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Quiescent Oct4$^+$ Neural Stem Cells (NSCs) Repopulate Ablated Glial Fibrillary Acidic Protein$^+$ NSCs in the Adult Mouse Brain

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Key Words. Adult stem cells • Neural stem cells (NSCs) • Stem cell cycle • Tissue regeneration

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Received December 11, 2016; accepted for publication June 13, 2017; first published online in STEM CELLS *EXPRESS* Month 00, 2017.

1066-5099/2017/$30.00/0

http://dx.doi.org/10.1002/stem.2662

ABSTRACT

Adult primitive neural stem cells (pNSCs) are a rare population of glial fibrillary acidic protein (GFAP)$^-$ Oct4$^+$ cells in the mouse forebrain subependymal zone bordering the lateral ventricles that give rise to clonal neurospheres in leukemia inhibitory factor in vitro. pNSC neurospheres can be passaged to self-renew or give rise to GFAP$^+$ NSCs that form neurospheres in epidermal growth factor and fibroblast growth factor 2, which we collectively refer to as definitive NSCs (dNSCs). Label retention experiments using doxycycline-inducible histone-2B (H2B)–green fluorescent protein (GFP) mice and several chase periods of up to 1 year quantified the adult pNSC cell cycle time as 3–5 months. We hypothesized that while pNSCs are not very proliferative at baseline, they may exist as a reserve pool of NSCs in case of injury. To test this function of pNSCs, we obtained conditional *Oct4* knockout mice, *Oct4$^{fl/fl}$;Sox1^{Cre}* (*Oct4^{CKO}*), which do not yield adult pNSC-derived neurospheres. When we ablated the progeny of pNSCs, namely all GFAP$^+$ dNSCs, in these *Oct4^{CKO}* mice, we found that dNSCs did not recover as they do in wild-type mice, suggesting that pNSCs are necessary for dNSC repopulation. Returning to the H2B-GFP mice, we observed that the cytosine β-D-arabinofuranoside ablation of proliferating cells including dNSCs-induced quiescent pNSCs to proliferate and significantly dilute their H2B-GFP label. In conclusion, we demonstrate that pNSCs are the most quiescent stem cells in the adult brain reported to date and that their lineage position upstream of GFAP$^+$ dNSCs allows them to repopulate a depleted neural lineage. STEM CELLS *2017; 00:000–000*

SIGNIFICANCE STATEMENT

Adult primitive neural stem cells (pNSCs) are rare cells in the mouse forebrain periventricular region that express a protein not expressed in other cell, a pluripotency gene called Oct4. Label retention experiments indicated that adult pNSCs are quiescent, dividing approximately once every 3–5 months. Transgenic mice without Oct4 did not generate pNSC-derived neurospheres, providing a pNSC loss of function model. After definitive NSCs (dNSCs) were ablated, transgenic mice had fewer dNSC-derived neurospheres, proliferating cells, and cells expressing neural stem cell proteins, suggesting that pNSCs are necessary for dNSC repopulation. In conclusion, pNSCs are quiescent and upstream of dNSCs, but proliferate following downstream lineage ablation to repopulate the neural lineage.

INTRODUCTION

Neural stem cells (NSCs) are a heterogeneous population residing within the first few layers of cells lining the lateral ventricles of the adult mouse brain. A majority of NSCs express *glial fibrillary acidic protein* (*GFAP*) and are identified through co-localization with other markers [1–3]. *GFAP*-expression includes many subtypes of NSCs, and we collectively refer to these cell types as definitive NSCs (dNSCs).

We previously identified an additional type of NSC called the primitive NSC (pNSC) [4], which does not express *GFAP* and is therefore missed if purifying on the basis of this marker. pNSCs are self-renewing, multipotent, GFAP$^-$ but Oct4$^+$ NSCs that give rise clonal neurospheres in leukemia inhibitory factor (LIF) [4, 5]. pNSCs can be isolated from the mouse embryo at embryonic day (E)5.5, in advance of dNSCs that arise at E7.5 and give rise to clonal neurospheres in fibroblast growth factor (FGF)-2 [6]. pNSCs persist into the adult mouse brain as rare cells that continue to generate clonal pNSC-derived neurospheres in the presence of LIF in vitro. pNSCs uniquely express low levels of *Oct4* based on quantitative polymerase chain reaction, *Oct4*-driven antibiotic resistance, Tg

(Oct4$^{IRES-GFP}$) flow cytometry, immunostaining, and morula aggregation experiments [4]. Different cell surface markers are expressed on pNSCs than on dNSCs [7], which can be used to target either population independently [5].

Adult GFAP$^+$ dNSCs form passageable neurospheres in the presence of epidermal growth factor (EGF), FGF, and heparin in vitro ("EFH") [8]. This self-renewing population of dNSCs is GFAP$^+$, multipotent, and comprises all GFAP$^+$ type B cells [1, 2, 9, 10]. In the adult mouse brain, GFAP$^+$ dNSCs divide to produce transit amplifying cells, which generate neuroblasts that migrate to the olfactory bulb and differentiate into GABAergic interneurons [1].

The number of pNSC neurospheres that can be derived from the forebrain periventricular region peaks postnatally, and it is unknown whether this is a result of a transient population of postnatal progenitors or whether they are pNSCs that become quiescent. We hypothesized that some pNSCs become quiescent in the adult brain, and cocultured adult-derived cells with subependymal zone (SEZ) cells from early postnatal animals to see whether the young niche might activate quiescent adult cells. We found an increase in the number of primary adult derived pNSC neurospheres suggesting they are present but quiescent. Using doxycycline (DOX)-inducible histone-2B (H2B)-green fluorescent protein (GFP) mice to assess the cell cycle times in pNSCs and dNSCs, label-retaining cells in the SEZ were found to be pNSCs, and label-retaining pNSC-derived neurospheres could still be obtained after a 1-year chase. From this, we calculated that adult pNSCs divide once every 3–5 months and, in agreement with previous studies, GFAP$^+$ dNSCs were found to divide once every 2–4 weeks.

In addition to pNSCs appearing earlier in development than dNSCs [6], pNSC-derived neurospheres passaged into EFH to give rise to dNSC-derived neurospheres, but dNSCs do not yield pNSC neurospheres if passaged into LIF [4]. Proliferating GFAP$^+$ dNSCs and dNSC-derived neurospheres are ablated by administration of AraC, an antimitotic agent that kills proliferating cells, but return over time. This repopulation was proposed to be due to GFAP$^+$CD133$^+$EGFR$^-$ NSCs [2], however, we find GFAP$^+$ dNSCs still return even after a combination of AraC to kill proliferating precursors and pull dNSCs into cycle and targeted killing of *GFAP*-expressing cells using ganciclovir (GCV) in mice that express thymidine kinase (tk) under the *GFAP* promoter (*GFAP*-tk) (as in Bush et al., 1990) [4, 10]. This suggests that the repopulation of GFAP$^+$ dNSCs is due to a cell that does not express *GFAP*, such as the pNSC.

To test whether Oct4$^+$GFAP$^-$ pNSCs are responsible for repopulation of the neural lineage, we sought a pNSC null mouse model. *Oct4*^{CKO} adult mice do not give rise to pNSC neurospheres in LIF and following AraC + GCV ablation exhibited significantly reduced repopulation of dNSC-derived neurospheres, fewer proliferating cells in the SEZ, and fewer Sox2$^+$GFAP$^+$ cells as compared to control mice. Furthermore, after administration of AraC to DOX-treated H2B-GFP mice, pNSCs significantly diluted their labeled histones compared with baseline, indicating that ablation of their downstream progeny caused them to proliferate.

Altogether, we show that pNSCs are a quiescent population upstream of GFAP$^+$ dNSCs that require *Oct4* for their function. pNSCs become activated from quiescence following GFAP$^+$ dNSC loss, and are responsible for the repopulation of dNSCs and the downstream neural lineage.

Materials and Methods

Mouse Strains

H2B-GFP mice were a kind gift from Dr. K. Hochedlinger [11]. *Oct4*^{fl} mice were a kind gift from Dr. A. Tomlin [12]. *Sox1*^{Cre} mice were a kind gift from Dr. S. Nishikawa [13]. *GFAP*-tk mice were a kind gift from Dr. M. Sofroniew [14]. Tg (Actin-B^{EYFP}) also known as 7AC5Nagy were obtained from Jackson Laboratories. Tg (Oct4$^{IRES-GFP}$) mice were a kind gift from Dr. Andras Nagy. C57BL/6 mice were purchased from Charles River. For detail on all mouse strains please see Supporting Information Figure S1. All mice were maintained in the Department of Comparative Medicine at the University of Toronto in accordance with the Guide to the Care and Use of Experimental Animals and approved by the Animal Care Committee.

