9-1-2018

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Received: 7 May 2018 | Revised: 23 July 2018 | Accepted: 26 July 2018

DOI: 10.1002/jsp2.1030

JOR Spine OPEN ACCESS

REVIEW

Latest advances in intervertebral disc development and progenitor cells

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Funding information
National Institute of Arthritis and Musculoskeletal and Skin Diseases, Grant/Award Number: R01AR065530R01AR066517; The Natural Sciences and Engineering Research Council of Canada and Canadian Institutes of Health Research, Grant/Award Number: CIHR New Investigator Award and Early Researcher A; The Research Council of Hong Kong, Grant/Award Number: T12-708/12-N

This paper is a concise review aiming to assemble the most relevant topics presented by the authors at ORS-Philadelphia Spine Research Society *Fourth International Spine Research Symposium*. It centers on the latest advances in disc development, its main structural entities, and the populating cells, with emphasis on the advances in pivotal molecular pathways responsible for forming the intervertebral discs (IVD). The objective of finding and emphasizing pathways and mechanisms that function to control tissue formation is to identify and to explore modifications occurring during normal aging, disease, and tissue repair. Thus, to comprehend that the cellular and molecular basis of tissue degeneration are crucial in the study of the dynamic interplay that includes cell-cell communication, gene regulation, and growth factors required to form a healthy and functional tissue during normal development.

KEYWORDS

development, stem cell, tissue-specific progenitor cells

1 | INTRODUCTION

This paper is a concise review aiming to assemble the most relevant topics presented by the authors at ORS-Philadelphia Spine Research Society *Fourth International Spine Research Symposium*. It centers on the latest advances in disc development, its main structural entities and the populating cells, with an emphasis on the advances in pivotal molecular pathways responsible for forming the intervertebral discs (IVD). The objective of finding and emphasizing pathways and mechanisms that function to control tissue formation is to identify and to explore modifications occurring during normal aging, disease, and tissue repair. Thus, a precise understanding of normal tissue development—including cell-cell communication, gene regulation, and growth factor dynamics—is imperative in identifying the cellular and molecular processes contributing to tissue degeneration, and for designing therapeutic interventions to curb and/or reverse these processes.

The development of the axial skeleton is a multistep process initiated by the formation of the notochord during early embryonic development. The notochord is laid down along the rostral-caudal axis, providing a primitive axial skeleton as well as secreted signals for the patterning of surrounding tissues. The vertebral column is formed by aggregation of the somitic mesenchyme around the notochord, which undergoes progressive patterning and differentiation to form the annulus fibrosus (AF), vertebral bodies, cartilage endplates, and ligaments. The notochord disappears where the vertebral bodies form but expands within the perichordal disc to form the nucleous pulposus (NP). In the following sections, we will provide a detailed overview of

IVD development, transcription factors, growth factors and/or morphogens, and the cell types that regulate the formation of the IVD.

2 | THE NOTOCHORD

2.1 | Embryonic origins of the notochord

conserved tissue that is necessary and sufficient for axis induction during gastrulation, and contributes to cells of the notochord and prechordal mesendoderm.[1] The dorsal organizer was originally identified in amphibians by Spemann and Mangold,[2] leading to the identification of homologous structures with conserved function in other species, including the embryonic shield in teleosts,[3] Hensen's node in the chick,[4] and the node in the mouse embryo.[5]

During gastrulation of the mouse embryo, a population of progenitor cells emerge from the anterior aspect of the primitive streak, termed axial mesoderm (also referred to as chordamesoderm or mesendoderm) and ingress to form the node.[6,7] The node is a transient, late organizer population consisting a few hundreds of cells that form a teardrop-shaped pit at the distal tip of the murine embryo at embryonic day (E) 7.5, responsible for establishing the left-right asymmetry of the body plan.[8,9] Motile cilia localized on the apical surface of cells within the node beat in a clockwise rotation to drive a leftward flow of extraembryonic fluid containing morphogens, such as Nodal, secreted by the columnar epithelial cells of ventral node (termed "nodal flow").[10–12] Disruption of either ciliogenesis[13–15] or cilia motility[16] results in abnormal left-right patterning of the mouse embryo. Although essential for proper embryo patterning, nodal flow is required only during a brief window of development, from 1 to 6 somite stage, which spans 6 to 7 hours of development in the mouse.[17,18] The TGFβ family proteins Nodal and Lefty-2, as well as the homeobox protein Pitx2 are essential to the establishment of left-right asymmetry in vertebrates.[18] Studies using targeted gene deletion in the mouse have identified key transcription factors expressed in the node and required for node morphogenesis and/or function, including FoxH1,[19] brachyury (T),[20] Lhx1,[21] FoxA2,[22] Tead,[23] Otx2,[24] and Noto.[25] Recent studies applied genome-wide analyses to characterize the gene regulatory networks driving the formation and function of the node and notochord,[26] demonstrating dramatic alterations in gene expression patterns as cells transition between developmental states and identifying key pathways and matrix components that may define these distinct stages.[27]

