Phillip Karpowicz, Cindi Morshead, Angela Kam, Eric Jervis, John Ramunas, Vincent Cheng, and Derek van der Kooy

Vol. 170, No. 5, August 29, 2005. Pages 721–732.

In the original version of this article, an author's name was misspelled. The correct spelling is John Ramunas.

Support for the immortal strand hypothesis: neural stem cells partition DNA asymmetrically in vitro

Phillip Karpowicz,[1] Cindi Morshead,[2] Angela Kam,[2] Eric Jervis,[4] John Ramunas,[4] Vincent Cheng,[2] and Derek van der Kooy[3]

[1]Institute of Medical Science, [2]Department of Surgery, and [3]Department of Medical Genetics and Microbiology, University of Toronto, Toronto, M5R 1A8, Canada
[4]Department of Chemical Engineering, University of Waterloo, Waterloo, N2L 3G1, Canada

The immortal strand hypothesis proposes that asymmetrically dividing stem cells (SCs) selectively segregate chromosomes that bear the oldest DNA templates. We investigated cosegregation in neural stem cells (NSCs). After exposure to the thymidine analogue 5-bromo-2-deoxyuridine (BrdU), which labels newly synthesized DNA, a subset of neural precursor cells were shown to retain BrdU signal. It was confirmed that some BrdU-retaining cells divided actively, and that these cells exhibited some characteristics of SCs. This asymmetric partitioning of DNA then was demonstrated during mitosis, and these results were further supported by real time imaging of SC clones, in which older and newly synthesized DNA templates were distributed asymmetrically after DNA synthesis. We demonstrate that NSCs are unique among precursor cells in the uneven partitioning of genetic material during cell divisions.

Introduction

A single cell can produce two dissimilar progeny in two fashions. A cell can undergo a symmetric division yielding identical daughters. If each daughter cell is then exposed to different microenvironments, after that division, either might cease to resemble its counterpart even though both had originally been spawned as equivalent cells and had been equivalent for a brief time. Alternatively, two daughter cells could be uniquely specified by inducing a mitotic cell to localize components on one side, and then separating such components by varying the cleavage plane during cytokinesis (Kusch et al., 2003). Thus each daughter would be primed to adopt a particular functional identity due to the uneven segregation of such components. There is increasing evidence that the latter of these may take place in dividing cells. Animal cells have been shown to unevenly segregate determinants of molecular programs before or during mitosis to specify the subsequent fate of their daughters. Both protein determinants (Freeman and Doe, 2001; Shen et al., 2002) and mRNA determinants (Lambert and Nagy, 2002) have been identified. *Saccharomyces cerevisiae* yeast have been shown to preferentially segregate their older, oxidatively damaged, proteins away from newly budding cells (Aguilaniu et al., 2003). Indeed, with the evidence that *S. cerevisiae* (Liakopoulos et al., 2003) and fruit fly germ cells (Yamashita et al., 2003) regulate the orientation of their plane of division, there is reason to suggest that the decision to divide asymmetrically takes place routinely.

Intriguingly, it has been suggested that DNA itself is segregated unevenly between recipient daughter cells. Such a separation would not be a reversible one, like unevenness in protein or mRNA distribution, both of which could theoretically be regulated after division so that dissimilar daughter cells might eventually establish an equivalence in certain biochemical pathways. Asymmetric DNA distribution would be an immutable physical discrepancy between daughter cells that would define a division as asymmetric by virtue of an inherent, and measurable, physical difference in cells containing original templates and cells containing newly synthesized DNA. Such a separation was first interpreted from the uneven distribution of ^{3}[H]thymidine in proliferating in vitro mouse embryonic fibroblasts (Lark et al., 1966), and later experiments suggested that stem cells (SC) in the intestinal epithelium of mice also segregated their chromosomes asymmetrically (Potten et al., 1978, 2002). Recent evidence continues to support chromosome cosegregation in mutated fibroblasts (Merok et al., 2002). This asymmetric distribution of chromosomes in dividing SCs was originally dubbed the immortal strand hypothesis (ISH; Cairns, 1975). Such a mechanism was envisioned to reduce the incidence of mutations arising from errors in DNA synthesis and repair in future progenitor cells derived from the SCs. An asymmetry in DNA inheritance between daughter cells might

Correspondence to P. Karpowicz: phillip.karpowicz@utoronto.ca

Abbreviations used in this paper: BrdU, 5-bromo-2-deoxyuridine; CFSE, carboxyfluorescein diacetate succinimidyl ester; DiI,1-dioctadecyl-3-tetramethylindocarbocyanine perchlorate; DIV, days in vitro; ESC, embryonic stem cell; GFAP, glial fibrillary acidic protein; ISH, immortal strand hypothesis; NSC, neural stem cell; SC, stem cell.

The Journal of Cell Biology, Vol. 170, No. 5, August 29, 2005 721–732
http://www.jcb.org/cgi/doi/10.1083/jcb.200502073

also retain sequence fidelity for genes conferring pluripotency to SCs. It has been suggested that SCs in somatic tissues actively suppress chromosome recombination events (Potten et al., 1978, 2002), and are exceptionally sensitive to DNA damage as demonstrated by the high incidence of apoptosis in irradiated SC populations. SCs are thus defined partially by their function to transmit a faithful copy of DNA template to future cell generations.

Many studies have failed to support the ISH in *S. cerevisiae* (Neff and Burke, 1991), mouse epidermal basal cells (Kuroki and Murakami, 1989), the proliferating cells of *Caenorhabditis elegans*, as well as murine embryos (Ito and McGhee, 1987; Ito et al., 1988). These positive and negative findings are equivocal as supporting and dissenting studies used distinct and contrasting cell types, at distinct and contrasting periods of an organism's development. Moreover, if such a mechanism manifests itself only in SCs, it may easily be overlooked as these comprise a minority in the cell population. Evidence of chromosome segregation in most studies to date has been undertaken retrospectively at the population level. Thus after three decades of research, it is still an open question if actively dividing SCs cosegregate older and newer DNA asymmetrically during mitosis.

According to the ISH, SCs cosegregate chromosomes to retain older DNA templates in one daughter SC but not the non-SC daughter (Fig. 1). Given that DNA replication is semiconservative, cosegregated chromosomes are distinguished because they contain one older strand, albeit one that is associated with a newer strand from one preceding round of DNA synthesis. We predicted that symmetric SC divisions would randomize segregation of chromosomes between daughter cells. The ISH was investigated in neural stem cells (NSCs) using a clonal cell culture system in which brain-derived colonies, arising from a single SC, are both self renewing and multipotent (Reynolds and Weiss, 1992; Morshead et al., 1994). The halogenated thymidine analogue, 5-bromo-2-deoxyuridine (BrdU) was used to label DNA strands. We asked: (a) would SCs retain BrdU(+) DNA strands in the absence of BrdU, if they divided symmetrically many times in the presence of BrdU (see Fig. 2 A); and (b) would SCs retain their original BrdU(−) strands, in the absence of BrdU, if they divided asymmetrically once and only once in the presence of BrdU (see Fig. 7).

It was expected that SCs would incorporate BrdU into newly synthesized DNA strands copied from unlabeled DNA templates during S-phase. A sufficiently long pulse of BrdU would ensure that at least some NSCs would contain mostly labeled DNA (see Fig. 2 A). If BrdU were removed after uptake, at least some SC's DNA strands would be labeled, and such cells would preferentially segregate these labeled strands as immortal strands. SCs could preferentially retain BrdU-labeled immortal strands if and only if such SCs divided symmetrically in the presence of BrdU, and thus selected and retained some BrdU-labeled strands as immortal strands through multiple asymmetric divisions. This long term labeling strategy was incorporated into the culture of NSCs (see Fig. 2 B). Alternatively, if SCs divided only asymmetrically once in the presence of BrdU label, they would lose the label during one asymmetric division event after analogue withdrawal. Such cells would specifically retain the original unlabeled DNA strands as immortal strands; having undergone no symmetric SC divisions, which might select newly synthesized DNA strands as immortal strands (see Fig. 7 A).

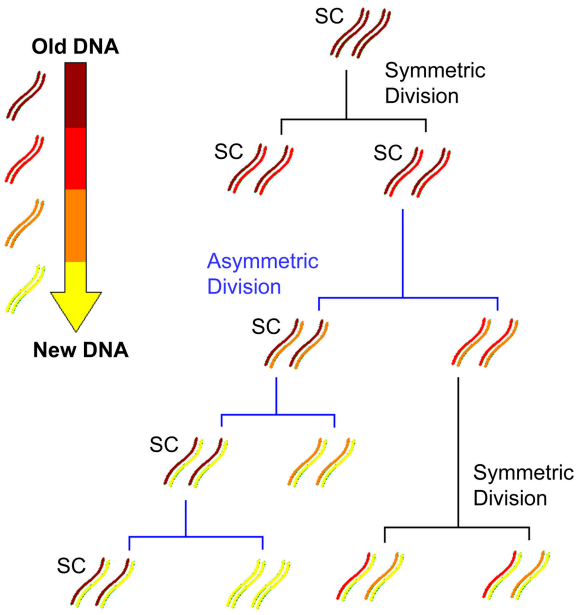


Figure 1. **Immortal strand hypothesis.** During asymmetric SC divisions, chromosomes containing oldest template DNA (dark red) are segregated to SCs. DNA is replicated semiconservatively, each chromosome contains one older template strand. Complements of old DNA–containing chromosomes are cosegregated through many rounds of asymmetric cell division, although symmetric SC divisions segregate chromosomes randomly. Thus over time, SCs contain proportionally more template-containing chromosomes than any other cells in the population, which contain mostly newer synthesized DNA (yellow).

