Single-Cell Transcriptomics Reveals Emergent Nucleus Pulposus Cell Subpopulations in the Postnatal Mouse Disc

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Introduction

Intervertebral disc degeneration is a major cause of low back pain, the leading cause of disability worldwide.[1] Emerging cell-based therapies targeting the disc nucleus pulposus (NP), particularly those employing adult stem cells, have shown promise in preclinical studies;[2] however, efficacy has been limited by the inability of these therapeutic cells to sufficiently mimic the phenotype of native NP cells, including survival in the nutrient poor disc microenvironment and production of a proteoglycan-rich extracellular matrix (ECM). During development, NP cells uniquely arise from the embryonic notochord,[3,4] transitioning from a role focused on tissue patterning through secretion of long-range morphogens, to one more focused on ECM production and maintenance.[5,6] The mechanisms underlying this transition in NP cell function remain poorly understood, and may provide important clues for optimizing adult stem cells for therapeutic, regenerative application. The objective of this study was to use single-cell transcriptomics to investigate emergent NP cell heterogeneity in the postnatal mouse disc. We hypothesized that the postnatal mouse disc contains multiple NP cell subpopulations with unique gene expression profiles that reflect distinct functional roles.

Methods

Single Cell RNA-Sequencing

To obtain notochord-derived NP cells for single cell RNA sequencing (scRNA-Seq), we used a Shh-cre;R26R-tdTomato mouse model, which leverages the fact that all cells of the embryonic notochord express Shh, and produces constitutive expression of tdTomato at the ROSA26 locus in these cells and their progeny (i.e. a fate map), including when Shh is no longer expressed. With IACUC approval, mice were euthanized at 30 days-of-age, and disc cells isolated via mechanical dissociation and brief collagenase digestion. TdTomato+ cells were then purified using FACS. Libraries were generated using the Chromium controller (10X Genomics) and sequencing was performed using the Illumina HiSeq platform. Three replicate sequencing experiments were performed. Unsupervised clustering was conducted using Seurat, and differentiation trajectory analysis was performed using Monocle

Histology

Lumbar spines were isolated from mice aged 0, 7, 14, 30 and 60 days, fixed in formalin, decalcified and processed for paraffin histology. Mid-sagittal sections were stained with Alcian blue and picrosirius red (ABPR) to demonstrate glycosaminoglycan (GAG) and collagen, respectively. Additionally, mid-sagittal, calcified cryosections were obtained from the lumbar spines of both Shh-cre;R26R:tdTomato and wild type mice at these same ages for fluorescent localization of NP cells and immunofluorescent localization of subpopulation-specific cell surface markers, respectively.

Results

Single Cell RNA-Sequencing

The three replicate scRNA-Seq experiments exhibited high reproducibility, and results were therefore pooled prior to further analyses. A total of 1116 notochord-derived NP cells (median 1445 genes/cell and 4854 UMIs/cell) were identified within the total sequenced cell population. UMAP plots revealed the presence of two distinct NP cell clusters exhibiting distinct gene expression profiles (Figure 1A). Differentiation trajectory analysis showed that cells in clusters 1 and 2 aligned along a pseudo-timeline (Figure 1B). Cluster-specific gene expression analyses demonstrated that NP cells in both clusters exhibited high expression of established NP markers including KRT18 and 19, and T, while expression of NP-specific ECM genes ACAN, COL2A1 and COL6A1 was confined to cells in cluster 2 (Figure 2). Based on the differential expression of these ECM genes, cells in clusters 1 and 2 were denoted early and