As the embryo elongates, the notochord development is initiated as trunk notochord precursors emerge from the node to form the notochordal plate at E8.0-E8.5 in the mouse.[28] The notochordal plate is continuous with the dorsal gut endoderm and positioned in the axial midline of the embryo; it is formed by three distinct cell types derived from the axial mesoderm—the prechordal plate, the anterior head process, and the node-derived notochordal precursors.[6,29,30] At E9.0, the notochord plate folds off the gut endoderm; cells of the prechordal plate contribute to the forebrain and rostral hindbrain, while cells of the anterior head process form the anterior notochord which rests in a central position in the mouse embryo flanked by the dorsal ridge of the neural tube (the floor plate) and ventrally by the gut endoderm (the endoderm plate).[31,32] Laterally, the notochord is flanked by the paraxial mesoderm, which will give rise to the somites and subsequently the AF and vertebrae. Live imaging of notochord formation in the mouse highlighted further differences in its cellular origins; the trunk notochord is derived from the node by mediolateral intercalation while the tail notochord is formed by node-derived cells that actively migrate toward the posterior and are maintained at the caudal end of the trunk notochord until incorporation at a later stage.[9]

The notochord forms a continuous rod-like structure in which cells display homogeneous morphology and gene expression patterns along the A/P axis. The distinction between the anterior head process and prechordal plate in the notochord is marked in the mouse by specific differences in genetic regulation, including dependence of the trunk notochord on expression of the transcription factors Noto[9] and T.[33] Conversely, loss of Nodal signaling[34] or loss of expression of the transcription factor Lim1[21] leads to complete loss of notochord formation. Formation of all levels of the notochord (including the anterior head process and prechordal plate) are dependent on the activity of the transcription factors Foxh1[19,35] and Foxa2.[22,36]

Similar to the organizer from which it is derived, the notochord is a transient structure in the developing embryo that serves at least two essential functions.[37] First, the notochord forms the primitive anterior/posterior axis of the embryo; a continuous rod-like structure that runs along the midline of the embryo, surrounded by the peri-notochordal basement membrane composed of extracellular matrix proteins.[38] The correct deposition and organization of this extracellular matrix is essential for notochord morphogenesis and maintenance of the rod-like structure of the notochord during early embryonic development.[39] In the mouse, formation of the peri-notochordal sheath is dependent on hedgehog signaling.[40] In its structural role, the notochord resembles cartilage; cells express the Sry-related HMG-box transcription factors Sox5, Sox6, and Sox9[41,42] and secrete an extracellular matrix rich in collagens, laminins, and aggrecan.[43–45] However, while chondrocytes secrete a hydrophilic extracellular matrix enabling the tissue to remain hydrated and resist compressive load,[46] studies in zebrafish and Xenopus demonstrate that notochord cells secrete a thick basement membrane sheath but retain hydrated materials in large intracellular vacuoles thereby allowing cells to resist compression.[47] Notochord vacuoles are generated by post-Golgi trafficking pathways and considered as the final step in notochord differentiation, relying on the preceding chordamesoderm specification, convergent extension, formation of the notochord sheath, and the spatiotemporal activation of vacuolating signals within the axial notochord.[48] The notochord remains in place until the development of the permanent axial skeleton. The second role of the notochord is to secrete morphogens, such as Shh and noggin, through which it regulates the patterning of surrounding tissues, including the neural tube,[49] the sclerotome of the somites,[50] the pancreas,[51] and the aorta.[52] It is important to underscore that our understanding of the pathways that regulate the formation and function of the embryonic notochord are largely based on studies in model organisms (including mouse, chick, zebrafish, and Xenopus); although several characteristics appear to be conserved, functional differences may exist between species, particularly, in humans where less detailed investigation has been undertaken.

2.2 | Fate of notochord-derived cells

During development of the axial skeleton, the notochord disappears in areas where the vertebral bodies form but expands within the perichordal disc, to form the central nucleus pulposus (NP).[53] Within the newly formed NP, notochord cells proliferate and produce a glycosaminoglycan-rich extracellular matrix that separates the original cell mass into a network of cell clusters. Using whole transcriptome analysis, recent studies have highlighted remarkable differences in signaling and cell biosynthesis associated with the transition from notochord (E12.5) to NP (postnatal day 0) in mice, including decreased expression of the Shh pathway and increased expression of the TGFβ and IGF-1 pathways.[27] In mice, the transcription factors Foxa1 and Foxa2 are required for proper formation of the NP from the embryonic notochord.[54] In most vertebrates, there is a progressive postnatal loss of large vacuolated notochord cells and the NP becomes instead populated by small chondrocyte-like cells.[55,56] In humans, cells of the NP change markedly with age; by skeletal maturity, despite maintaining expression of notochord markers (including brachyury, galectin-3, and CD24),[57] cells of the NP assume distinct phenotypic and molecular characteristics.[58–62] The loss of notochord cells from the NP is associated with the onset of degenerative changes in the IVD, suggesting that these cells are required for NP maintenance.[55,63,64] A number of recent studies have begun to characterize the NP cell molecular phenotype and changes associated with age and degeneration in humans.[57,65,66]

