Deciphering the role of Neurog2-Ascl1 co-expression in the retina

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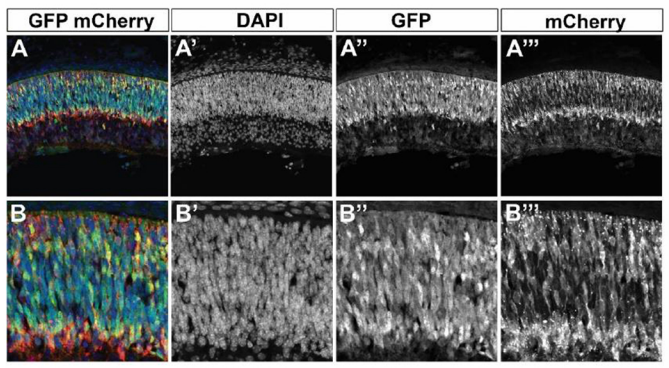
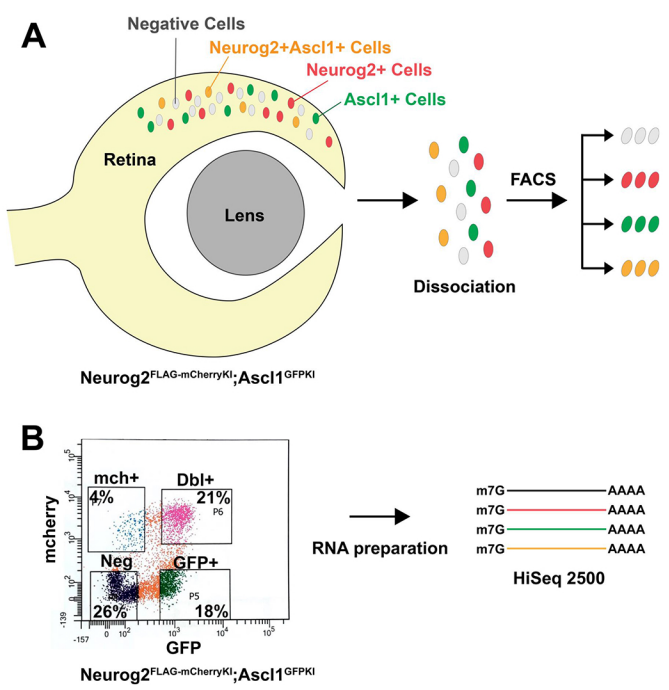
Abstract: During development, multipotent retinal progenitor cells (RPCs) undergo temporal identity transitions to give rise to neurons and glia in a defined temporal sequence. However, rather than distinct waves of differentiation for each retinal cell type (i.e., ganglion, horizontal, amacrine, bipolar, cones, rods, Muller glia), retinal cells are born in overlapping temporal windows. The temporal competence model states that RPCs are multipotent and stochastically give rise to all cell types with few exceptions (e.g., rod only clones, Olig2$^+$ RPCs biased towards rods and amacrine cells, Ascl1$^+$ RPCs give rise to all retinal cells except ganglion cells). Here we investigated whether the proneural transcription factors Neurog2 and Ascl1, which specify neural cell fates in other regions of the nervous system, also specify distinct cell fates in the retina. We found that a subset of embryonic RPCs express Neurog2 and Ascl1 either alone or in combination. To assess how these RPCs differ, we sorted Neurog2/Ascl1 negative, single, and double positive retinal cells from embryonic day (E) E16.5 *Neurog2^{mCherryKI};Ascl1^{GFPKI}* transgenics and performed RNA-seq. PCA and ANOVA-like analysis showed that Neurog2/Ascl1 double$^+$, single$^+$ and negative cells are distinguished by eight distinct clusters defining the 7,145 differentially expressed genes. Neurog2/Ascl1 double$^+$ retinal cells preferentially express genes that are involved in the differentiation of early-born retinal cell types (e.g., amacrine cells and horizontal cells), such as *Pax6, Neurod1*, *Neurod4* and *Onecut1*. To examine the fate and function of Neurog2/Ascl1 double$^+$ RPCs during retinal development *in vivo*, we used a novel split-Cre system, in which Cre recombinase is only active in Neurog2/Ascl1 double$^+$ cells. In *split-Cre;Rosa-zsGreen* reporter mice, we discovered that Neurog2/Ascl1 double$^+$ RPCs exclusively give rise to amacrine cells. Moreover, amacrine cell number were significantly reduced in *split-Cre;Rosa-DTR* 'deletor' mice. In summary, Neurog2/Ascl1 co-expresion defines a new distinct population of RPC that give rise to amacrine cells and supports the idea that some RPCs are lineage restricted. Taken together, these data bring new insights into how amacrine cell fates are specified and reveal the existence of another lineage-restricted RPC pool.

