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VIEWPOINT

A tale of two cousins: Ependymal cells, quiescent neural stem cells and potential mechanisms driving their functional divergence

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Recent work has suggested that stem cells exhibit far greater heterogeneity than initially thought. Indeed, their dynamic nature and shared traits with surrounding niche cells have made prospective identification of adult neural stem cells (NSCs) challenging. Refined fate mapping strategies and single-cell omics techniques have begun to clarify functionally distinct states within the adult NSC pool, the molecular signatures that govern these states, and the functional contributions/interactions with neighboring cells within the subventricular niche. Ependymal cells are the epithelial cells which line the ventricular system and reside in the same niche as NSCs. Our own work has revealed that, despite sharing similar embryonic origins with NSCs and close geographic proximity, ependymal cells are transcriptionally distinct and fail to exhibit stem cell function *in vivo*, even following injury. Intriguingly, comparison of ependymal cells with qNSCs revealed transcriptional signatures that are largely overlapping, suggesting that post-transcriptional regulation might underlie their divergent phenotypes. Additional analysis of ependymal versus qNSC gene regulatory network activation supports this notion. This Viewpoint summarizes the historical confusion regarding the identity of NSCs within the lateral ventricle niche and describes recent work that provides greater appreciation for the diverse functional states within the NSC niche.

Defining the NSC: decades of debate

The existence of a neural stem cell (NSC) in the adult mammalian brain was heralded in 1992 when Reynolds and Weiss showed that cells isolated from the striatum were able to self-renew and undergo multi-lineage differentiation [1]. Proliferation studies done decades earlier suggested that adult NSCs were most likely resident within the wall of the lateral ventricles [2,3], however, the actual identity of the adult NSC was less clear. Microseparation of the adult periventricular zone and subsequent *in vitro* self-renewal and differentiation assays suggested that NSCs were limited to the subependymal layer [4]. Yet, contradictory work reported that ciliated ependymal cells (forming the epithelial interface between the ventricular lumen and

Abbreviations
aNSCs, active NSCs; bHLH, basic helix-loop-helix; GO, gene ontology; GRNs, gene regulatory networks; Hes5, Hes Family BHLH Transcription Factor 5; Id3, DNA-binding protein inhibitor; IKK, IκB kinase; NSCs, neural stem cells; PAGODA, pathway and gene set overdispersion analysis; qNSCs, quiescent NSCs; Shh, sonic hedgehog; SVZ, subventricular zone; TF, transcription factor; V-SVZ, ventricular–subventricular zone.

the underlying brain parenchyma) also exhibited proliferative capacity in vitro and could give rise to new neurons in vivo. Unfortunately, this latter study used nonspecific dye labeling techniques, potentially also labeling subependymal cells, thus muddying their interpretation [5]. Indeed, since then, several lines of evidence showed that it was specialized GFAP+ astrocytes within the subependymal region of the anterior lateral ventricle that gave rise to new neurons and repopulate the olfactory bulb throughout adulthood [6–8]. Interestingly, fate mapping of radial glial cells (the primary pool of cortical neural progenitors during brain development) revealed that both astrocytic neural stem cells and ependymal cells share a common ancestry [9]. To some, this encouraged the notion that ependymal cells might harbor latent neural stem cell features [10,11].

Since their discovery, a major impediment to studying NSCs and ependymal cell biology has been a lack of robust tools to selectively identify these two cell types *in vivo*. Previous attempts to fate map ependymal cells relied on the human-Foxj1 or CD133 gene promoters, and found NSC-like functions in ependymal cells, however, subsequent studies showed that these promoters were inappropriate for fate mapping ependymal cells [12–15]. Indirect studies of ependymal cell function also failed to observe ependymal cell neurogenesis [16]. Recently, we identified a novel transgenic fate mapping model for ependymal cells, based on the unique expression of αSMA within ependymal cells in the subventricular zone (SVZ) niche, and its absence in neural lineages [17] which has now been independently replicated [18]. We were able to directly test the response of ependymal cells to injury, independent of NSCs, and assemble the first true molecular profile of ependymal cells and the neural stem cell lineage from the adult SVZ [17]. Indeed, this work confirmed a lack of overt stem cell function for ependymal cells and provided a compelling comparison to uncover fundamental insights into the molecular signatures underlying NSCs at various stages of activation.