The fate of the notochord cells within the NP has long been debated. It has been proposed that small chondrocyte-like NP cells were mesenchyme-derived, populating the NP following migration from the surrounding cartilage endplate (CEP)[67] or originating from transient amplifying cells in the perichondrium at the periphery of the disc.[68] In this context, notochord cells were postulated to direct mesenchyme cell migration and stimulate matrix synthesis prior to undergoing apoptosis or necrosis at the completion of disc formation.[69,70] Alternatively, notochord cells were proposed to serve as IVD-specific progenitors undergoing terminal differentiation to give rise to the small cells of the NP.[63,71,72] In the mouse, genetic strategies for lineage-tracing have demonstrated that all cells of the adult NP are notochord-derived.[53,73]

3 | THE ANNULUS FIBROSUS

Carlier[74] evaluated the embryonic development of the sheep IVD, and described the tissue surrounding the notochord to be composed of undifferentiated embryonic cells that produce a matrix, which becomes irregular-shaped and localized in fibrils, and subsequently converts into fibrous, arranging themselves in lamellae. This structure was called as the AF. Besides the NP, the AF is another crucial component of the IVD. It forms orthogonal layers of collagen-rich fibrils surrounding the proteoglycan-rich and gelatinous NP while connecting the two adjacent levels of the vertebral bodies forming a strong joint.

3.1 | Embryonic origin of AF

AF originates from the somitocoele. The dorsal epithelium of the somite gives rise to the dermo-myotome, while the ventral region gives rise to the sclerotome starting at stage III of somitogenesis.[75] Before giving rise to the sclerotome, the somitocoele undergoes epithelium to mesenchymal transition (EMT). The dorsolateral domain of early sclerotome gives rise to another compartment of the somite called "syndetome".[76] Syndetome is characterized by the expression of Scleraxis (*Scx*).[76,77] *Scx* is a basic helix-loop-helix transcription factor. All tendon/ligament lineage of cells express *Scx*.[78] Using Scx^{Cre}; $R26^{LacZ}$ reporter line for fate-mapping studies, it was demonstrated that entire AF is derived from *Scx*-expression cells.[79] Scx^{GFP} expression was detected as early as E12.5 in the AF of the mouse embryo. The Scx^{Cre}-$R26^{Ali4}$ fluorescent reporter also marked the Scx^{GFP} cells at E12.5 suggesting their lineage from syndetome.[80] Vertebral bodies did not have *Scx+* cells showing the specificity of these reporters. Although previously it was thought that AF arises from the sclerotome region of the somite, Murchison et al,[81] detected endogenous Scx^{GFP} expression in the AF of the E18.5 mouse embryo, showing that it stays on as the AF develops This conclusion was mainly due to the markers used to fate-map, which were not exclusive to sclerotome. In one such study using $Tbx18^{Cre}$; $R26^{LacZ}$ line for fate-mapping studies, *Tbx18*-derived cells were observed in AF at E16.5.[82] However, $Tbx18^{Cre}$ marks several other embryonic structures including myocardial cells in early embryo at E9.5.[83] Also, a few NP cells were observed to be *Tbx18+*, although Choi et al[53] and McCann et al.[73] have shown that all NP cells derive from a homogenous population of notochordal cells. The $Tbx18^{Cre}$ used in the Bruggeman et al[82] study is not inducible, and hence, will also mark syndetome, which in turn originates from the sclerotome. Therefore, it is not clear from the Bruggeman study whether the *Tbx18+* cells in the AF came from syndetome or sclerotome. Similarly, other studies also used markers that did not distinguish between the cells derived exclusively from sclerotome and/or syndetome. However, fate-mapping studies using *Scx* driver lines, which markers only "syndetome" compartment of the somite, clearly shows that the *Scx*-derived cells form the AF.

3.2 | Regulation of AF development and its maintenance

3.2.1 | Sonic hedgehog signaling

The induction of somitocoele to form sclerotome is driven by Sonic hedgehog (Shh) produced by the notochord and floorplate which upregulates Pax1 expression in the sclerotome.[50,84–87] However, Choi et al showed that Shh expression from the notochord and not floor plate is sufficient for maintenance of Pax1 expression in the ventral sclerotome.[88] Pax1 is a marker of ventral sclerotome, while Pax9 is a marker of dorsal sclerotome. Bone morphogenetic protein (BMP) signaling antagonizes Shh signaling in this process, while Noggin and Gremlin1, in turn, antagonizes BMP, allowing Shh to regulate sclerotome differentiation.[89,90] In addition to Pax1, Shh also regulates the expression of genes required for the development of axial skeleton like Pax9, NKx3.2, and Sox9.[85,91–94] Pax1 and Pax9 are crucial for

axial skeleton development, as mutations in these genes cause defects in the formation of the vertebral column.[95–97] Bapx1 is known to be downstream of Sox9,[98] and is activated by Pax1 and Pax9 in the sclerotome.[99,100] Deletion of Bapx1 resulted in vertebral defects. With the induction of sclerotome, Pax3 and Pax7 expression is downregulated. The notochord-derived NP continues to express Shh in the postnatal stages, and its blockade by small molecule cyclopamine in vitro, or conditional targeting in vivo, decreased the expression of hedgehog signaling targets PTCH1 and GLI1 in the AF, suggesting that AF responds to Shh produced by the NP cells.[101]