1. Identification of Neurog2/Ascl1 co-expression in Neurog2$^{FLAG\text{-}mcherryKI}$;Ascl1^{GFPKI} RPCs



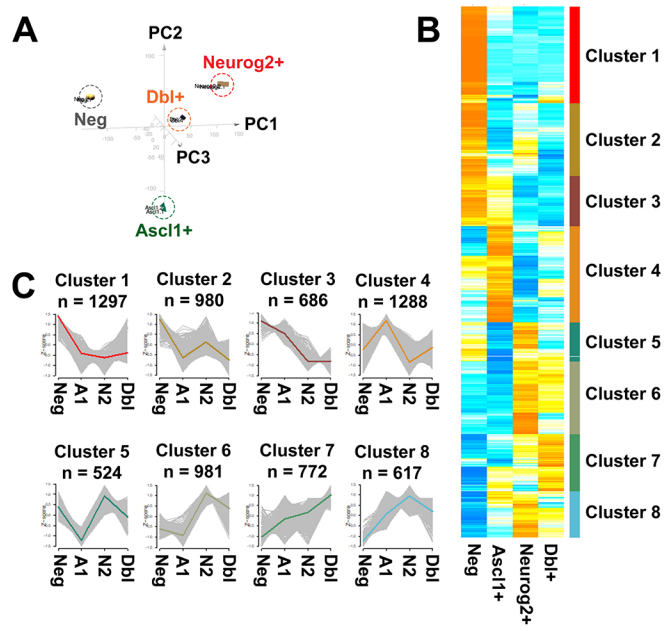
(A-A''') Immunostaining of mCherry and GFP in e17 stage transgenic Neurog2$^{mCherryKI;}$Ascl1^{GFPKI} (B) higher magnification of the image in A.

2. RNAseq strategy for proneural negative, Ascl1+, Neurog2+ and Neurog2+Ascl1+ retinal cells



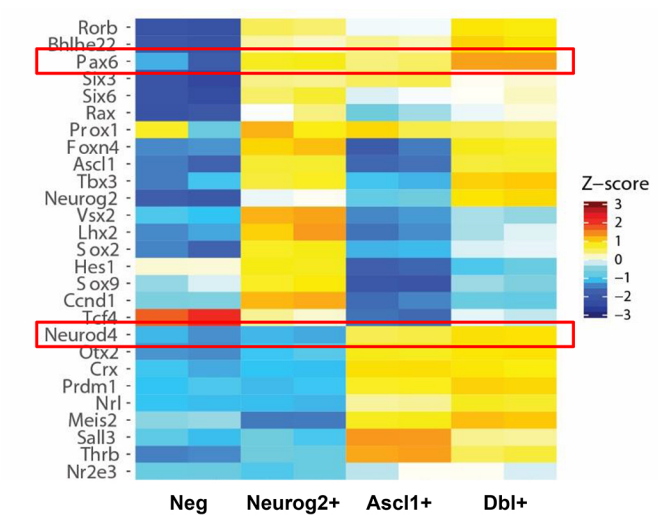
(A) Schematic illustration of the isolation method for proneural negative, mCherry single+, GFP single+ and mCherry/GFP double+ retinal cells from Neurog2$^{FLAG\text{-}mCherryKI;}$Ascl1^{GFPKI} transgenic mouse. (B) The representative result of florescence activated cell sorting(FACS) of Neurog2$^{FLAG\text{-}mCherryKI;}$Ascl1^{GFPKI} transgenic mouse retina for RNAseq.