Label Retention Assay

H2B-GFP mice received DOX continually in the drinking water (2 mg/mL in 1% sucrose) for 6 weeks starting at 6–10 weeks of age, followed by 1-, 4-, and 12-month chase periods without DOX exposure [11].

Ablation Paradigms

Oct4^{CKO} and *Oct4*^{CKO}*;tk* adult mice were anesthetized with 3%–5% isoflurane and injected with Ketoprofen (3 mg/kg). A cannula was implanted into the lateral ventricle (+0.2 mm anterior, +0.7 mm lateral, depth of 2.5 mm below the skull, relative to bregma) and connected to a mini osmotic pump placed subcutaneously on the back (Alzet 1007D, Direct Corp.). Cytosine β-D-arabinofuranoside (AraC) is an antimitotic agent used here to ablate proliferating cells. It is rapidly converted to cytosine arabinoside triphosphate, which is structurally similar to cytosine triphosphate and gets incorporated into the DNA, but cannot serve as a template for further DNA elongation, thereby leading to cell death. In mice expressing tk transgene under the *GFAP* promoter, GCV is phosphorylated and metabolized to toxic GCV triphosphate, which gets incorporated into replicating DNA strands and inhibits DNA synthesis. Therefore, all dividing, *GFAP*-expressing cells are killed in the presence of GCV. *Oct4*^{CKO} received a 14-day infusion of 4% AraC (Sigma) at 0.25 μL/hour. *Oct4*^{CKO}*;*tk mice received a 7-day infusion of 2% AraC at 0.5 μL/hour followed immediately by a 3-day infusion of 200 μM GCV at 1 μL/hour (Sigma). H2B-GFP mice that received AraC were allowed 3 days to recover after DOX exposure and then infused for 7 days with 2% AraC at 0.5 μL/hour. Surgeries were performed initially on large numbers of mice to combat the AraC-induced toxicity, particularly on the hematopoietic and intestinal systems, and small numbers of mice survived to the experiment endpoints.

Proliferation Analysis

EdU (Life Technologies A10044) was administered to mice continuously via their drinking water (0.2 mg/mL in 1% sucrose) for 7 days during the last week of recovery after AraC and GCV infusion in *Oct4*^{CKO}*;*tk and *Oct4*^{Ctl}*;*tk mice.

Primary Dissections and Neurosphere Cultures

The neurosphere assay was performed as described previously [15]. In brief, periventricular cells surrounding the lateral ventricles were enzymatically and mechanically dissociated to single cells and plated at a density of 10 cells per microliter, which was previously shown to be sufficiently low density for growing clonal neurospheres [16] in 24-well culture plates (Nunclon). pNSC-derived neurospheres and dNSC-derived neurospheres are grown from periventricular cells from the same dissection, but plated separately in different growth factors. To yield pNSC-derived neurospheres, single primary cells were plated in serum-free media (made in-house from GIBCO Dulbecco's modified Eagle's medium and F12) supplemented with LIF (10 ng/mL) (recombinant). For dNSC-derived neurospheres, single cells from the same dissection were plated in SFM supplemented with EGF (20 ng/mL; Sigma E4127), basic FGF (bFGF2, 10 ng/mL; Sigma F0291) and heparin (2 μg/mL; Sigma H3149). All cells from the periventricular zone dissection were plated in both growth factor conditions separately, and resulting neurospheres were counted after 7–10 days in vitro. Stringent size criteria were used when counting neurospheres: primitive neurospheres were defined as 50 μm or larger in diameter and definitive neurospheres as 100 μm or larger in diameter. All experiments were repeated at least in triplicate.

Immunohistochemistry

Mice were killed with an overdose of sodium pentobarbital and perfused transcardially with cold phosphate-buffered saline followed by 4% PFA. Brains were post-fixed overnight at 4°C then cryoprotected in 30% sucrose. Coronal sections (14 μm) were prepared on a cryostat (–16°C). Tissue was permeabilized with 0.5% TritonX and blocked in 10% normal goat serum. Primary antibodies, chicken anti-GFP (Aves Lab #GFP-1020, 1:500), rabbit anti-SOX2 (Abcam ab97959, 1:1000), and mouse anti-GFAP (Millipore MAB3402, 1:500), were incubated overnight at 4°C. Secondary antibodies, 488 goat anti-chicken (Alexa, A11039, 1:400), 568 goat anti-rabbit (Alexa A11036, 1:400), and 647 goat anti-mouse (Alexa A21236, 1:400) were incubated for 30 minutes at room temperature. EdU was detected with Click-iT EdU Alexa Fluor 488 Imaging Kit (Life Technologies C10337). Nuclei were counterstained with Hoechst. Staining was imaged on an Olympus Fluoview FV1000 confocal laser scanning microscope.

Statistics

Data are represented as means ± SEM unless otherwise stated. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA) and Microsoft Excel. All experiments were performed at least in triplicate. Analysis of variance with Bonferroni's multiple comparison tests and Student's *t* test were performed with a significance level of 0.05.

Results

pNSCs Are a Quiescent Stem Cell Population in the Adult Mouse Brain

We previously observed that the number of pNSCs that can be isolated from the periventricular region in a neurosphere assay seems to decrease from the early postnatal period to adulthood [4, 5], but that the absolute number of Oct4-GFP$^+$ cells from a primary dissection of pup and adult brains is roughly equivalent when quantified by flow cytometry [5]. We hypothesized that this is due to a decrease in the number of adult pNSCs that form neurospheres, rather than a decrease in the total number of adult pNSCs. To test this, we cocultured Tg(ActinB^{YFP}) adult and wildtype CD1 post-natal day 7 primary periventricular cells to test whether a young environment could activate non-dividing adult pNSCs. As predicted, adult cells in an overwhelmingly young environment (1:9 ratio of adult to post-natal cells) led to a 5.5 times increase in the number of YFP$^+$ adult pNSC neurospheres compared with majority of adult cultures (8.25 vs. 1.49 adult pNSC neurospheres/40,000 cells; Fig. 1A). This suggested that the young environment enabled otherwise non-sphere-forming pNSCs to proliferate to form neurospheres. Adult dNSCs were not altered by coculture with young cells (Fig. 1B), although dNSC neurospheres from aged animals were demonstrated to increase in an early post-natal coculture [17].

That some adult pNSCs do not readily divide to form neurospheres suggested the pNSC population may contain quiescent stem cells. To compare the cell cycles of pNSCs and dNSCs, we performed a long-term label retention experiment by administering DOX to adult H2B-GFP mice via the drinking water for 6 weeks to label cells. H2B-GFP mice have transgenic H2B-GFP under the control of a tetracycline-responsive promoter element (TRE;tetO) whereby exposure to DOX induces expression of the stable H2B-GFP fusion protein. After DOX removal, cells dilute the GFP-labeled histones as they proliferate (Fig. 1C), thus leaving quiescent cells labeled over long chase periods [11]. The H2B-GFP system allows for unbiased label retention studies due to replication-independent initial labeling, unlike BrdU or EdU based assays. Immediately following 6 weeks of DOX exposure (time 0), cells lining the lateral ventricle were labeled (Fig. 1D), as were pNSC-derived and dNSC-derived neurospheres. Any amount of labeling in a clonal neurosphere indicated the sphere-initiating cell was labeled, and mottled expression was observed as expected since the H2B-GFP label dilutes as the cells proliferate in culture to form a neurosphere. Mottled GFP staining in clonal neurospheres suggests different frequencies of division of cells within the neurospheres.

For these experiments, we assumed that: (a) pNSCs and dNSCs were initially labeled equally in vivo. (b) All cells dilute the label by half with every cell division. (c) There is symmetric segregation of histones. (d) Labeled pNSCs and dNSCs form neurospheres where even single GFP$^+$ cells can be visualized.