3.2.2 | HMG-box transcription factors

Both embryonic notochord and ventral sclerotome express Sox9 by E8.5 and 10.5, respectively, during mouse development.[102] Brent et al[73] showed that the syndetome in early mouse embryo had mixed cell population, some expressed Scx, while others were Sox9, Sox5, and Sox6 positive.[77] It is known that Sox9 is upstream of Sox5 and Sox6 in chondrogenesis.[103,104] While Sugimoto et al[79] showed overlapping Scx and Sox9 expression in syndetome at E10.5, Brent et al showed that in Sox5/Sox6 mutants the Sox9 expression remains unchanged, and the Scx expression, as well as the tendon progenitors, increased in number, suggesting that cartilage differentiation mediated through Sox5/ Sox6 is stimulated by suppressing tendon development.[77] In 2003, Brent et al found that Shh negatively regulates tendon lineage through Pax1.[76] By targeting Sox9 by ScxCre, only in the syndetome-derived AF, it was shown that Sox9 is crucial for the formation of proper AF.[79] However, Ck19Cre-mediated targeting of Sox9 in the notochord, showed the absence of AF from the IVD by E15.5.[102] This effect could be due to loss of rostrocaudal notochord from the *CK19*^{Cre}; *Sox9*$^{flox/flox}$ mutants E10.5 onwards, and failure to initiate AP-segmentation of perinotochordal sclerotome by E11.5.[102] Conversely, short fragments of functional notochord can start cartilage differentiation in Sox9+ perinotochordal sclerotome resulting in a metameric-like pattern resembling regular vertebral column during development. These studies point out the importance of notochord, and notochordal signals in the maintenance of AF. Sox9 continues to play an essential role in the IVD after skeletal maturity. Conditional targeting of Sox9 using Agc1-CreERT2 in 2-month-old mice severely affected the entire disc structure and extracellular matrix remodeling 1 month later.[105] Yet, Agc1CreERT2 targets Sox9 in the NP, AF, cartilaginous endplate and adjacent growth plate chondrocytes. By Chip-on-Chip analysis, Ctgf was identified as a direct target of Sox9 in rat NP cells. The role of Sox9 in the regulation of Ctgf was validated by conditional targeting of Sox9, at 2 weeks of age in mice, using Col2CreER driver line and analyzing the CTGF expression by 2 months of age and severe structural defects were also observed. Loss of Sox9 and CTGF resulted in severe structural defects in these mice.[106] Blockade of Shh in vitro and targeting its conditional allele in vivo, in the NP, resulted in loss of Sox9, and extracellular matrix markers like collagen 1, collagen 2, chondroitin sulfate, and keratan sulfate in the AF, indicating that Shh, from notochord-derived NP, continues to regulate AF development and differentiation in the postnatal stages.

3.2.3 | TGFβ signaling

Using *Col2a*^{Cre} Baffi et al targeted *Tgfβr2* using its conditional allele to block response to TGFβ signaling and showed that the development of IVD and AF was affected at E13.5, E15.5, and E17.5.[107] Profiling studies from cultured sclerotome at E11.5 to identify the targets of TGFβ and BMP signaling in vitro showed that Scx, Sox5, Sox6, and Sox9 were few of TGFβ signaling targets. This study showed that TGFβ signaling is crucial for differentiation of AF from sclerotome. Jin et al[108] used the tamoxifen-inducible allele of *Col2*^{CreER} to targeted *TGFβr2* expressing cells in the neonatal stages and showed that Col10a1, MMP13, ADAMTS4, and ADAMTS5 are negatively by TGFβ signaling. Based on the reporter data *Cre*-mediated recombination in this allele was observed in the inner AF and growth plate chondrocytes only. In the postnatal stages, TGFβ signaling is downstream of Shh signaling in neonatal mouse IVDs.[101] Hayes and Ralphs[109] showed that TGFβ1 alone or in combination with IGF1 stimulated sulphated glycosaminoglycan, Col1, and Col2 secretion by AF cells.[109] Using the mouse model of spondylocarpotarsal synostosis (SCT) Zieba et al[106] showed that Filamin b (*Flnb*) null mice have early onset of degenerative disc defects, especially in the AF. SCT is an autosomal recessive disorder with loss of function mutation in Filamin B (*FLNB*) gene resulting in progressive vertebral fusions. FLNB was detected as early as E14.5 in mouse IVD. This study also showed an increase in TGFβ and BMP signaling in the AF of Flnb-null mice. This raises the issue of the specific role of TGFβ in postnatal disc maintenance.[106] Figure 1 is a schematic illustration depicting key stages of IVD development, highlighting the growth factors, morphogens, and transcription factors.