Here we present in vitro evidence that old and new DNA templates are distributed asymmetrically in NSC divisions in clonal population studies and at the single cell level.

Results

Clonal neural precursor colonies are heterogeneous for BrdU

NSCs from the forebrain ventricles of adult mice can be induced to divide in vitro when they are cultured in the presence of proliferation-inducing mitogens. These form clonally derived neurospheres, spherical colonies of coalescent cells, which can be induced to terminally differentiate only upon the removal of mitogens and the addition of serum. SCs in these colonies comprise a minority of the total cells present, the majority of cells in colonies being committed neuronal or glial progenitors that do not posses the ability to self-renew (Morshead et al., 1998). In vivo such cells are thought to divide mainly asymmetrically (Morshead et al., 1998). We examined the distribution of BrdU in SC colonies grown at clonal density. It has been shown that murine cells do not take up detectable BrdU during DNA repair, at concentrations 300-fold higher than in our conditions of 0.6 μM (Palmer et al., 2000)

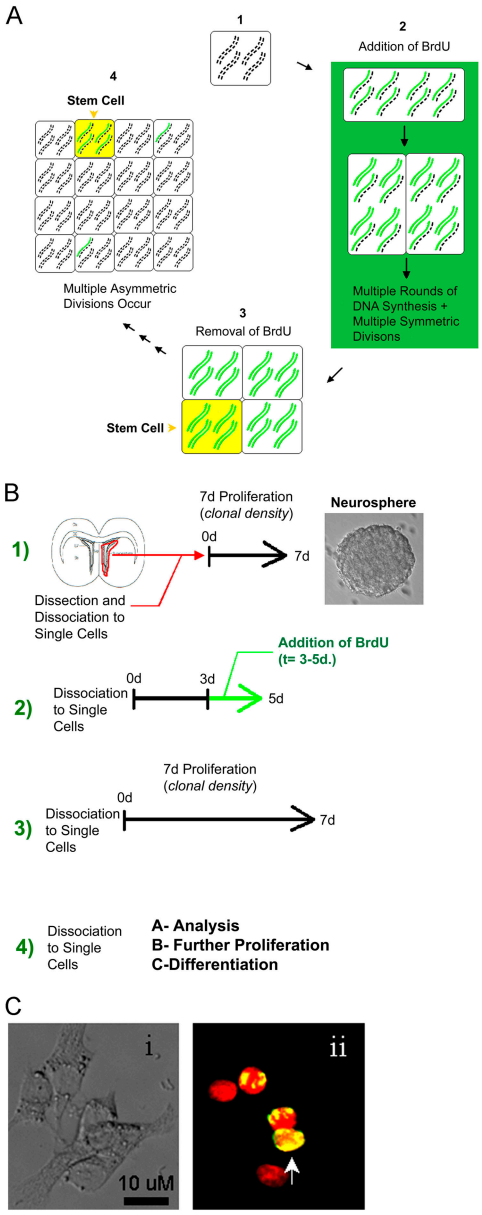


Figure 2. **After a 2-d exposure to BrdU, cells from adult murine forebrain ventricles demonstrate heterogenous BrdU labeling in vitro.** (A) BrdU retention strategy: (1) Each double strand (1 chromosome) represents 10 chromosomes of a mouse cell. Cells are unlabeled for BrdU (black). (2) During multiple rounds of DNA synthesis, BrdU (green) is taken up and distributed in both symmetric and asymmetric divisions in the presence of BrdU. (3) BrdU is removed and the daughter cells now undergo DNA synthesis in the absence of BrdU. (4) BrdU should be retained if labeled chromosomes are cosegregated as immortal strands into SCs. (B) BrdU–neurosphere assay. (1) Cells from adult forebrain lateral ventricles are cultured for 7 d at clonal density. (2) After dissociation, cells are pulsed with BrdU for 2 d, at 3 DIV. (3) BrdU is removed and cells are passaged at clonal density for an additional 7 d. (4) Cells are examined (A), passaged (B), or differentiated (C). (C) 10 d after BrdU exposure, cell clones still contained heavily BrdU(+) cells (arrows) and BrdU(−) cells. The retention of BrdU(+), in cells seeded at clonal density, suggests that BrdU(+) cells give rise to both labeled and unlabeled progeny. (i) Bright field shows 3 d cell clumps; (ii) histone-labeled nuclei are red, BrdU-labeled cells are green; merge shows overlap as yellow.

making it highly unlikely that cells would take up detectable analogue during DNA repair. After a BrdU pulse of 2 d, we found that 98.6 ± 0.2% of all cells were BrdU(+). Cells that were not labeled were either postmitotic, or had a cell cycle >2 d.

Treatment of cells with BrdU did not alter SC phenotype. BrdU(+) cells retained the ability to self-renew, as demonstrated by subcloning secondary, tertiary, and quaternary BrdU-treated cell clones after BrdU exposure (unpublished data). Untreated clones grew to an average diameter of 144 ± 8 microns at 7 d, similarly to their BrdU-treated counterparts, which were 149 ± 5 microns in diameter. We determined that a clonally derived 149 micron BrdU-pulsed colony represented 3,075 ± 91 cells in total.

When primary SC colonies were passaged twice in the absence of BrdU, tertiary colonies generally had one or a few BrdU(+) cells 10 d after BrdU exposure (Fig. 2 C). In all cases, such colonies arose clonally from single BrdU(+) cells, that had not diluted out BrdU label over 10 d. This suggested either that: the proliferating founder BrdU(+) cells were cycling at a slow rate relative to their progeny; or, were postmitotic cells that had arisen in the first division of an actively proliferating BrdU(+) founder cell, which itself kept dividing to dilute out BrdU label; or, alternatively, were a result of heterogeneity in chromosome segregation.

Neural precursors retain BrdU in contrast to embryonic stem cells and fibroblasts

Neurosphere cells were exposed to BrdU and then proliferated in the absence of BrdU. Population expansion was assessed simultaneously with the presence of BrdU label in dissociated cells up to 10 d after BrdU withdrawal (Fig. 3 A). This period of time spanned an estimated 9 to 10 population doublings. However, this is likely to be an underestimate of the actual number of cell divisions as there is considerable cell death in clones that was not taken into account (unpublished data). Indeed, as the average colony contains over 3,000 cells after 1 wk of culture, this represents ~12 population doublings in the absence of cell death. Over time we observed an attenuation of BrdU signal in many cells. At day 7, most cells (33.6 ± 2.0% BrdU[+]) ceased to possess any detectable BrdU signal. This is likely to be a result of the attenuation of BrdU signal via cell divisions to a threshold at which the presence of BrdU is so slight that it cannot be detected by immunocytochemistry using our detection protocol. Despite this severe loss, there was a striking perseverance of BrdU-labeled cells at 10 population doublings, day 10, with 8.7 ± 1.3% of cells exhibiting varying levels of BrdU(+) signal.

We repeated this exact experiment using the R1 embryonic stem cell (ESC) line (Nagy et al., 1993). R1's are an SC population, possessing the characteristics of self-renewal in vitro and multipotentiality in vivo. R1 cells did not retain BrdU (Fig. 3 B). Over a 9-d period, we observed approximately eight to nine doublings. This demonstrated that the population doubling rate of BrdU-treated R1 cells was similar to that of neurosphere cells. By day 7 (six doublings) only 1.3 ± 0.4% of cells

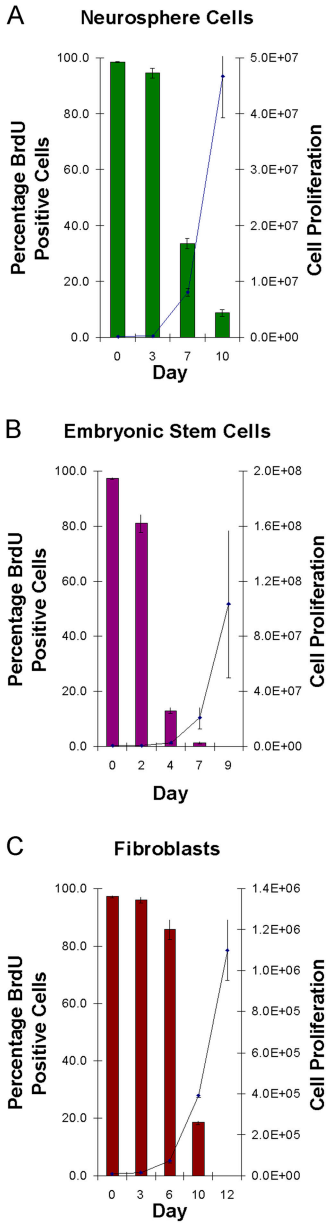


Figure 3. **Neurosphere cells retain BrdU in contrast to ESCs and fibroblasts.** (A) Proportion of BrdU-labeled cells (bars) and population expansion (line) in adult neurosphere culture. 98.6 ± 0.2% of 62,500 cells plated are BrdU(+). At day 3, cells have achieved two population doublings, and at 7 d, seven doublings. 8.7 ± 1.3% of cells retain BrdU signal at the 10 d time point of 10 population doublings. (B) Proportion of BrdU-labeled cells (bars) and population expansion (line) in the R1 ESC line. Between 2 and 4 d, embryonic cells have reached the threshold during which BrdU is lost, demonstrated by a dramatic decrease from 81.1 ± 3.2% to 13.0 ± 1.2% cells labeled. ESCs lose all BrdU signal after seven doublings evidenced by day 7 (six population doublings). (C) Proportion of BrdU-labeled cells (bars) and population expansion (line) in the STO fibroblast cell line. At day 6, cells achieve three populations doublings demonstrating that fibroblasts have 2× the cell cycle time as neural precursors. At day 12, and with less than seven doublings, fibroblasts' BrdU signal is abolished.

possessed traces of the analogue, and this was extinguished completely at day 9.