Validating our first assumption, immediately after 6 weeks of DOX exposure 44% ± 4% of dNSCs and 41% ± 9% of pNSCs were labeled (Fig. 1E). The equal proportion of initially labeled pNSC- and dNSC-derived neurospheres supports the unbiased, replication-independent labeling in the H2B-GFP mouse model, which makes it better suited for comparing different cell populations than methods that require cells enter S phase for labeling to occur. In addition, the equal percentages of pNSC- and dNSC-derived neurospheres initially labeled after DOX exposure indicates that in vitro cell divisions and differences in pNSC and dNSC neurosphere sizes do not affect our ability to detect GFP$^+$ cells within the sphere.

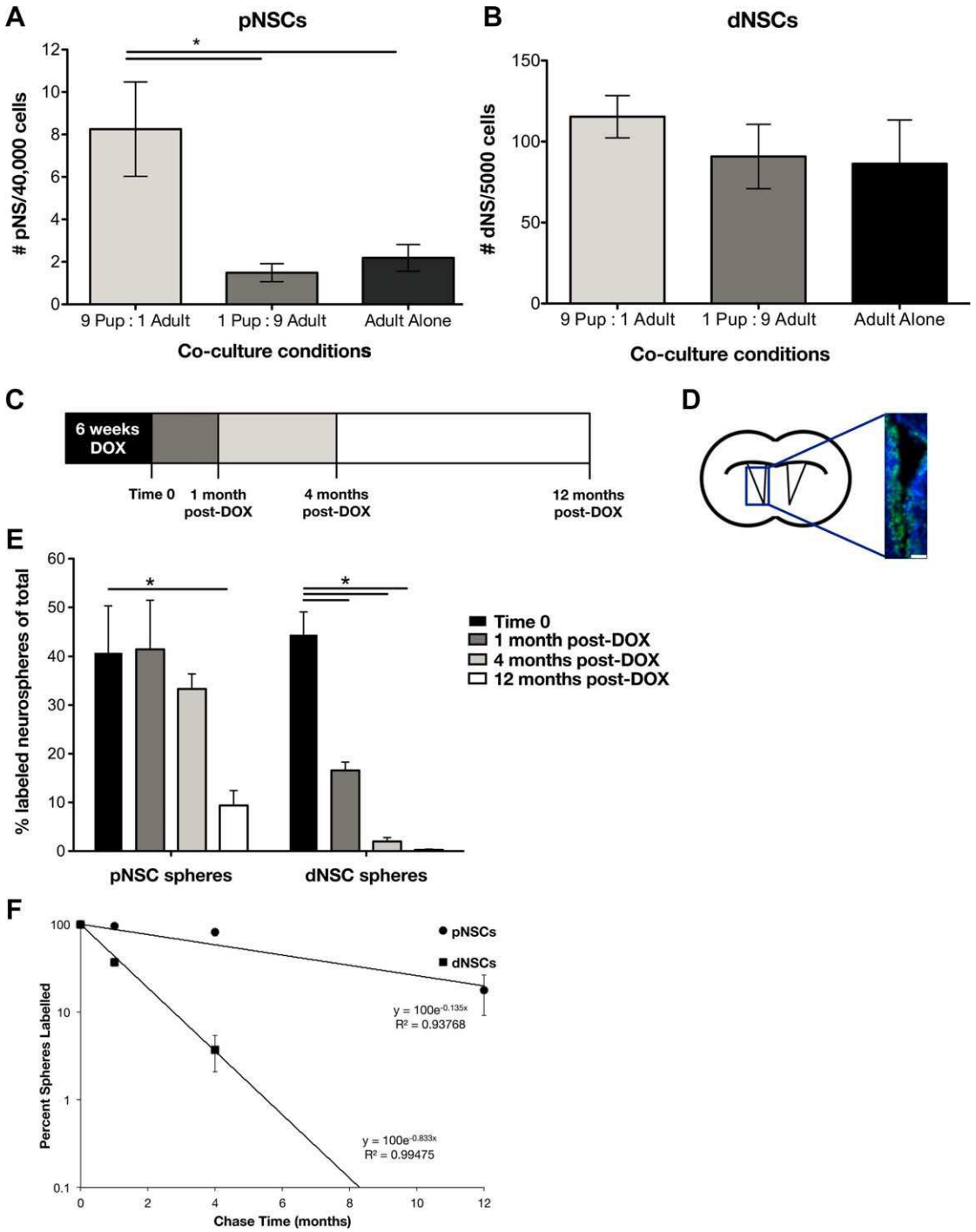


Figure 1. Label retention in primitive neural stem cells (pNSCs) and definitive NSCs (dNSCs) in histone-2B (H2B)–green fluorescent protein (GFP) mice. **(A)**: Quantification of YFP^+ pNSC-derived neurospheres in leukemia inhibitory factor (LIF) after coculture with postnatal day 7 (pup) periventricular cells (one-way analysis of variance [ANOVA] $F(2,6) = 7.525$, $p = .0042$, $n = 7$). pNS = pNSC neurospheres. **(B)**: YFP^+ dNSC-derived neurospheres in epidermal growth factor, fibroblast growth factor 2, and heparin after coculture with pup periventricular cells ($n = 3$–6 experiments). dNS = dNSC neurospheres. **(C)**: Doxycycline (DOX) was delivered in the drinking water for 6 weeks followed by 1-, 4-, and 12-month chase periods. **(D)**: DOX-induced GFP labeling in cells surrounding the lateral ventricle of H2B-GFP mice (scale bar = 50 μm). **(E)**: During the chase periods, dNSCs divided more frequently and diluted their GFP label while pNSCs retained labeling longer, expressed as a percentage of total neurospheres isolated (two-way ANOVA $F(3,54) = 3.8$, $p = .001$, $n = 6$–10 per timepoint). **(F)**: GFP-labeled neurospheres expressed as a percentage of the neurospheres initially labeled immediately after DOX exposure. Abbreviations: dNSCs, definitive neural stem cell; DOX, doxycycline; pNSCs, primitive neural stem cells.

The H2B-GFP model indicated that dNSCs quickly diluted out their label, while pNSCs retained their label over longer chase periods (Fig. 1E). dNSCs significantly diluted their label from 44% labeled at baseline to 17% ± 2%, 2% ± 1%, and 0.2% ± 0.1% after a 1-, 4-, and 12-month chase, respectively. pNSCs did not reduce their label during the first month of the

chase period, then it decreased to 33% ± 3% and 9% ± 3% labeling after a 4- and 12-month chase, respectively (Fig. 1E).

To calculate pNSC and dNSC cell cycle times from these long-term label retention data, we expressed the percentages of labeled neurospheres as a percentage of spheres initially labeled. For example, after 1 year, 9% of the total population of pNSC-derived neurospheres was GFP^+, which is approximately 22% of the population of pNSC-derived neurospheres that was initially labeled immediately after DOX exposure (Fig. 1F). Since at 12 months post-DOX exposure, the percentage of labeled dNSC-derived neurospheres was 0.2% ± 0.1% (1 GFP^+ neurosphere from 1 of 14 mice analyzed) and not significantly different from 0%, we presumed that the dilution of label happened well before 12 months in dNSCs. Excluding this dNSC 12-month time point, the R^2 value of the line of best fit increases from 0.95 to 0.99. The GFP dilution was plotted on a logarithmic scale to confirm that the label diluted exponentially, further demonstrating label retention in pNSCs (Fig. 1F). From the lines of best fit on the dilution graph (R^2 value of 0.94 for pNSCs), we set the *y* intercept to 50% to calculate the amount of time (*x*) required to dilute the H2B-GFP label by half. We calculated the cell cycle of dNSCs to be 24 days (0.8 months) and pNSCs to be 5.1 months. If we account for the difference in sphere sizes between pNSC- and dNSC-derived neurospheres, dNSCs divide 50% more than pNSCs (10–11 cell divisions vs. 7 cell divisions, respectively) to form a neurosphere. Therefore, the rate of dilution in pNSCs was 50% less than dNSCs in culture and the cell cycle time of pNSCs reduces from 5.2 months to a more conservative estimate of 3.4 months. Based on these two methods of analyses, we conclude that the cell cycle time of pNSCs is 3–5 months, whereas dNSCs divide every 2–4 weeks.

