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Piggybac Transposon-Based Lineage Tracing to Reveal Ependymogenesis

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Piggybac Transposon-Based Lineage Tracing to Reveal Ependymogenesis

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M.B.B.S., Dhaka Medical College (Dhaka University), 2016

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Piggybac Transposon-Based Lineage Tracing to Reveal Ependymogenesis

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Abbreviations

CNS - Central Nervous System
CSF - Cerebrospinal Fluid
EdU - 5’-Ethynyl-2’-deoxyuridine
BrdU - 5’-Bromo-2’-deoxyuridine
GFAP - Glial Fibrillary Acidic Acid
EGFP - Enhanced Green Fluorescent Protein
i.p. - Intraperitoneal
IUE - In Utero Electroporation
NSC - Neural Stem Cell
NSPC – Neural stem/progenitor cell
RGC - Radial glial cell
PBS - Phosphate Buffered Saline
Pbase - PiggyBac Transposase
SVZ - Subventricular Zone
WM - Wholemount
TABLE OF CONTENTS

PAGE

Abstract .......................................................................................................................... vi
Introduction/Background ......................................................................................... 1
Materials and Methods ............................................................................................... 5
Results .......................................................................................................................... 10
Figure 1 ....................................................................................................................... 11
Figure 2 ....................................................................................................................... 13
Figure 3 ....................................................................................................................... 19
Figure 4 ....................................................................................................................... 23
Table 1 ......................................................................................................................... 25
Figure 5 ....................................................................................................................... 27
Discussion .................................................................................................................. 29
References .................................................................................................................. 34
Abstract

The lateral walls of the lateral ventricle of the brain are home to one of the two main neurogenic niches of the postnatal and adult mammalian brain, the ventricular-subventricular zone (V-SVZ). During late embryonic development (E14 to E16 in mouse brain), a subpopulation of radial glial cells from the neuroepithelial lining of lateral ventricle wall starts ependymogenesis to establish an ependymal cell lining of the ventricular system. Ependymal cell maturation and ciliogenesis continues after birth and the remaining radial glia, designated neural stem cells after birth, are established within the ependymal lining by retention of a thin apical process. The postmitotic multi-ciliated ependymal cells provide structural and trophic support to the V-SVZ stem cell niche. We sought to investigate changes to the V-SVZ stem cell niche over the course of mouse brain development and in particular to determine the association between ependymogenesis, stem cell number and stem cell niche organization at the ventricle surface. Our piggybac transposon-based lineage tracing method, through sparse labelling, shows neural stem cells and ependymal cells have a common origin and ependymal cells can arise from radial glial cells via both symmetric and asymmetric division. Using IUE in conjunction with thymidine analog double-labelling, we propose that from a single radial glial cell multiple rounds of division are involved in the process of ependymogenesis and terminal symmetric ependymal generation from neural stem cells reduces neural stem cell numbers. At the time of ependymal cell formation, the new ependymal cell lining displaces the remaining radial glia/stem cell somata to the subventricular zone (SVZ). These remaining ventricular subventricular zone (V-SVZ)
stem cells, are arrayed in clusters and maintain only a thin apical process at the ventricle surface. In the en face wholemount view of the lateral wall of the lateral ventricle, the stem cell apical processes surrounded by ependymal cells are referred to as ‘pinwheels’ and represent regenerative units. To further understand the composition of the distinct stem cell retention pattern in the form of a pinwheel in the lateral wall of the lateral ventricle of the rodent brain, we have used the piggybac multicolor fluorophore together with in utero electroporation technique for lineage tracing analysis.
Introduction/Background

Cerebro-ventricular system:

The cerebral ventricles are a series of interconnected, fluid-filled cavities that include the two lateral ventricles, the third ventricle, cerebral aqueduct, and the fourth ventricle. These cavities lie in the core of the forebrain and brainstem, acting as a dynamic pressure system that has biophysical and biochemical consequences to the surrounding brain parenchyma (Johanson et al. 2011; Sakka et al. 2011). A membranous network of fenestrated blood capillaries and cells called the choroid plexus, produces most of the cerebrospinal fluid (CSF) that fills the ventricular system and provides hydro-mechanical protection to the brain (Liddelow et al. 2015). The CSF also plays a crucial role in maintaining homeostasis through elimination of potentially toxic catabolites, regulation of electrolytes, and circulation of active molecules and neuropeptides (Mori et al. 1990; Veening and Barendregt 2010). The CSF percolates through the ventricular system and then flows into the subarachnoid space through perforations in the thin covering of the fourth ventricle and then eventually gets absorbed by specialized structures called arachnoid villi or granulations, returning to venous circulation. As the CNS is the only organ of the body that lacks anatomically defined lymphoid tissues, it has developed a unique adaptation for achieving fluid balance and interstitial waste removal that is called glymphatic system, a glial-dependent perivascular network that subserves a pseudo-lymphatic function in the brain. Within the glymphatic pathway, cerebrospinal fluid (CSF) enters the brain via periarterial spaces, passes into the interstitium via perivascular astrocytic aquaporin-4, and then drives the perivenous drainage of interstitial fluid (ISF)
and its solute. In addition to its traditionally identified role providing buoyancy to the brain, the CSF has also been suggested to function as a part of the glymphatic system, a glial-dependent perivascular network that subserves a pseudo-lymphatic function in the brain by driving the perivenous drainage of interstitial fluid (ISF) and its solute, within the glymphatic pathway (Plog et al. 2018, Louveau et al. 2017).

