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Notochord to Nucleus Pulposus Transition

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Abstract A tissue that commonly deteriorates in older vertebrates is the intervertebral disc, which is located between the vertebrae. Age-related changes in the intervertebral discs are thought to cause most cases of back pain. Back pain affects more than half of people over the age of 65, and the treatment of back pain costs 50–100 billion dollars per year in the USA. The normal intervertebral disc is composed of three distinct regions: a thick outer ring of fibrous cartilage called the annulus fibrosus, a gel-like material that is surrounded by the annulus fibrosus called the nucleus pulposus, and superior and inferior cartilaginous end plates. The nucleus pulposus has been shown to be critical for disc health and function. Damage to this structure often leads to disc disease. Recent reports have demonstrated that the embryonic notochord, a rod-like structure present in the midline of vertebrate embryos, gives rise to all cell types found in adult nuclei pulposi. The mechanism responsible for the transformation of the notochord into nuclei pulposi is unknown. In this review, we discuss potential molecular and physical mechanisms that may be responsible for the notochord to nuclei pulposi transition.

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Introduction

The notochord is a rod-like structure that runs along the midline of vertebrate organisms. It forms in the midline of embryos 7.5 days after fertilization (E7.5) from the axial mesoderm [1, 2]. The role this structure plays in patterning the neural tube, somites, and other nearby tissues has been extensively documented in a number of excellent reviews [3–5]. This review will focus on the fate of notochord cells and the role they play in formation of the intervertebral discs and potentially disease.

Intervertebral discs are located between adjacent vertebrae. They are fibrocartilaginous joint-like structures that connect and cushion the vertebrae in the axial skeleton. Intervertebral discs are comprised of three functionally distinct regions that, when healthy, work together to allow for fluid and painless movement of the spine. The gel-like nucleus pulposus comprises the middle region of the disc and is surrounded by the fibrocartilaginous annulus fibrosus. These tissues are attached to each vertebra by cartilaginous endplates.

In damaged or degenerating discs, the boundary between the annulus fibrosus and the nucleus pulposus becomes less defined and the nucleus pulposus becomes more fibrotic and less gel-like [6, 7]. Age-related changes in the intervertebral discs are thought to cause most cases of back pain (reviewed in [8, 9]). Back pain affects more than half of the people over the age of 65 and the treatment of back pain costs 50–100 billion dollars per year in the USA [9–11]. Presently, there is no cure for disc degeneration.

Nuclei Pulposi are Derived from the Embryonic Notochord

Exquisite pictures of developing human embryos published by Walmsley in 1953 [12] suggested that nuclei pulposi formed from cells located in the notochord. Molecular conformation that nuclei pulposi were derived from notochord cells would not occur for another 55 years. In the mid-2000s, experiments performed using the mouse model system demonstrated that all cells located in nuclei pulposi are derived from cells located in the notochord [13••, 14•]. These experiments took advantage of the ability to "fate map" cells. In fate mapping experiments, cells are irreversibly marked by their ability to express an easily detectable protein (usually LacZ or a florescent protein like GFP, YFP, or TOMATO). When a marked cell divides, all its descendants continue to express the marker. Using this approach, the fate of individual cells and their descendants can be followed throughout the life of the mouse.

Marking of notochord cells determined that all cells located in the embryonic nucleus pulposus were comprised of cells that had originated from the embryonic notochord [13••, 14•]. By following the fate of these cells throughout the approximately 2-year lifespan of a mouse, it was determined that cells located in newly formed nuclei pulposi produced all the cells found in nuclei pulposi throughout the life of the animals. In particular, cells were not observed to migrate into nuclei pulposi from the surrounding annulus fibrosis or end plates. These data suggest that the notochord is the sole source of cells found in nuclei pulpous throughout the entire mouse lifespan [13••, 14•]. Although fate mapping experiments are not possible in humans, it is likely that human nuclei pulposi form through a similar mechanism.