4 | PROGENITOR CELLS IN THE INTERVERTEBRAL DISC

Tissue maintenance and repair require the presence of local progenitor cells or stem cells, or the recruitment of appropriate cell types into the damaged site(s) for repair. In tissues that requires a constant turnover of cells in normal maintenance, replenishment is strictly controlled from specialized region(s) within the tissue, referred to as the stem cell niche. Example of localized niche is the intestinal crypt[110,111] with stem cells and supporting cells that continue to replenish the enterocytes as they are shed. Others include stem cells in the hair follicles[112,113] and the kidney nephrons.[114] These are self-renewing and proliferative stem cells with specialized requirements in tissues for high-throughput cell replenishment. In tissues that do not require a high maintenance, stem cells are activated or recruited into the damaged sites as required. Bone fracture is a good example where progenitor cells are recruited from multiple regional sites to the damaged site for repair.[115] Like articular cartilage, IVD do not repair well once damaged and the reason is unknown.[116,117] It could be related to the avascular nature of these tissues or the presence, location and ability to activate regional stem cells. There are many studies addressing the presence of stem/progenitor cells in the IVD in human and animal models, mostly using in vitro approaches, with limited in vivo findings. In addition, while the developmental and cellular processes in the formation of the IVD may be conserved in the different mammalian

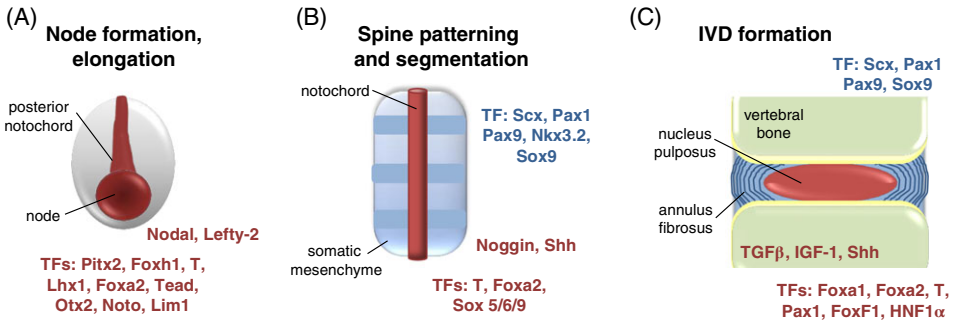

FIGURE 1 Schematic illustration depicting key stages of intervertebral disc development, highlighting the growth factors, morphogens, and transcription factors. Depiction of key stages in axial skeletogenesis, including (A) node formation and elongation in the early embryo; (B) aggregation of the somatic mesenchyme around the notochord to form a continuous perichordal tube with metameric condensation of the axial mesenchyme (depicted by darker blue bands) leading to spine segmentation; and (C) formation of intervertebral discs. Notochord/nucleus pulposus derived structures are colored in red, and structures contributing to the annulus fibrosus are colored in blue. At each stage, selected growth factors, morphogens, and transcription factors (TFs) known to be required for IVD development are indicated, with notochord/nucleus pulposus associated factors indicated in red, and annulus fibrosus associated factors indicated in blue

models, the cellular compositions of the adult discs are vastly different in the animal models analyzed to that of human; in particular the NP.[118] Thus, in understanding of these studies, one needs to place the findings in context, from the perspective of the detection and cell isolation methods, source of the cells, and their differentiation potentials.

4.1 | Presence of mesenchymal stem cells in the IVD

Potential stem cells with mesenchymal stem cells' (MSC) characteristics have been isolated from human IVDs. Given the difficulties of obtaining healthy tissues and the need to address the repair potentials of IVDs, many studies have used degenerated human IVD tissues in the search for progenitor cells. A seminal study used an explant culture approach in the isolation of cells with MSC characteristics from both the AF and NP.[119] This approach relied on the migration and proliferation of cells selected as they detached from the explants and adhered to the plastic surface of the culture plate. From this, cells expressing typical MSC markers, such as CD105, CD166, CD63, CD49a, CD90, CD73, p75 low-affinity nerve growth factor receptor, and CD133/1, were identified.[119]

It is interesting that MSCs with very similar characteristics can be isolated from both AF and NP tissues, even though their developmental origin is different,[120] suggesting that these MSCs may not be of embryonic origin persisting from the development of the IVD. This would be consistent with the finding that pericytes, cells lining blood vessels shared similar cell surface markers with MSCs in situ,[121] and the conceptual commentary that all MSCs may be of pericyte origin.[122] Whether pericytes could find their way into the NP is questionable as the IVD is an avascular tissue. One possibility is external recruitment as exogenous human MSCs were shown to have a capacity to migrate through the CEP to the NP in ex vivo whole organ cultures using bovine IVDs, under culturing conditions that would induce degeneration such as excessive mechanical loading, limiting nutrition, and physical injury from a needle puncture.[123,124] However, CD146, a marker for MSCs with pericyte origin was not identified in progenitor cells isolated from mouse and human IVDs,[125] but the existence of MSCs of pericyte origin cannot be excluded.