**Ependymal cells and function of the ependymal cell monolayer**

A specialized type of glial cell called an ependymal cell forms a monolayer lining the cerebral ventricles and the central canal of the spinal cord (Bruni et al., 1985, Del Bigio 1995; Lennington et al. 2003; Conover and Shook 2011). Although these cells are of a glial lineage, but they have many epithelial characteristics including an associated basement membrane and multiple types of junctional protein complexes (Lippoldt et al., 2000b; Lippoldt et al., 2000a; Kuo et al., 2006). By linking 10-30 neighboring ependymal cell gap junctions, ependymal cells may participate in synchronizing their activity (Bouille et al., 1991; Rodriguez et al., 2010; Sival et al., 2011). The tight barrier created by zona occludens (tight junctions) and zona adherens (adherens junctions) typically excludes molecules greater than 1.9kDa from diffusing between neighboring ependymal cells (Sarnat, 1995; Liddelow et al., 2009). This tight barrier requires molecules to travel across both the apical and basal membranes in order to move between the CSF and parenchyma, giving the ependymal monolayer a filtering role (Kuchler et al., 1994; Del Bigio, 1995). Neural stem cells that retain a ventricle-contacting apical process also have apical adherens and tight junctions with neighboring ependymal cells and other stem cells (Jacquet et al., 2009; Mirzadeh et al., 2008; Paez-Gonzalez et al., 2011), supporting barrier and structural functions along the lateral wall.
The presence of multiple motile (9 + 2 microtubule arrangement) cilia on their apical surface contribute to fluid circulation and brain homeostasis (Del Bigio, 1995). Coordinated beating of cilia between neighboring ependymal cells leads to a directional flow of CSF across the surface of the ependymal monolayer, distributing signaling molecules and removing debris (Scott et al., 1974; Ibanez-Tallon et al., 2004; Sawamoto et al., 2006; Riquelme et al., 2008). Ependymal-generated CSF flow establishes gradients of chemo-repellents that is thought to guide the migration of young neurons in the SVZ (Sawamoto et al., 2006; Han et al., 2008; Ihrie and Alvarez-Buylla, 2011). In addition to aiding in molecule circulation, ependymal cells have been shown to express Noggin, a molecule that promotes neurogenesis over gliogenesis by inhibiting BMPs in the SVZ (Lim et al., 2000).

**Transformation of the ventricular zone into the V-SVZ neural stem cell niche**

The neuroepithelium gives rise to radial glial cells; both cell types line the ventricular lumen and are the main neural progenitors during embryonic development. The earliest stem cells/precursors of the developing brain are a pseudostratified layer of proliferative neuroepithelial cells that give rise to radial glial cells, which become the predominant progenitors during neurogenesis and gliogenesis (Barry et al., 2005; McDermott et al., 2005). New neurons are initially generated by neuroepithelial cells, and then by descendant radial glia, and outer radial glia in human and non-human primates, via their progeny intermediate progenitor cells (Hansen et al., 2010; LaMonica et al., 2012; Lui et al., 2011; Malik et al., 2013). Radial glia has a distinct radial morphology where the cell bodies are located in the ventricular zone and their basal process radiates to the pial...
surface. Radial glia also generates a monolayer of ependymal cells that lines the ventricles (Jacquet et al., 2009; Mirzadeh et al., 2008; Spassky et al., 2005). In adult mammalian brain, the adult neural stem cells' ventricle contacting apical process is surrounded by the mature multiciliated ependymal cells in a unique distinctive pattern referred to as "pinwheel" organization (Mirzadeh et al., 2008; Shen et al., 2008; Tavazoie et al., 2008). Similarly, human V-SVZ stem cells are organized and maintained in similar units along the ventricle surface (Coletti et al., 2018). But how this transition from embryonic VZ to V-SVZ results in mature ependymal cells surrounding a cluster of neural stem cells (e.g., pinwheel organization/regenerative units) is unknown.
Materials and Method*


Animals:

CD-1 mice (Mus musculus) (Charles River Laboratories, Wilmington, MA, USA) were used to study. Housing, breeding, handling, care and processing of the animals were carried out in accordance with regulations approved by the Institutional Animal Care and Use Committee of the University of Connecticut. The day the vaginal plug was detected was designated as E0.

Mouse lateral ventricle reconstruction and analysis*

For coronal sections, P7 and P30 mice were anesthetized with isoflurane, then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA). The extracted brains were fixed overnight in 4% PFA at 4°C. E13, E16, and P1 mice were anesthetized with isoflurane, heads were removed and fixed overnight in 4% PFA at 4°C. After removing the skin, embryonic and P1 brains were removed from the skull using a Leica MZ95 stereomicroscope. All brains were washed for 3×10 min in PBS before dissection and vibratome sectioning for 3D reconstructions.
To generate 3D reconstructions of the mouse brain and ventricles, coronal sections from E13 (42 µm), E16, P1, P7 and P30 (all 50 µm) brains were sectioned on a vibratome (VT-1000S, Leica). Mouse brain tissue sections were stained with β-catenin overnight (rabbit polyclonal anti-β-catenin, 1:100; Cell Signaling Technology, #9562), secondary antibody for 1 h (donkey anti-rabbit 546, 1:500; Invitrogen, #A10040), nuclear stain DAPI (300 mM; Molecular Probes, #D-1306) for 10 min and imaged on a Zeiss Axio Imager M2 microscope with ApoTome (Carl Zeiss MicroImaging) with a Hamamatsu Photonics ORCA-R2 digital camera (C10600). Alternating coronal sections were imaged, and the contours of the lateral ventricle walls and surface of the brain were traced to generate 3D reconstructions, as described (Acabchuk et al., 2015). Volume and surface area analysis were performed using StereoInvestigator and Neurolucida Explorer software (MBF Bioscience).

