The transcription factors regulating intervertebral disc development

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Abstract
Damage to the intervertebral discs (IVDs) occurs due to aging or excessive mechanical stress, causing a series of IVD-related degenerative diseases, such as spinal disc herniation and spondylosis. These IVD-related diseases are difficult to cure, partially because the regeneration ability of IVDs is not sufficient. As a novel strategy for treatment of IVD-related diseases, mesenchymal stem cell transplantation to the damaged discs has been reported in animal studies. To further develop and improve this approach, it is necessary to gain a better understanding of the molecular network regulating IVD development by critical transcription factors. Recent findings reveal that during IVD development, nucleus pulposus and annuls fibrosus differentiation is coordinated by a series of transcription factors, such as Mkx, Pax1, Shh, Foxa1, T-Brachyury, and Sox5, 6, 9. The combination of mesenchymal stem cell transplantation with the regulation of these molecules may provide a novel strategy for treatment of degenerative disc diseases.

KEYWORDS
annulus fibrosus, intervertebral disc, mesenchymal stem cells, nucleus pulposus, transcription factor

1  |  INTRODUCTION

Intervertebral discs (IVDs) are fibrocartilaginous structures connecting adjacent vertebrae in the spinal column.1 Degeneration or damage of IVDs due to aging or excessive mechanical loading could result in lumbar spine diseases, such as intervertebral disc herniation, spinal spondylosis, and spinal canal stenosis.2, 3 Patients with these diseases suffer from severe pain, which limits their productivity and daily activities. IVD is among the largest avascular tissues in the body and has poor self-healing potential,4 which makes damage to IVDs irreversible, leading to degenerative spondylosis. There are only a few therapeutic approaches available to IVD-related diseases. In many cases, therapies for IVDs related diseases may be limited to relieving pain. Surgical approaches, such as discectomy of IVD herniation, also provide relief from severe pain; however, this operation does not affect the progression of the diseases.4 To develop an innovative regenerative therapy for IVD-related diseases, knowledge of the molecular network in IVD development and homeostasis should be useful. In particular, identification of specific transcription factors regulating IVD development is key to uncover the gene expression network (Figure 1). These critical transcription factors could be targets or tools to develop regenerative medicine for IVD-related diseases. Toward this end, we summarize the recent progress on the analysis of the critical transcription factors in IVD development and repair.

2  |  THE STRUCTURAL ANALYSIS AND DEVELOPMENTAL PROGRAM OF IVDs

IVDs consist of three major components: the nucleus pulposus (NP), the annulus fibrosus (AF), and the cartilaginous endplate (EP). NP is a jelly-like material located in the center of an IVD. The main function of NP is to stabilize the IVD against mechanical stress.5 AF is the fibrous tissue...
surrounding the NP. AF is composed of the outer AF (OAF) and the inner AF (IAF). The OAF is a highly organized collagenous structure consisting mainly of type I collagen, whereas the IAF contains organized type I and type II collagen. EP comprises cartilaginous tissues between the IAF and the NP. Mechanical pressure to the IVDs is well absorbed and balanced into the NP and the AF structure. Capillary vessels in the EP provide nutrition to the entire IVD. The developmental origins of these components are different. Based on cell fate tracing analysis in mice, the notochord is the origin of the NP and the sclerotome is the origin of the AF, EP, and vertebral body. At E10.0, a notochord sheath is formed around the notochord and vertebral body formation begins. At E12.5, sclerotome cells migrate and condense around the notochord (Figure 2A). In a somite pattern, the dense part of the sclerotic cells forms the vertebral body and the sparse part becomes the annulus. The notochord region that expands within the future IVD forms the NP (Figure 2B).

In this way, the formation of the vertebral body, endplate, nucleus pulposus, and annulus fibrosus is completed to form the adult IVD (Figure 2C). The origin of development differs for each tissue constituting the intervertebral disc, and therefore transcription factors important for development vary depending on each constituent tissue. Since the embryonic origin of each component is different from one another, the transcription factors involved in each specific cell type differentiation are different.

