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Whole-Transcriptome Profiling of Notochord-Derived Cells during Embryonic Nucleus Pulposus Formation

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Introduction

Intervertebral disc degeneration is implicated as a major cause of low back pain,[1] necessitating 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. The objective of this study was to establish changes in global mRNA expression profiles of resident cells as the notochord transforms into the NP using whole-transcriptome sequencing (RNA-Seq), focusing on signaling pathways that regulate patterning, growth, differentiation, structural extracellular matrix (ECM) molecules, and putative NP cell-specific markers.

Methods

For these studies (IACUC approved), we used the Shh-cre;ROSA:YFP mouse model,[3] which takes advantage of the fact that all notochordal cells express the morphogen Sonic Hedgehog (SHH), while the cells of the surrounding mesenchyme do not. In this model, SHH-expressing cells also express YFP, enabling isolation of pure populations of notochord-derived cells at any developmental stage. Two key developmental stages were examined: embryonic day 12.5 (E12.5, immediately prior to initiation of the notochord to NP transformation) and postnatal day 0 (P0, fully formed NP) (Figures 1A, B). Each biological replicate ($n = 4$, both groups) consisted of pooled embryos or pups (~6) from one litter. E12.5 RNA was extracted from isolated notochords, and P0 RNA was extracted from YFP-positive cells isolated using fluorescence-assisted cell sorting (Figure 1C). High quality total RNA (RIN > 7) was isolated from each sample and RNA-Seq libraries prepared using the TotalScript Kit (Illumina; San Diego, CA). Single-end, 100-base sequencing was performed (Illumina HiSeq 2500) and results aligned to the mouse genome. Differential gene expression was established using DESeq2[5] with significance as $p < 0.05$.

Results

Principal component analysis (PCA) revealed clear differences in global mRNA abundance between E12.5 and P0 (Figure 2). There were > 4600 genes significantly differentially expressed with fold-changes greater than 2. There was significantly higher mRNA abundance of ECM structural genes, including proteoglycans (aggrecan (ACAN); brevican (BCAN); biglycan (BGN); decorin (DCN)) and collagens (COL1A1, COL6A1, COL10A1), at P0 compared to E12.5 (Figure 3A). Examining signaling pathways known to regulate skeletal patterning, growth, and differentiation, there was significantly lower mRNA abundance of Shh pathway activators including ligand (SHH), receptors (Patched1 (PTCH1); Smoothened (SMO)), and transcription factors (GLI1, 2, 3) (Figure 3B). A large number of genes associated with the TGF-β pathway were

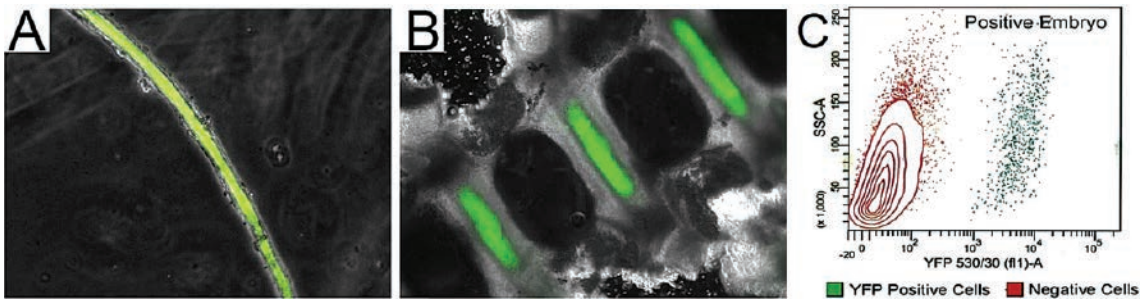

Figure 1. (A) E12.5 YFP-positive notochord. (B) P0 spine with YFP-positive NP. (C) FACS plot of isolated YFP-positive notochord-derived cells.

