to form focal adhesion complexes [79]. Syndecan 4 was expressed on cell surface of AF cells at early stages of development. Then, when the AF cells started to organize, Syndecan 4 was expressed highly on cell surface in oAF. The expression pattern was similar to Fibronectin,

which is known to be critical for the initiation of lamellar structure. The results imply that Syndecan 4 could be an important protein promoting lamellar structure in AF.

Conclusion

Understanding how the axial skeleton develops provides important information that can be used to develop repair, regeneration, and tissue engineering strategies for IVD disease. Mimicking normal developmental processes in vitro to generate new tissues was recently termed developmental engineering [80–83]. The idea is to emphasize the process that goes into making a tissue. Focus on the process of development provides a rational step-wise framework for regeneration and tissue engineering. Developmental engineering has already been used to generate cartilage and bone organs in culture [38, 84, 85]. The IVD is more complex than cartilage or bone. Nevertheless, the use of a step-wise processes based on information about embryonic development would facilitate tissue engineering for IVD. Special consideration would have to be given to gradients within the tissue and integration with the surrounding end plate and ligaments. As described here, the pathways controlling these aspects of IVD development are starting to be elucidated.

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** Of moderate importance

*** Of major importance

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Figure 1.
A) Overview of development of the axial skeleton.
The IVD is derived from two embryonic structures: the notochord and the sclerotome. The notochord develops into the nucleus pulposus. The sclerotome will form all of the other connective tissues of the spine including the annulus fibrosus (both the inner and outer annulus fibrosus), the vertebral body including the end plate, as well as tendons/ligaments (not shown).
NT: Neural Tube, SC: Sclerotome, NT: Notochord, VB: Vertebral Body, IVD: Intervertebral disc, iAF: inner Annulus Fibrosus, oAF: outer Annulus Fibrosus, NP: Nucleus Pulposus, CEP: Cartilage Endplate.
B) Factors that regulate development of the IVD.
Transcription factors and signaling molecules regulating conversion of the notochord to the nucleus pulposus (NC to NP), sclerotome to vertebral body (SC to VB) and sclerotome to annulus fibrosus (SC to AF) discussed in this review.
NKX3.2: NK3 Homeobox 2, Bapx1: Bagpipe 1, BMP: Bone morphogenic factor, SCX: Scleraxis, Tnmd: Tenomodulin, TGFβ: Transforming growth factor b, FLNB: Filamin B, HIF1-a: Hypoxia-inducible factor 1-alpha