Growth Factor and Extracellular Matrix Expression and Localization during Nucleus Pulposus Formation

Sun Peck, PhD[1]
Kendra McKee, MD[2]
Neil Malhotra, PhD[1]
Brian Harfe, PhD[2]
Lachlan Smith, PhD[1]

[1]University of Pennsylvania
Philadelphia, PA

[2]University of Florida
Gainesville, FL

Introduction

Intervertebral disc degeneration is implicated as a major cause of low back pain[1]. Current available treatment options primarily focus on relieving pain rather than regenerating disc tissue, and thus, there is a need for new therapeutic strategies that alleviate symptoms as well as restore disc structure and mechanical function. The earliest degenerative changes occur in the central nucleus pulposus (NP), where altered composition initiates a cascade that compromises mechanical function and culminates in structural failure. An impediment to the development of cell-based strategies for NP repair is the unique developmental origin of the NP, as NP cells are derived from the notochord and not the mesenchyme[2-4]. Improved understanding of embryonic NP formation may enable recapitulation of developmental signals that might drive therapeutic cell types, such as mesenchymal stem cells, towards an NP cell-like phenotype to optimize adult disc regeneration. Previously, we established changes in global mRNA expression profiles of resident cells as the notochord transforms into the NP using whole-transcriptome sequencing (RNA-Seq), and found that key signaling pathway elements that regulate patterning, growth, differentiation, as well as structural extracellular matrix (ECM) molecules, showed significant differential gene expression across this embryonic developmental window[5]. In this study, our objectives were to build on these findings by examining protein expression of growth factors and ECM molecules implicated in our RNA-Seq results at key developmental stages as the notochord transforms into the NP.

Methods

For these IACUC approved studies, we used the Shh-cre;ROSA:YFP mouse model[3], where all Sonic Hedgehog (Shh) expressing notochord-derived cells express YFP throughout the life of the mouse (i.e. creates a fate map). We examined two key developmental stages representing the immediate, opposite ends of the notochord to NP transformation: E12.5 (fully formed, intact notochord) and P0 (fully formed spine with distinct disc space). Whole embryos (E12.5) or isolated spines (P0) were fixed in formalin, and processed into paraffin. Midsagittal, 8 μm thick sections were stained with Alcian blue/picrosirius red (ABPR) for GAG and collagen respectively, hematoxylin and eosin (H&E) for cellularity, or immunostained with antibodies specific to proteins-of-interest (ECM: Collagens I, II and VI, and aggrecan; growth factors: Shh, transforming growth factor β1 (TGF-β1), and insulin-like growth factor 1 (IGF-1)) and counterstained with hematoxylin. Staining intensity in the notochord/NP and associated tissues was semi-quantitatively assessed.

Results

At E12.5, there was a discrete notochordal structure with a GAG-rich inner core and outer sheath, both of which were relatively acellular compared to the rest of the notochord (Figure 1). GAG-rich mesenchymal condensations in regions that will form future vertebral bodies were clearly present (Figure 1). At P0, the spine was fully formed with distinct vertebral bodies and disc spaces, including clear boundaries between the annulus fibrosus and the NP (Figure 1).

Extracellular matrix components collagens I, II, and VI, and aggrecan showed diffuse staining in non-cellular regions (core and sheath) of the E12.5 notochord. At P0, these molecules exhibited intense staining at the outer boundary of the NP. SHH, TGFβ1, and IGF1 all showed

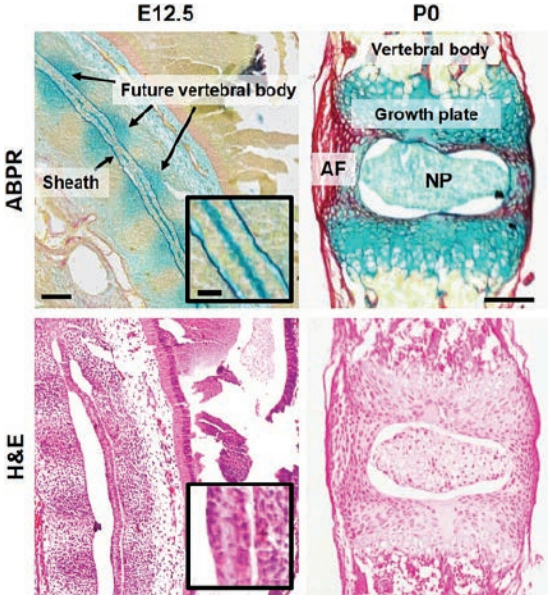

Figure 1. Overall morphology at E12.5 (notochord) and P0 (disc). AF: annulus fibrosus; NP: nucleus pulposus. Scale bar = 100 μm; inset scale bar = 20 μm.