**Plasmids/ PiggyBac transposon**

PiggyBac mediated transgenesis can label the progeny of neural progenitors and transgenes are stably maintained in progeny. The piggyBac transposon system is a binary system with a helper plasmid (e.g. pCAG-PBase) providing piggyBac transposase (PBase), and the donor plasmid (e.g. pPBCAG-eGFP) providing the pCAG-eGFP (fluorescence reporter) transgene between the 5′ and 3′ terminal repeats (TRs) of the donor plasmid (Chen et al. 2012). We have used pPBCAG-eGFP (cytoplasmic enhanced GFP), pPBCAG-mCherry (cytoplasmic), pPBCAG-Cerulean (cytoplasmic), pPBCAG-mCitrine (cytoplasmic) and pGLAST-PBase that were received as gifts from Dr. Joseph loturco lab. pPBCAG-Cerulean were purchased from addgene. pCAG-PBase was constructed by replacing eGFP with PBase sequence (Wu et al., 2007) in pCAG-eGFP.
using EcoRI and NotI sites. pGLAST-PBase was made by inserting PBase downstream of GLAST promoter provided by Dr. D.J. Volsky (Kim et al., 2003).

**In utero electroporation (IUE)**

In utero electroporation was performed as described (Chen et al., 2012). We conducted IUE at E15 and E16 and harvested the brains at P1, P7 or P14. Pregnant mice were anesthetized with a mixture of ketamine/xylazine (120/8 mg/kg i.p.). Metacam analgesic was administered daily at a dosage of 1 mg/kg s.c. just before surgery and 2 days following surgery. To visualize the plasmid during electroporation, plasmids were mixed with 2 mg/ml Fast Green (Sigma). In all conditions, pPBCAG-eGFP were used at a final concentration of 1.0 μg/μl, while pGLAST-PBase were used at a final concentration of 1.5 μg/μl. During surgery, the uterine horns were exposed and one lateral ventricle of each embryo was pressure injected with 1–2 μl of plasmid DNA. Injections were made through the uterine wall and embryonic membranes by inserting a pulled glass microelectrode (Drummond Scientific) into the lateral ventricle and injecting by pressure pulses delivered with a Picospritzer II (General Valve). Electroporation was accomplished with a BTX 8300 pulse generator (BTX Harvard Apparatus) and BTX tweezer-trodes. Five consecutive electric square wave pulses (37V, E15; 50 ms duration) were applied to each embryo, after which the uterine horns were replaced into the abdominal cavity. Dams were placed in a clean cage to recover and they were monitored closely.

**Thymidine analog (EdU and BrdU) administration**

To determine the spatial disposition of newborn ependymal cells in a pinwheel organization by labelling dividing cells during S phase a single injection of EdU was
administered at E16, 24 hours after the IUE with piggybac transposon tagged fluorescent protein with a GLAST promoter at E15. 100 mg/kg body weight (10 mg/ml stock, dissolved in filtered PBS) of EdU: 5′-Ethynyl-2′-deoxyuridine (Thermo Fisher Scientific) was administered to pregnant mice by intraperitoneal injection. To validate the idea of successive division of RGC, we have used another thymidine analog BrdU: 5′-Bromo-2′-deoxyuridine (Thermofisher scientific) injected intraperitoneally 24 hours after at E17 keeping in mind the cell cycle duration of radial glial cell and actively dividing neural stem cell of the V-SVZ region is 17 hrs (S-phase duration 4.5 hrs) (Morshead et al.,1992; Ponti et al., 2012).

**Mouse brain tissue wholemount dissection**

For wholemount sections, P7, P14 and P30 mice were anesthetized with isoflurane, then transcardially perfused with 0.9% saline. After decapitation, brains were removed from the skull using a Leica MZ95 stereomicroscope. Lateral ventricle wall wholemounts were prepared as described (Mirzadeh et al., 2008). Dissected whole-mount preparations were placed in PFA with 0.1% Triton X-100 overnight. P1 mice were anesthetized with isoflurane, heads were extracted and placed in 4% PFA with 0.1% Triton X-100 overnight and wholemounts were then prepared (Mirzadeh et al., 2010a).