### 3 | TRANSCRIPTION FACTORS RELATED TO NP DEVELOPMENT

**Sonic Hedgehog (Shh)** is specifically expressed in the notochord at E9.5 in mice and plays an important role in the formation and maintenance of the notochord.** Smootherned (Smo) functions to activate Shh signaling. It was reported that mice with conditional knockouts of Smo and Shh do not form a notochord sheath and therefore, cannot form the NP.** A recent report revealed that the expression of Shh at E12.5 is notably higher than that at P0 with notochord-derived cells. It indicates Shh functions mainly in the developmental phase. In addition, Shh signaling activates Wnt (a portmanteau of Wingless and Int-1) signaling and increases T-Brachyury and Aggrecan expression within adult NP (Table 1). Thus, Shh is considered to be an important transcription factor not only in NP development but also in NP maintenance. Foxa1 and Foxa2 are expressed in the notochord at E8.5 in mice (Figure 2A). Foxa1 single knockout mice have severely deformed IVDs and Foxa2 single knockout is fatal in early development, and hence notochord formation cannot be analyzed in these mice. However, in Shh-CreERT: Foxa2/Foxa1 knock out mice, notochord formation is disturbed. These results indicate the possibility of functional redundancy between Foxa1 and Foxa2 and suggest that these transcription factors together are essential in notochord formation. These transcription factors are involved in the expression of Shh in the notochord (Table 1). To date, the expression pattern of Foxa1 and Foxa2 in the adult phase is not reported yet and it is of interest to test the potential functions of these genes in the adult.

**T-Brachyury** is a transcription factor that plays a critical role in embryonic mesoderm development, particularly for the formation of the notochord (Figure 2A) (Table 1). Recent studies have shown that T-Brachyury is expressed and functions in the notochord at E10.5. Shh Cre inducible T-Brachyury shRNA expressing T-Brachyury knockdown mice show a phenotype with a costal level of vertebral malformation or loss because of the loss of the notochordal cells, which indicates that T-Brachyury is essential for normal spine formation. Even after the completion of the NP formation, T-Brachyury is still strongly expressed in the NP cells, particularly in the notochordal cells. It is also reported that T-Brachyury regulates the expression of fibroblast growth factors 8 and Axin 2, which are related to disc degeneration. Thus, T-Brachyury has a function in not only NP development but also NP maintenance (Table 1).

**Sox5, 6, and 9** are related to the formation of NP and IAF. Sox9 knock out mice show complete absence of cartilage; therefore, Sox9 is
considered as a master transcription factor in chondrogenesis.\textsuperscript{18} Sox5 and Sox6 are also known to act as transcription factors in chondrogenesis by enhancing the function of Sox9.\textsuperscript{19} In view of IVD development, Sox5 and Sox6 are expressed in the sclerotome and the notochord at E11.5 in mice (Figure 2A).\textsuperscript{20} They regulate the expression of type II collagen and aggrecan and are involved in the formation of the vertebral body and the IAF. In Sox5/Sox6 double-knockout mice, the formation of the vertebral body and the IAF was impaired, resulting in the inhibition of NP formation.\textsuperscript{20} Furthermore, Sox9 was also expressed in the sclerotome and the notochord at E10.5 in mice and was involved in the formation of the NP, IAF, and vertebral body (Table 1).\textsuperscript{21}

**TABLE 1** Summary of transcription factors related to NP and AF developments

| Name          | Expression site                  | Onset of expression (mice) | Development              | Homeostasis                  |
|---------------|----------------------------------|-----------------------------|---------------------------|------------------------------|
| Shh           | Notochordal cells                | E9.5-                        | Formation of notochordal sheath and the NP\textsuperscript{10} | Regulation of the expression of Brachyury and Aggrecan\textsuperscript{12} |
| Foxa1, Foxa2  | Notochordal cells                | E8.5-                        | Adjustment of the expression of Shh\textsuperscript{13} | unknown                      |
| T-Brachyury   | Notochordal cells                | E10.5-                       | Formation of notochord\textsuperscript{14, 15} | Regulation of the expression of FGF8 and Axin\textsuperscript{16, 17} |
| Sox5,6,9      | Notochordal and sclerotome cells | Sox5,6: E11.5- Sox9: R10.5- | Formation of the NP and the IAF\textsuperscript{20} | Regulation of the expression of Col2 and Aggrecan\textsuperscript{18} |
| Pax1, 9       | Sclerotome cells (early stage)   | E10.5-                       | Regulation of the cartilage related genes\textsuperscript{26} | Unknown                      |