We assayed a second group of cells: the STO/SNL fibroblast cell line, transformed cells derived from embryonic mice that were passaged >40 times. Fibroblasts are not thought to be SCs. Like R1's, STO fibroblasts did not retain BrdU (Fig. 3 C). By five to six population doublings, at day 10, only 18.6 ± 0.7% of the cells retained any BrdU signal, and this was completely eradicated at day 12, which corresponds to less than seven doublings.

In both ESCs and fibroblasts, BrdU extinction was noted when cells were expanded over seven population doublings. A mouse cell, which contains 40 BrdU(+) chromosomes and which halved chromosomes containing BrdU label in each division symmetrically would indeed dilute this number to one single chromosome after five to six division events. On average, seven cell divisions in the absence of BrdU are sufficient to extinguish the label if cells partition BrdU-labeled chromosomes randomly. In contrast to fibroblasts and ESCs, which are thought to divide only symmetrically, NSC colonies contained cells able to retain the analogue well past this seven-division dilution threshold.

We attempted to see if NSCs would eventually dilute out all BrdU through symmetric divisions. BrdU-exposed SC colonies were passaged four times in the absence of BrdU. Overall this represents >25 doublings, and indeed in only a few cases were we able to find BrdU-labeled cells in colonies maintained past 14 such doublings (unpublished data). NSCs do not divide asymmetrically exclusively, but can certainly divide symmetrically, as evidenced by the formation of multiple secondary colonies arising from a single subcloned NSC colony plated at clonal density (Morshead et al., 1994). Only cells that have not divided symmetrically more than seven times retain BrdU at a detectable level. Symmetric divisions in neurosphere culture may account for the eventual loss of all BrdU signal in all cells, and asymmetric divisions may explain the retention of the BrdU signal in contrast to ESCs and fibroblasts.

BrdU-retaining cells are not quiescent

If BrdU-retaining cells were postmitotic or relatively mitotically stagnant, such heterogeneity in cell cycle within the neurosphere cell population would explain the contrast between these cells and embryonic cells or fibroblasts.

1-Dioctadecyl-3-tetramethylindocarbocyanine perchlorate (DiI) is a lipophilic fluorescent dye that associates with cell membranes and carboxyfluorescein diacetate succinimidyl ester (CFSE) is a cytosolic dye that renders cells fluorescent upon uptake. We reasoned that cells initially positive for either such dyes would subsequently halve their fluorescent dye intensity after each division, as the dye was redistributed among the daughter cells. This would enable the separation of fractions of cells that were dividing quickly from their quiescent counterparts, before such cells were examined for the presence of BrdU. DiI has already been proven amenable to FACS analysis (Malatesta et al., 2000) and there is no evidence that DiI can be passed between adjacent cells. Nonetheless, we cocultured neurosphere cells that had been exposed to DiI, with GFP(+)/DiI(−) neurosphere cells in high cell density aggregated colonies for 1 wk. We confirmed that none of the GFP(+) cells took up DiI label, confirming that the dye cannot be shared between adjacent cells (unpublished data).

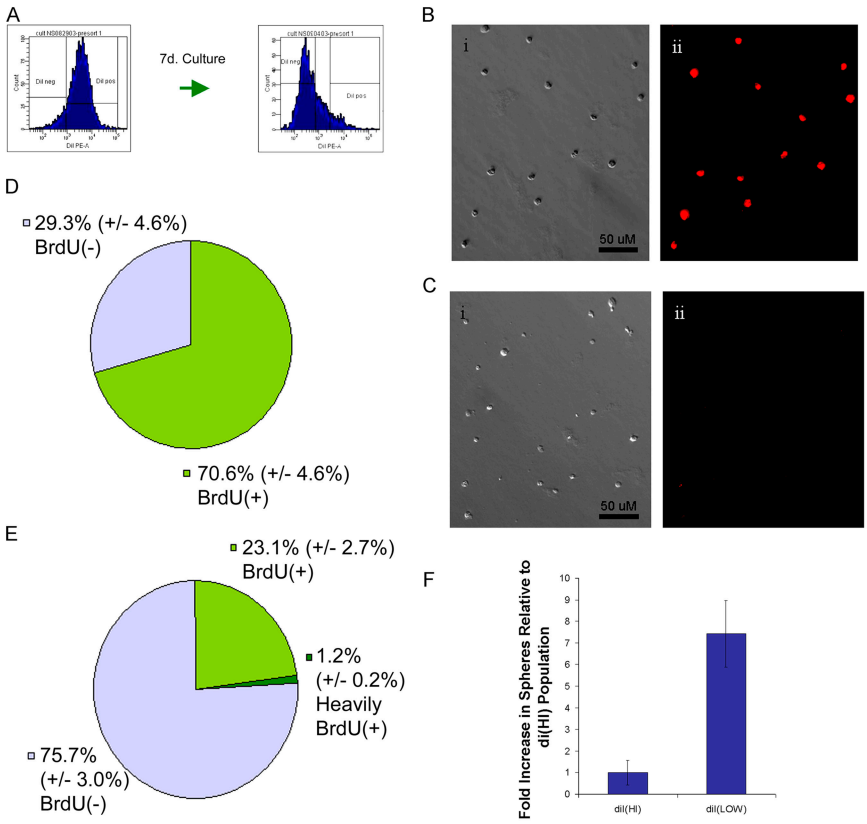


Figure 4. **NSCs are fast dividing cells in vitro, and show BrdU retention.** (A) Distribution of DiI, initially and after 1 wk in vitro. DiI signal decreases as a result of DiI dilution via cell proliferation. (DiI pos) DiI(HI+) fraction; (DiI neg) DiI(LOW+) fraction. (B) DiI(HI+) fraction of slowly dividing neurosphere cells where DiI signal is vivid. (i) Dissociated cells in bright field; (ii) DiI signal in red. (C) DiI(LOW+) fraction of rapidly dividing neurosphere cells where DiI signal is noticeably lower than in DiI(HI+) fraction. (i) Dissociated cells in bright field; (ii) DiI signal in red. (D) Data shows DiI(HI+) population (10% of total). As expected, slowly cycling cells do not greatly attenuate BrdU or DiI. The BrdU(−) population may be the same 1% of cells that are BrdU(−) immediately after BrdU exposure. (E) Data shows DiI(LOW+) population (63% of total). A subset of BrdU(+) cells are DiI(LOW+) after extended cell proliferation in vitro. BrdU retention in rapidly cycling cells suggests these are cosegregating their DNA. (F) Comparison in clonal sphere formation between DiI(HI+) and DiI(LOW+) fractions. The majority of neurospheres arise from the fast-cycling DiI(LOW+) population (7.5-fold increase over DiI[HI+]). This suggests SCs are in this actively proliferating fraction.

Neurosphere cells were exposed to BrdU and then immediately pulsed with DiI. We sorted cells to confirm that these cells were also DiI(+). 97.6 ± 0.7% of cells emitted a high DiI signal by FACS. These results confirmed our starting population was positive for both indicators of cell proliferation.

The BrdU(+)/DiI(+) cells were proliferated for 1 wk in the absence of BrdU and then sorted into DiI(HI+) and DiI(LOW+) fractions. In 1 wk, the DiI signal was diminished in most of the cells as shown by the shift in DiI intensity (Fig. 4 A). We collected 9.7 ± 2.2% of the cells as a DiI(HI+) fraction and 63.1 ± 7.4% of all cells as the DiI(LOW+) fraction, leaving a buffer fraction of ~30% cells between the two groups to reduce contamination between them. DiI signal was assessed by visual inspection to confirm that DiI(HI+) cells were indeed strongly positive for the membrane dye (Fig. 4 B), whereas DiI(LOW+) cells displayed no signal (Fig. 4 C).

Of the DiI(HI+) group 70.6 ± 4.6% of cells were BrdU(+) (Fig. 4 D), which was expected as slow cycling cells that did not dilute DiI through cell divisions, might fail to dilute BrdU though cell divisions. The remaining 29.3 ± 4.6% of the DiI(+) cells were BrdU(−). These cells could be postmitotic cells that did not synthesize DNA during BrdU exposure, and also did not divide to dilute the DiI label. As these cells occupy 2.9% of the total cell population, it is conceivable they are the same cells as the 1.4% of cells that did not label with BrdU in neurosphere culture immediately after exposure to the analogue.