3. Gene Ontology analysis for 8 clusters



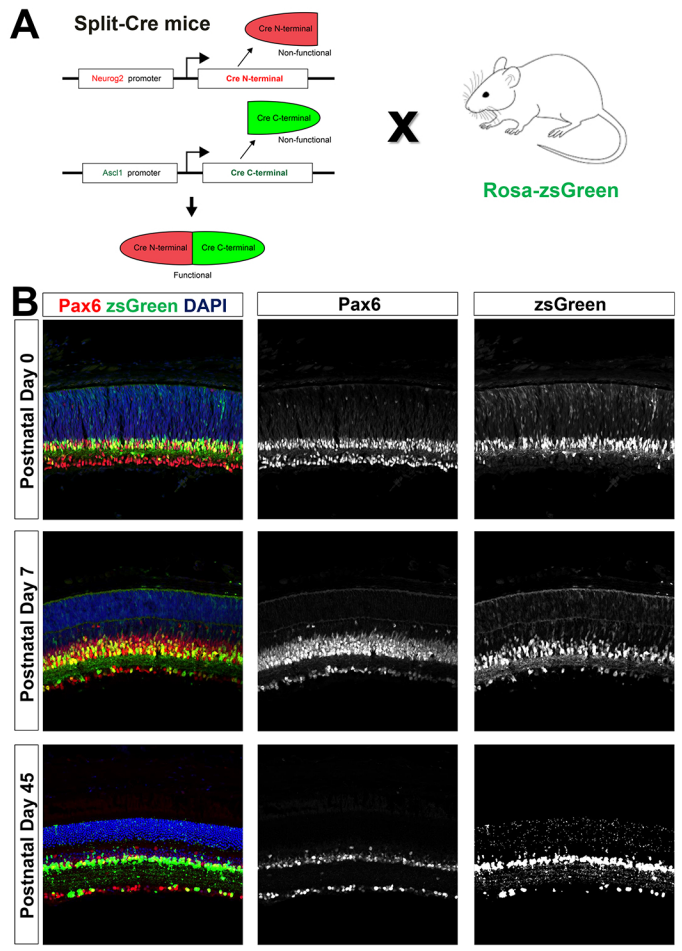
(A) Principle component analysis of RNAseq data from negative, Neurog2+, Ascl1+, and double+ retina cells, showing the distinct overall transcriptomes in each population. (B) Heatmap with 8 clusters that shows all differential expressed (DE) genes from ANOVA-like analysis in the dataset (Fold Change (log2): ≥1, FDR (Benjamini-Hochberg) < 0.01). Total DE genes: 7,145. (C) Graph showing gene expression pattern of the 8 clusters from clustering analysis using 7,145 DE genes.

4. Heatmap analysis for cell type specific gene



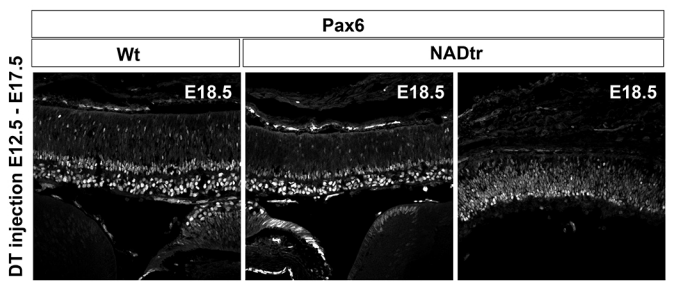
Heatmap analysis for the RNAseq data showing Transcription factors involved in RPCs and different cell types determination in negative, Neurog2+, Ascl1+, and double+ retinal cells. Amacrine specific genes, *Pax6* and *Neurod4* are highlighted with red boxes.

5. Split-Cre Lineage Tracing Analysis reveals that double+ RPCs give rise to Amacrine cells



(A) Schematic Illustration of Neurog2/Ascl1 split-Cre strategy for lineage tracing double positive cells and their progeny. (B) Lineage trace of the double positive cells at e15, p0, p7 and p45 reveals that double positive cells give rise to pax6 positive amacrine cells.

6. Split-Cre Ablation of the double+ RPCs results in partial Amacrine cells loss



Ablation of the double positive cells in Split-Cre;Rosa-DTR (NADtr) using diphtheria toxin injection from E12.5-E17.5 leads to a significant reduction of the pax6 positive amacrine cells at E18.5.

Conclusions and Future directions

1. Retinal RPCs have four distinct pools: proneural negative, Ascl1+, Neurog2+ and Neurog2+Ascl1+
2. RNAseq analysis revealed that 4 populations differ from one another
3. Amacrine specific genes, *Pax6* and *NeuroD4*, are highly expressed in Neurog2/Ascl1 double positive cells.
4. Lineage tracing analysis showed Neurog2/Ascl1 double positive cells give rise to Pax6+ amacrine cells
5. Ablation of double+ cells using split-Cre;Rosa-DTR mice with DT injection showed that loss of Neurog2/Ascl1 double+ cells result in a partial amacrine cell depletion.
6. Ongoing analysis aims to identify whether double positive RPCs generate specific amacrine cell types during retinal development.

Funding Acknowledgements

Grant funding (CIHR) to CS and Studentship support from the Department of Ophthalmology and Visual Sciences to SH through a Vision Science Research Program.

VISION SCIENCE RESEARCH PROGRAM
Ophthalmology & Vision Sciences UNIVERSITY OF TORONTO
CIHR IRSC
Sunnybrook HEALTH SCIENCES CENTRE