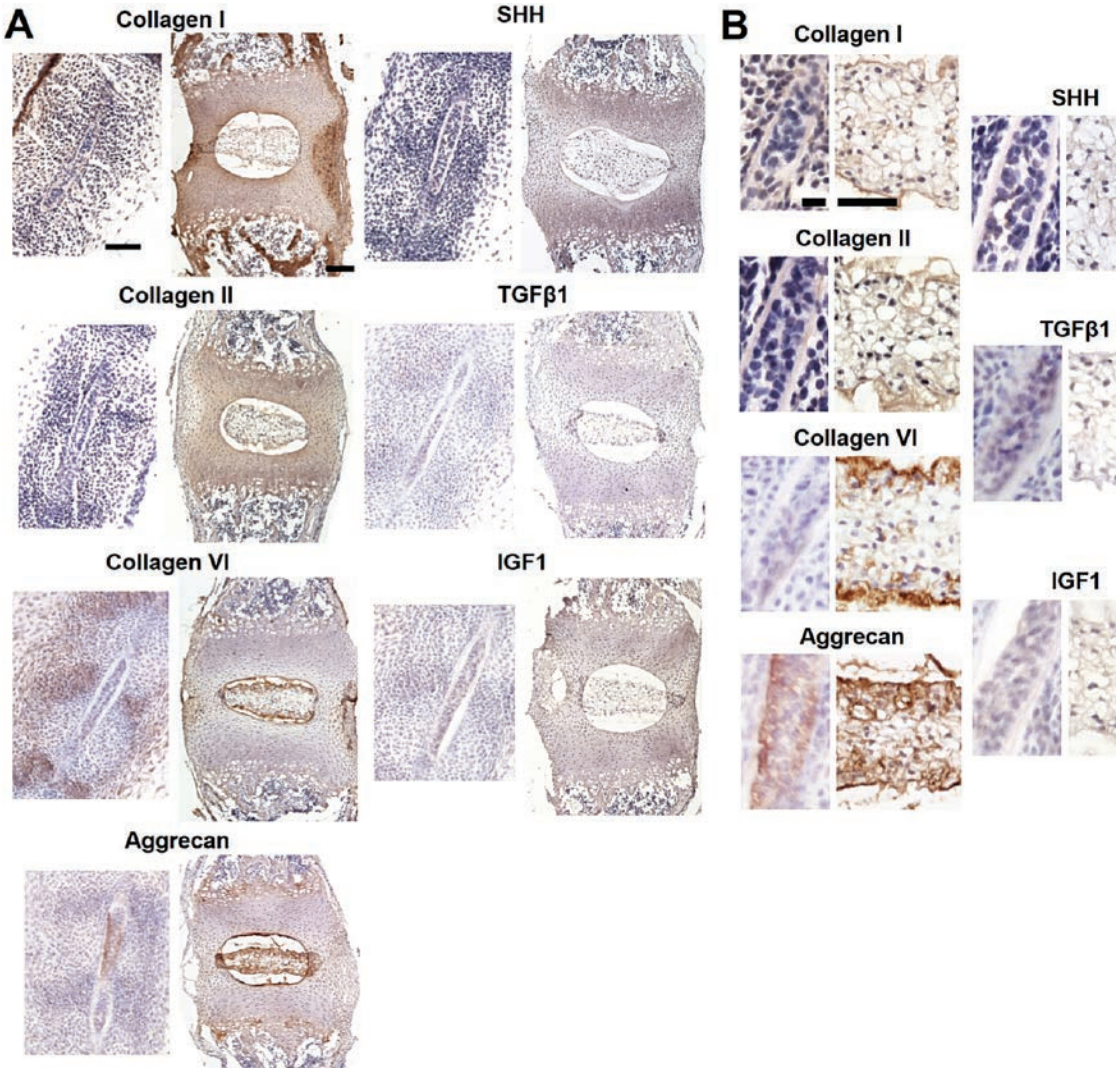

Figure 2. Representative immunostaining of ECM and growth factors at E12.5 and P0. For each pairing, the left image is E12.5, and the right image is P0. Panels in B represent higher magnification images of notochord/NP from A. Scales in A: E12.5 = 50 μm, P0 = 100 μm, and B: E12.5 = 10 μm, P0 = 50 μm.

Table 1 Developmental Staae and Region

	E12.5			P0			
Molecule	**N**	**DC**	**VC**	**NP**	**IAF**	**OAF**	**E**
SHH	**	-	-	*	-	-	-
TGFβ1	**	**	*	**	*	*	**
IGF1	**	**	*	*	-	-	**

Table 2 Developmental Stage and Region

	E12.5			P0			
Molecule	**N**	**DC**	**VC**	**NP**	**IAF**	**OAF**	**E**
Aggrecan	**	*	*	***	*	*	**
Collagen I	*	***		**		***	
Collagen II	*	*	*	*	**	*	***
Collagen VI	*	***	-	***	-	**	*

cellular expression in the E12.5 notochord. At P0, expression of TGFβ1 and IGF1 by NP cells was heterogeneous (strongly by some cells, weakly by others). SHH expression in the NP was weaker at P0 than at E12.5. In both the E12.5 and P0 samples, positive immunostaining of non-notochord/NP tissues for many of these molecules was also observed. Semi-quantitative scoring of protein localization is presented in Tables 1 and 2.

Discussion

In our previous whole-transcriptomic profiling study, we found a large number of differentially expressed growth factor and ECM genes at P0 compared to E12.5[5], which are largely reflected on the protein level in our current results. We demonstrated marked changes in protein localization and expression levels between E12.5 and P0. As mRNA and protein levels do not always directly correlate in expression, ongoing work is focused on elucidating regulatory and functional roles of these genes on both the transcriptional and translational levels. The changes observed most likely reflect a switch from patterning (decreased Shh signaling) to growth (increased TGFβ1, IGF1, and ECM structural genes) as the NP develops into a functional, load-bearing tissue. Heterogeneous expression within the NP at P0 suggests that resident cells may be undergoing progressive phenotypic changes to accommodate evolving functional requirements. Interestingly,

we also observed staining of non-notochord derived tissue in our studies, which will help to inform future studies exploring the roles of these molecules in embryonic spine development as a whole. Overall, these data support our long-term goal to establish and recapitulate the specific developmental signals required for embryonic NP formation in order to improve cell-based therapeutic strategies for disc regeneration.

Significance

Low back pain associated with intervertebral disc degeneration is a significant global health and economic burden. The results from this study further our knowledge and understanding of NP development and serves to inform development of improved cell-based therapeutics for disc regeneration.

References

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