Deep Sequencing of Notochord-Derived Cells During Embryonic Formation of the Nucleus Pulposus: Preliminary Findings

[1]Lachlan J. Smith, PhD
[1]Joseph A. Chiaro, BS
[2]Kendra K. McKee, BS
[1]Neil R. Malhotra, MD
[1]Robert L. Mauck, PhD
[2]Brian D. Harfe, PhD

[1]University of Pennsylvania, Philadelphia, PA, USA

[2]University of Florida, Gainesville, FL, USA

Introduction

Intervertebral disc degeneration is strongly implicated as a cause of low back pain.[1] Current surgical and conservative treatments for discogenic low back pain only treat the symptoms, and there is a pressing need for new therapeutic strategies to restore native disc structure and mechanical function by potentiating native tissue regeneration. Identification of such strategies may be achieved by studying aspects of intervertebral disc development. During embryonic development, the mesoderm-derived notochord undergoes a transformation from a rod-like structure that acts as signaling center involved in patterning the axial skeleton, to form the disc nucleus pulposus (NP), the proteoglycan-rich structure at the center of the disc that performs a crucial role in resisting compressive loads.[2] While previous studies have shown that all cells comprising the adult mouse NP are notochord-derived,[3,4] little is known about the molecular mechanisms which drive the transformation from notochord to NP. The objective of this study was to use whole transcriptome deep sequencing (RNA-Seq) to define differences in the global mRNA expression profiles of notochord-derived cells during embryonic formation of the NP, and to identify the key anabolic factors driving this process. We hypothesized that differences between the global mRNA expression profiles of embryonic notochords and postnatal discs would reflect a transformation from a signaling center (notochord) to an extracellular matrix-rich and functional load-bearing tissue (NP).

Methods

Animal studies were performed following IACUC approval. Mice used in this study were Shh-cre;ROSA:YFP.[3] This system takes advantage of the fact that all cells of the notochord express the morphogen sonic hedgehog (SHH), whereas those of the surrounding mesenchyme do not. This allows for FACS isolation of pure populations of notochord-derived cells. Two developmental stages were examined in this study: embryonic day 12.5 (E12.5), immediately before the notochord-NP transformation commences (Figure 1, top); and postnatal day 0 (P0), when the transformation is complete and the NP is fully formed (Figure 1, bottom). Each biological replicate (E12.5: n=3; P0: n=1) was comprised of cells pooled from all embryos/pups in a litter (average of n=6 per litter). For E12.5, discrete notochords were dissected from embryos for immediate RNA isolation. For P0 samples, spines, were dissected free of posterior elements, including dorsal root ganglia, minced and digested for 2 hours in collagenase. YFP-

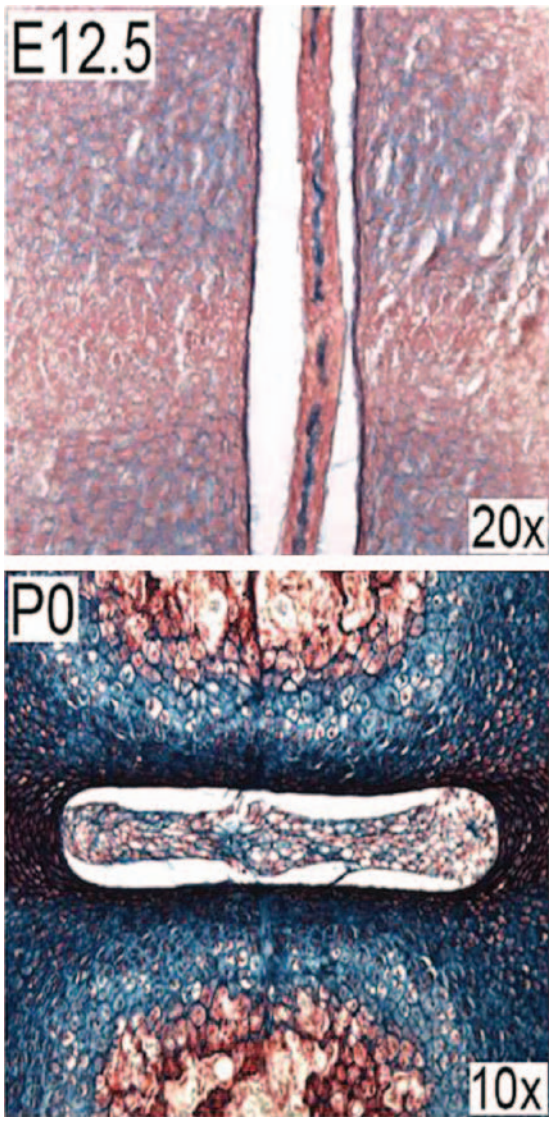

Figure 1. Histological appearance of the embryonic spine at E12.5 showing intact notochord (top), and at P0 showing fully formed NP (bottom).