Within the DiI(LOW+) group we observed many BrdU(−) cells (75.7 ± 3.0%; Fig. 4 E), which was expected as the neurosphere cells had already demonstrated a loss of BrdU label through symmetric divisions, and the extinction of both cell division indicators was predicted. Intriguingly, this actively dividing fraction also contained 23.1 ± 2.7% weakly or moderately BrdU(+) cells, and 1.2 ± 0.2% heavily BrdU(+) cells. Altogether these occupied 15% of the total cell population. Together with the 7.1% of BrdU(+)/DiI(HI+) cells above, this is lower than the 33.6 ± 2.0% of cells we originally gathered during our analysis of BrdU retention at day 7 of neurosphere culture (Fig. 3 A). Nonetheless, 1.2% of these cells had BrdU(+) signals at a strength that was qualitatively equivalent with that of cells immediately after BrdU withdrawal. The retention of DNA label in fast proliferating cells during 1 wk of culture was suggestive of the cosegregation of BrdU(+) chromosomes during asymmetric cell divisions.

We reproduced these results using CFSE instead of DiI (unpublished data). In addition, we quantified the intensity of fluorescence emitted by cells immediately after CFSE exposure whose average was found to be >4,000 higher than that diluted by cells proliferated for 7 d. Indeed we calculated that the average level of intensity emitted by even the highest CFSE fluorescent cells, at 7 d culture, reflected at least 12 population doublings. This is the number of divisions one would expect in a single neurosphere clone of >3,000 cells at this time point. Moreover, when we diluted the concentration of initial CFSE dye applied to cells to approximate seven population doublings (seven halvings of that concentration), we found that cells treated with this concentration were still, on average, >250 times more fluorescent than those exposed to undiluted dye and allowed 7 d to dilute it. We thus confirm that in 7 d prolifera-

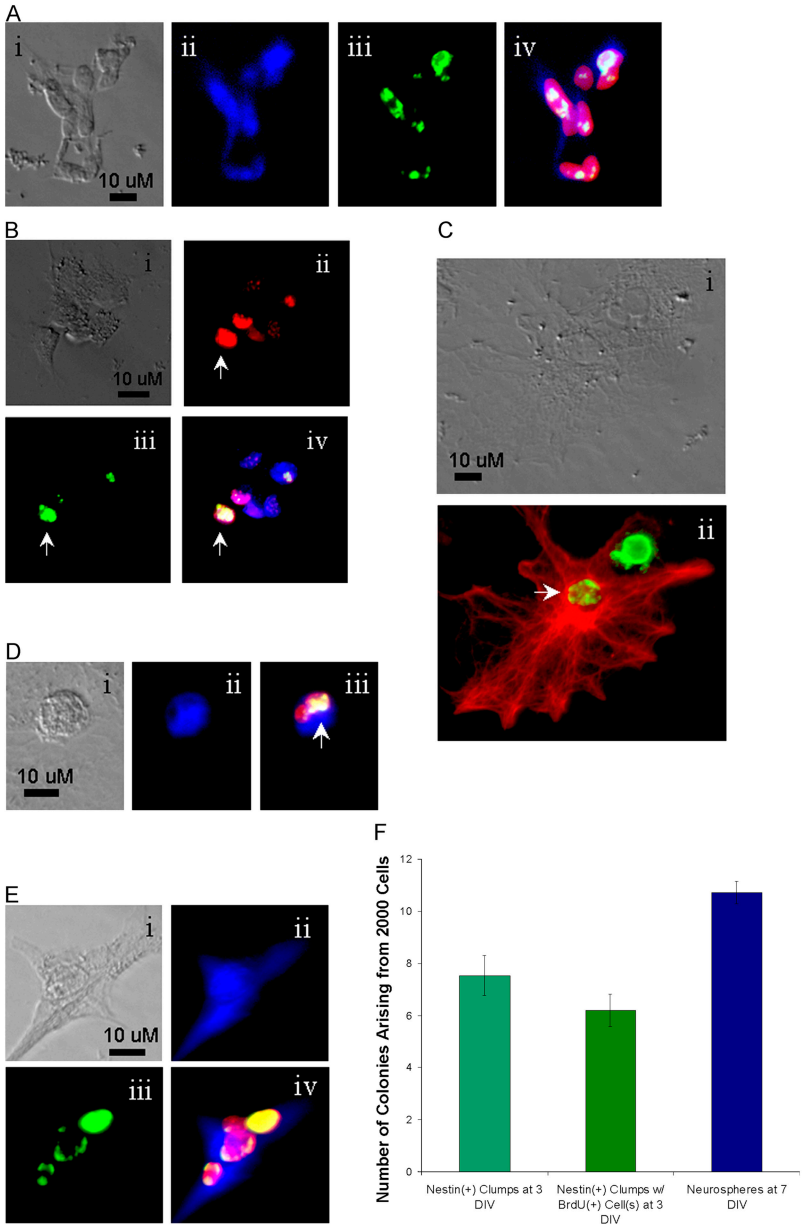


Figure 5. **A proportion of BrdU-retaining cells possess markers of proliferating and undifferentiated neural progenitors.** (A) All clones at 4 d after BrdU exposure are Nestin(+). (i) Bright field shows 4 d clone; (ii) Nestin is blue; (iii) BrdU-labeled nuclei are green; (iv) merge shows Nestin in blue, histone-labeled nuclei in red, and BrdU-labeled cells in green. (B) All clones 10 d after BrdU exposure contain Ki67(+) cells. Note strong Ki67 positivity of BrdU(+) cell (arrow). (i) Bright field shows clone; (ii) Ki67 expression is red; (iii) BrdU-labeled nuclei are green; (iv) merge shows Ki67 in red, BrdU-labeled cells are green, Hoechst-labeled nuclei are blue. (C) 14 d after BrdU differentiated clone, with arrows indicating GFAP(+) cell nucleus. (i) Bright field; (ii) merge shows GFAP in red and BrdU in green. (D)14 d after BrdU differentiated clone, with arrows indicating Nestin(+) cell nucleus. (i) Bright field; (ii) Nestin is blue; (iii) merge shows Nestin in blue, histone-labeled nuclei in red, and BrdU-labeled cells in green. (E) Clones arising from 7 d differentiated spheres (total of 17 d after BrdU). Cells show high Nestin(+) and undifferentiated cell morphology. (i) Bright field shows clone; (ii) Nestin is blue; (iii) BrdU-labeled nuclei are green; (iv) merge shows Nestin in blue, histone-labeled nuclei are red, and BrdU-labeled cells are green. (F) Numbers of Nestin(+) clones arising, from neurospheres exposed to differentiation conditions after BrdU removal. 3 DIV refers to clones that have been 17 d without BrdU, and 7 DIV clones have been without BrdU for 21 d. Nearly all clones at 3 DIV arise from BrdU(+) cells. The total number of SC colonies is slightly higher than this number (some taking longer to start proliferating), suggesting that all clones at 3 DIV are SC colonies.

tion conditions, most neurosphere cells are proliferating and undergo over seven population doublings, at which BrdU fluorescence is diluted past the threshold of detection in ESCs and fibroblasts. However, a subset of proliferating neurosphere cells retain BrdU.

In vivo, SCs are thought to divide slowly (Morshead et al., 1994), but based on our evidence we predicted this was not the case in vitro. If SCs divided slowly, it would follow that they would be enriched in the DiI(HI+) fraction. We assessed each fraction for secondary colony forming ability (Fig. 4 F), which is indicative of SC presence via self renewal. The DiI(LOW+) population gave rise to 7.4 (±1.5) times as many spheres as the DiI(HI+) population at clonal density. This suggested that this fast-dividing DiI(LOW+) fraction contained most if not all of the SCs. Subcloning the DiI(LOW+) and DiI(HI+) fractions revealed that not one secondary sphere arose from the DiI(HI+) sphere cells though many secondary spheres arose from the DiI(LOW+) sphere cell population. This suggests that the DiI(HI+) spheres arose from progenitor cells that were unable to self renew whereas self-renewing SCs were fully restricted to the DiI(LOW+) fraction. What is more, 24% of the DiI(LOW+) population contained BrdU(+) cells.