4.1.1 | MSC markers in the healthy and degenerate IVD

The identification of CD133 positive (CD133+) cells is interesting as CD133 is a cell marker for many types of progenitor cells. However, it seems that CD133+ cells isolated from degenerated human IVD cells did not exhibit better adipogenic or chondrogenic differentiation capacity than CD133 negative (CD133−) cells,[119] suggesting that in a degenerative state of an IVD, the environment could alter the functionality of the progenitor cells. The notion that a "local niche" could influence MSC potential is supported by a study comparing progenitor cells isolated from the NP and bone marrow (BM) MSCs from the same individual with degenerated NP that show a diminished adipogenic differentiation potential of the MSCs isolated from the degenerated NP.[126] Furthermore, while using a common method for the isolation of MSCs from explant cultures of human IVDs, variation exists in the differentiation potential of the MSCs isolated.[119,126] While the reason is unclear, it is again likely to be related to changing niche of a degenerating IVD with age and severity of degeneration as confounding factors. Adding to the complexity is the identification of a small population of cells with MSC characteristics (CD105, CD90, and STRO-1) from degenerated human IVDs that also co-express OCT3/4 (a primitive marker for multipotency) and NOTCH1 (a signaling marker associated with cell fate determination).[127] This represents only a minor population of NP cells which is as expected for progenitor cells in adult tissues.

4.2 | Progenitor cells' isolation and functional assays

The explant approach to isolated MSCs presents a heterogeneous population of cells derived from degenerated IVDs that can differentiate along the mesenchyme lineage.[119,126,127] Thus, it is also important to assess whether there are single-cell progenitors in the IVD. This requires the approach of the colony-forming assay (CFA), and requires the dissociation of the tissues to release cells as singletons and assess their potential to form colony-forming units (CFUs) from a single cell.

A recent study assessed the CFUs of MSCs isolated from porcine NP of normal and degenerated (induced by annular puncture)

conditions from the same animal.[128] While both sources of MSCs (expressing MSC markers, CD29, CD90, and CD44) could differentiate into the classic mesenchymal lineages (osteoblasts, chondrocytes, and adipocytes), the CFUs were different, being higher from degenerated NP, and with increased proliferation rates in vitro. On the other hand, MSCs from the healthy NP showed a better chondrogenic differentiation potential and higher expression of NP extracellular matrix such as aggrecan and type II collagen.[128] As the IVD degenerates, the changes in mechanical property can also influence the local environment/niche of progenitor cells. Indeed, adjusting extracellular matrix stiffness and elasticity can influence the differentiation lineage of progenitor cells in vitro,[129,130] and the fate of NP derived progenitor cells.[131]

Potential progenitor cells with MSCs characteristics have been isolated from the CEP from degenerated human IVDs, harvested through posterior discectomy procedure.[132] Cells were cultured in 2% low-melting-point agarose in a 3D environment, allowing the formation of cell clusters. Large cell clusters were selected and expanded in monolayer culture. These cells express the typical markers for MSCs including CD105, CD73, CD90, CD44, CD166, and Stro-1,[132] and able to differentiated efficiently long the mesoderm lineage to osteoblasts, chondrocytes, and adipocytes. It is important to bear in mind that these are not clonal cells, but cells that form a cluster. Given the potential IVD progenitor cell niche is adjacent to the CEP and the connection between CEP and AF, it is possible that the progenitor cells in CEP[132] and AF[119,133,134] identified in the in vitro studies are originated from the IVD niche. More recently, cells expressing CD146 was found to be localized along the surface of the outer AF, a potential migratory route of the progenitor cells in the IVD niche and as part of the progression for differentiation.[135] Interestingly, CD146 appears to define a commitment of AF cells for a contractile phenotype in vitro, in their ability to contract a collagen gel. This may be related to the CD146 expression in response to TGFβ1 stimulation and higher expression of SM22α and elastin, both associated with contractile property of tissues.[135]