**4 | THE TRANSCRIPTION FACTORS RELATED TO AF DEVELOPMENT**

Whereas there are substantial reports to elucidate the NP development, there are few reports that explore AF development. AF consists of two different types of tissues, the IAF and the OAF, and the molecular network for each tissue’s development should be determined. Pax 1 and Pax 9 play a significant role in the development of IVDs and are strongly expressed in the sclerotome at E10.5 in mice (Figure 2A). The expression of these genes is under control of Shh from the notochord.\textsuperscript{22} Pax1 is expressed throughout sclerosis in the early stage of development, but its expression gradually decreases in the vertebral...
body part, and in the later stage of development, expression is limited to the AF, especially the OAF.\textsuperscript{23, 24} Pax1 knockout mice have vertebral and intervertebral disc dysplasia and rib dysplasia, and the notochord cannot form the NP structure even in the late stage of development.\textsuperscript{25} Recently, it was reported that Pax1 functions mainly in the IAF in the early-to-middle developmental stage and showed Pax1-mediated signaling to the notochord and its role in the regulation of cell proliferation.\textsuperscript{25} Another report showed that Pax1 and Pax9 regulated the expression of the cartilage-related genes known to be regulated by Sox5, Sox6, and Sox9 in the early developmental stage.\textsuperscript{26} Moreover, Pax1 and Pax9 are downregulated by way of a negative feedback mechanism through Sox5, Sox6, and Sox9 expression (Table 1).\textsuperscript{26} Thus, Pax1 and Pax9 function mainly for the IAF development as separation of the IAF and the OAF. However, it is still unknown why the expression of Pax1 and Pax9 is kept in the OAF with warranting further analysis.

Recently, Mohawk (Mkx) has been reported as an essential transcription factor for OAF development.\textsuperscript{27} Mkx is a member of the three-amino-acid loop superclass of atypical homeobox genes belonging to the Iroquois family.\textsuperscript{28} The expression of Mkx in the syndetome is detectable at E12.5 and its expression is maintained even in matured ligament cells.\textsuperscript{29} In IVD, Mkx is mainly expressed in the OAF at the early developmental phase until well after maturity (Figure 2B).\textsuperscript{27} In Mkx knockout mice, the AF was found to be thinner than that in the wild-type mice, and it was also confirmed using electron microscopy that the diameter of collagen fibrils had reduced in Mkx knockout mice.\textsuperscript{27} Moreover, in the OAF cells, multiple genes associated with ligament tissue synthesis were downregulated in Mkx knockout mice.\textsuperscript{27} Taken together, these phenotypes show that Mkx plays an essential role in OAF formation (Table 1).

Mkx is known as a transcription factor that has an essential role in tendon and ligament development. Mkx knockout mice show a reduced tendon mass but no decrease in the number of tendon cells (the same phenotype was seen in the AF).\textsuperscript{27, 29} In Mkx knockout rats, the heterotopic ossification of the tendon has occurred via failed tenogenesis.\textsuperscript{30} Furthermore, some reports focused on the function of Mkx after tendon and ligament maturation, and they showed that the reduction of Mkx expression induces ligament degeneration, and that appropriate mechanical stress, applied via Mkx expression in vitro and in vivo, was essential for tendon homeostasis.\textsuperscript{31} From these reports, it can be predicted that Mkx has an essential function not only in tendon maturation but also in the maintenance of tendon homeostasis. In the OAF, the expression of Mkx is also kept after its maturation\textsuperscript{24}; thus, we can hypothesize that Mkx plays a role in maintaining OAF homeostasis, and further analysis is expected in the future.