**Whole Mount Immunostaining**

Wholemounts were immunostained with the following primary antibodies: mouse monoclonal anti β-catenin (1:250; BD Biosciences, #610154), rabbit polyclonal anti-γ-tubulin (1:500; Sigma-Aldrich, #T5192), goat polyclonal anti-GFAP (1:250; Abcam,
#ab53554) and mouse monoclonal anti-FOXJ1 (1:250; Invitrogen, #14-9965-80). Alexa Fluor dye-conjugated polyclonal secondary antibodies (1:500, Invitrogen) were used: donkey anti-mouse 488 (#21202), donkey anti-mouse 546 (#A10036), donkey anti-rabbit (#A21206), donkey anti-goat-647(#A21447) and donkey anti-rat 647 (#A18744). Blocking solutions contained 1% Triton X-100. Validations of all commercial antibodies are available from the manufacturer’s datasheets. Whole mounts were incubated in blocking solution, 1% Triton X-100/10% normal donkey serum/PBS, for 1-2 hr at room temperature. Primary antibodies were incubated for 24-48 hr at 4C, followed by appropriate secondary antibodies for 24-48 hrs at 4C, depending on target antigens. The EdU incorporation was detected using the Click-iT EdU Alexa Fluor imaging kit (Thermo Fisher Scientific for Alexa Fluor 488, 594 or 647 staining), according to manufacturer's protocol. Briefly, V-SVZ wholemounts Lateral ventricle were permeabilized in blocking solution with 0.5% Triton X-100 for 20 minutes and washed with 10% normal donkey serum in 1X PBS twice. After washing with PBS, sections were incubated for 1 hour with the Click-iT reaction cocktail, protected from light. After these immunostainings, a 200-300mm thick section of the lateral wall was carefully dissected away from the remaining brain by removing cortex and overhanging thalamus. Lateral wall tissue wholemounts were coverslipped with Aqua-Poly/Mount (Polyscience) and imaged on a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems). Whole mounts were left to settle for at least twelve hours prior to imaging.
**Results**


**Caudal to rostral progression of mouse ependymogenesis and persistence of stem cells only along the lateral wall of the lateral ventricle***

For the assessment of brain development in the mouse we used serial coronal sections to generate three-dimensional (3D) reconstructions of both total brain and lateral ventricle volumes at five discrete stages of embryonic to postnatal brain development: E13, E16, postnatal day P1, P7 and P30 (Fig. 1, left column). In addition, whole-mount preparations of the lateral and medial wall of the lateral ventricles were prepared for each of the five stages of development (Doetsch et al., 1997; Mirzadeh et al., 2008; Shook et al., 2012). Changes in cell coverage along the entire extent of both the medial and lateral walls of the lateral ventricle were examined using immunohistochemistry to distinguish radial glia [γ-tubulin+ basal body of single cilium, GLAST+ (also known as SLC1A3), FOXJ1−], radial glia that are transitioning to immature ependymal cells (2-5 γ-tubulin+ basal bodies of cilia, FOXJ1+), mature ependymal cells (multi-cilia γ-tubulin+ clusters, FOXJ1+) and neural stem cells (single cilium γ-tubulin+ basal body, GFAP+) (see Fig. 1A) (Jacquet et al., 2009; Mirzadeh et al., 2010b, 2008).
**Figure 1.** Ependymogenesis proceeds caudal to rostral along lateral ventricle wall during mouse brain development. (A-E) 3D reconstructions at E13 (A), E16 (B), P1 (C), P7 (D) and P30 (E) show lateral ventricles and whole-brain contours (left column). Schematics of representative microscope images (second column, lateral wall) highlight ependymal cell development along caudal, middle and rostral regions of the lateral ventricle wall. Wave of caudal-to-rostral ependymal cell formation is illustrated on 2D projections of the ventricle wall. Scale bars: 20 μm in top whole-view schematic; 1 mm in E13 and E16 2D projections; 500 μm in P1, P7 and P30 2D projections. Pie charts (third column) indicate average percentage of radial glia, immature ependymal cells, V-SVZ stem cells and mature ependymal cells along caudal, middle and rostral regions of the lateral ventricle wall (n=3) at each developmental stage.
Figure 2: Characterization of mouse V-SVZ cell types, pinwheel organization, and medial wall ependymogenesis

(A) Characterization of radial glia cells [γ-tubulin+ single cilium, GLAST+, GFAP+, FOXJ1- (not shown)], immature ependymal cells [γ-tubulin+ two or more cilia, GLAST+, GFAP-, FOXJ1+ (not shown)] and a pinwheel unit (right), comprised of mature ependymal cells [γ-tubulin+ multicilia clusters, β-catenin+, FOXJ1+ (not shown)] surrounding a core of V-SVZ stem cells (GFAP+, γ-tubulin+ single cilium, β-catenin+) (scale bar = 10 μm). (B) Traces of lateral ventricles (2D projections) at E16, P1 and P30 show caudal to rostral wave of ependymogenesis (E16 scale bar = 1mm, P1, P30 scale bar = 500 μm). Immunohistochemistry of representative microscope images (scale bars = 10 μm) and their associated schematics (scale bar = 20 μm) highlight ependymal cell development along caudal, middle, and rostral regions of the lateral ventricle wall. Basal bodies of cilia were used to identify apical surface cell types. Pinwheel organization is highlighted in purple. (C) Schematic representations of microscope images show ependymal cell development along caudal, middle, and rostral regions (13,567.59 μm² areas) of the medial wall of the lateral ventricle (scale bar = 20 μm). By P30, the entire wall is covered by mature ependyma, shown by a representative rostral region. Schematic Key: Radial glia (blue), immature ependymal cells (pale yellow), stem cells (green), mature ependymal cells (orange).
In Fig. 1, renderings of representative microscope images along the lateral wall detail cell organization at the ventricle surface (Fig. 1, second column, Fig. 2B). Cell type ratios (Fig. 1, third column), the average percentages of each cell type at three locations along the lateral wall for each developmental time point, were determined based on counts of a 13,567.59 µm² area for each rostral, middle and caudal sample (n=3 animals). Before E13, radial glia covers the surface of the entire ventricular system surface (data not shown) (Kriegstein and Alvarez-Buylla, 2009). At E13 and E16 (Fig. 1 A, B), immature ependymal cells, which make up ∼35% of total cells at the surface of the ventricle, were found primarily in the caudal-most aspects of the lateral ventricle lateral wall. Immature ependymal cells in the middle and rostral regions comprised only ∼11% and ∼7%, respectively, of the total cell number. By P1 (Fig. 1C), mature ependymal cells, which are characterized by a large tightly clustered array of multiple cilia, cover most of the caudal wall (60%) and stem cells that are organized in the core of pinwheel units made up the remainder (Fig. 2B). Immature and mature ependymal cells make up 34.2% of the middle lateral wall (22.1% immature ependymal cells and 8.5% mature ependymal cells), and only immature ependymal cells (20.4%) and radial glia (79.6%) line the rostral-most wall.