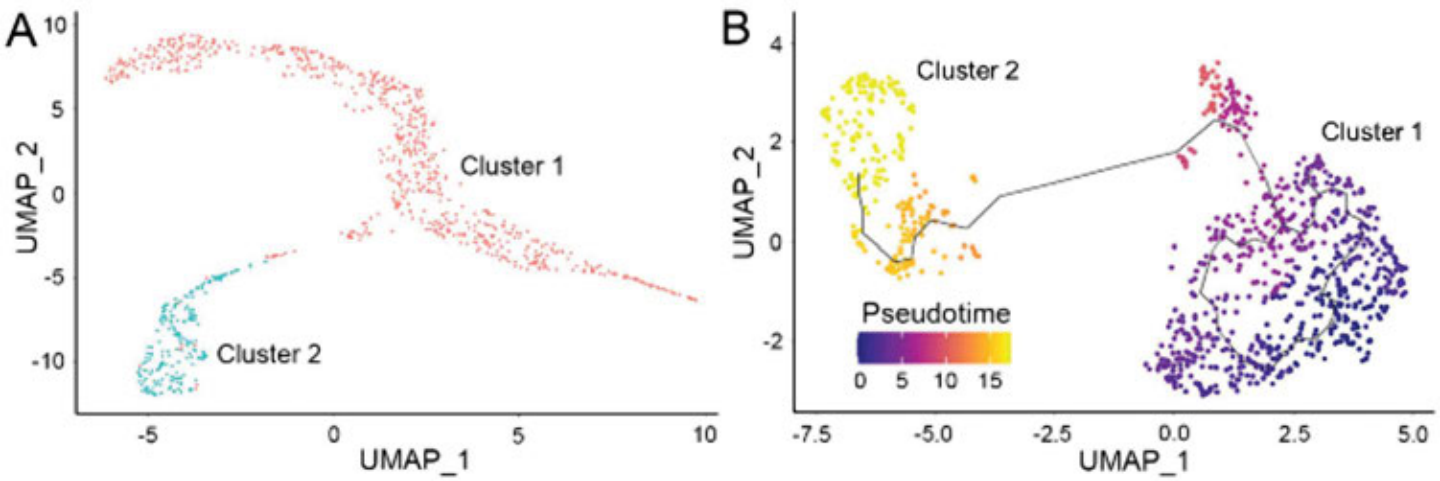

Figure 1. (A) UMAP plot showing clustering of two distinct populations of notochord-derived NP cells. **(B)** Differentiation trajectory analysis showing the pseudo-temporal transition from cluster 1 to 2 cells.

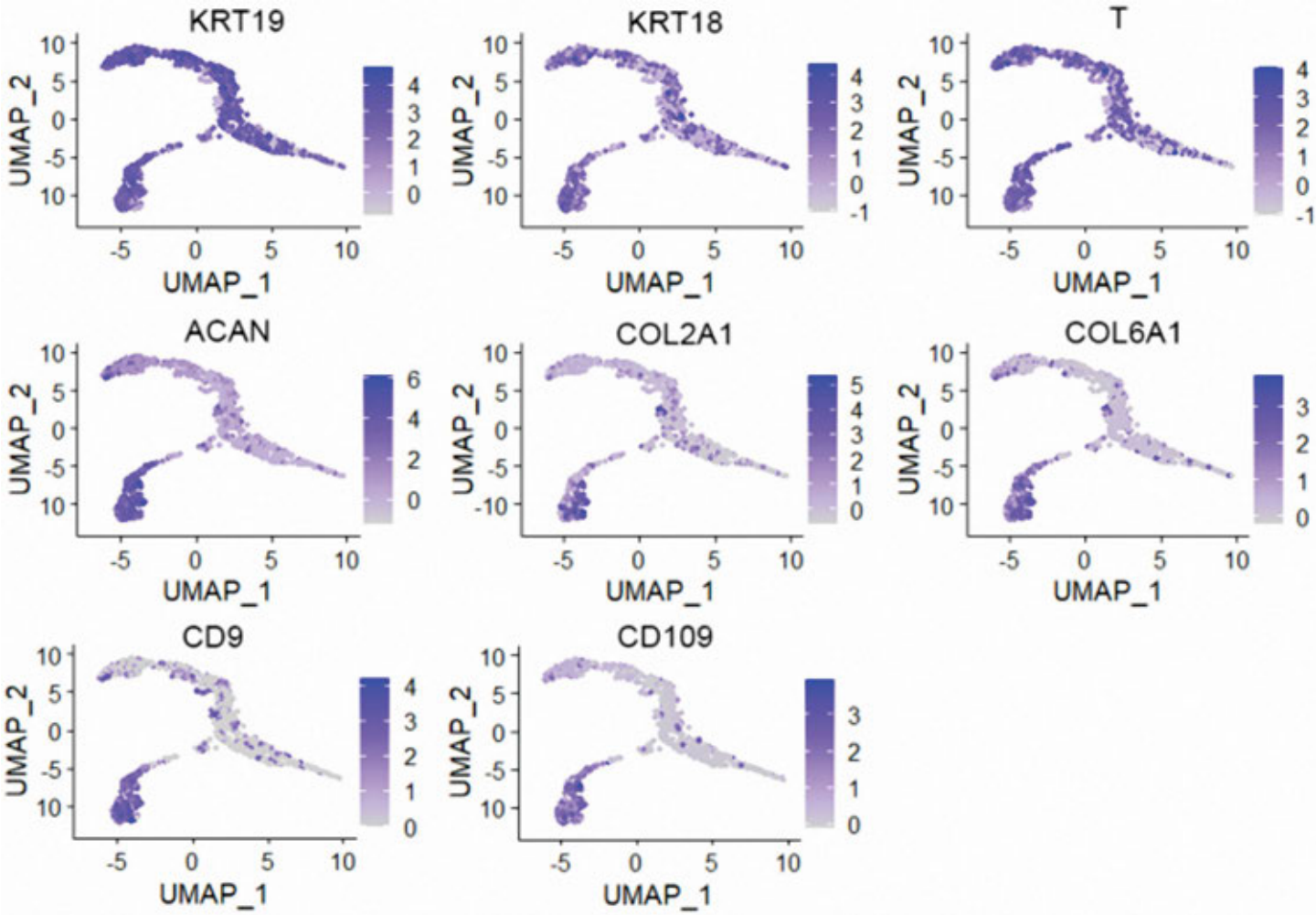

Figure 2. UPAP plots showing relative espression of NP marker, ECM and cell surface marker genes in each cell cluster.