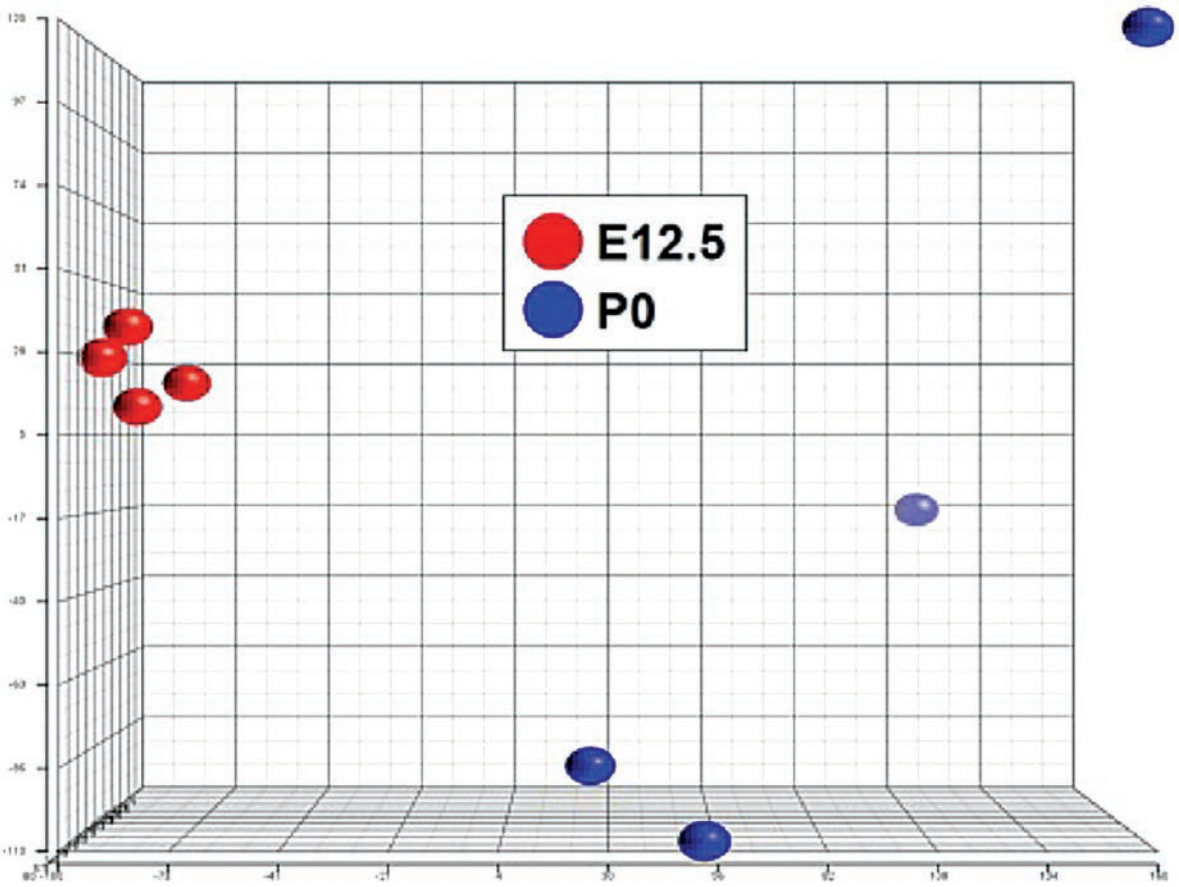

Figure 2. RNA-Seq PCA plot. N = 4.

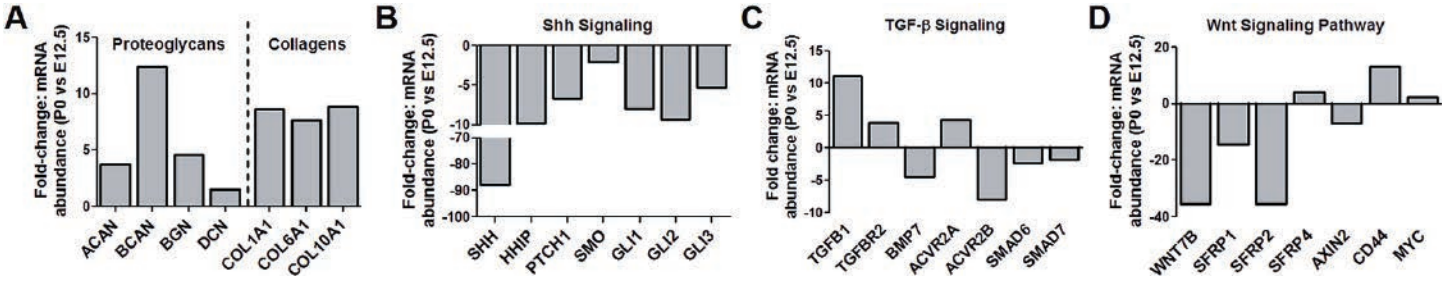

Figure 3. Fold-change in mRNA expression of P0 vs E12.5 cells. A. ECM structural genes. B. Shh pathway genes. C. TGF-β pathway genes. D. Wnt pathway genes. N = 4; all $p < 0.05$.

also differentially expressed at P0 compared to E12.5, including 11.2 and 3.9-fold increases in TGFB1 and TGFBR2, respectively (Figure 3C). Furthermore, many genes of the Wnt signaling pathway were differentially expressed at P0, including pathway ligands such as WNT7B, modulators such as SFRPs (secreted frizzled-related proteins), and downstream target genes, AXIN2, CD44, and MYC (Figure 3D). Finally, we examined differential expression of molecules considered to be specific markers of the NP cell phenotype.[6] Many such markers exhibited stable expression across the E12.5 to P0 developmental window, including brachyury (T), keratins 8 and 18 (KRT8, KRT18), and hypoxia-induced factor (HIF1A). Others exhibited significant changes in expression from E12.5 to P0, including keratin 19 (KRT19, 2.9-fold increase), carbonic anhydrase 3 (CAR3, 8.3-fold increase), carbonic anhydrase 12 (CAR12, 4.6-fold decrease), and vimentin (VIM, 5.7-fold increase).

Discussion

The large number of differentially expressed genes at P0 compared to E12.5 is not surprising given the substantial morphological changes occurring in this developmental window. Changing expression levels of ECM and signaling molecules likely reflect a switch from patterning (altered Shh and Wnt signaling) to growth (increased TGF-β signaling and ECM) as the NP develops into a functional, load-bearing tissue. TGF-β signaling has previously been identified as critical for disc development,[7] and our findings support those results. Differential expression of many Shh and Wnt pathway genes supports the changing role of these pathways at the intersection of the embryonic and postnatal phases of development.[8] Analysis of putative NP markers[6] also showed significant differential expression at P0 when compared to

E12.5. Ongoing work will validate these RNA-Seq results and establish the effects of targeted activation or inactivation of these signaling molecules on embryonic disc formation and postnatal growth. NP markers found to exhibit stable expression throughout embryonic development may be the most faithful indicators of a cell's notochordal origin, although the importance of many of these markers in the context of adult function and regenerative therapeutics remains to be fully elucidated. Our long term goal is 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 issue. The results from this study will inform the development of improved cell-based therapeutics for disc regeneration.

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