Some BrdU-retaining cells express markers of proliferating, multipotent precursors

In vivo neural precursor cells are positive for Nestin, a filament protein that is present in proliferating neural precursors (Lendahl et al., 1990). We observed that all cells in proliferating clones were Nestin(+) at 4 d after BrdU removal, and that every clone contained one or more BrdU(+) cells (Fig. 5 A). At 4, 7, and 10 d under proliferation conditions, all colonies derived from BrdU-exposed cells were composed entirely of Nestin(+) cells, and contained no glial fibrillary acidic protein

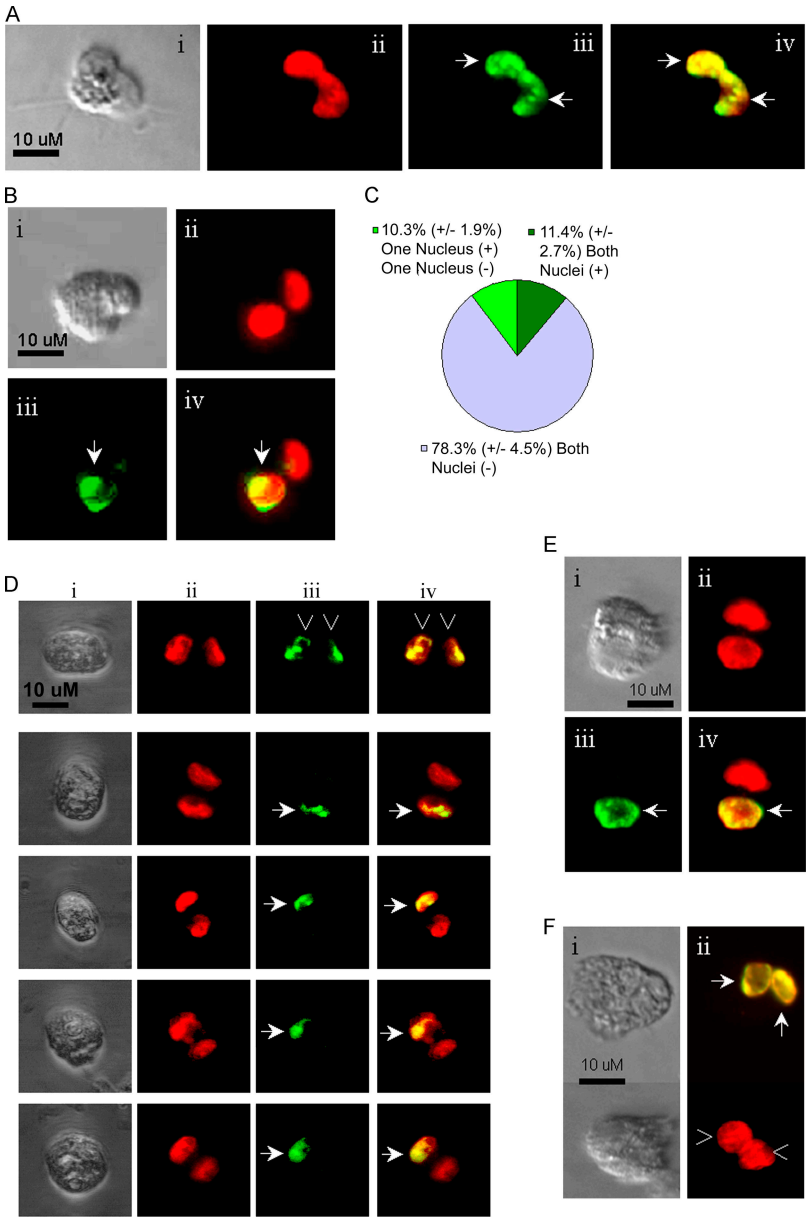


Figure 6. **Cell division inhibition of BrdU-retaining cells suggests asymmetric DNA partitioning.** (A) BrdU distribution in a cell arrested during cytokinesis, 10 d after BrdU. Arrows indicate symmetric BrdU(+) nuclei in same cell. (i) Bright field shows binucleate cell; (ii) histone-labeled nuclei are red; (iii) BrdU is green; (iv) merge of histone and BrdU. (B) BrdU distribution in a cell arrested during cytokinesis, 10 d after BrdU. Arrows indicate BrdU(+) nucleus, adjacent to BrdU(−) nucleus, in same cell. (i) Bright field shows binucleate cell; (ii) histone-labeled nuclei are red; (iii) BrdU is green; (iv) shows merge of histone and BrdU. (C) BrdU distribution in binucleate cell population treated with cytochalasin D. Uneven segregation of labeled DNA to daughter nuclei occurs in 10% of the binucleate cell population. (D) Confocal microscopy of BrdU-exposed cells arrested during karyokinesis, 10 d after BrdU exposure. Upon removal of inhibitor, cells were timed for fixation at late anaphase or telophase. Mitotic cells were observed segregating labeled DNA nonrandomly to one daughter in top row (arrows), as opposed to the even segregation of BrdU in bottom examples (arrowheads). BrdU labeling was confirmed at all focal planes. (i) Bright field shows mitotic cells; (ii) histone-labeled nuclei are red; (iii) BrdU is green; (iv) merge of histone and BrdU. (E) Cell doublets arising from 10 d after BrdU cells inhibited during karyokinesis. Cells released from inhibition were allowed to complete mitosis. Uneven labeling of BrdU(+/−) daughter nuclei was again apparent (arrows). (i) Bright field shows two cells; (ii) histone-labeled nuclei are red; (iii) BrdU is indicated by green; (iv) merge of histone and BrdU. (F) Cell doublets arising from 10 d after BrdU cells inhibited during karyokinesis. Cells released from inhibition were allowed to complete mitosis. Some doublets displayed evenly labeled BrdU(+) daughters (arrows) or unlabeled, BrdU(−) doublets (arrowheads). (i) Bright field shows binucleate cells; (ii) merge of histone-labeled nuclei (red) and BrdU (green).

(GFAP)(+) cells (a marker of astrocytes), or β-3-tubulin(+) cells (a marker of neuronal cells; unpublished data). We then stained cells at 10 d after BrdU removal for Ki67, a cell proliferation marker, and found that 79.1 ± 7.5% were Ki67(+). At both these time points we confirmed that every single cell colony contained Ki67(+) cells and that BrdU(+) cells also displayed Ki67 positivity (Fig. 5 B). We found similar results using proliferating cell nuclear antigen (PCNA; also known as DNA polymerase clamp), another marker of proliferation (unpublished data). This data suggests that under proliferation conditions all colonies are composed of cycling Nestin(+) cells.

1 wk after BrdU removal, individual colonies were isolated and exposed to differentiation conditions for an additional week. This span of time encompassed a total of 2 wk since BrdU exposure. Clones were examined for the presence of BrdU and Nestin in conjunction with GFAP. GFAP(+) cells displayed BrdU(+) nuclei (Fig. 5 C) as did Nestin(+) cells (Fig. 5 D). Within a single differentiated neurosphere there were 1.9 ± 0.4% Nestin(+) cells, and of these 63.4 ± 6.7% were BrdU(+). All coexpressed GFAP. If the cosegregation of BrdU-labeled DNA occurs in SCs, then these results are consistent with previous findings, which demonstrate the coexpression of Nestin and GFAP by NSCs (Doetsch et al., 1999; Imura et al., 2003; Morshead et al., 2003). This data shows that the majority of cells possessing markers of undifferentiated, proliferating neural precursors, also retain BrdU label, during 7 d differentiation and 14 d after exposure.

We took differentiated neurospheres from differentiation conditions and replated them at clonal density in proliferation conditions, to see if these undifferentiated cells would subclone. Most cells subcloned, remained differentiated as assessed by their obvious glial, or neuronal morphology. Such cells did not divide and displayed low, if any, Nestin positivity. Interestingly, a proportion of cells did not appear to be differ-

entiated neurons or glia by morphology, displayed high Nestin(+), and divided rapidly (Fig. 5 E). Of 2,000 cells removed from the differentiation substratum and examined at 3 d in vitro (DIV), approximately eight secondary clones arose, and of these six had at least one BrdU(+) cell present despite a total of 17 d culture having elapsed since BrdU exposure (Fig. 5 F). Hence, we suggest that secondary clones obtained after 1 wk of differentiation arise from BrdU(+) cells, which themselves persist as undifferentiated BrdU(+)/Nestin(+) precursors under differentiation conditions. On average 11 neurospheres, arising from differentiated colonies, were produced for every 2,000 cells plated. We suggest that these neurospheres at 7 d are the same cell clones examined at 3 d.

This data reveals that self-renewing NSCs persist in differentiation conditions, and that it is likely that some of these retain BrdU. It is extraordinary that undifferentiated and cycling Nestin(+)/BrdU(+) cells would persist in clones composed of an average of 3,000 cells, but which in some cases grew to large 300 micron clones numbering up to 15,000 cells. All clones contained BrdU(+) cells. Such a phenomenon is strongly suggestive of chromosome cosegregation in NSCs, because such cells must have already achieved more than seven cell doublings, a time point at which we have shown BrdU to be no longer detectable in symmetrically dividing STO or R1 cells.