Oct4 Conditional Knockout Mice Serve as a pNSC Loss of Function Model

As *Oct4* expression is unique to pNSCs, we sought to determine whether loss of *Oct4* expression affects pNSCs using *Sox1*-driven *Cre* expression to induce a conditional loss of *Oct4* in all neural cells (Fig. 2A). $Oct4^{CKO}$ mice appeared phenotypically normal, however, pNSC-derived neurospheres were absent from $Oct4^{CKO}$, while heterozygote $Oct4^{wt/fl};Sox1^{Cre}$ $(Oct4^{Ctl})$ mice generated a normal abundance of pNSC-derived neurospheres not different from wildtype controls (Fig. 2C). This suggests that *Oct4* is required by pNSCs, at least for neurosphere formation. In contrast, $Oct4^{CKO}$ mice gave rise to a normal abundance of dNSC-derived neurospheres (Fig. 2D). Since *Sox1* is not initially expressed until ~E7.5 [18] and this is after pNSCs and dNSCs first appear in the developing embryo [6]. The loss of *Oct4* does not affect the initial generation of dNSCs in development and they appear able to maintain their own population under baseline conditions in $Oct4^{CKO}$ mice.

To further understand the impact of a loss of *Oct4* on NSCs, we quantified neurospheres from $Oct4^{CKO}$ and control mice at E12, E17.5, postnatal day 7, 14, and 8-week old adults. Unlike the findings from the adult brain (Fig. 2C), $Oct4^{CKO}$ mice at earlier developmental ages gave rise to similar number of pNSC neurospheres in LIF as controls (Fig. 2E). However, we previously described that some embryonic and postnatal-derived neurospheres are progenitor-derived and do not self-renew [19]. To test for the cardinal stem cell property of self-renewal to determine whether pNSC neurospheres from $Oct4^{CKO}$ mice were progenitor-derived, we performed single sphere passaging and found that indeed $Oct4^{CKO}$ mice pNSC neurospheres did not form secondary neurospheres in LIF (Fig. 2G). Since $Oct4^{Ctl}$ neurospheres could self-renew (Fig. 2G), we concluded that any neurospheres forming perinatally from $Oct4^{CKO}$ mice are derived from transient progenitors and not pNSCs. Similar to the adult data for dNSC neurospheres in EFH (Fig. 2D), we found no significant differences between EFH neurospheres in $Oct4^{CKO}$ mice and controls (Fig. 2F). From these data we conclude that pNSCs are not functional when *Oct4* is conditionally knocked out as they cannot form self-renewing neurospheres at any age measured.

Oct4-Expressing pNSCs Are Required for dNSC Neurosphere Repopulation after dNSC Ablation

The lack of pNSC-derived neurospheres observed in $Oct4^{CKO}$ animals suggests *Oct4* is required for pNSCs survival, proliferation, or cell identity. By crossing $Oct4^{CKO}$ mice to a strain with transgenic $Oct4^{IRES\text{-}GFP}$, we reasoned we could determine whether pNSCs were still present (and unable to proliferate) or whether no cells able to activate the transgenic Oct4 promoter (pNSCs) remained. Using $Oct4^{fl/fl}$;Tg($Oct4^{IRES\text{-}GFP}$) mice lacking *Cre* as controls, we did not find a difference in the proportion of Tg($Oct4^{IRES\text{-}GFP}$)-positive cells in the periventricular region of $Oct4^{CKO}$;Tg($Oct4^{IRES\text{-}GFP}$) mice (0.05% of total cells GFP^+ in the conditional knockout versus 0.04% in controls; Fig. 3A) by flow cytometry. From this, we infer that pNSCs are not lost without *Oct4*, but are unable to proliferate.

We previously observed that ablated dNSCs return over time [4], which we suggest is due to their repopulation by upstream pNSCs. Using the $Oct4^{CKO}$ mice where pNSCs do not proliferate as described above, we repeated the same ablation paradigms to test whether the $GFAP^+$ dNSC population could still return. AraC was infused directly into the lateral ventricle of $Oct4^{CKO}$ and $Oct4^{Ctl}$ mice continuously for 2 weeks to ablate all proliferating cells, including neurosphere-initiating dNSCs (Fig. 3B). After AraC treatment, the ability of pNSC loss of function $(Oct4^{CKO})$ mice to repopulate dNSCs after a 2- and 4-week recovery period was assessed using a neurosphere assay. The dNSC-derived neurosphere repopulation was reduced significantly after 4 weeks of recovery in $Oct4^{CKO}$ mice versus $Oct4^{Ctl}$ control mice, which recovered to 24% ± 5% and 91% ± 8% of naïve baseline, respectively (Fig. 3B). Therefore, pNSCs must proliferate to repopulate ablated dNSCs.

AraC infusion did not induce a complete loss of dNSCs, and since even a few proliferative $GFAP^+$ dNSCs could be the source of a recovered dNSC population [9], we sought a more comprehensive ablation protocol. We crossed the $Oct4^{CKO}$ strain to *GFAP*-tk mice to take advantage of a more extensive ablation of $GFAP^+$ cells using GCV [4]. We infused AraC for 7 days to ablate all dividing progenitor cells and bring $GFAP^+$ dNSCs into cycle to increase the efficiency of the subsequent 3-day GCV infusion (Fig. 3C). After initial ablation, dNSC-derived neurospheres were completely absent after a 2-week recovery in $Oct4^{CKO}$;tk mice and had returned to just 2.7% ± 1.8% of untreated control neurosphere levels after a 4-week recovery, a significant reduction compared with heterozygous $Oct4^{Ctl}$;tk mice (Fig. 3C). Strikingly, half of the $Oct4^{CKO}$;tk mice who received this treatment had 0 dNSC-derived