4.2.1 | Disialoganglioside 2 and tyrosine kinase receptor

Two novel NP progenitor cell markers: A detailed analysis of cells isolated from the NP of mouse and human IVDs showed the presence of potential "stem cells" with self-renewal potentials in vivo, and progenitor cells with specific cell surface markers that could inform a hierarchical differentiation progression for progenitor cells to a mature NP cell.[125] These were initially identified in the mouse NP assessed using the colony-forming assay with isolated NP cells cultured in a methylcellulose semi-solid medium[136] that identified adhesive fibroblast colonies and nonadhesive sphere forming colonies. Focusing on cells from the sphere-forming colonies (positive for type II collagen and aggrecan expression), two novel NP progenitor cell markers, disialoganglioside 2 (GD2) and tyrosine kinase receptor (Tie2) were identified. Importantly, it was shown that Tie2+/GD2− cells behave as dormant stem cells; Tie2+/GD2+ double positive cells have stem cell properties with self-renewal potentials, with Tie2−/GD2+ as potential NP cell progenitors; the same Tie2−/GD2+ population showed expression of the "NP marker" CD24, suggesting possible committed NP progenitors; and finally, loosing expression for both Tie2 and GD2, but maintaining CD24 expression, define mature cells in the NP.[125] Interestingly, in human IVDs, the combined number of Tie2+/GD2− and Tie2+/GD2+ cells, as well as the CFUs of NP cells, decline rapidly with age at around 40 years old, coincide with the age of onset and severity of IVD degeneration.[125] It was further shown that angiopoietin-1 as a ligand of Tie2 may have an important in maintaining these progenitor cells and protecting cells in the NP from apoptosis.[125] These findings by Sakai et al[125] are supported by two additional studies. From young bovine coccygeal discs, Tekari et al[137] sorted NP cells for Tie2 and showed that Tie2+ cells characteristics of progenitors able to differentiate into the osteogenic, chondrogenic, and adipogenic lineages in vitro, forming spheroid colonies although with a decline during expansion.[137] In another study, NP-derived cells harvested from patients undergoing discectomy were subjected to cell sorting based on the Tie2 and GD2 co-expression. The analyzed Tie2+/GD2+ population showed similar properties in colony-forming ability, cell proliferation, and stem cell gene expression compared to BM-derived MSCs from the same subjects. Interestingly, Tie2+/GD2+ cells differentiated into osteoblasts similar to BM-MSCs, were found to be superior in chondrogenic differentiation but inferior in adipogenesis, compared to BM-MSCs.[138] Of interest is the work reported by Rodrigues-Pinto et al.[66] Human embryo and fetal spines (notochord and somites/sclerotome) were isolated by microdissection to follow the spatio-temporal expression of the believed human notochordal markers. Expression of Tie2, as well as KRT8, KRT18, KRT19, T, GAL3, CD24, CD55, CD90, BASP1, CTGF, and E-Cad was assessed by immunohistochemistry. Their findings showed that Tie2, but also CD90 and E-Cad, were not expressed in the early developing spine between the studied period of 3.5 to 18 weeks post-conception, suggesting Tie2 is expressed later in IVD development and may be considered as an NP progenitor cell marker.

To summarize, these "progenitor cells" do show heterogeneity in their differentiation potential, and their presence in "healthy" human IVD has yet to be thoroughly studied, and their relationship to the adhesive MSCs in this and other studies also need to be addressed. It is possible that they are from notochord or sclerotome source and differ in embryonic or postnatal origins. In Figure 2, we summarize the to date identified cells in the healthy IVD, and their evolution in function of time.

4.3 | In vivo cell tracing

Using pulse-chase labeling with BrDU has been reported for the IVD in New Zealand white rabbits.[139] Few proliferating cells can be identified in the NP and AF during the labeling period, but following a prolong chase period, BrDU label-retaining cells (potential stem/progenitor cells) are detected, concentrating in a region close to the perichondrium at the junction of the outer AF and the vertebral body,[139] suggesting the presence of a stem cell niche. This niche is analogous to a region known as the "Groove of Ranvier" in long bones, where progenitor cells have been identified to reside in this region that serves to replenish chondrocytes in the cartilage growth plate.[140,141] These cells, identified in the IVD express MSCs markers, including Notch1, Stro-1, and c-KIT.[139] Similar cells were identified in