What underlies functional divergence in the adult NSC niche?

We made a direct transcriptional comparison of NSCs to ependymal cells—both cells reside within the same microenvironment, share the same ancestral origin [19] and mutually express multiple 'stem cell' associated markers (e.g., Sox2, Nestin, CD133), yet one exhibits definitive stem/progenitor properties while the other does not. In this context, our work has highlighted the striking transcriptional similarities between ependymal cells and quiescent NSCs (qNSCs). Hierarchical gene clustering placed qNSCs closest to ependymal cells relative to active NSCs (aNSCs) or neuroblasts, despite ependymal cells lacking any clear stem cell-like abilities [17]. In total, 2375 genes were shared by ependymal cells and qNSCs ($P > 0.01$ or log2 fold change < 1) with only 321 genes uniquely segregating within either cell type. In pathway and gene set overdispersion analysis (PAGODA), we identified two shared aspects between ependymal cells and qNSCs enriched in neural developmental-related and microtubule-associated gene ontology (GO) terms. A shared ancestry within the radial glial lineage [9] and shared microenvironment may be the source of much of these similarities. Despite the transcriptional overlap seen between ependymal cells and qNSCs, there are a select number of defining genes for qNSCs that might be responsible for their definitive function. Notably, the top three gene sets driving the qNSC-specific aspect in our PAGODA analysis were all de novo gene sets: that is, these genes were not enriched in any GO terms but shared a common expression pattern across our sample. The first de novo gene set (gene set #1) revealed homogeneity in the expression of this gene set across all qNSCs and was enriched for glutamine family amino acid metabolism and nervous system development terms. A handful of the genes we identified within this gene set have been previously associated with qNSCs. For example, DNA-binding protein inhibitor (Id3) negatively regulates the basic helix-loop-helix (bHLH) transcription factors and inhibits transcriptional activity in qNSCs, possibly acting as a significant contributor to maintaining quiescence in qNSCs [20]. Another, Hes Family BHLH Transcription Factor 5 (Hes5), is a transcriptional repressor of genes that require a bHLH protein, playing an important role as a negative regulator of neurogenesis [21,22].

In contrast to the homogeneity seen in de novo gene set #1, de novo gene sets #2 and #3 were largely heterogeneous within qNSC cells. Whether this heterogeneity represents a possible divergence in individual qNSC function within the larger qNSC pool [23] or whether this variation is simply a product of microenvironment or subtle differences in differentiation state is unclear. Nevertheless, support for individual qNSCs subsets exhibiting an intrinsically directed commitment to defined neuronal fates prior to any detectable differentiation has been suggested [24,25]. When heterotopically grafted or grown in vitro, postnatal NSCs from distinct regions of the ventricular–subventricular zone (V-SVZ) generated different types of neurons,

suggesting that NSCs may harbor greater diversity and fate restriction than traditionally thought [24,26]. This does not appear to be regulated by their geographic residence because isolation of NSCs from distinct locale and subsequently exposing them to ectopic microenvironments had little effect on their differentiation to a specified neuronal fate, further suggesting the presence of intrinsic biases within the NSC pool. Cayouette and Raff [27] also suggested that retinal progenitor cells exhibit intrinsically defined differentiation, as demonstrated when early retinal progenitor cells are transplanted into late retinal environments and continue to produce cells with similarly early fates [27]. Such findings suggest that broad neural potency may not be a generalizable trait across adult NSCs. Considering these studies together with our work show that qNSCs exhibited a large degree of transcriptional diversity, the notion that additional mechanisms beyond gene transcription may be responsible for acquisition or fine-tuning of NSC function, is appealing.

One such additional mechanism responsible for controlling NSCs is extrinsic signaling [28,29]. For example the ectopic activation of sonic hedgehog (Shh) signaling pathways in the dorsal SVZ can induce dorsal NSCs to respecify their progeny to ventrally derived cell types, showing that regional Shh signaling is a key factor in the specification of NSC identity [30]. Recently, Paul *et al*. [31] demonstrated long-range regulation of NSCs in the anterior ventral SVZ from hypothalamic projections that promoted NSC proliferation and the generation of granule neurons; and notably, Ortega *et al*. [26] demonstrated that canonical Wnt signaling in primary cultures of adult SVZ treated with Wnt3a protein increases oligodendrogenesis, without affecting the number of astroglia or neurons in culture.