Cell cycle arrest and real time imaging confirm asymmetric segregation of older and newly synthesized DNA

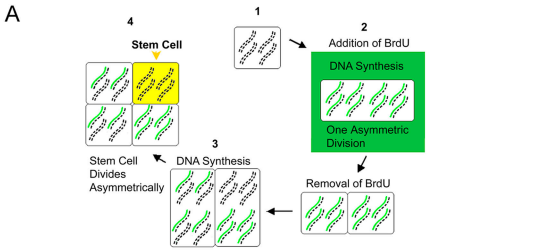
We asked whether in vitro neural precursors could distribute DNA asymmetrically using cytokinetic and karyokinetic inhibitors and immunofluorescence. 10 d after BrdU cells were exposed to an actin binding protein, cytochalasin D, to arrest them during cytokinesis, although karyokinesis had already occurred. Such treatment resulted in the recovery of many binucleate cells, composing approximately half of the total cell population. The complete dissociation of cells, including binucleate cells, into a single cell suspension was verified on a hemocytometer. Though most BrdU(+) binucleate cells displayed equivalent BrdU signal in Fig. 6 A we found instances of cells that had one labeled nucleus and one unlabeled nucleus (Fig. 6 B). Such cells had been arrested by mitotic inhibition over a period of 24 h, meaning that it is likely that many of these cells had a cell cycle of $<$24 h. Thus, it is likely that at least 10 divisions occurred in these cells over 10 d in neurosphere culture. Notwithstanding 10 consecutive divisions, a subset of cells cosegregated BrdU-labeled chromosomes into one nucleus and remained positively labeled in contrast to the majority of cells examined at this time point. Quantification revealed that 78.3 $\pm$ 4.5 binucleate cells had two unlabeled nuclei, 11.4 $\pm$ 2.7% had equally labeled nuclei, and 10.3 $\pm$ 1.9% exhibited BrdU signal in only one of the daughter nuclei (Fig. 6 C). No evidence of uneven BrdU(+) signal in fibroblast cells treated with cytochalasin D was found (unpublished data), in contrast to neural cells.

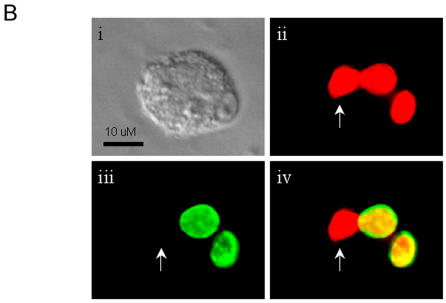
A similar experiment was repeated, but this time using the mitotic inhibitor nocodazole. Upon removal of mitotic inhibitor, cells were allowed to continue mitosis during which they were fixed at ten minute intervals from 10 to 60 min. Mitotic stages were identified by nuclear and chromosome morphology. BrdU localization was assessed at late anaphase or telophase, when chromosomes were condensed and a complete separation of forthcoming daughter cell chromosomes was evident. We observed the uneven distribution of BrdU in the chromosome of cells arrested during mitosis by high-power confocal microscopy (Fig. 6 D), although inhibitor inefficiency and orientation of nonadherent neurosphere cells impeded quantification. As well, we cannot resolve whether the demonstration of asymmetric BrdU localization in single mitotic cells is due to a dilution of BrdU to the threshold of detection in some of these cases.

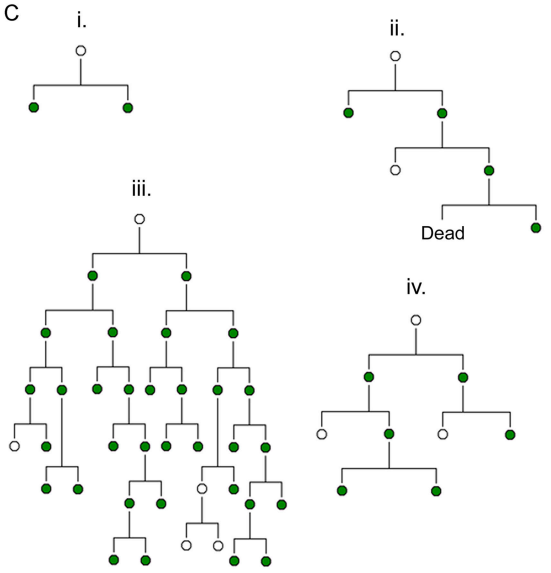
We continued the above experiment, but allowed single dissociated cells 2 h to complete division upon removal of nocodazole. Consequently, single mitotic cells became cell doublets. We again found unevenly labeled daughter cells similar to the results obtained with cytochalasin D–treated cells (Fig. 6 E), as well as cell doublets, which were BrdU(−) or evenly BrdU(+) (Fig. 6 F).

Thus far our results did not examine asymmetric DNA partitioning within living individual mitotic cells. We made use of a real time imaging system to track cell division within clones arising from single neurosphere cells. Clones were filmed and then fixed at varying time points, and cell lineages were reconstructed. Unlike our studies so far, we did not expose cells for an extended time to BrdU. Instead, cells were plated in BrdU-containing medium and allowed to undergo DNA synthesis to divide exactly once. We reasoned that, just before mitosis, each mouse cell would contain 40 pairs (4N) of BrdU(+) chromosomes to be distributed to both daughters. After division in BrdU, both daughter cells would contain 40 chromosomes, half unlabeled with the original unlabeled DNA template strand and half labeled with the new and BrdU(+) synthesized strand (Fig. 7 A). Therefore each cell daughter would be positive for BrdU signal at the two cell stage, when BrdU was removed. Whereas in our previous work we inferred immortal strand retention in SCs by the presence of analogue, here we examined the loss of newly synthesized BrdU(+) DNA and retention of older, unlabeled DNA in SCs. Two asymmetric divisions, the first in the presence of BrdU, the second in the absence of BrdU, would result in a dissociation of labeled and unlabeled DNA strands. Daughter SCs would retain the original unlabeled strands.

15 clones were traced and their lineages retrospectively established. The progeny of each clone were stained for the presence of BrdU (Fig. 7 B). In 6 out of 15 clones we noted at least one division event in which asymmetric DNA segregation occurred. Indeed, in 5 out of 15 clones, segregation was observed within only one division event after BrdU removal (Fig. 7 C). This data suggests unlabeled DNA templates originally present in the founder cell that have been cosegregated in cells, as immortal strands one round of DNA synthesis after BrdU uptake. The complete dissociation of a mouse cell's 40 BrdU(−) and 40 BrdU(+) DNA chromosomes in one such event, has a calculated probability of just 1.8×10^{-10} percent, and should not occur at this high frequency if segregation is







D

Clone	Number Cells Total	Number BrdU(+)	Number BrdU(-)	Asymmetry
1	8	8	0	no
2	19	16	3	yes (2 events)
3	18	18	0	no
4	5	5	0	no
5	4	2	1	yes (1 event)
6	2	2	0	no
7	6	6	0	no
8	7	4	2	yes (2 events)
9	3	1	1	yes (1 event)
10	5	5	0	no
11	3	3	0	no
12	5	5	0	no
13	5	3	2	yes (2 events)
14	5	5	0	no
15	5	2	3	yes (2 events)

Figure 7. **Live cell imaging supports nonrandom segregation of DNA.** (A) Schematic showing BrdU imaging strategy. (1) Each double strand (1 chromosome) represents 10 chromosomes of a mouse cell. Cells are unlabeled for BrdU (black). (2) During DNA synthesis, BrdU (green) is taken up for exactly one division in the presence of BrdU. (3) BrdU is removed and the daughters enter a second round of DNA synthesis in the absence of BrdU. (4) Division events after the second round of DNA synthesis should show BrdU asymmetry if groups of unlabeled chromosomes are cosegregated as immortal strands into SCs. (B) A clone imaged in real time. After one division event, BrdU was removed, and colony was fixed after two further cell divisions in the absence of BrdU. Arrow indicates a cell that has cleared all BrdU signal. (i) Bright field shows clone; (ii) histone-labeled nuclei are red; (iii) BrdU is indicated by green; (iv) merge of histone and BrdU. (C) Lineage diagrams from four clones traced (ii–iv show asymmetric DNA partitioning). Clone (ii) is the same shown in B. Each lineage represents divisions of one single cell, plated in the presence of BrdU, which is taken up during the first division initially labeling daughter nuclei (green), as demonstrated in clone (i). BrdU was removed after this one division, and cells continued proliferating until analysis. Note, the presence of BrdU(+) is inferred in parental cells from their offspring. Dead cells were observed to disintegrate while imaging, before analysis. (D) Summary of clones traced. 6 out of 15 clones demonstrated asymmetric partitioning of new and old DNA.

random. Our results are summarized in Fig. 7 D. That cells remain labeled for up to five divisions upon BrdU uptake (Fig. 7 C), shows that asymmetric BrdU partitioning observed in these experiments is not due to cell nuclei being at the threshold of BrdU dilution in this experiment.

Using the same strategy, we plated cells in the microwells of Terasaki plates in the presence of BrdU. We confirmed that single cells were initially present in each well, and after overnight incubation, wells containing cell doublets were scored before the removal of BrdU. Cells were then allowed to proliferate for 4 d before the removal of mitogens and addition of serum and substrate to initiate cell differentiation. Colonies were then assessed for BrdU, β-3-tubulin, and Nestin. We examined 29 clones and found that 9 of these showed asymmetric BrdU partitioning, whereas 13 showed asymmetry in cell fate, containing a mixture of both Nestin and β-3-tubulin(+) cells (unpublished data). Strikingly, eight of the nine clones with asymmetric BrdU partitioning also demonstrated concomitant asymmetric cell fate. Within asymmetric clones, all β-3-tubulin(+) cells were BrdU(+), but only 37.8 ± 12.2% of the Nestin(+) cells colabeled with BrdU, suggesting that some progenitors and/or SCs shed newly synthesized DNA preferentially. None of the clones in this experiment produced the number of offspring expected from seven population doublings, the number at which BrdU would be reaching its threshold of dilution. These results suggest that: (a) asymmetric DNA partitioning is correlated with asymmetric cell fate; and (b) only undifferentiated precursors cosegregate and retain their original unlabeled DNA, whereas labeled DNA is passed on to cells destined to differentiate.