As essential transcription factors for tendon and ligament development, Scleraxis (Scx) and Egr1 have also been studied extensively. Scx is a helix-loop-helix (bHLH) transcription factor that is expressed in tendon progenitors.\textsuperscript{32, 33} Scx is also expressed in the AF during the developmental phase.\textsuperscript{33} The OAF and ligaments are both fibrous tissues that consist of mainly type I collagen and both perform the same function, that is, to connect bone to bone and contribute to the stability between them. Therefore, we can predict that these tissues are similar in view of development. Interestingly, in Scx knockout mice, significant hypoplasia of the tendon is seen whereas the IVDs structure is normal. This difference may indicate an underlying property that differentiates the OAF and ligaments from tendons. Egr1 is a member of the Egr family of C2H2-type zinc finger transcription factors.\textsuperscript{34} Egr1 and Egr2 are expressed in the developing tendon and play important roles in tendon formation.\textsuperscript{35} Unfortunately, the function of Egr1 in the development of IVDs is not well defined. In Egr1 knockout mice, the tendon was found to be hypoplastic and the expression of Scx was impaired, whereas the expression of Mkx was maintained. Therefore, it can be predicted that Mkx and Egr1 have different pathways for tendon development.\textsuperscript{36} In the future, it may be attractive to explore this aspect by focusing on the function of Egr1 toward the AF.

5 | STEM CELL THERAPY INDUCED BY TRANSCRIPTION FACTORS

To develop regenerative therapy for treating IVD damage, a number of cell transplantation studies into IVDs have been reported. These studies can be divided into three categories: cell induction using growth factors, cell transplantation using mature cells, and cell transplantation using stem or progenitor cells. Among them, mesenchymal stem cell (MSC) transplantation for NP regeneration has been well-developed by many groups.\textsuperscript{37-41} The clinical studies of MSC transplantation in NP have already been conducted and have yielded successful results to some extent.\textsuperscript{42-46} However, whether transplanted MSCs could successfully differentiate to NP cells to reconstruct IVDs remains unclear. In this regard, identification or induction of more tissue-specific progenitor cells for IVDs may improve the therapy. One study reported that TIE 2 and GD 2 are markers of IVD progenitor cells.\textsuperscript{47} Another study attempted to induce the formation of notochordal cells from iPScells.\textsuperscript{48} These cells could be applied to stem cell therapy for NP in the near future.

Regarding the reconstruction of the damaged OAF, the transplantation of OAF cells or MSCs into IVD injury sites has been reported in animal models.\textsuperscript{49-51} However, the transplanted OAF cells did not maintain the OAF cell characteristics and were not able to synthesize sufficient collagen fibers.\textsuperscript{50} This would be partly because the differentiation of MSCs to the OAF is not well-directed in the transplantation region.\textsuperscript{49, 51} To overcome this issue, MSCs modified by IVD-specific transcription factor expression could be applied. MSCs that over-express Mkx acquire the ability to produce multiple tendon- and ligament-associated proteins and synthesize ligament-like tissues.\textsuperscript{24, 52, 53} Based on these findings, the transplantation of these cells into the IVD injury in the animal model results in ligament-like tissue synthesis that has sufficient physical properties.\textsuperscript{24} This successful model builds a case for understanding the function of a transcription factor in tissue development and using it as a therapeutic tool.

There are also reports on methods of inducing MSCs to NP cells using growth factors.\textsuperscript{54, 55} One study utilized the pellet culture of human mesenchymal stem cells and human adipocyte-derived stem cells with GDF6, and successfully induced the expression of Sox9 and...
The developmental mechanism of the IVD has recently been uncovered with identifications of critical transcription factors in IVD development. In the next stage, it is essential to reveal the transcriptional networks coordinated by these transcription factors during IVD development. For this purpose, chromatin immunoprecipitation and/or single cell analysis should be performed. As for clinical application, cell therapy or chemical compounds targeting these transcription factors could be tested to repair IVDs (Figure 3).

6 | CONCLUSION AND FUTURE DIRECTION

The developmental mechanism of the IVD has recently been uncovered with identifications of critical transcription factors in IVD development. In the next stage, it is essential to reveal the transcriptional network coordinated by these transcription factors during IVD development. For this purpose, chromatin immunoprecipitation and/or single cell analysis should be performed. As for clinical application, cell therapy or chemical compounds targeting these transcription factors could be tested to repair IVDs (Figure 3).

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CONFLICT OF INTEREST

The authors declare no conflicts of interests.

AUTHOR CONTRIBUTIONS

R. N. and H. A. wrote the paper.

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