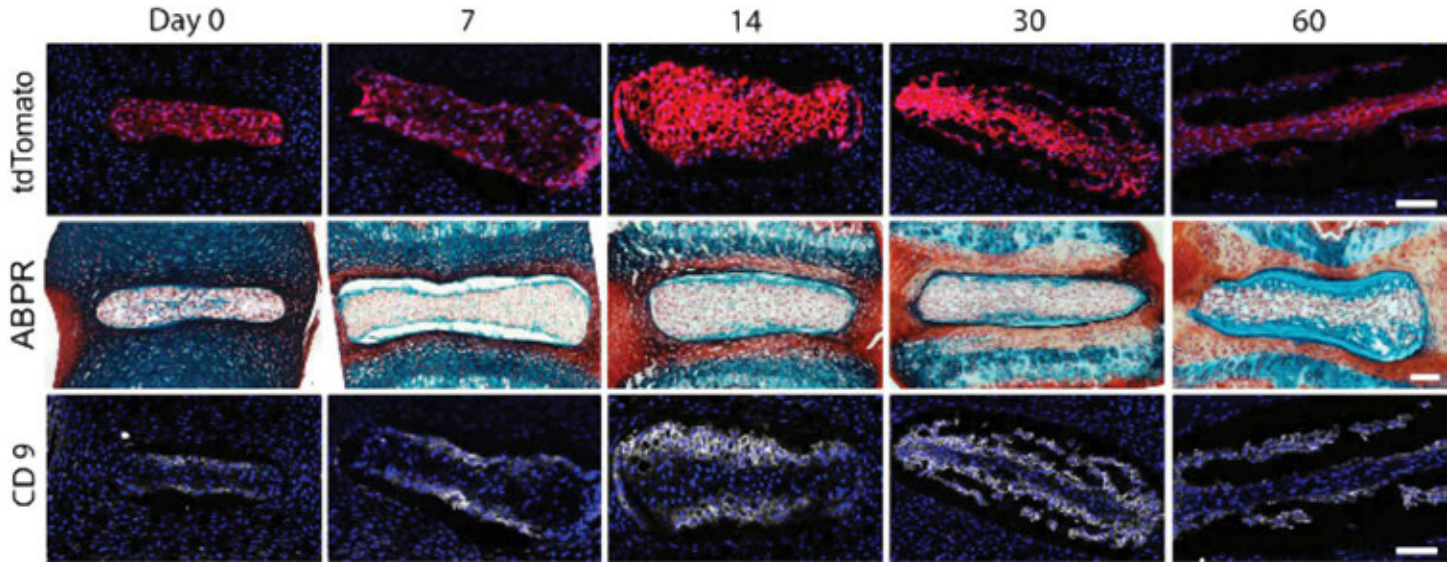

Figure 3. TdTomato+ cells in the NPs of discs from Shh-cre;R26R:tdTomato mice aged 0 to 60 days **(top).** Alcian blue/picrosirius red-stained sections showing progressive accumulation of GAG-rich ECM in the peripheral regions of the NP with increasing age **(middle).** Immunoflorescent staining showing CD9-positive cells confined to the peripheral regions of the NP **(bottom).** Scale = 100μm.

late-stage NP cells, respectively. In addition to high ECM gene expression, late-stage NP-cells uniquely expressed the cell surface markers CD9 and CD109

Histology

Fluorescence imaging revealed tdTomato expression throughout the NP. ABPR staining showed progressive ECM deposition in the NP from day 0 to 90. Notably, an emergent halo of GAG-rich ECM was apparent surrounding a central region of vacuolated cells. Immunofluorescent staining for CD9 demonstrated that late-stage NP cells co-localized with this emerging halo of GAG-rich ECM at all ages examined.

Discussion

In this study we provide the first evidence of emergent heterogeneity amongst NP cells in the postnatal mouse disc. Specifically, we established the existence of early and late-stage NP cells, which exhibit distinct gene expression profiles reflecting unique functional roles. Findings suggest that early-stage NP cells residing at the center of the NP give rise to late-stage NP cells that reside at the periphery, and are responsible for producing the halo of proteoglycan-rich ECM. This ECM is crucial for maintaining disc hydrostatic pressure and resisting axial compressive forces. Importantly, our results also identified candidate surface markers, CD9 and CD109, which are uniquely expressed by late-stage NP cells. Histological findings suggest that the transition from early to late-stage NP cells occurs progressively during postnatal growth.This may explain the eventual depletion of early stage (historically referred to as "notochordal" cells) in other species, including humans. Ongoing work will confirm these findings by examining additional ages in the mouse using scRNA-Seq. Our ultimate goal is to leverage these findings to develop an optimized, cell-based therapy for disc regeneration.

Significance

Stem cell-based therapies for disc degeneration hold significant promise for patients with chronic low back pain. The results of this study expand understanding of the cellular mechanisms underlying postnatal disc formation, and suggest target characteristics for therapeutic stem cells.

Acknowledgements

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