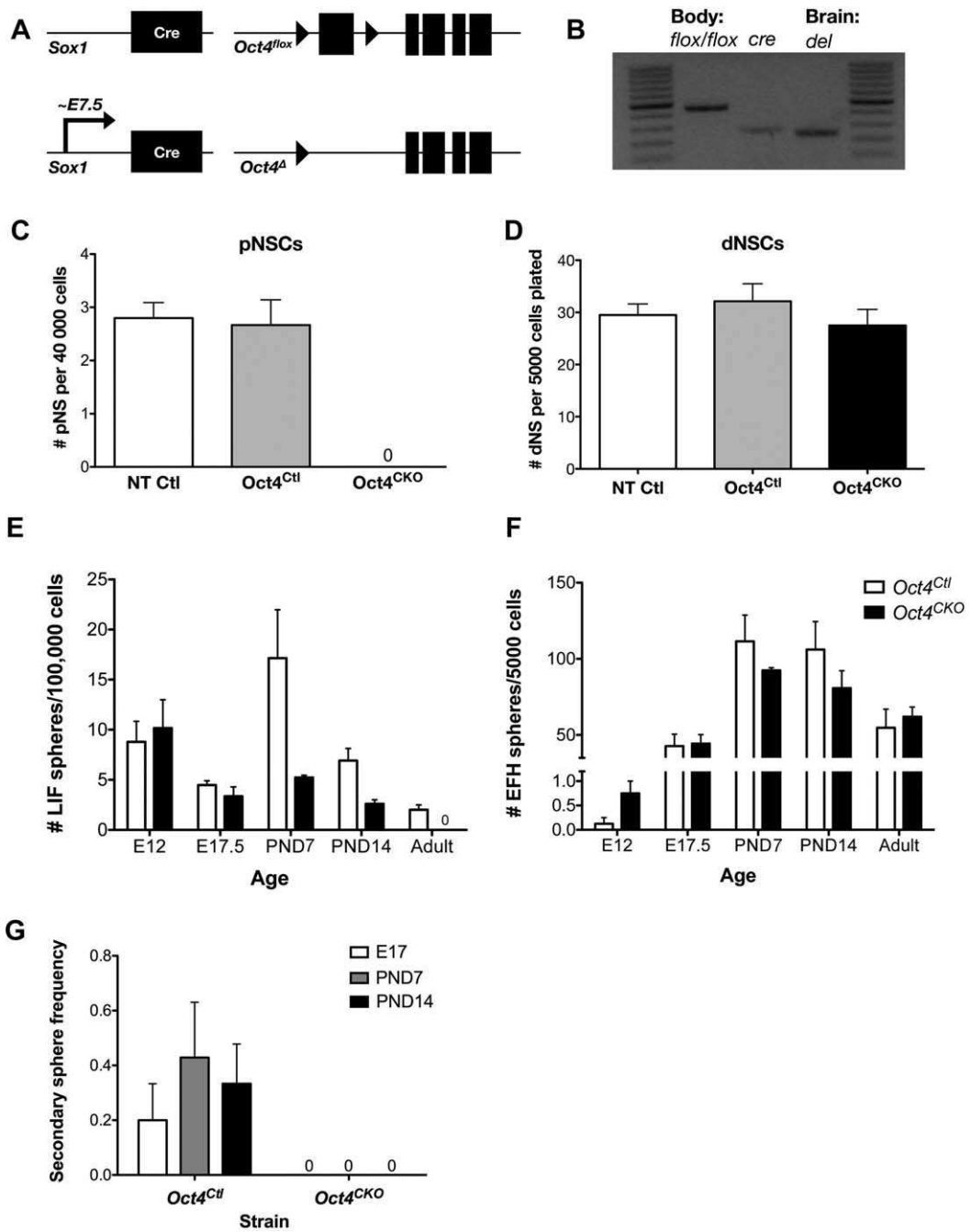


Figure 2. *Oct4*^{CKO} mice are a primitive neural stem cell (pNSC) loss of function model. **(A)**: Mice are homozygous for *Cre recombinase* insertion to the *Sox1* locus, and the *Oct4*^{fl} allele that has the promoter and exon 1 flanked by *loxP* sites. Constitutive *Cre* expression, driven by the *Sox1* promoter, drives excision of the critical exon of *Oct4*, causing the conditional knockout (adapted from ref. [12]). **(B)**: Representative polymerase chain reaction of one *Oct4*^{CKO} animal with mutations in (A) who has the recombination event resulting in a conditional loss of *Oct4*. The first lane shows homozygosity for *Oct4*^{fl} alleles when probing tissue from the body with primers to detect *Oct4*^{fl} (450 bp), *Oct4*$^{+}$ (415 bp), or *Oct4*$^{\Delta}$ (245 bp). Lane 2 shows the same animal positive for *Oct4*^{fl} also carried *Sox1*^{Cre} (269 bp). Lane 3 shows this combination and leads to recombination event in the brain resulting in *Oct4*$^{\Delta}$. **(C)**: pNSC-derived neurospheres were absent from *Oct4*^{CKO}, while *Oct4*^{Ctl} are similar to non-transgenic (NT) control mice (2.67 vs. 2.80 neurospheres per 40,000 cells, respectively). $n = 8$. **(D)**: Definitive NSC (dNSC)-derived neurospheres were not affected by loss of *Oct4* expression. A total of 29.5 neurospheres per 5,000 cells in NT control animals versus 32.1 and 27.5 in *Oct4*^{Ctl} and *Oct4*^{CKO}, respectively. $n = 8$. **(E)**: Quantification of pNSC neurospheres per 100,000 cells in *Oct4*^{Ctl} and *Oct4*^{CKO} mice from embryonic, perinatal, and adult brains. $n = 2$–5 mice per genotype and timepoint. **(F)**: Quantification of dNSC neurospheres per 5,000 cells in *Oct4*^{Ctl} and *Oct4*^{CKO} mice from embryonic, perinatal, and adult brains. $n = 2$–5 mice per genotype and timepoint. **(G)**: Single sphere passaging of pNSC neurospheres from *Oct4*^{Ctl} and *Oct4*^{CKO} mice to test for self-renewal in LIF. *Y*-axis indicates number of pNSC neurospheres derived from each single passaged sphere. $n = 7$–13 neurospheres per genotype per timepoint. Abbreviations: Ctl, control; dNSCs, definitive neural stem cell; EFH, epidermal growth factor, fibroblast growth factor 2, and heparin; LIF, leukemia inhibitory factor; NT, non-transgenic; PND, postnatal day; pNSCs, primitive neural stem cells.

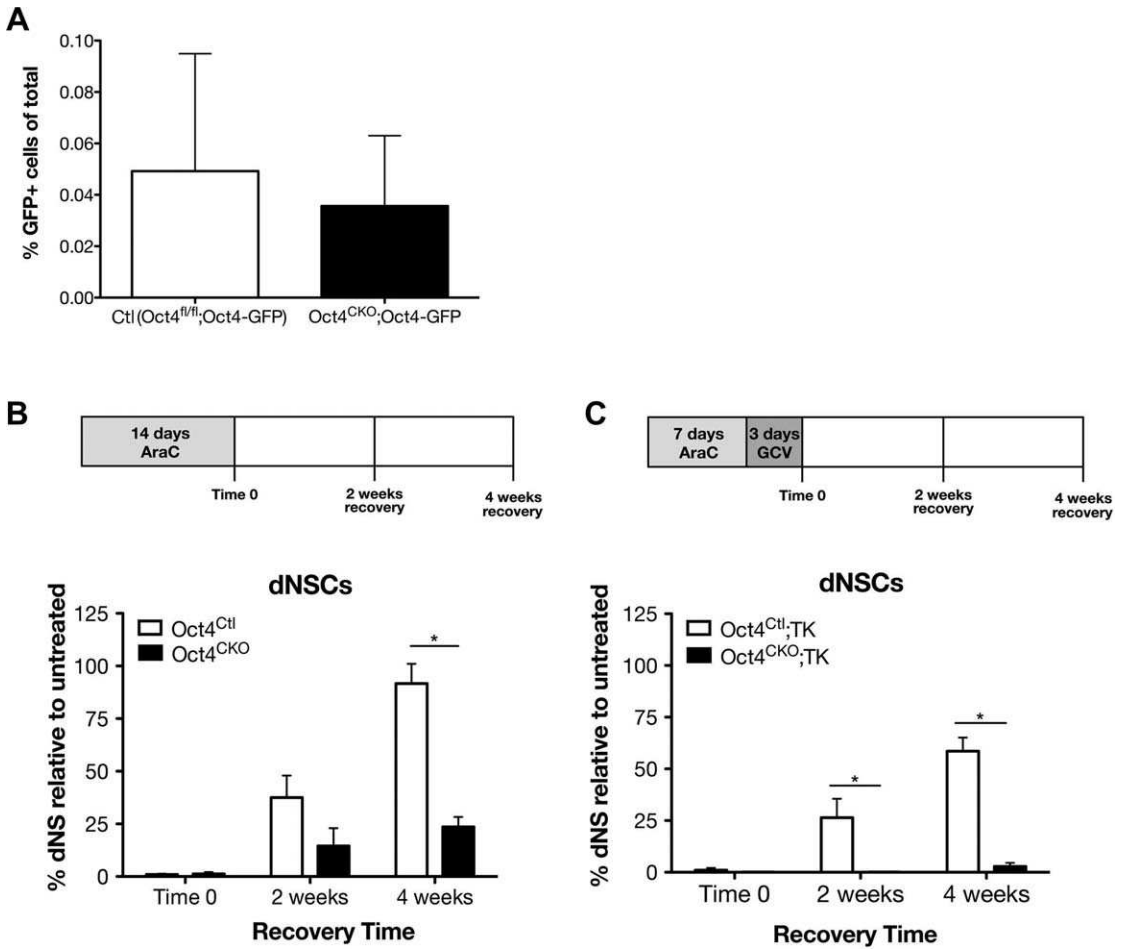


Figure 3. Primitive neural stem cells (NSCs) require *Oct4* for their ability to repopulate an ablated definitive NSC (dNSC) pool. **(A)**: Flow cytometry of Oct4-green fluorescent protein (GFP)$^+$ primary cells from the periventricular region of Tg(Oct4$^{IRES\text{-}GFP}$) animals crossed to other *Oct4$^{fl/fl}$* or *Oct4^{CKO}* mice. **(B)**: *Oct4^{CKO}* and *Oct4^{Ctl}* mice received a 2-week cytosine β-D-arabinofuranoside (AraC) infusion to ablate dNSCs and progenitors. *Oct4^{CKO}* had significantly reduced repopulation of dNSC-derived neurospheres compared with *Oct4^{Ctl}* (two-way analysis of variance [ANOVA] $F(2,18) = 12.45$, $p = .001$, $n = 4$). dNSC-derived neurospheres are quantified per 5,000 cells plated and expressed relative to untreated control. **(C)**: Ablation in *Oct4^{CKO}*;tk mice with 7 days of AraC infusion followed by 3 days of ganciclovir infusion induced a further reduction in the dNSC repopulation. *Oct4^{CKO}*;tk did not exhibit any dNSC repopulation after 2-week recovery and significantly reduced repopulation after 4-week recovery compared with *Oct4^{Ctl}*;tk (two-way ANOVA $F(2,13) = 11.42$, $p = .001$, $n = 4$). Abbreviations: AraC, cytosine β-D-arabinofuranoside; dNSCs, definitive neural stem cell; GCV, ganciclovir; GFP, green fluorescent protein.

neurospheres 4 weeks post-ablation, and the other half only gave rise to rare dNSC neurospheres (average of 5 neurospheres per 40,000 cells, a 2.5% recovery). This same effect was seen when we allowed mice to recover after ablation for 8 weeks, suggesting this lack of repopulation without functioning pNSCs is permanent and that Oct4$^+$ pNSCs are required for the repopulation of GFAP$^+$ dNSCs.