As the caudal-to-rostral wave of newly differentiated ependymal cells begins to cover the ventricle surface, clusters of radial glia/neural stem cells (V-SVZ stem cells) were found to retain only a small apical process at the ventricle surface, whereas stem cell somatas were displaced below the newly generated ependymal cell monolayer, as previously described (Mirzadeh et al., 2008). By P7 (Fig. 1D), only mature ependymal cells and clusters of stem cell apical processes, classic ‘pinwheel’ units (Mirzadeh et al., 2008), make up the caudal (58.4% mature ependymal cells, 41.6% stem cell processes) and
middle (41.3% mature ependymal cells, 58.7% stem cell processes) aspect of the lateral wall. In the rostral-most aspect of the lateral wall, radial glia (40.3%) and immature ependymal cells (5.6%) were still detected. By P30 (Fig. 1E, Fig. Fig. 2B), all regions of the lateral wall were covered with organized pinwheel units. Cell counts at P30 indicate that the majority of cells at the ventricle surface are mature ependymal cells (∼60%), with stem cells making up ∼40% of the total cell count. However, as stem cell somatas are displaced to the SVZ, the ventricle-contacting apical process takes up only ∼10% of the ventricle surface area compared with ependymal cells (see also Spassky et al., 2005).

Ependymogenesis along the medial wall also proceeds as a caudal-to-rostral wave (Fig. 2C). At E13, the medial wall is covered by radial glia, with immature ependymal cells present only in the caudal-most region (not shown). By E16, differentiation of immature ependymal cells progresses rostrally along the medial wall and, after birth (P1), the caudal and middle regions were covered predominantly by mature multi-ciliated ependymal cells, whereas the rostral region was still lined primarily with radial glia. At P30, the medial wall was covered by mature multi-ciliated ependymal cells: stem cells were not observed along the medial wall. Others report small clusters of stem cells only along the rostral-most aspect of the medial wall in postnatal mice (Mirzadeh et al., 2008), but, as we have found, these are subsequently lost in early adulthood (Fig. 2C).

Here, we highlight the conversion of neuroepithelial cells to an ependymal monolayer that is interspersed with clusters of stem cells along the lateral wall only, not the medial wall. These data support earlier findings that describe the caudal-to-rostral wave of ependymogenesis along the lateral ventricle lateral wall (Mirzadeh et al., 2008; Spradling et al., 2001, Coletti et al., 2018)
Ependymogenesis uses both symmetric and asymmetric modes of division

In order to directly observe clonal relationships among cells in the lateral ventricle epithelial lining of the mouse brain, we used the Piggybac transposon-based lineage-tracing method. This clonal analysis is based on the electroporation of piggybac transposon donor constructs (encoding fluorophore reporter protein with either nuclear or cytoplasmic localization), as well as plasmids encoding piggyBac transposase (PGLAST-PBase). By combining pGLAST-PBase with PB CAG-eGFP it was possible to integrate the fluorophore into the GLAST genome of radial glial cells by in utero electroporation during early ependymogenesis (at E15) and successfully label all subsequent lineages of an individual radial glial cell (Chen et al, 2012). We used sparse labelling (<1µg/µg of plasmid), as sparsely labelled radial glial cells allow capture of unique events.

Ependymal cells and radial glial/stem cells (NSC) were identified based on their immunohistological appearance. We used β-catenin and γ-tubulin to define the ependymal cell adherens junctions and basal bodies of cilia, respectively, and individual apical NSC processes were identified based on the presence of a single primary cilium (a single γ-tubulin+ puncta compared to a cluster of γ-tubulin+ puncta denoting motile cilia typical of ependymal cells) and expression of GFAP. β-catenin staining also helped us to identify cell borders and to confirm ependymal cell or NSC cell identity based on the size of their apical domain.