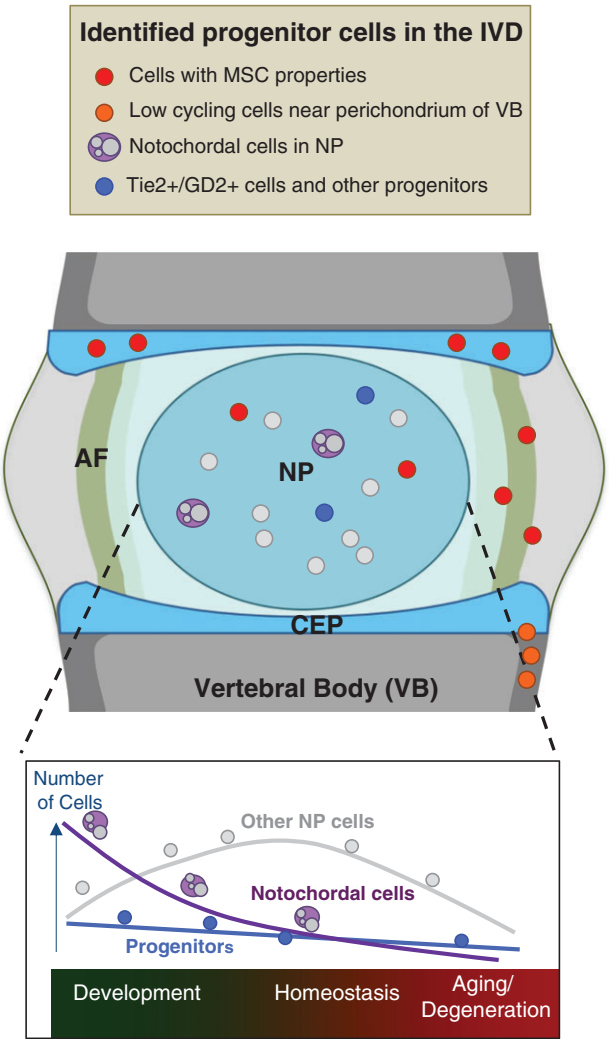

FIGURE 2 Identified progenitor cells populating the IVD and the changes in their numbers as a function of time, from development, through homeostasis and to aging. Depiction of cells with MSC properties, low-cycling cells near the perichondrium of the vertebral body, notochordal and Tie2+/GD2+ and other progenitor cells

Sprague-Dawley rats, Gottinger minipigs and degenerated human IVDs.[139] A model was proposed for the presence of progenitor cells in this niche that undergo a transition of amplifying cells, and finally, differentiated cells of the IVD. Furthermore, it may be possible that these cells migrate into the IVD during growth and repair. Indeed, possible "migration routes" of these progenitor cells in the outer AF were studied, from an analysis of a cell adhesion and migration marker (β1 integrin) and EMT markers (Snail-1 and -2) in young and aged rabbits.[142] Activation of EMT would be consistent with a change in the migratory property of a cell. In EMT, the cytoskeleton of the cells is rearranged to a flattened phenotype to facilitate the migration of cells to a different location whereby members of the SNAIL superfamily of transcription factors are activated. As such, this pool of cells is suggested to be a source of progenitor cells for the maintenance of the AF during adult life.[142] However, how this relates to the MSCs identified in degenerative human IVDs from the in vitro analyses is unclear, that will require careful cell tracing analysis using specific genetic tools in mice.

4.4 | Summary

Together, the in vitro and in vivo studies support the presence of resident progenitor cell populations in the IVD. While in vitro studies can provide useful information, it is important to understand the limitation in the isolation and expansion approaches, as the possibility of cellular dedifferentiation cannot be excluded. Bona fide dedifferentiation of human articular chondrocytes was showed with a gradual unregulated expression of MSCs markers in during monolayer cultures; including CD90, CD166, CD49c, CD44, CD10, CD26, CD49e, CD151, CD51/61, and CD81,[143] that can be accelerated by FGF2 supplement.[144] Whether of IVD cells dedifferentiate in monolayer culture has not been specifically addressed. Furthermore, the relevance of the progenitor cells in human needs substantial validation as many studies uses degenerated IVDs, and why the IVD continues to degenerate even in the presence of these progenitor cells is not clear at all. The opportunities are available to decipher the presence, location, and fate of these progenitor cells in animal models prior to validation in human tissues. Given the vast mouse tools available to study cell fate, it is highly feasible if appropriate gene markers can be identified to track and localize potential progenitor cells or cells providing homeostasis support of the IVD tissues. A golden opportunity is the application of single-cell transcriptomic analyses to interrogate the cell types present in the different components of the IVD, and to identified presence of potential progenitor cells without in vitro culture and cell expansion. The technologies are available and the cost is becoming affordable.[145,146] Once identified, the in situ identification and localization in the IVD tissue can be validated. This is clearly important in the formulation of therapeutic treatments of symptomatic IVD degeneration, for exogenous cell therapy, or activation of endogenous progenitor cells for repair.

ACKNOWLEDGEMENTS

Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Awards Number RO1AR065530 (CLD) and R01AR066517 (ZG). The content is solely under the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The Natural Sciences and Engineering Research Council of Canada and Canadian Institutes of Health Research funded C.A.S., who is also supported by a CIHR New Investigator Award and Early Researcher Award from the Ontario Ministry of Research and Innovation. The Research Council of Hong Kong under the Theme-based Research Scheme (T12-708/12-N) provided funding support to DC. The authors thank Dr Wilson Chan for his help in the preparation of Figure 2.

Author contributions

C.A.S., D.C., C.L.D., and Z.G. contributed to the concept of the paper and wrote the manuscript. All authors have read and approved the final submitted manuscript.

Conflict of interest

The authors declare not to have any conflict of interest.

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How to cite this article: Séguin CA, Chan D, Dahia CL, Gazit Z. Latest advances in intervertebral disc development and progenitor cells. *JOR Spine*. 2018;1:e1030. https://doi.org/10.1002/jsp2.1030