Discussion

NSCs are Nestin(+) mitotic cells that incorporate BrdU in vitro and clonally give rise to great numbers of Nestin(+) neural precursors, which can be differentiated into neural cells. Colony cells are functionally heterogeneous; only a subset have the ability to form secondary colonies in culture, demonstrating that SCs divide symmetrically to produce equivalent progeny. We have shown that: (a) During long-term BrdU exposure, symmetric divisions result in the uptake and subsequent dilution of BrdU signal, a phenomenon that is consistent with semi conservative DNA replication and the ISH. Yet in vitro NSCs retain BrdU unlike ESCs, fibroblasts, and some of the neural progenitors arising from NSCs. (b) Under proliferation conditions neurosphere cells are actively dividing, though they exhibit some heterogeneity in cell cycle. (c) NSCs are

fast-dividing cells in vitro. Under proliferation conditions, cells that retain BrdU are mitotically active and it is the fast-dividing cell population that produces SC colonies. (d) Under differentiation conditions, a proportion of BrdU(+) cells retain Nestin, and the majority of colonies subcloned from differentiated colonies are founded by Nestin(+), BrdU-retaining cells. (e) Cell division symmetry can be partially defined by DNA inheritance, and is correlated with asymmetry in the fate of cells arising within a clone. Asymmetric divisions are exhibited by asymmetric BrdU localization in single mitotic cells. Real time imaging reveals that BrdU-labeled DNA is asymmetrically partitioned one division immediately after BrdU uptake; a time point at which a mitotic cell would have to segregate in the range of 40 BrdU(+) chromosome to one daughter but not the other. The great discrepancy between BrdU(+) and BrdU(−) nuclei observed in these real time imaging experiments, and the high frequency at which they occur, argue against a stochastic nature to this process. Asymmetric DNA segregation explains how a single clone of 3,000 cells, representing 12 cell doublings, retains BrdU label. We conclude that in vitro, NSCs are unique in the uneven partitioning of genetic material during some division events.

The expectation that cells would retain a full complement of labeled chromosomes has been used to invalidate the ISH (Ito et al., 1988). This expectation is false, because cells dilute BrdU-containing chromosomes randomly through symmetric divisions, and may segregate varying ratios of BrdU-labeled and -unlabeled chromosomes as immortal strand–bearing chromosomes. Indeed, BrdU heterogeneity among immortal DNA strands is entirely consistent with the cosegregation phenomenon. For example, if a single SC had one half of each chromosome strand labeled with BrdU, it would possess 40 one-half–labeled, and 40-nonlabeled chromosomes immediately after DNA synthesis had taken place. If such a cell was to divide symmetrically, it would randomly obtain between 0 to 40 of the labeled chromosomes to be consequently segregated as immortal strands. Thus, a cell will not be indelibly marked with analogue as a result of DNA synthesis according to the ISH. This is consistent with our results.

It has been suggested that chromosome cosegregation confers a means for SCs, that arise in the embryo and, which divide in the animal until senescence, to avoid passing potentially deleterious mutations, occurring as a result of errors in DNA synthesis and DNA repair to their progeny (Cairns, 2002). SCs may also need to avoid recombining chromosomes, as recombination might obviate or at least attenuate any benefits accrued through the segregation of older chromosomes. Suppression of recombination in SCs would itself provide a mechanism to avoid loss of heterozygosity events that could lead to cell transformation (Tischfield and Shao, 2003).

Studies on the mollusc (Tomasovic and Mix, 1974) demonstrated a surprising retention of incorporated DNA label in cells within continuously regenerating tissues of the adult animal. Cells from the adult mouse have been found to retain thymidine analogue (Potten et al., 1978, 2002). However, investigations of cosegregation during development have failed to observe DNA label retention in murine blastocysts or morula in vivo (Ito et al., 1988), nor in the embryos of *C. elegans* (Ito and McGhee, 1987). Similarly, our evidence does not support the ISH in ESCs derived from the 3.5-d blastocyst of early murine embryos. Further work needs to address this discrepancy explicitly.

Template strand retention might attenuate the end replication problem in telomeres during DNA synthesis and thus allow a SC greater divisions without the need for telomerase or other related mechanisms. The occurrence of symmetric divisions in SCs means that the end replication problem would still apply to SCs, although these may possess an overall greater potential number of total divisions, before becoming senescent, relative to non-SCs. Consequently, we hypothesize that SCs cosegregating older chromosomes possess a greater proliferative capacity than non-SCs in the absence of enzymatic telomere length maintenance.

Mechanisms involving microtubule/centrosomal asymmetry in both protein (Liakopoulos et al., 2003) and mRNA (Lambert and Nagy, 2002) localization have been shown to occur during cell division. It is possible that older chromosome strands could be associated with the mitotic spindle apparatus that would cosegregate immortal strand–bearing chromosomes in an intrinsic fashion. A molecular basis for such an uneven chromosome segregation is unknown, but a theoretical model for such a system has been proposed, which would involve sequence recognition of either leading or lagging templates in dividing cells (Jablonka and Jablonka, 1982). It is possible that leading versus lagging DNA synthesis might prime chromosomes for separation during synthesis itself. The yeast, *Schizosaccharomyces pombe*, uses a DNA strand–specific imprinting mechanism to produce daughter cells, where only one of the two changes its mating cell type by the process of mating type switching (Dalgaard and Klar, 2001). Such an asymmetry is conferred at the template level, where an imprint is installed only during lagging strand replication, but not in that of the leading strand. The lagging strand imprint permits subsequent DNA recombination by a double strand repair mechanism, which differentiates daughter cell chromosomes. In this system it appears that inheritance of specific chain of the parental chromosome is crucial for cellular differentiation and asymmetry between daughter cell progeny. Without the imprint cells do not maintain the multipotent lineage and can only produce differentiated progeny, behaviors that bear intriguing similarity to the multipotent nature of self-renewing SCs in multicellular eukaryotes. We envision such a mechanism to be primarily epigenetic, though progeny arising asymmetrically from the SC lineage might carry sequence differences as a result of errors in DNA synthesis. The uneven segregation of DNA pattern in an endlessly cycling cell might be sufficient to define the epigenetic persistence of the SC itself.

The pioneering efforts of Meselson and Stahl (1958), demonstrating that the semiconservative replication of DNA resulted in equal partitioning of genetic material, overall have suggested a fundamentally random nature to the distribution of genetic copy between generations. It has been generally assumed that eukaryotic chromosomes are randomly distributed to daughter cells and that daughter cell asymmetry is not a re-

sult of DNA asymmetry, but a rather a result of genetic product differences. This may not apply to all mitotic inheritance; our results here support the hypothesis that a small population of neural cells retain their original DNA when dividing asymmetrically; and that these cells possess NSC characteristics.

Materials and methods

Dissection and cell culture
CD1 mouse forebrain ventricles were dissected as previously described (Morshead et al., 1994). Neurosphere cells were cultured (Reynolds and Weiss, 1992) at a low density of two to five cells, or one cell per well, as previously described (Tropepe et al., 1997). Cultures were discarded at any time past passage 4. R1 embryonic cells and STO fibroblasts were donated by Janet Rossant and Andras Nagy from the Sammuel Lunenfeld Research Institute (Mount Sinai Hospital, Toronto, Ontario, Canada). ESCs were grown on mitotically inactivated fibroblasts as previously described (Nagy et al., 1993). Fibroblast cells were similarly grown in DME but containing 10% fetal calf serum (Hyclone), no growth factors, essential amino acids, sodium pyruvate, or β-mercaptoethanol.

Differentiation
Neurospheres were isolated and transferred to 24-well plates (Nunclon) coated with 15.1 mg/ml MATRIGEL basement membrane matrix (Becton Dickinson) diluted 1:25. Alternatively, 5-cm Petri dishes (Nunclon) were coated with MATRIGEL and clones were transferred in bulk. Cells were differentiated for 7 d in serum-free media containing 1% FBS (Hyclone). Cells were removed from MATRIGEL, using 0.25% porcine trypsin–EDTA solution (Sigma-Aldrich) applied for 5 min at 37°C.

BrdU and dye labeling
0.6 μM BrdU (Sigma-Aldrich) was used to label synthesized DNA. To remove BrdU, cells were centrifuged, washed, and reconstituted in fresh media. BrdU was applied at the same concentration and time interval in ESCs and fibroblasts. Vybrant DiI (Molecular Probes) was administered to neurosphere cells after dissociation using 5 μl/ml of DiI stock for 5 min at 37°C. CFSE (Molecular Probes) was used according to manufacturer's instructions. Cells were washed three times using serum-free media to remove dyes.