We analyzed the eGFP+ cells from the most apical surface of whole mounts at P14 and found eGFP+ β-catenin+ multiciliated ependymal cell pairs in animals injected at E15 (Fig 3A). Interestingly, we also found pairs formed by one eGFP+ β-catenin+ ependymal cell
with one eGFP+GFAP+ neural stem cell (Fig 3B). *In utero* electroporation at E15 followed by 5-Ethynyl-2'-deoxyuridine (EdU) injection at E16 also showed the presence of both types of pairs after immunostaining of the P14 brain. By using another more photostable red fluorescent protein mCherry (derived from DsRed of *Discosoma* sea anemone) we showed EdU+mCherry+β-catenin+ ependymal cell doublets (Fig 3C) and doublets of one EdU+mCherry+ GFAP+ neural stem cell with one EdU+mCherry+β-catenin+ ependymal cell (Fig 3D) revealed that these pairs of EdU tagged cells must have been derived from a recombined progenitor cell that divided soon after EdU administration.
**Figure 3: Ependymal cells can be derived from both symmetric and asymmetric division**

(A) Following in utero electroporation of E16 embryo with PBCAG-eGFP and PGLAST-PBase, the lateral wall wholemount from P14 mice immunostained for β-catenin (red), γ-tubulin and GFAP (blue), and eGFP (green) shows eGFP+/β-catenin+ ependymal cell doublets indicating symmetric division (indicated by yellow arrow) in a pinwheel organization (white outline in merged image, apical view); scale bar = 20µm. (B) P14 lateral wall wholemount shows pairs of 1 β-catenin+ ependymal cell and 1 GFAP+ neural stem cell (indicated by white arrow) indicating asymmetric division; β-catenin (red), γ-tubulin (red), GFAP (blue), and eGFP (green), scale bar = 20µm (C) Thymidine analog (EdU) injection at E16 after E15 IUE with PB CAG-mCherry and PGLAST-PBase analysis at P14 brain wholemount shows mCherry+EdU+β-catenin+ symmetrically generated pairs of ependymal cells; β-catenin and EdU (green), GFAP (Blue), mCherry (Red), scale bar = 20µm. (D) In the same P14 brain, presence of a pair formed by one β-catenin+ ependymal cell and one GFAP+ neural stem cell (indicated by white arrow) represents asymmetric division of a neural stem cell; β-catenin (green), EdU (purple), GFAP (blue), and mCherry (red), scale bar = 20µm.
In P14 brains, doublets of ependymal cells (symmetric division) are predominant compared to a single ependymal cell and an associated stem cell generated via asymmetric division.

After in utero electroporation of PBCAG-eGFP with PGLAST-PBase in pregnant mice with E16 embryos and brain harvest at postnatal day 14 (P14), we quantified the eGFP+ doublets to determine regional differences along the caudal to rostral aspect of the lateral wall of lateral ventricle and whether doublets were comprised of one eGFP+ β-catenin+ ependymal cell and one eGFP+GFAP+ stem cell (asymmetric division) or two eGFP+ β-catenin+ ependymal cells (symmetric division). The percentages of eGFP+ doublets of each division type (symmetric vs asymmetric) including ependymal singlets at three locations along the lateral wall of lateral ventricle for P14 mice were determined based on counts of a 13,567.59 µm² area for each rostral, middle and caudal region.

The schematic representations of microscope images show eGFP+ ependyma and neural stem cells along caudal, middle, and rostral regions (13,567.59 m² areas) of the lateral wall of the lateral ventricle in Figure 4A.

In Table 1, the percentage of eGFP+ symmetric division, asymmetric division and the ependymal singlets from caudal (in 5 different mice), middle (in 6 different mice) and rostral (in 9 different mice) regions are shown and from the graph we can see the average percentages of the symmetric divisions from rostral (26.12%), middle (25.98%) and caudal (25.67%) region tends to be higher than the average percentages of asymmetric divisions from the rostral (13.60%), middle (8.12%) and caudal (8.33%) region of the lateral wall of lateral ventricle.
We also found single eGFP+ β-catenin+ ependymal cells in the rostral (21.40%), middle (28.52%) and caudal (32.89%) region, with no other labeled cells in their proximity (see Fig. 4B). These cells are likely derived by uptake of plasmid at the time of the in utero electroporation by immature ependymal cells that were still GLAST+. 
A

Rostral  Middle  Caudal

Mature Ependymal Cell
Radial Glial Cell
GFP+ Ependymal Cell
GFP+ Stem Cell

Rostral  Middle  Caudal

β-catenin
γ-tubulin
GFAP
GFP

B

% of GFP-labeled Events

Rostral  Middle  Caudal

Symmetric Divisions
Asymmetric Divisions
Immature Ependymal Cells
Figure 4: eGFP+ lineages along the apical surface of the lateral wall of the lateral ventricle from wholemount preparations of the caudal, middle and rostral regions.

(A) The schematic representation of the confocal traces (2D projections) of P14 wholemount show examples of symmetric and asymmetric division in rostral, middle and caudal regions (13,567.59 μm² areas). White arrows denote asymmetric divisions and dashed boxes indicate symmetric divisions. IUE (throughout check that you use either IUE or in utero electroporation, don’t alternate between the two) performed at E16 using pBCAG-eGFP and pGLAST-PBase. Top row shows schematics of and the immunohistochemistry of representative microscope images are shown at the bottom; β-Catenin (red), Y-Tubulin (red), GFAP (blue), GFP (green). Schematic Key: mature ependymal cells (Dark yellow), GFP+ mature ependymal cells (Green), GFP+ stem cells (blue), Neural stem cells (Red). (B) Average percentages of the eGFP+ symmetric, asymmetric and ependymal singlets count from all three (caudal, n=5; middle, n=6 and rostral, n=9) regions in P14 brain lateral wall of lateral ventricle.
Table 1: Average percentages of eGFP+ symmetric and asymmetric doublets and ependymal cell singlets.