Paper No: 0238
2014 Annual Meeting
Orthopaedic Research Society
lachlans@mail.med.upenn.edu

positive (notochord-derived) cells were collected using FACS (Figure 2), and RNA isolated. RNA was converted to RNA-Seq libraries, sequenced (Illumina HiSeq, ~50 million reads/transcript) and aligned to the mouse genome. Fold changes (E12.5=>P0) in extracellular matrix molecules, signaling factors and known notochordal/NP markers were assessed. Analysis was limited to the top 5000 expressed genes for each group. Pathway analysis was performed using Ingenuity Pathway Analysis software. Specifically, upstream analysis was used to predict those growth factors potentiating cell differentiation and driving embryonic formation of the NP.

Results

Analysis of fold changes revealed increased expression of extracellular matrix molecules at P0 relative to E12.5 (Figure 3A). Specifically, this included proteoglycans (ACAN: +3; LUM: +5; FMOD; +8; BGN: +42; DCN: +11; ASPN: +31; and PRG4: +76), and collagens (COL2A1: +2; COL1A1: +5 and COL6A1: +7). Expression of the morphogen SHH was dramatically decreased at P0 relative to E12.5 (−164). With respect to putative NP cell markers, some were decreased (KRT8: −5; KRT18: −11; HBB: −54), and others were unchanged (VIM, KRT19, PAX1) (Figure 3B). Several chondrogenic markers also showed increased expression, including COMP (+72) and TNC (+10). Upstream analysis of global fold changes in mRNA expression predicted that the anabolic cytokines most likely driving embryonic formation of the NP include TGFB1, TGFB3, BMP6, CTGF and EGF.

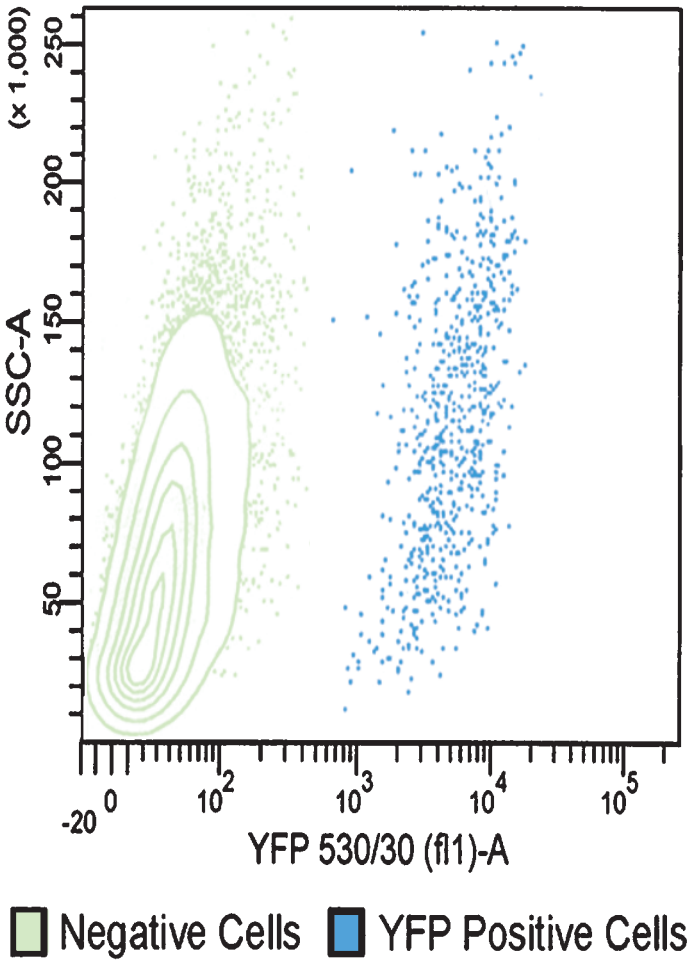

Figure 2. Representative cell sorting result showing a distinct population of YFP-positive cells from Shh-cre;ROSA:YFP embryos.