Suppression of neurogenic capacity in ependymal cells may be largely due to post-transcriptional modification. Translational repression of primed radial glial progenitors is involved in neuronal fate specification [32], and similar mechanisms may be involved in divergent fate specification in the subventricular zone. The notion that radial glial progenitors are subject to a mix of post-transcriptional modifications is supported by multiple perspectives: as neurogenesis occurs over a relatively short time frame during development, mRNAs already present in precursors would provide a rapid mechanism of simple derepression to allow for subsequent differentiation; as introduced above, transcriptional priming would facilitate rapid extrinsic signaling controlling cell differentiation in the highly dynamic environment of the SVZ [32]. Given the transcriptional overlap between qNSCs and ependymal cells, secondary mechanisms such as post-transcriptional modification [32,33] are likely necessary to drive functional divergence within this lineage.

We reassessed our single-cell RNAseq dataset to unearth the gene regulatory networks (GRNs) controlling NSCs compared to ependymal cells (SCENIC) [34,35] (Fig. 1; Fig. S1, Script S1-2, File S1-2). Although expression of most transcription factors (*Sox2, Sox9, Klf9, Hes1* etc.) was similar across ependymal cells and qNSCs [17], there were several highly discrepant GRNs between these cell types (Fig. 1B–D). This supports the notion that divergent transcription factor activity likely drives acquisition of different phenotypes and also suggests that the mere presence of TF mRNA within a given cell does not faithfully reflect TF activity (binding DNA to activate downstream effectors). Along with a number of other cell-type–specific GRNs (Fig. 1E), our analysis reveals that the GRNs governed by Sox9 were largely expressed in ependymal cells, whereas the GRNs governed by *Klf9* and *Hes1* were largely specific to qNSCs (Fig. 1 B–D). By regulating the expression of large gene sets, transcription factors act as powerful fate determinants. Interestingly, our analysis also revealed that the GRNs associated with the transcription factor, *Sox2* were largely expressed in the ependymal cell and qNSC populations but not in the aNSC population (Fig. 1A) or in neuroblasts (not shown) [36]. Because we have demonstrated the presence of Sox2 protein in ependymal cells, qNSCs, aNSCs, and neuroblasts [17], as well as the expression of *Sox2* mRNA in these populations (Fig. 1A), it may be that TF activity is regulated post-translationally perhaps at the level of chromatin remodeling. Whether this is the case for other TFs within the NSC niche remains to be determined.

Ependymal cells as a source of NSCs for brain repair?

Exciting work has recently demonstrated that resident astroglial cells residing within the brain parenchyma or responding to an injury can be redirected to acquire a neuronal fate via transcriptional re-programming [37]. If ependymal cells could be coaxed to acquire alternative fates, this may hold significant clinical potential given ependymal cells greatly outnumber NSCs in the adult brain [38]. As well, their apposition to the ventricular lumen also facilitates their accessibility for therapeutic activation. Although mature ependymal cells are highly differentiated and postmitotic, their transcriptional signature retains a striking resemblance