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Radial glial/neural stem cells undergo multiple rounds of division in the process of ependymogenesis

Following electroporation, P14 wholemounts revealed that most clones were composed of 2 eGFP+ cells; however, we also found some clones of 3-4 eGFP+ cells, as shown in Figure 5. Clones containing 3 to 4 cells can be explained by 2 or 3 successive cell divisions from a labeled radial glial cell. Interestingly, a majority of these clones contained more ependymal cells than NSCs, suggesting that symmetric ‘consuming’ divisions giving rise to 2 ependymal cells, might be the reason for depletion of NSC within the V-SVZ of developing brain. Previously Redmond (2019) showed in lineage tracing experiments and clonal analysis of double-labelled cell (tdtomato+BrdU+) comparison (between E14.5 and E17.5), the presence of higher number of symmetric divisions at E17.5 brain whereas at E14.5 there is higher number of asymmetric divisions, that suggests ependymal cells derive from radial glial cell mostly through asymmetric division in earlier developmental timepoints. A similar idea of ‘disposable stem cell model’ has been proposed by Encinas et al. to explain age-related loss of mouse hippocampal neural stem cells and the appearance of new astrocytes, where quiescent neural progenitor divided asymmetrically to generate active neural progenitor cells and finally through symmetric division the active neural progenitor cell caused the decline of NSC pool with aging. In line with this idea, we propose the possible model of radial glial cell division giving rise to ependymal cells in early embryonic life through 1-3 successive asymmetric self-renewing divisions and then a final symmetric terminal division.
Figure 5: Successive divisions of single radial glial cell generates ependymal cell clones of 3-4 cell

In the top panel (A) sparse labelling reveals the presence of a clone formed by 2 eGFP+β-catenin+ ependymal cells and one eGFP+GFAP+ stem cell suggesting the possibility of one round of asymmetric division of a radial glial cell followed by another round of asymmetric division of the daughter stem cell. (B) Clone of 3 eGFP+ ependymal cells suggest the early asymmetric and terminal symmetric division of the daughter stem cell. (C) Three eGFP+ ependymal cells with one stem cell indicates the possibility of 3 successive asymmetric divisions. (D) Adjacent four ependymal cells can be explained in two ways. Either RGC divided symmetrically to two NSCs and each of them symmetrically divided to generate four ependymal cells or RGC divided twice asymmetrically and terminally differentiated in a symmetric 'consuming' division; β-Catenin (red), Y-Tubulin (blue), GFAP (blue), GFP (green); scale bar = 20µm. In the bottom panel, I propose a possible model of stem cell division during development in which asymmetric division predominates during early development (steps 1-3) and transitions to symmetric division (step 4) later in development.
During early development of the mammalian brain, the pseudostratified layer of proliferative cells lining the fluid-filled ventricular system in the forebrain contributes to the robust expansion of the cerebral cortex. Initially the neuroepithelial cells, and then their descendant radial glia and outer radial glia, take part in neurogenesis via their progeny, intermediate progenitor cells (Hansen et al., 2010; LaMonica et al., 2012; Lui et al., 2011; Malik et al., 2013). Eventually, radial glia generates a monolayer of ependymal cells that lines the ventricles (Jacquet et al., 2009; Mirzadeh et al., 2008; Spassky et al., 2005) and displaces the remaining radial glia cell body to the subventricular zone. In the lateral wall of lateral ventricle of the adult mice brain, ependymal cells surrounding the thin apical process of neural stem cells are organized into what have been labeled ‘pinwheels’ at the ventricle surface. Neural stem/progenitor cell projections forming the ‘pin’ of the pinwheel of ependymal cells (Mirzadeh et al., 2008). In this way, the embryonic ventricular zone transforms into the largest stem cell niche of the adult rodent forebrain brain.

In rodents, V-SVZ neurogenesis continues to provide new neurons to the olfactory bulb throughout adulthood. However, with increasing age stem cell numbers are reduced and neurogenic capacity is significantly diminished, but new olfactory bulb neurons continue to be produced even in old age. Humans, in contrast, show little to no new neurogenesis after two years of age. In adult rodent forebrain, the persistence of an active subventricular zone neural stem cell niche supports its continued role in the production of new neurons and in generating cells to function in repair through adulthood. The
reorganization of the V-SVZ stem cell niche and its relationship to ependymogenesis has not been well characterized in the human brain.

To compare and contrast with human brain development, an assessment of brain development in the mouse works as an appropriate model. To generate three-dimensional (3D) reconstructions of both total brain and lateral ventricle volumes we have used serial coronal sections at five discrete stages of embryonic to postnatal brain development: E13, E16, postnatal day P1, P7 and P30 and the changing cellular organization was analyzed along both the medial and lateral wall of the lateral ventricle. In studies of the medial wall, our studies showed that the ependymogenesis also proceeds in a caudal-to-rostral wave similar to lateral wall and in both cases, the newly generated immature ependyma starts to replace the radial glia at around E13-E14 - eventually covering the whole ventricular wall with an immature ependymal lining. The appearance of mature multiciliated ependymal cells was detected after birth at approximately P1. Here we focused on the conversion of neuroepithelia to an ependymal monolayer that is interspersed with clusters of stem cells as found only along the lateral, and not the medial wall.