The "Pressure" Model

A huge gap in our knowledge of disc formation is the lack of understanding of how the notochord transforms into nuclei pulposi. A number of laboratories, including our own [15], have documented the physical transition of the rod-like notochord into nuclei pulposi. In mice, this transition occurs over the course of approximately a day and a half. In this review, we propose two models to explain how this invariant process might occur.

Histological sections obtained during the transition of the notochord into nuclei pulposi revealed that formation of vertebrae and the disc occurs concurrently (Fig. 1 and [12, 15, 16]). In regions of the vertebral column where mesenchymal cells were found to condense into vertebrae, notochord cells were excluded. Analysis of histological sections revealed that the loss of notochord cells in these regions were not due to massive cell death [17, 18]. In addition, extensive cell proliferation was not observed in regions of the vertebral column where nuclei pulposi were forming [17, 18]. These data lead to the hypothesis that notochord cells were being "squeezed" or "pushed" into regions of the vertebral column where the discs were forming (Fig. 1 and [18]).

In support of the "pressure" model, mutant mice that lacked the acellular notochordal sheath, which normally surrounds notochord cells, but contained normal vertebrae resulted in the scattering of notochord cells throughout the vertebral column [18]. A mouse model that lacked the notochordal sheath and condensing vertebra retained a rod-like notochord [18]. These data suggest that the formation of vertebra around regions of the notochord may play a central role in the formation of nuclei pulposi.

While the ability of the condensing vertebrae to push notochord cells into regions of the vertebral column where disc formation will commence could explain the invariant and repetitive intervertebral disc pattern observed in all vertebrates, there is currently no evidence that nuclei pulposi cells move or are under any type of pressure during disc formation. In vivo imaging studies are needed to conclusively demonstrate that notochord cells migrate into areas of the vertebral column where discs form. These types of imaging studies have been hindered by the location of the notochord/intervertebral discs in a relatively inaccessible region of the embryo. The use of thick histological sections coupled with confocal microscopy has failed to date, possibly due to the absence of complete vertebra on specimens used in these studies, to document notochord cell movement (our unpublished results).

The data suggesting that the forming vertebra exert pressure on the notochord, resulting in cell movement, has been obtained through the analysis of mutant mice. We have proposed that the removal of vertebrae and the resulting absence of notochord cells transiting into nuclei pulposi are due to the absence of this putative force-producing structure [18]. In these experiments, the signaling molecule *Sonic Hedgehog* (*Shh*) was removed. It is possible that the removal of *Shh* directly or indirectly halted the formation of nuclei pulposi independent of the proper formation of vertebrae. Experiments in which the notochordal sheath or vertebrae are physically removed during normal development could lead to confirmation of the key role vertebrae formation may play in disc patterning. While the chick model system is ideal for tissue manipulations and contains a notochord surrounded by a sheath, unfortunately, chickens do not appear to contain nuclei pulposi [16]. The notochord is assumed to ultimately undergo cell death in chickens, although this has not been proven [16].

The "Repulsion/Attraction" Model

During formation of a number of tissues, cell movement is driven by the expression of attractant/repulsive proteins. A
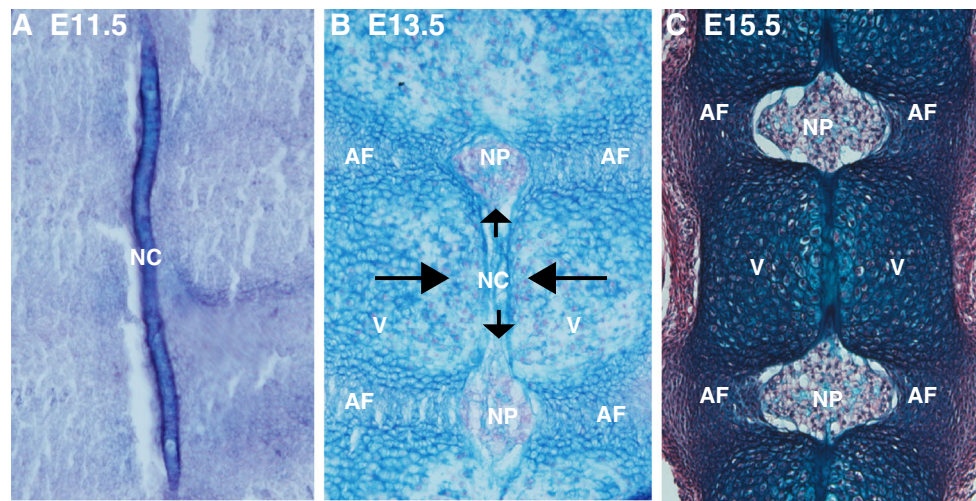
Fig. 1 Model of formation of nuclei pulposi from the notochord. In E11.5 (**a**) mouse embryos, no nuclei pulposi are present. The notochord is a rod-like structure that runs through the midline of the embryo. At E13.5 (**b**), nuclei pulposi are forming. The *large arrows* represent pressure being applied to the notochord from the surrounding vertebrae. *Smaller arrows* denote putative movement of cells within the notochord into the forming nuclei pulposi, which are located between each vertebra. By E15.5 (**c**), well-formed discs are present. The acellular notochordal sheath is still observed between each vertebra. *NC* notochord, *NP* nucleus pulposus, *V* vertebrae, *AF* annulus fibrosis. All sections shown are frontal sections stained with alcian blue and picrosirius red