Fewer EdU$^+$ and SOX2$^+$GFAP$^+$ Cells Are Present in *Oct4^{CKO}*;Tk Mice after Ablation

After AraC/GCV ablation in *Oct4^{CKO}*;tk mice, EdU was administered in the drinking water during the last week (days 21–28) of recovery to label proliferating cells (Fig. 4A). Non-ablated wildtype mice, ablated *Oct4^{Ctl}*;tk mice and ablated *Oct4^{CKO}*;tk mice received EdU to compare the amounts of proliferation in the SEZ. EdU, SOX2, and GFAP were quantified in the periventricular region after a 4-week recovery from AraC/GCV ablation. After 4-week recovery, *Oct4^{CKO}*;tk mice had significantly fewer EdU$^+$ cells in the SEZ of the forebrain as compared to *Oct4^{Ctl}*;tk and untreated controls (Fig. 4B, 4C). Quantification of SOX2$^+$GFAP$^+$ cells revealed significantly fewer double positive cells in proximity to the lateral ventricle in *Oct4^{CKO}*;tk mice compared with *Oct4^{Ctl}*;tk mice and untreated controls (Fig. 4D, 4E). The number of SOX2$^+$GFAP$^+$ cells includes the dNSC population, and is consistent with the reduced repopulation of dNSC-derived neurospheres in *Oct4^{CKO}*;tk following AraC/GCV treatment (Figs. 3C, 4E).

SOX2$^+$GFAP$^+$ cells that were also EdU$^+$ were quantified to identify proliferating dNSCs. A greater proportion of SOX2$^+$GFAP$^+$ cells that were present were EdU$^+$ in *Oct4^{CKO}*;tk mice compared with *Oct4^{Ctl}*;tk (Fig. 4F). There were fewer EdU$^+$ proliferating cells and fewer SOX2$^+$GFAP$^+$ cells, but of the SOX2$^+$GFAP$^+$ cells that were present a higher percentage were proliferating compared with *Oct4^{Ctl}*;tk. This increased proliferation within the reduced SOX2$^+$GFAP$^+$ population suggests that in the absence of functioning pNSCs, SOX2$^+$GFAP$^+$

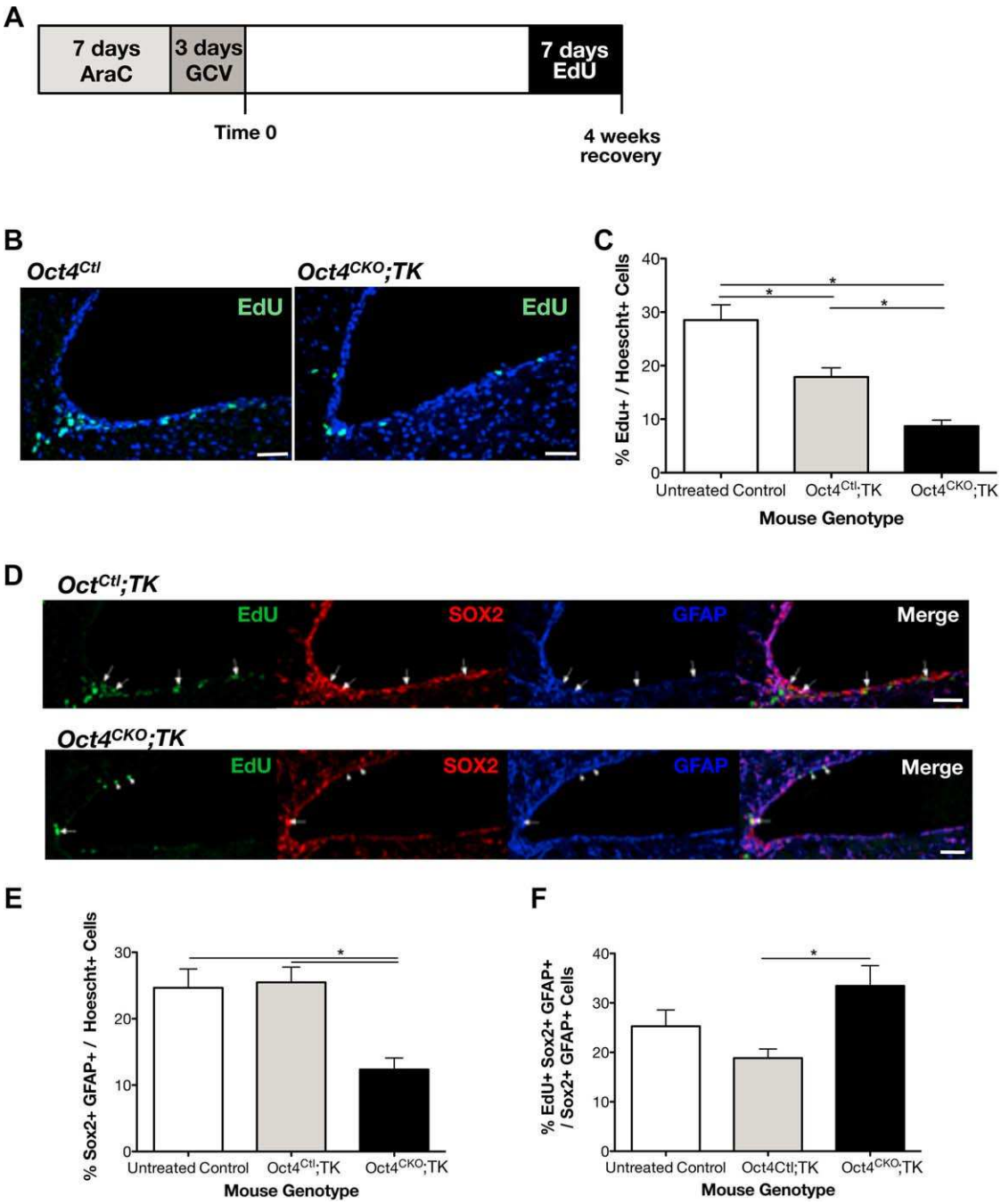


Figure 4. Reduced proliferation and definitive neural stem cell (dNSC) recovery in the periventricular region of *Oct4*^{CKO};tk mice after ablation. **(A)**: Mice received cytosine β-D-arabinofuranoside/ganciclovir ablation followed by a 4-week recovery with EdU delivered in the drinking water during the last week of recovery (days 21–28). **(B, C)**: *Oct4*^{CKO};tk mice had fewer EdU^{+} cells in the periventricular region as compared to *Oct4*^{Ctl};tk after recovery (two-way analysis of variance [ANOVA] $F(2,13) = 21.31$, $p = .0001$, $n = 5$). **(D)**: Visualization of glial fibrillary acidic protein (GFAP)$^{+}$SOX2^{+} cells and EdU^{+} coexpression. Regular arrow indicates EdU^{+}SOX2^{+}GFAP^{+}, short arrow indicates EdU^{+}SOX2^{+}GFAP^{-}, arrowhead indicates EdU^{+}SOX2^{-}GFAP^{+}. **(E)**: Fewer GFAP^{+}SOX2^{+} cells were observed in *Oct4*^{CKO};tk mice as compared to *Oct4*^{Ctl};tk and wildtype control (two-way ANOVA $F(2,11) = 7.40$, $p = .01$, $n = 5$). **(F)**: An increased proportion of SOX2^{+} GFAP^{+} cells were EdU^{+} in *Oct4*^{CKO};tk compared with *Oct4*^{Ctl};tk suggesting that dNSCs proliferate in an attempt to contribute to their repopulation (two-way ANOVA $F(2,12) = 5.17$, $p = .02$, $n = 5$). Abbreviations: AraC, cytosine β-D-arabinofuranoside; EdU, 5-ethynyl-2′-deoxyuridine; GCV, ganciclovir; GFAP, glial fibrillary acidic protein; TK, thymidine kinase.

