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Postmitotic Fate Refinement in the Subplate

Mohammed A. Mostajo-Radji[1,2] and Alex A. Pollen[1,2,*]
[1]Department of Neurology, University of California San Francisco, San Francisco, CA 94143, USA
[2]The Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, University of California San Francisco, San Francisco, CA 94143, USA
*Correspondence: alex.pollen@ucsf.edu
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How is the astonishing diversity of cortical neurons specified? In this issue of *Cell Stem Cell*, Ozair et al. (2018) leverage hPSC neural differentiation to show that projection neurons undergo prolonged sojourns in the subplate before migrating to deep layers, suggesting that pausing in the subplate may enable integration of intrinsic and extrinsic cues during postmitotic fate refinement.

The cerebral cortex is a six-layered structure that expanded dramatically during primate evolution. Although cortical layers contain distinct classes of neurons, the stages, cell types, and anatomical compartments that specify this diversity are still being unraveled. Prior to birth, radial glia in the germinal zone generate neurons that migrate outward to form cortical layers in an "inside-out" pattern. Molecular programs in radial glia contribute to projection neuron diversity as the fate of daughter neurons becomes progressively restricted over time. However, the majority of subtype-specific decisions appear to occur postmitotically (McKenna et al., 2015). Notably, overexpression of a single transcription factor can alter fate in postmitotic projection neurons during embryonic development (Ye et al., 2015), leaving open the possibility that cues received during neuronal migration and maturation influence cell fate.

In this issue of *Cell Stem Cell*, Ozair et al. (2018) discover an unexpected link between human cortical neuron fate specification and the subplate. The subplate is a transient structure beneath the cortex composed of some of the earliest-born cortical neurons. These neurons coordinate the first connections between thalamus and cortex and are required for forming normal sensory maps. Although the subplate expanded during primate brain evolution, these enigmatic neurons are thought to largely disappear shortly after birth through programmed cell death, leaving behind only a remnant population of corticothalamic projection neurons in layer 6b and interstitial white matter neurons (Hoerder-Suabedissen and Molnár, 2015). Interestingly, some transient structures, such as the hippocampal intermediate zone, may serve as a "waiting compartment" where newborn neurons receive synaptic innervation before migrating to their final locations (Altman and Bayer, 1990). However, subplate neurons have been difficult to isolate due to the mixing of resident subplate cells with later-born migratory neurons and their transcriptional similarities to deep-layer neocortical projection neurons.

To better characterize the function of human subplate during cortical fate specification, Ozair et al. performed immunostaining for transcription factors that define distinct classes of cortical neurons. Surprisingly, subplate neurons in post-conception week (PCW) 15 frontal lobe co-expressed the conserved subplate marker NURR1 with markers of callosal (SATB2), subcerebral (CTIP2), and corticothalamic (TLE4) projection neuron identity and with markers of neuronal maturation (MEF2C). The observation that competing molecular programs for distinct neuronal subtypes co-exist in individual cells suggests that maturing subplate neurons retain the potential to form multiple classes of projection neurons. Indeed, the early co-expression of these class-specific transcription factors is consistent with the complex molecular circuits that guide development of projection neuron connectivity patterns (McKenna et al., 2015).

To directly study the fate of human subplate neurons, Ozair et al. use human pluripotent stem cell models of neuronal development. After patterning the stem cells to forebrain lineage progenitors, the authors use a Notch signaling inhibitor to generate a synchronized cohort of postmitotic neurons at stages when subplate neurons are produced. By day 75, these *in vitro* neurons co-express NURR1 with SATB2, CTIP2, and TLE4 as observed in frontal lobe subplate. Contrary to models of programmed cell death in the subplate, these neurons survived in long-term culture and gradually lost expression of canonical subplate markers. By day 120, as NURR1 expression declined, neurons resolved into discrete groups that expressed either SATB2 or CTIP2, but not both markers together. Interestingly, the SATB2 neurons from these cohorts did not co-express CUX2 upon maturation, indicating that these represent deep-layer callosal neurons. These findings suggest that mature human subplate neurons do not undergo cell death after coordinating afferent connections from thalamus, but instead may undergo a fate refinement process to form distinct neuronal subtypes that occupy deep cortical layers.