similar mechanism could be driving the transition of the notochord into nuclei pulposi. Regions of the vertebral column where the discs form may be expressing attractant molecules which would be sensed by notochord cells causing these cells to aggregate in disc-forming regions of the vertebral column. A second possibility is that repulsive signals emanating from the vertebrae-forming regions of the vertebral column result in exclusion of notochord cells. In either model, notochord cells would preferentially end up residing in between the forming vertebrae.

The Eph/ephrin family is required for the patterning of numerous tissues in the developing embryo [19–21]. Eph proteins comprise the largest class of receptor tyrosine kinases and bind membrane-bound ligands called ephrins. Binding of Eph proteins to ephrins initiates signaling cascades in both the Eph and ephrin-producing cells. This pathway is required for setting and maintaining tissue boarders in a number of regions of the developing embryo, and this pathway has been demonstrated to be sufficient to segregate a mixed population of cells [22–24].

During formation of nuclei pulposi, the Eph/ephrin pathway could be responsible for sorting notochord cells into regions of the embryo where disc formation occurs. In support of this model, the EphA4 receptor is expressed in the notochord while ephrins are found in the surrounding mesenchyme [22]. If the Eph/ephrin pathway functions during disc formation, it may not be the sole mechanism responsible for the transition of the notochord into nuclei pulposi. The Eph/ephrin pathway requires direct cell-cell contact, and all notochord cells may not be in contact with the surrounding mesenchyme [25]. The Eph/ephrin pathway could play an essential role in consolidating notochord cells in the forming disc and inhibiting them from mixing with mesenchymal cells, which forms the surrounding annulus fibrosis [16].

The Robo/Slit signaling pathway is a second pathway that could play a role in the notochord to nuclei pulposi transition. This pathway plays essential roles in axon path finding, neural crest migration, and development of the diaphragm and kidney [26–29]. Recently, *Robo1* and *Robo2* were shown to be essential for the correct positioning of the stomach during embryogenesis [30]. In mice lacking both *Robo1* and *Robo2*, the stomach was mis-positioned in the thoracic instead of the abdominal cavity. This phenotype resembled congenital diaphragmatic hernia cases in humans, and suggested that organ position can be regulated by Robo/Slit signaling [30].

Robo genes were initially identified in a *Drosophila* screen for mutants in which commissural axons incorrectly migrated [28, 31]. Subsequently, Slit was found to be secreted by midline glial cells and bind Robo receptors [26, 27, 32–34]. Robo receptors are part of the immunoglobulin superfamily of cell adhesion molecules. Slit proteins are secreted glycoproteins and have been shown to be the major ligand for Robo receptors [28, 34, 35]. Robo/Slit interactions have been extensively characterized during nerve growth. Both in vivo and in vitro assays have demonstrated that Slit protein can repel cells expressing Robo [30, 36–38]. In vivo, the Robo/Slit pathway is essential for controlling both neuron and organ positioning [29, 30, 39–42].