Based on our comprehensive comparative spatiotemporal analyses of cytoarchitectural changes along the mouse and human ventricle surface, our lab has also uncovered a distinctive stem cell retention pattern in humans as ependymal cells populate the surface of the ventricle in an occipital-to-frontal wave. During perinatal development, ventricle-contacting stem cells are reduced and by 7-months few stem cells are detected, paralleling the decline in neurogenesis (Coletti et al., 2018).
One focus of my research was to track the mechanism of stem cell division at different
developmental time points during the process of ependymogenesis. As it remained to be
determined whether the stem cell pool expands when a stem cell gives rise to two stem
cells (symmetric division), stays the same size by giving rise to one stem cell and one
differentiated cell (asymmetric division), or decreases when a stem cell generates two
differentiated daughter cells (symmetric differentiative division), we tried to investigate if
there is any spatiotemporal change in division pattern during ependymogenesis and how
stem cell division may support stem cell retention within pinwheels. I used PiggyBac in
utero electroporation (PB-IUE) lineage tracing to label stem cells and their progeny along
the ventricular surface. Immunohistochemistry of whole mount sections of the lateral
ventricle allowed me to visualize eGFP+ progeny of electroporated radial glial cells. In our
study, I showed that the two main cell types in the epithelial lining of lateral wall of lateral
ventricle, multi-ciliated ependymal cells and neural stem cells are generated from a single
progenitor radial glial cell (Redmond et al., 2019, Ortiz-A’lvarez et al., 2019, Stratton et
al., 2019). Scoring of asymmetric (1 stem cell and 1 ependymal cell) versus symmetric (2
ependymal cells) division of stem cells in postnatal development, allowed me to observe
the predominant mode of division prevailing among adult P14 mice brains. Our finding
revealed that, in adult mice, the majority of the neural stem cells gave rise to ependymal
cells through symmetric division rather than asymmetric division. Similar findings have
been proposed by Ortiz-A’lvarez et al., (2019) that adult neural stem cells are sister cells
to ependymal cells, whereas most ependymal cells arise from the terminal symmetric
divisions of the lineage and antagonist regulators of DNA replication, GemC1
overexpression favors the formation of pure ependymal clones through symmetric
division whereas Geminin favors the formation of B1 (NSC) cell-containing clones at E14.5.

In the case of neurogenesis, Obernier and colleagues have shown, by doing different clonal lineage-tracing methods and ex vivo imaging, in the adult V-SVZ that symmetric division is the primary mode of division. Similarly, our study of ependymogenesis reveals higher average percentages of the symmetric divisions in rostral (26.12%), middle (25.98%) and caudal (25.67%) regions, compared to average percentages of the asymmetric division from the rostral (13.60%), middle (8.12%) and caudal (8.33%) region of the lateral wall of lateral ventricle in the P14 brain.

On the other hand, during embryonic brain development the primary progenitor radial glia, predominantly undergo asymmetric division to generate neurons directly or via intermediate progenitors (Noctor et al., 2004). Similarly, during ependymogenesis, more numbers of asymmetric division of radial glial cells have been observed at embryonic 14.5 day than later developmental time points which aligns with our suggested model for embryonic/perinatal ependymogenesis (Fig. 5) (Redmond et al., 2019). Asymmetric cell division is the predominant form of division by radial glia in embryonic development (Kriegstein and Alvarez-Buylla, 2009), but it is a process that will not support the stem cell pool over extended periods of time because of stem cell exhaustion (Hormoz, 2013; Obernier et al., 2018; Shahriyari and Komarova, 2013). Also, the presence of 3-4 cells clone in our sparse labelling experiment, lead us to think about the possibility of multiple successive divisions during ependymogenesis and pinwheel formation that can be studied using a thymidine analog double-labelling birthdating technique in conjunction to piggybac transposon based IUE (or write out!). Further study is needed to evaluate
whether the prevalence of symmetric division over asymmetric division correlates well with the reduction in total neural stem cell number that occurs with development. Also, the choice of stem cell division strategy may change based on: 1) stage of development, 2) number of prior divisions a stem cell undergoes, and 3) pathology (e.g., hydrocephalus). My results can provide insight into normal stem cell development, which can be examined in mouse models of hydrocephalus to understand how the V-SVZ niche is altered by pathological ventricle enlargement to better understand the pathologic outcome and possible treatment.
References:


Banizs, Boglarka, Martin M. Pike, C. Leigh Millican, William B. Ferguson, Peter Komlosi, James Sheetz, Phillip D. Bell, Erik M. Schwiebert, and Bradley K. Yoder. "Dysfunctional cilia lead to altered ependyma and choroid plexus function, and result in the formation of hydrocephalus." Development 132, no. 23 (2005): 5329-5339.


dependent gene expression is required for differentiation of radial glia into ependymal cells and a subset of astrocytes in the postnatal brain. Development 136, 4021-4031.


Rodríguez, Esteban M., Juan L. Blázquez, and Montserrat Guerra. "The design of barriers in the hypothalamus allows the median eminence and the arcuate nucleus to enjoy private milieux: the former opens to the portal blood and the latter to the cerebrospinal fluid." Peptides 31, no. 4 (2010): 757-776.