To examine whether human subplate neurons later incorporate into deep layers of the cortical plate, the authors returned to primary tissue samples. Previous studies in macaque observed downward dispersion of subplate neurons likely caused by incoming thalamocortical afferents (Duque et al., 2016). However, Ozair et al. observed an increased number of NURR1-expressing cells in deep layers of cortical plate at PCW19 compared to PCW15, suggesting that subplate neurons may invade the cortical plate at stages when their identity is refined (Figure 1). The authors further show that PCW15 subplate neurons express the migratory neuron marker PRDM8, and that *in vitro*-derived neurons expressing

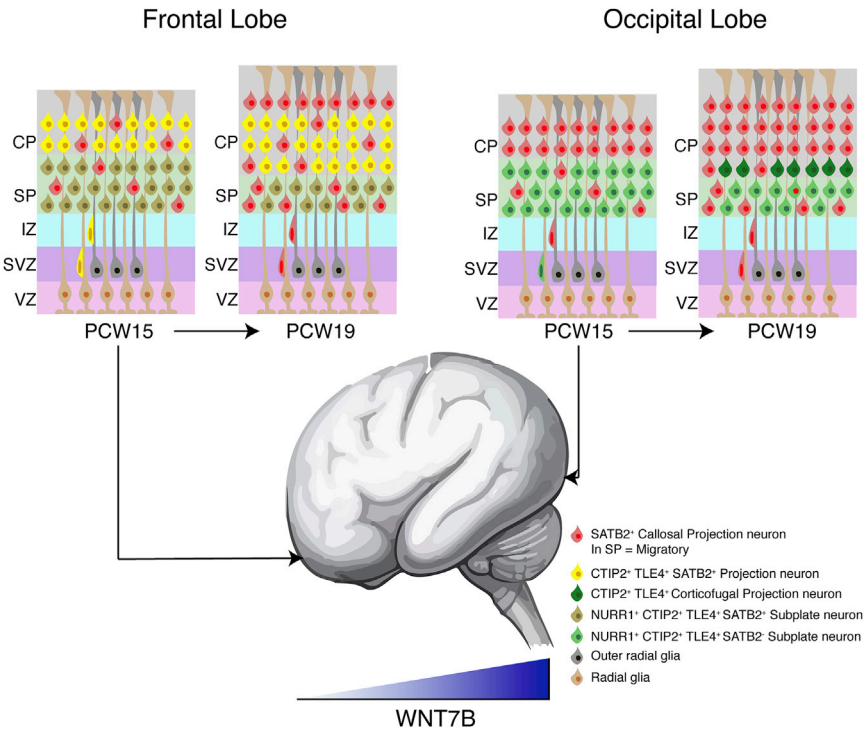

Figure 1. Neuronal Identity Is Refined in the Subplate

At post-conception week (PCW) 15, frontal lobe postmitotic subplate neurons (NURR1-positive) express markers of callosal (SATB2) and corticofugal (including subcerebral CTIP2 and corticothalamic TLE4) projection neuron markers. In the occipital lobe, NURR1-positive subplate neurons express CTIP2 and TLE4, but not SATB2. WNT7B is expressed in a caudal-rostral gradient in subplate and may repress SATB2. By PCW19, immunohistochemistry indicates that subplate neurons in the occipital lobe lose NURR1 expression and may migrate into deep layers of the cortical plate. CP, cortical plate; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

NURR1 and PRDM8 migrate prior to adopting pyramidal neuron morphology. These results support the intriguing possibility that subplate neurons may actively migrate to deep layers of the cortex.

What factors might contribute to refinement of neuronal identity as subplate neurons migrate into cortex? Based on the enriched expression of Wnt pathway genes in subplate neurons, the authors examined the role of this pathway in fate refinement, finding that Wnt signaling represses SATB2 expression in stem cell-derived subplate neurons. To examine the *in vivo* relevance of Wnt signaling in fate refinement, the authors focused on WNT7B, which is also enriched in corticofugal projection neuron programs (Ye et al., 2015). WNT7B and SATB2 show mutually exclusive expression in subplate neurons, consistent with repression acting through autocrine signaling. WNT7B expression is also highest in the occipital lobe, and SATB2 expression is largely absent in occipital lobe subplate neurons. These findings highlight WNT7B as a strong candidate factor for shaping postmitotic fate refinement in corticofugal neurons. In addition, the gradient of WNT7B expression in human cortex provides a possible mechanism for the recent observation of a narrow transition from predominant co-expression of SATB2 and CTIP2 in frontal lobe to mutually exclusive expression in parietal and occipital lobes (Nowakowski et al., 2017).