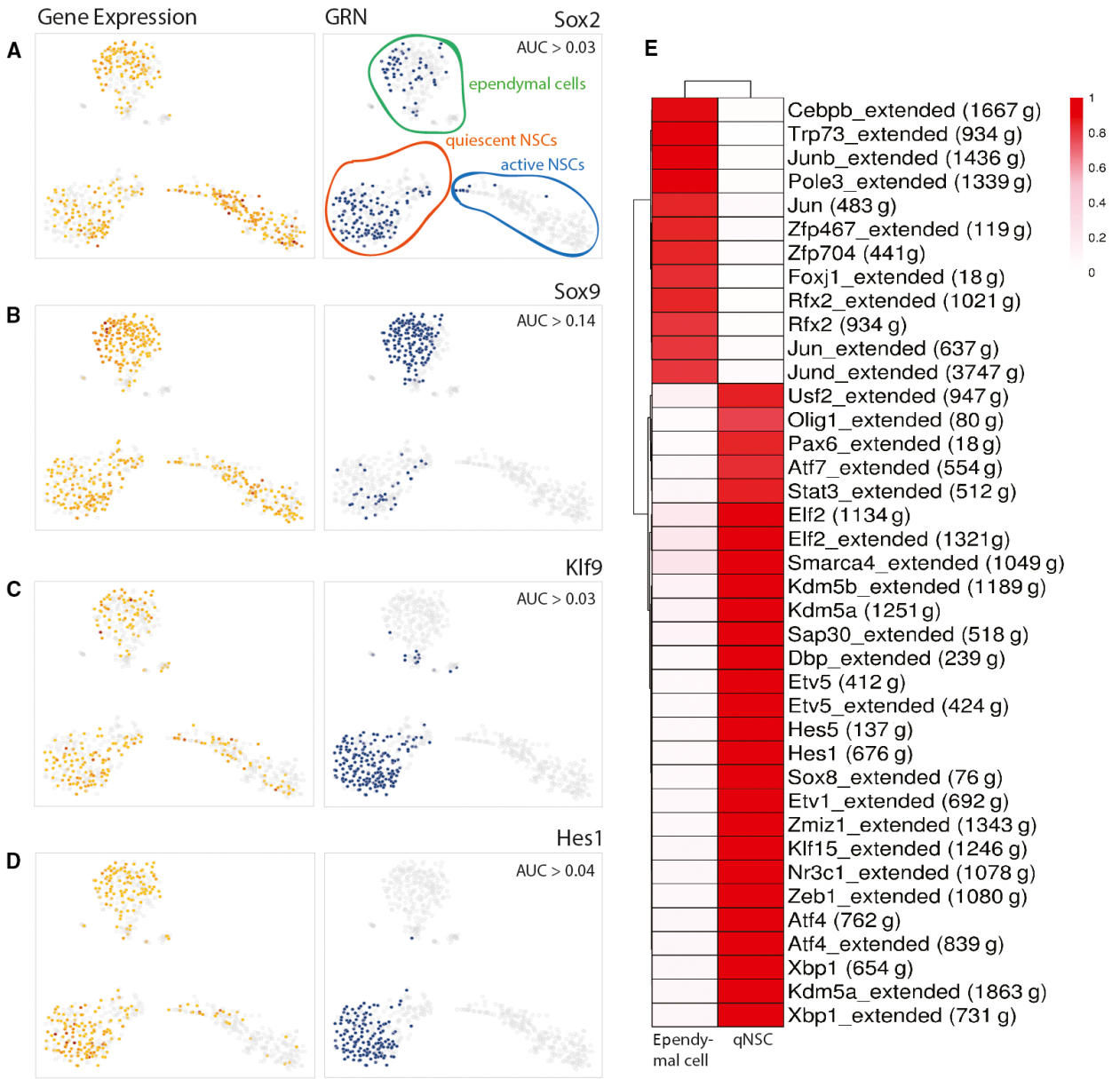

Fig. 1. Gene regulatory network (GRN) analysis suggests distinct transcription factor (TF) activity patterns underlying divergent NSC and ependymal cell functions. Reassessment of our single cell RNAseq dataset using SCENIC revealed distinct GRN patterns (A–E). The expression of transcription factor mRNA for Sox9 (B), Klf9 (C), and Hes1 (D) were similar across ependymal cells and quiescent NSCs (qNSCs), but showed discrepant GRNs. Along with a number of other cell-type–specific GRNs (E), our analysis reveals that the GRNs governed by Sox9 were largely expressed in ependymal cells, whereas the GRNs governed by Klf9 and Hes1 were unique to qNSCs (B–D). Interestingly, our analysis also revealed that the GRNs associated with Sox2 were largely expressed in both ependymal cell and qNSC populations but not in the active NSC (aNSC) population (A).

to the quiescent NSC and a number of NSC protein 'markers' are also shared (e.g., Sox2, Nestin, CD133, are all expressed in both ependymal cells and in NSCs) and can exhibit a modest level of plasticity [12,16,39] in response to injury or chemical activation. Young and colleagues [16] demonstrated that the number of ependymal basal processes extending into the subependymal parenchyma significantly increases after hypoxic injury partly reminiscent of a radial glia-like phenotype; and recently Muthusamy *et al.* [12] noted elongation of ependymal processes stretching toward the site of a cortical ischemic injury. Perhaps most

intriguing, Abdi and colleagues recently demonstrated that ependymal cells could be experimentally induced to dedifferentiate by manipulating IκB kinase (IKK) activity resulting in the degradation of a key transcription factor involved in cilia motility. Using IKK2 inhibitors, a cocktail of viral vectors and growth factors, they were able to artificially induce ependymal cell dedifferentiation, proliferation, and subsequent regeneration of multiciliated ependymal cells. Yet, despite acquiring this proliferative state, they never observed neurogenesis from the ependymal cell population [39].