Four *Robo* and three *Slit* genes have been identified in the mouse genome [27, 28, 43]. All three *Slit* genes are expressed in the notochord [30]. While the expression pattern of *Robo* receptors has not been extensively investigated in the vertebral column, it is clear that at least *Robo1* and *Robo2* are expressed in mesenchymal cells surrounding the notochord [30].

The intriguing expression patterns of *Slit* and *Robo* genes raises the possibility that this signaling pathway may play a key role in the movement of notochord cells during disc formation. Robo-expressing mesenchyme cells in the vertebral column could by repelled from notochord-expressing Slit cells. In this model, *Robo*-expressing mesenchyme cells would be actively pushed away from regions of the vertebral column where Slit was present. This pathway could play a key role in inhibiting the mixing of Robo-expressing mesenchymal cells and Slit-expressing notochord cells during the transition of the notochord into nuclei pulposi.

The Notochord to Nuclei Pulposi Transition and Chordoma

Chordoma is a rare cancer that affects ~1 in a 1,000,000 people [44–46]. It usually occurs in the bones of the spine and/or skull. Due to chordoma having many molecular characteristics similar to notochord cells, and their proximity to the vertebral column, this type of cancer is proposed to arise from "notochordal remnants" that reside outside nuclei pulposi [47–49].

Consistent with the hypothesis that chordomas arise from notochord cells, others and we have shown that not all notochord cells end up residing in nuclei pulposi [13••, 14•]. Some notochord cells are found in fully formed adult vertebrae ("notochordal remnants"). We hypothesis that a small number of notochord cells may not migrate/been pushed fast enough into the forming discs during embryogenesis, and as a result, they become stuck during formation of vertebrae.

In mice, every adult vertebra contains a small number of cells derived from the embryonic notochord [13••, 14•]. An analysis of human cadavers indicates that the majority of human vertebra also contains notochord cells [50]. The low prevalence of chordoma in humans (and mice) suggests that the vast majority of notochord cells residing in mature vertebrae do not result in disease.

Recently, duplications of *T* (*brachyury*) were shown to occur in four families that had at least three cases of chordoma [51••]. In addition, *T* (*brachyury*) is expressed in most sporadic chordoma cases, suggesting that this locus may play a key role in chordoma formation [52]. It is unknown how alterations in the *T* (*brachyury*) locus may cause chordoma. While *T* (*brachyury*) is expressed in the notochord during normal development, notochord cells present in the vertebrae of adult mice do not express *T* (*brachyury*) [18]. Duplications of this locus may result in elevated expression of *T* (*brachyury*) during embryonic development and/or ectopic expression of this gene in notochord cells present in adults.

We propose that an increase in the number of notochord cells in the adult vertebral column may result in an elevated risk of chordoma. The presence of additional cells of notochord origin that are poised to undergo a mutation activating *T* (*brachyury*), and potentially other loci, may increase the risk of developing chordoma. The presence of additional notochord cells in the adult vertebral column could result from improper migration of notochord cells into nuclei pulposi and/or the presence of "holes" in the sheath surrounding the embryonic notochord during nuclei pulposi formation.

Conclusion

The two models described above are not mutually exclusive. It is possible that a combination of pressure exerted by the forming vertebrae coupled with an attraction/repulsive signaling system emanating from discrete regions of the vertebral column are responsible for the formation of nuclei pulposi. Experiments that can either directly measure pressure exerted on the notochord and/or experiments designed to determine gene expression changes that directly correlate with cells under compressive pressure are needed to determine the potential mechanism(s) responsible for the transition of the rod-like notochord into nuclei pulposi. These experiments will only be possible by encouraging close interactions between the diverse disciples of Developmental Biology and Engineering.

Compliance with Ethics Guidelines

Conflict of Interest   The authors of this paper declare they have no conflicts of interest

Human and Animal Rights and Informed Consent   Dr. Lawson has nothing to declare.

All studies by Dr. Harfe involving animals were performed after approval by the appropriate institutional review boards.

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