The mechanisms underlying the specification of neuronal diversity in the neocortex are among the most fascinating topics in developmental neuroscience. Ozair et al. provide a blueprint for tackling these questions in human development by combining quantitative descriptions of primary tissue samples with analysis of experimentally tractable pluripotent stem cell models. Meanwhile, single-cell RNA-sequencing is helping us to understand the scale of the problem. For example, 19 molecularly distinct subtypes of projection neurons exist in adult mouse visual cortex alone (Tasic et al., 2016), yet only a few broad clusters of maturing excitatory neurons emerge in developing human cortex (PCW10–22), largely based on birthdate and cortical area (Nowakowski et al., 2017). The subplate could represent an anatomical location in which intrinsic genetic programs interact with thalamocortical activity to influence neuronal fate. Indeed, subplate morphology varies widely across cortical areas (Hoerder-Suabedissen and Molnár, 2015), and thalamocortical inputs shape the class-specific identity of layer 4 excitatory neurons in mouse somatosensory cortex (Pouchelon et al., 2014). Additionally, prenatal spontaneous thalamic waves control the patterning of cortical areas (Moreno-Juan et al., 2017). The integration of multiple signals in postmitotic subplate neurons could refine neuronal fate by resolving competition among nascent class-specific regulatory factors. Future studies across model systems and incorporating new technologies such as high-throughput measurements of lineage relationships and connectivity patterns will be necessary to study how the fine interplay between intrinsic programs and activity shapes neuronal diversity across cortical areas and layers.

DECLARATION OF INTERESTS

A.A.P. is on the scientific advisory board of System1 Biosciences.

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Genotoxic Lemons Become Epigenomic Lemonade

Stefan F. Cordes[1] **and Cynthia E. Dunbar**[1,*]
[1]Hematology Branch, National Institutes of Health, Bethesda, MD 20892, USA
*Correspondence: dunbarc@nhlbi.nih.gov
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Active regulatory elements in hematopoietic stem cells (HSCs) are incompletely characterized, since extant approaches immunophenotypically define and isolate rare HSCs. In the current issue of *Cell Stem Cell*, Wünsche et al. (2018) use γ-retroviral insertion sites from a human gene therapy trial to identify the active enhancer landscape of functionally characterized HSCs.

Hematopoiesis requires exquisite regulation that balances HSC self-renewal with differentiation in order to maintain homeostasis. Understanding the regulatory landscape controlling these processes is central to designing interventions when they go awry in hematological malignancies, as well as efforts to generate HSCs from induced pluripotent stem cells or expand HSCs *in vitro* for clinical applications. Direct study of HSCs is made difficult by the fact that HSCs are rare and immunophenotypic markers characterize HSCs only incompletely. Currently the most refined population of candidate human adult HSCs (Doulatov et al., 2012) contains only between 2% and 20% cells with long-term repopulating activity in xenografted NSG mice, a model difficult to directly extrapolate to the function of HSCs in humans long-term.

Chromatin structure and DNA methylation lay the foundation for transcriptional gene regulation. Previous work (Corces et al., 2016) profiled the chromatin accessibility landscape in human HSCs via an Assay for Transposase Accessible Chromatin using sequencing (ATAC-seq). Farlik et al. (2016) performed single-cell methylome sequencing to study progressive DNA methylation during hematopoiesis. Both studies relied on the immunophenotypic characterization of HSCs and used assays that were optimized for small numbers of input cells. Corces et al. found chromatin accessibility to be more specific for hematopoietic cell type than transcriptome analysis and introduced "enhancer cytometry" as a strategy to deconvolve individual cell types from an ensemble profile. This approach was able to discriminate clearly between all normal hematopoietic cell types except between multipotent progenitor cells and HSCs.

Murine γ-retroviral vectors have been used to heritably deliver genes into long-term repopulating HSCs (LT-HSCs) for the treatment of a variety of human diseases. Insertion sites (ISs) are essentially unique to each transduced engrafting stem or progenitor cell, and they are conferred with high fidelity to their progeny. Since retroviral ISs preferentially target regulatory elements of active genes (De Ravin et al., 2014), Wünsch and colleagues hypothesized that information about the enhancer landscape of true functionally defined LT-HSCs may be recovered by mapping the genomic locations of ISs recovered from cells of the mature hematopoietic lineages from patients enrolled in a clinical gene therapy trial during stable long-term hematopoiesis. They analyzed IS data retrieved via linear-amplification-mediated PCR (LAM-PCR) from ten patients who underwent autologous transplantation of gene-corrected $CD34^+$ cells for Wiskott-Aldrich syndrome (WAS), a serious immunodeficiency disorder (Boztug et al., 2010). In order to consistently define a transition from short-term to long-term hematopoiesis, Wünsch et al. computed a (regularized) odds ratio for the presence or absence of ISs for pairs of time points and identified a transition to pairwise positive associations for each patient, generally at time points later than 1 year post-transplantation, in agreement with previous estimates of the time frame for the appearance of stable contributions from LT-HSCs (Biasco et al., 2016; Koelle et al., 2017). Wünsch et al. then used this IS data to create a genome-wide catalog of regulatory regions in