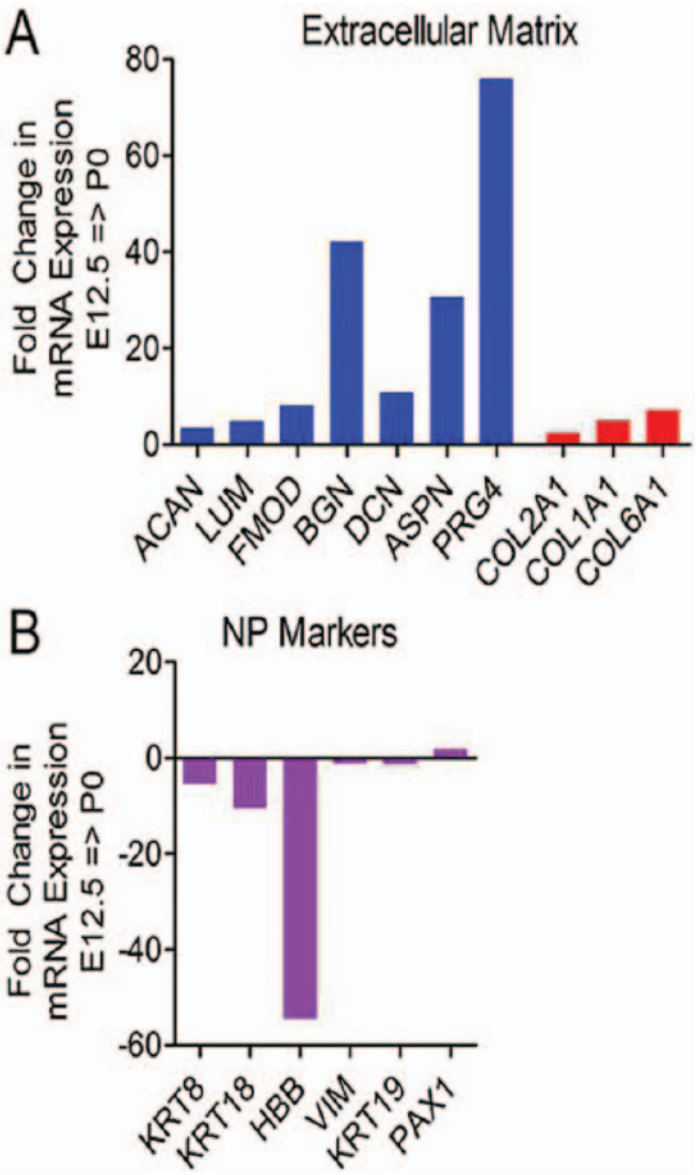

Figure 3. A) Fold changes in mRNA expression of key extracellular matrix components, and B) NP cell markers, from E12.5 to P0.

Discussion

These results, while preliminary, support the conclusion that during embryonic formation of the NP, notochord cells differentiate toward a more chondrogenic phenotype, adopting a molecular expression profile necessary to synthesize and maintain an extracellular matrix-rich, functional, load-bearing tissue. Reduction in SHH expression is consistent with a diminished postnatal signaling role for the NP. Ongoing work will confirm these findings with

additional biological replicates and complementary assays (including verification by real time PCR, in situ hybridization and immunohistochemistry). Identification of key anabolic factors responsible for notochord-derived cell differentiation and embryonic formation of the NP may lead to novel growth factor regimens to direct stem cell differentiation towards the NP phenotype or be injected therapeutically to promote intervertebral disc regeneration.

Significance

Low back pain resulting from intervertebral disc degeneration is a significant socio-economic burden. The results of this study may lead to new biological therapies for structural and functional regeneration of the disc.

Acknowledgments

This work was funded by grants from the NIH (NIAMS and NIA), the Penn Center for Musculoskeletal Disorders, and the Department of Veterans Affairs. The authors acknowledge the technical assistance provided by staff members of the Next Generation Sequencing Core at the University of Pennsylvania.

References

1. Andersson GB. Epidemiological features of chronic low-back pain. *Lancet* 354, 581–585 (1999).

2. Smith LJ, Nerurkar NL, Choi KS, et al. Degeneration and regeneration of the intervertebral disc: lessons from development. *Dis. Model. Mech.* 4, 31–41 (2011).

3. Choi KS, Cohn MJ, Harfe BD. Identification of nucleus pulposus precursor cells and notochordal remnants in the mouse: implications for disk degeneration and chordoma formation. *Dev. Dyn.* 237, 3953–3958 (2008).

4. McCann MR, Tamplin OJ, Rossant J, et al. Tracing notochord-derived cells using a Noto-cre mouse: implications for intervertebral disc development. *Dis. Model. Mech.* 5, 73–82 (2012).