precursors may undergo more proliferation to attempt to repopulate the neural lineage. Despite attempts through increased proliferation of the few remaining Sox2^{+}GFAP^{+} cells, the periventricular region of *Oct4*^{CKO};tk pNSC loss of function mice are unable to repopulate the ablated dNSCs.

pNSCs Become Activated and Proliferate after Antimitotic Ablation

While pNSCs do not seem to be necessary for dNSC maintenance under baseline conditions, we have shown that they are required for repopulating dNSCs and their progeny

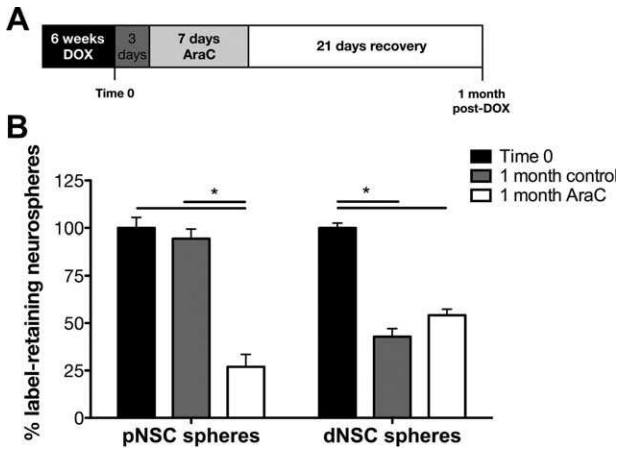


Figure 5. Primitive neural stem cells (pNSCs) exit quiescence and proliferate following cytosine β-D-arabinofuranoside (AraC) infusion. **(A)**: Histone-2B–green fluorescent protein mice received doxycycline (DOX) in the drinking water for 6 weeks, then after a 3-day rest, a 7-day intraventricular AraC infusion ablated proliferating cells, followed by a 21-day recovery. **(B)**: pNSCs do not dilute their label within 1 month without ablation, but after exposure to AraC significantly diluted their label (two-way analysis of variance $F(2,12) = 36.22$, $p = .001$, $n = 3$). Definitive NSCs did not show reduced labeling after AraC treatment compared with control animals 1 month post-DOX. Abbreviations: AraC, cytosine β-D-arabinofuranoside; DOX, doxycycline; pNSC, primitive neural stem cell.

following ablation, in a process that requires *Oct4*. To confirm that it is quiescent pNSCs being activated by downstream dNSC ablation, we returned to the H2B-GFP mouse model. Mice were exposed to DOX to label cells, and 3 days later, AraC was infused into the lateral ventricles for 7 days, followed by recovery for 21 days (Fig. 5A). In this paradigm, cells activated to proliferate following AraC infusion will dilute their label compared with control mice that did not receive AraC. While pNSCs in control (non-ablated) mice do not demonstrate label dilution after 1 month, mice that received AraC gave rise to significantly fewer labeled pNSC-derived neurospheres (Fig. 5B). This indicates that pNSCs were induced to proliferate following AraC infusion. In contrast, dNSC-derived neurospheres were equally labeled from 1-month control mice and 1-month with AraC mice (Fig. 5B). This does not preclude the proliferation of dNSCs, but rather suggests that the dNSC repopulation after AraC treatment arose from labeled pNSC precursors that passed on their labeling in vivo. The dilution of the H2B-GFP label in pNSCs but not dNSCs after AraC treatment supports a lineage model where *Oct4*-expressing pNSCs are upstream of $GFAP^+$ dNSCs and proliferate to repopulate dNSCs after ablation.

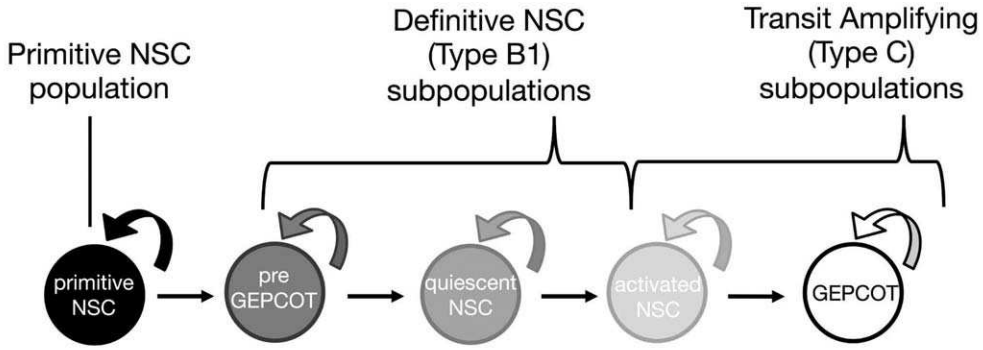
Discussion

The detection of *Oct4* in adult somatic stem cells has been widely challenged (Lengner et al., 2008) [20]. We previously reported that adult-derived pNSCs express *Oct4* [4] and the present report demonstrates that *Oct4* is necessary for pNSC proliferation. Another study reported the absence of *Oct4* expression in the mouse brain and that conditional inactivation of the *Oct4* alleles did not result in any phenotypic abnormalities or changes in subependymal $Ki67^+$ labeling [21]. However, due to the rarity of pNSCs, it would be easy to miss *Oct4* expression and this study did not induce an injury and ablate downstream cells, which we demonstrate was required to observe the phenotype. In agreement with ref. [21], we did not observe a difference in the abundance of EdU^+ cells in the periventricular region of untreated $Oct4^{CKO}$;tk mice versus C57BL/6 mice. In contrast, we previously reported *Oct4* expression specifically within the adult subependyma [4] and herein demonstrate that *Oct4* expression is crucial to pNSC function and the repopulation of lost dNSCs. This is the first time that Oct4 expression has been demonstrated in the adult mouse brain and the first time it has been demonstrated to be necessary for the function of an adult cell. Although Oct4 was reported to be expressed in an adult stem cell population, very small embryonic-like cells [22], this finding has not been replicated (Danova-Alt et al., 2013) [23, 24]. This leaves the quiescent pNSC as the only adult stem cell to express and more interestingly, to require, Oct4 for its function.

In many stem cell lineages, quiescence is hypothesized to be a mechanism of preventing stem cell exhaustion and/or protecting against replication-related mutations [25–28]). This is well characterized in the rare long-term repopulating hematopoietic stem cells (HSCs), which reside at the top of the HSC hierarchy [11, 29, 30].

Long-term label retention indicated that pNSCs are quiescent in the adult mouse brain and we estimate that they divide once every 3–5 months, which corresponds to 5–8 divisions in the lifetime of a mouse. This is similar to label-retaining primitive HSCs (also referred to as long-term or dormant HSCs) that divide once every 4.8 months [11, 29]. These primitive HSCs are normally quiescent, but become activated following exposure to 5-fluorouracil, an antimitotic drug, to repopulate the downstream hematopoietic lineage [29]. Primitive HSCs are very rare but have the greatest repopulation ability amongst blood stem cells after transplantation [31]. In addition to the commonalities of rarity and long cell cycle times of these two stem cell populations, primitive HSCs are negative for the common HSC marker CD34 [30, 31]. This may be considered similar to pNSCs being negative for the traditional NSC marker, GFAP, and could reflect a mechanism whereby lineage-related brain stem cells have different characteristics to limit their sensitivity to similar stresses and maintain a reserve population. Therefore, pNSCs fit into a stem cell hierarchy where uniquely marked, rare, and quiescent cells reside at the top of the adult stem cell hierarchy.