Since ependymal cells and astrocytic NSCs are derived from radial glia progenitors [8], one possible future approach to improve repair could involve purposeful reintroduction of transcriptional programs to initiate a 'de-programming' event that would enable acquisition of a radial glia-like progenitor phenotype, capable of generating new neurons and/or oligodendrocytes. If the induction of these pseudoradial process extensions can be enhanced and are indeed functional, they may provide a striking substrate to assist progenitor cell migration from the subventricular zone to brain-injured sites—a process reminiscent of embryonic neural development. The aforementioned experiments suggest that ependymal cells can be chemically directed to reacquire a proliferative state, and so future studies should focus on understanding the precise molecular drivers that are required to initiate this de-programming and then modify this to enable acquisition of a (multipotent) neural progenitor state.

Role of ependymal cells in pathogenesis of neurodegenerative disease

One of the most intriguing findings from our own work was the striking enrichment of genes involved in lipid and glucose metabolism within the ependymal population. Indeed, this complements recent findings by Hamilton and colleagues, and Muthusamy and colleagues describing the prevalence of lipid accumulation and defective barrier function within the V-SVZ of the aged or demented brain [40,41]. We propose that in addition to their sensory functions within the ventricle, the ependymal cells provide a dynamic interface between the CSF and brain parenchyma that may be a major contributor to metabolic homeostasis within the brain and their dysfunction may lead to pathogenesis of various neurodegenerative disease states. During normal aging, the ependymal layer thins, the density of motile cilia on the apical surface of ependymal cells is reduced, and ependymal cells accumulate lipid droplets [41]. This latter phenomenon becomes largely augmented in neurodegenerative disorders, such as Alzheimer's disease, where lipid droplets enriched with oleic acid side chains selectively accumulate in ependymal cells, ultimately resulting in the deterioration of the SVZ neurogenic niche and further contributing to the symptoms associated with such neurodegenerative conditions [40].

Conclusion

In comparison to NSCs, its cousin the ependymal cell, has been understudied and overlooked. After 20 years since the first suggestion of ependymal cells being a putative NSC [5], we only now have reliable transgenic tools to elucidate the precise functions of ependymal cells, insight into the regulatory mechanisms that guide their functional specification within the NSC niche, and their potential contributions to both brain homeostasis and brain pathology. Indeed, many exciting suggestions about the capabilities of ependymal cells now await testing.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

JAS, PS, EC, and JB wrote the manuscript. SS performed SCENIC analysis.

Data accessibility

Research data pertaining to this article are located at figshare.com: https://doi.org/10.6084/m9.figshare.7811576

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. A full binarized heatmap of active regulons comparing E Cells with quiescent NSCs. Color scale indicates percentage of cells of that type with active regulon.

Script S1. R Script demonstrating initialization of SCENIC settings for E Cell comparisons reported. Modified from: https://rawcdn.githack.com/aertslab/SCENIC/a0a00644b2f3589a3e2bc65486fc5f6cc00f48e1/inst/doc/SCENIC_Running.html

Script S2. R Script demonstrating SCENIC Steps completed for E Cell comparisons reported. Modified from: https://rawcdn.githack.com/aertslab/SCENIC/a0a00644b2f3589a3e2bc65486fc5f6cc00f48e1/inst/doc/SCENIC_Running.html

File S1. A Hierarchical Data Format (HDF5)-based loom file containing normalized gene expression matrix and SCENIC regulons comparing E Cells with activated Neural Stem Cells (NSCs) and quiescent NSCs. Users can upload this .loom file onto SCope (http://scope.aertslab.org) to visualize gene expression and regulon activity as colored projections on tSNE coordinates.

File S2. A Hierarchical Data Format (HDF5)-based loom file containing normalized gene expression matrix and SCENIC regulons comparing E Cells with quiescent NSCs. Users can upload this .loom file onto SCope (http://scope.aertslab.org) to visualize gene expression and regulon activity as colored projections on tSNE coordinates.