Immunofluorescence and microscopy
Dissociated cells or colonies were coated with MATRIGEL for 30 min at 37°C. Cell attachment was assessed by gently tapping plates under microscope. Cells were also attached using CELL-TAK (Becton Dickinson) according to manufacturer's instructions. Cells were fixed using 4% paraformaldehyde (Sigma-Aldrich) dissolved in cold Stockholm's phosphate-buffered saline (pH 7.3) for 15 min. Neurospheres were equilibrated in 30% sucrose (Sigma-Aldrich) and StPBS overnight at 4°C, embedded in cryoprotectant (Thermo Electron Corporation), and sectioned on a Jencon's OTF5000 cryostat. To detect BrdU, cells were exposed to 4 N HCl for 30 min. Cells were blocked using 10% normal goat serum (Sigma-Aldrich) in StPBS, pH 7.3, 0.3% Triton (Sigma-Aldrich) for 45 min at room temperature. Primary antibodies were applied overnight in StPBS, 1.0% NGS, 0.3% Triton (Sigma-Aldrich). Anti-BrdU Bu1/75 (1:500; Abcam), anti-Nestin (1:1,000–2,000; Chemicon), antiglial fibrillary acidic protein (1:400; Biomedical Technologies), anti–β-tubulin isotype III (1:500; Sigma-Aldrich), anti-Ki67 (1:10; Becton Dickinson), proliferating cell nuclear antigen (1:10; Zymed Laboratories), and anti–pan-histone (1:500; Chemicon) were used. Secondary antibodies were applied at 37°C for 50 min in StPBS 1.0% normal goat serum. TRITC, FITC, and CY3-conjugated antibodies (1:250; Jackson ImmunoResearch Laboratories) or secondary 350 and 568 nm Alexa Fluor antibodies (1:300; Molecular Probes) were used. Nuclei were sometimes counterstained with 10 μg/ml Hoechst 33258 (Sigma-Aldrich). Cells were photographed in StPBS or Gel Mount (Biomeda Corp.). Cells were visualized at 40×/0.55 (dry lens) objective using a Nikon DIAPHOT 200 microscope, and a 40×/0.60 Olympus IX81 microscope with the Olympus Microsuite version 3.2 analysis imaging system software (Soft Imaging Systems Corp.). Cell nuclei were counted within a known area of 15 μm thickness to calculate cell density. For confocal microscopy, cells were visualized at 100×/0.30 (oil immersion lens) objective using a Zeiss Axiovert 100 LSM410 with LSM version 3.993 imaging software (Carl Zeiss MicroImaging Corp.). Photos were processed using Adobe Photoshop 6.0 software.

Cell division inhibition
Cells were exposed to 2 μM of cytochalasin D (Sigma-Aldrich) or 0.1 μg/ml Nocodazole (Sigma-Aldrich) for 24 h at 37°C. Nocodazole inhibition was removed by aspirating media containing mitotic inhibitor, washing with serum-free media, and resuspending cells in medium containing FGF2, heparin, and EGF. Cells were then fixed 25–30 min later.

FACS
Cells were sorted on FACS DiVa (Becton-Dickinson) system. Cells were sorted at ~9,000 events per second and fractions were kept on ice until plated. For each sample, freshly pulsed DiI or CFSE cells were used to confirm positivity at the outset of each sort.

Cell imaging
Cells were imaged at 40×/0.75 (dry lens) magnification using an Axiovert 200 inverted microscope (Carl Zeiss MicroImaging, Inc.). Samples were illuminated every 2 min during image acquisition and images were captured with Sony XCD-SX900 digital camera, using ImageJ software (National Institutes of Health). Cells were loaded in BrdU-containing media and filmed until one division had occurred. BrdU was then immediately removed and fresh media substituted.

Submitted: 11 February 2005
Accepted: 15 July 2005

References

Aguilaniu, H., L. Gustafsson, M. Rigoulet, and T. Nystrom. 2003. Asymmetric inheritance of oxidatively damaged proteins during cytokinesis. *Science.* 299:1751–1753.

Cairns, J. 1975. Mutation selection and the natural history of cancer. *Nature.* 255:197–200.

Cairns, J. 2002. Somatic stem cells and the kinetics of mutagenesis and carcinogenesis. *Proc. Natl. Acad. Sci. USA.* 99:10567–10570.

Dalgaard, J.Z., and A.J. Klar. 2001. Does *S. pombe* exploit the intrinsic asymmetry of DNA synthesis to imprint daughter cells for mating-type switching? *Trends Genet.* 17:153–157.

Doetsch, F., I. Caille, D.A. Lim, J.M. Garcia-Verdugo, and A. Alvarez-Buylla. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell.* 97:703–716.

Freeman, M.R., and C.Q. Doe. 2001. Asymmetric Prospero localization is required to generate mixed neuronal/glial lineages in the *Drosophila* CNS. *Development.* 128:4103–4112.

Imura, T., H.I. Kornblum, and M.V. Sofroniew. 2003. The predominant neural stem cell isolated from postnatal and adult forebrain but not early embryonic forebrain expresses GFAP. *J. Neurosci.* 23:2824–2832.

Ito, K., and J.D. McGhee. 1987. Parental DNA strands segregate randomly during embryonic development of *Caenorhabditis elegans*. *Cell.* 49:329–336.

Ito, K., J.D. McGhee, and G.A. Schultz. 1988. Paternal DNA strands segregate to both trophectoderm and inner cell mass of the developing mouse embryo. *Genes Dev.* 2:929–936.

Jablonka, P., and E. Jablonka. 1982. Non-random sister chromatid segregation by cell type. *J. Theor. Biol.* 99:427–436.

Kuroki, T., and Y. Murakami. 1989. Random segregation of DNA strands in epidermal basal cells. *Jpn. J. Cancer Res.* 80:637–642.

Kusch, J., D. Liakopoulos, and Y. Barral. 2003. Spindle asymmetry: a compass for the cell. *Trends Cell Biol.* 13:562–569.

Lambert, J.D., and L.M. Nagy. 2002. Asymmetric inheritance of centrosomally localized mRNAs during embryonic cleavages. *Nature.* 420:682–686.

Lark, K.G., R.A. Consigli, and H.C. Minocha. 1966. Segregation of sister chromatids in mammalian cells. *Science.* 154:1202–1205.

Lendahl, U., L.B. Zimmerman, and R.D. McKay. 1990. CNS stem cells express a new class of intermediate filament protein. *Cell.* 60:585–595.

Liakopoulos, D., J. Kusch, S. Grava, J. Vogel, and Y. Barral. 2003. Asymmetric loading of Kar9 onto spindle poles and microtubules ensures proper spindle alignment. *Cell.* 112:561–574.

Malatesta, P., E. Hartfuss, and M. Gotz. 2000. Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development.* 127:5253–5263.

Merok, J.R., J.A. Lansita, J.R. Tunstead, and J.L. Sherley. 2002. Cosegregation of chromosomes containing immortal DNA strands in cells that cycle with asymmetric stem cell kinetics. *Cancer Res.* 62:6791–6795.

Meselson, M., and F.W. Stahl. 1958. The replication of DNA. *Cold Spring Harb. Symp. Quant. Biol.* 23:9–12.

Morshead, C.M., B.A. Reynolds, C.G. Craig, M.W. McBurney, W.A. Staines, D. Morassutti, S. Weiss, and D. van der Kooy. 1994. Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron.* 13:1071–1082.

Morshead, C.M., C.G. Craig, and D. van der Kooy. 1998. In vivo clonal analyses reveal the properties of endogenous neural stem cell proliferation in the adult mammalian forebrain. *Development.* 125:2251–2261.

Morshead, C.M., A.D. Garcia, M.V. Sofroniew, and D. van der Kooy. 2003. The ablation of glial fibrillary acidic protein-positive cells from the adult central nervous system results in the loss of forebrain neural stem cells but not retinal stem cells. *Eur. J. Neurosci.* 18:76–84.

Nagy, A., J. Rossant, R. Nagy, W. Abramow-Newerly, and J.C. Roder. 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA*. 90:8424–8428.

Neff, M.W., and D.J. Burke. 1991. Random segregation of chromatids at mitosis in *Saccharomyces cerevisiae*. *Genetics.* 127:463–473.

Palmer, T.D., A.R. Willhoite, and F.H. Gage. 2000. Vascular niche for adult hippocampal neurogenesis. *J. Comp. Neurol.* 425:479–494.

Potten, C.S., W.J. Hume, P. Reid, and J. Cairns. 1978. The segregation of DNA in epithelial stem cells. *Cell.* 15:899–906.

Potten, C.S., G. Owen, and D. Booth. 2002. Intestinal stem cells protect their genome by selective segregation of template DNA strands. *J. Cell Sci.* 115:2381–2388.

Reynolds, B.A., and S. Weiss. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science.* 255:1707–1710.

Shen, Q., W. Zhong, Y.N. Jan, and S. Temple. 2002. Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts. *Development.* 129:4843–4853.

Tischfield, J.A., and C. Shao. 2003. Somatic recombination redux. *Nat. Genet.* 33:5–6.

Tomasovic, S.P., and M.C. Mix. 1974. Cell renewal in the gill of the freshwater mussel, Margaritifera margaritifera: an autoradiographic study using high specific activity tritiated thymidine. *J. Cell Sci.* 14:561–569.

Tropepe, V., C.G. Craig, C.M. Morshead, and D. van der Kooy. 1997. Transforming growth factor-alpha null and senescent mice show decreased neural progenitor cell proliferation in the forebrain subependyma. *J. Neurosci.* 17:7850–7859.

Yamashita, Y.M., D.L. Jones, and M.T. Fuller. 2003. Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. *Science.* 301:1547–1550.