Here, we show that quiescent pNSCs can proliferate to repopulate the downstream neural lineage, including ablated $GFAP^+$ dNSCs. pNSCs cannot proliferate in $Oct4^{CKO}$ mice, thus these mice were used as a pNSC loss of function mouse model to test their role in repopulation of ablated dNSCs and downstream progenitors. $Oct4^{CKO}$;tk mice treated with AraC to kill proliferating cells and bring $GFAP^+$ dNSCs into cycle, then GCV to kill all dividing $GFAP^+$ cells, exhibited a significantly reduced dNSC repopulation, and significantly fewer EdU^+ cells and $SOX2^+GFAP^+$ cells (comprising dNSCs) after recovery. We previously demonstrated that *GFAP*-expressing cells that do not divide during GCV exposure will still be killed upon division up to 21 days post-GCV [4], likely due to retention of metabolized GCV exclusively in *GFAP*-expressing cells with the transgenic tk. This supports the interpretation that



	pNSC	pre GEPCOT	qNSC	dNSC	aNSC	GEPC OT	TA
Oct4	+	–	–	–	–	–	–
GFAP	–	–/+	+	+	+	–	–
EGF-R	–	–	–	+	+	+	+
nestin	–	–	–	+	+	+	+
% pop	0.08	6	2.5	0.2-0.4	2.1	3	10
cell cycle	3-5 months	33 days	5-6 days	15 days	3 hours	6 hours	12.7 hours
% NIC	0.004	.06	1	0.6	20	27	0
self-renewal	✓ SS	✓ Bulk	✓ Bulk	✓ SS	✓ Bulk	✓ Bulk	N/A
multipotent	✓ SS	✓ Bulk	✓ Bulk	✓ SS	✓ Bulk	✓ Bulk	N/A
reference	Sachewsky et al., 2014	Mich et al., 2014	Codega et al., 2014	Morshead et al., 1994, 1998	Codega et al., 2014	Mich et al., 2014	Morshead et al., 1992

Figure 6. Proposed unified theory of the adult neural stem cell (NSC) lineage. We present a hypothesis integrating our data on primitive NSCs (pNSCs) reported here and in ref. [4], recent reports of ref. 2, 3, and past reports of definitive NSCs/type B cells [9] (Morshead et al., 1998). We used data presented within these reports as best possible to generate data for comparisons across the cell populations to generate a proposed NSC lineage. % pop indicates percentage of population within the periventricular region. % NIC indicates percentage of neurosphere initiating cells in the neurosphere assay (all cultured in epidermal growth factor, fibroblast growth factor 2, and heparin except for pNSCs, which are cultured in leukemia inhibitory factor). Glial fibrillary acidic protein: [2], Figure 3A, 3C; [3] Figure 4A. % pop: [2], Figure 3A, 3B; [3], Figures 1B and 3E. cell cycle: [2], Figure 3E (quiescent NSCs were 0.8% labeled after 1 hour BrdU pulse = 100% labeled after 5.2 days), Figure 3E (activated NSCs were 35.5% labeled after 1 hour BrdU pulse = 100% labeled after 3 hours); [3], Figure 1D (GEPCOT cells were 35% labeled after 2 hours BrdU pulse = 100% after 6 hours), Figure 4C (pre-GEPCOT cells were 3% labeled after 24 hours BrdU pulse = 100% labeled after 33 days). % NIC: [2], Figure 5K; [3], Figures 1C and 4B. Abbreviations: aNSC, activated NSC; EGF-R, epidermal growth factor receptor; GFAP, glial fibrillary acidic protein; NIC, neurosphere initiating cell; NSC, neural stem cell; qNSC, quiescent NSC; SS, single-sphere passaging; TA, transit amplifying cell.

the repopulation of $GFAP^+$ dNSCs is due to a precursor cell that does not express *GFAP*, rather than due to a quiescent $GFAP^+$ population.

Only two pNSC loss of function mice had a few dNSC neurospheres (2.7% of controls) 4 weeks after AraC + GCV, and this likely resulted from dNSCs that were not ablated in the AraC + GCV paradigm, whereas the $Oct4^{CKO}$;tk mice that had zero dNSCs 4 and 8 weeks post-ablation likely represent mice with complete ablations. Regardless, the significant difference in recovery between $Oct4^{CKO}$;tk mice and $Oct4^{Ctl}$ mice suggests that $Oct4^+$ pNSCs are responsible for the vast majority of repopulation of dNSCs and the neural lineage. In addition, increased $SOX2^+GFAP^+EdU^+$ cells in $Oct4^{CKO}$;tk compared with $Oct4^{Ctl}$;tk suggest that surviving $SOX2^+GFAP^+$ precursors proliferate in the absence of pNSCs, although it is insufficient to repopulate dNSCs.

To confirm the proliferation of pNSCs in response to ablation of dNSCs, AraC was administered to H2B-GFP mice and demonstrated significantly reduced label retention in the pNSCs indicating an exit from quiescence to repopulate the downstream lineage. Therefore, these data support that pNSCs fit into a stem cell hierarchy that is similar to those reported in other lineages where rare, quiescent cells reside at the top of the lineage and act as a reserve stem cell pool [27, 28].

Recovery of ablated dNSCs and downstream progenitors after AraC or AraC/GCV in $Oct4^{CKO}$;tk mice supports a NSC

hierarchy whereby pNSCs give rise to dNSCs [4]. It appears that dNSCs are capable of maintaining their own population in the absence of ablation, as untreated *Oct4*^{CKO} did not give rise to any pNSC-derived neurospheres but generated a normal abundance of dNSC-derived neurospheres. Despite the ability to maintain their own population at baseline, dNSCs require pNSCs for their repopulation. We predict that had excision of *Oct4* occurred earlier in development than after ~E8 when driven by the *Sox1* promoter in *Oct4*^{CKO} mice, the initial establishment of GFAP^{+} dNSCs would have been impaired, resulting in the absence of most of the brain.

Despite reports of ependymal cells activated after injury to act as stem cells [32, 33], pNSCs are not ependymal-derived, based on the subependymal position of Tg(Oct4$^{IRES-GFP}$) cells in periventricular whole mounts and on the findings that pNSC-derived neurospheres self-renew and are not ciliated [4, 5]. We performed cell cycle analysis on pNSCs and dNSCs with the H2B-GFP mouse model, which holds great benefit over BrdU or EdU label retention models, as the H2B model does not require a cell to be proliferating to take up the initial label. This advantage of the H2B system has allowed us to study cells with such long cell cycle times that they would have been missed in BrdU-, EdU-, and CldU-based experiments [2, 3, 34].

Furthermore, we propose that pNSCs are upstream of the proposed quiescent (q)NSCs described in ref. 2 and pre-GEPCOT cells [3], which would both fit into our definition of dNSCs given their *GFAP* expression (Fig. 6). pNSCs are the only NSC type that are Oct4^{+} and GFAP^{-}; they are the most rare, slowest proliferating, and have the lowest rate of neurosphere formation. Pre-GEPCOT cells were reported to be upstream of GEPCOT cells, which are Glast^{mid}EGFR^{high}PlexinB2^{high}CD24$^{-/low}$O4/PSA-NCAM$^{-/low}$Ter119/CD45^{-} [3]. Pre-GEPCOT cells are slowly proliferating with low neurosphere-forming abilities and have mixed *GFAP* expression and are the most abundant in the niche [3]. Quiescent NSCs (qNSCs) as defined by Codega et al. are the first cell population in the lineage to be exclusively GFAP^{+}, maintain a low rate of neurosphere formation and we suggest that they have a shorter cell cycle of 5–6 days (based on BrdU pulse data [2]). Therefore, pNSCs, Pre-GEPCOTS, and qNSCs are all characterized by low neurosphere forming ability (Fig. 5), although it remains possible that only a subpopulation of cells positive for the classifying cell markers function as stem cells.

Downstream, activated NSCs are more mitotically active, have EGF-R expression, and mixed mid/high GFAP expression [2]. GEPCOT cells have lower GFAP expression than activated NSCs and slightly slower cell cycle times (based on our calculation from their BrdU pulse data, Fig. 6) [3]. It remains unclear which of these populations are independent or overlapping cell populations, and whether some cell types may be different states of the same cell population.

We present a unified hypothesis of the NSC lineage (Fig. 6), and it remains of great interest to investigate these NSC hierarchies in relation to type B[1] and type C cells [35] described at the electron microscopic level. We suggest that pNSCs reside at the top of the NSC lineage, and as an adult somatic cells expressing *Oct4*, may have interesting implications for their ability to repopulate the adult neural lineage.

Future studies, including lineage tracing under baseline conditions, would be beneficial to consolidate our report with these other populations of NSCs to directly illuminate their lineage relationship.

Acknowledgments

We thank Nadia Sachewsky and Wenjun Xu for their insight and helpful discussion regarding the pNSC project, the members of the van der Kooy laboratory for their contribution to the manuscript, and Brenda Coles for technical assistance. This work was supported by CIHR, CFREF (Medicine By Design), Canadian Stem Cell Network, Ontario Brain Institute and NeuroDevNet NCE.

Author Contributions

R.L.R.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; S.Z.Y.: collection and assembly of data, data analysis and interpretation, manuscript writing; C.M.M.: conception and design, data analysis and interpretation; D.v.d.K.: conception and design, financial support, data analysis and interpretation, manuscript writing.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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