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Origin of New Glial Cells in Intact and Injured Adult Spinal Cord

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SUMMARY

Several distinct cell types in the adult central nervous system have been suggested to act as stem or progenitor cells generating new cells under physiological or pathological conditions. We have assessed the origin of new cells in the adult mouse spinal cord by genetic fate mapping. Oligodendrocyte progenitors self-renew, give rise to new mature oligodendrocytes, and constitute the dominating proliferating cell population in the intact adult spinal cord. In contrast, astrocytes and ependymal cells, which are restricted to limited self-duplication in the intact spinal cord, generate the largest number of cells after spinal cord injury. Only ependymal cells generate progeny of multiple fates, and neural stem cell activity in the intact and injured adult spinal cord is confined to this cell population. We provide an integrated view of how several distinct cell types contribute in complementary ways to cell maintenance and the reaction to injury.

INTRODUCTION

A major challenge in developmental biology and regenerative medicine is to understand the origin of cells. Once development is completed, the number of cells in adult tissues can be maintained by duplication of differentiated cells or by the generation of new cells from stem or progenitor cells. Most tissues are composed of a variety of differentiated cells of different lineages and it is poorly understood how their numbers are maintained in adult tissues during homeostasis and in response to injury.

The adult central nervous system was considered a comparatively static tissue with little cell turnover. It is well established today that there is more plasticity than previously thought and that new neurons are produced continuously from stem cells in the subventricular zone of the lateral ventricles and the dentate gyrus of the hippocampus (Zhao et al., 2008). In most parts of the central nervous system, however, neurons are not added in adulthood and there is limited cell turnover. Nevertheless, cells with neural stem cell properties can be isolated from most regions of the adult central nervous system, including, for example, the spinal cord (Johansson et al., 1999; Shihabuddin et al., 1997; Weiss et al., 1996).

Spinal cord injury is often mentioned among the first conditions for which stem cells may provide a new therapy. There are several studies indicating that transplantation of neural stem cells or cells derived from embryonic stem cells to the injured spinal cord have a beneficial effect on functional recovery in experimental models, although the mechanism is poorly understood (Barnabé-Heider and Frisén, 2008). Improved functional recovery after transplantation of in vitro expanded neural stem cells derived from the adult spinal cord (Hofstetter et al., 2005) demonstrates that there are stem cells in the adult spinal cord that can exert beneficial effects after injury, but fail to do so efficiently under normal conditions. Identification of stem and progenitor cells in the adult spinal cord and an understanding of their response to injury could allow the development of pharmacological strategies to modulate these cells in situ to produce desired progeny after injury as an alternative to cell transplantation.

Oligodendrocyte progenitors, ependymal cells, and astrocytes have all been suggested to act as neural stem cells in the adult spinal cord (Horner et al., 2000; Johansson et al., 1999; Martens et al., 2002; Meletis et al., 2008; Ohori et al., 2006). Most studies have focused on the highly proliferative oligodendrocyte progenitor population. Indirect data from viral labeling of proliferating cells and lineage analysis from embryonic stages have led to the suggestion that oligodendrocyte progenitors in the adult spinal cord have neural stem cell properties and produce cells of multiple lineages in vivo (Horky et al., 2006; Ohori et al., 2006; Sellers et al., 2009; Zhu et al., 2008a, 2008b), but this has not been addressed directly. Ependymal cells give rise to differentiated progeny after injury and constitute the main cell population in the intact spinal cord with in vitro neural stem cell properties (Meletis et al., 2008), but the origin of the increased number of neural stem cells after injury (Moreno-Manzano et al., 2009; Xu et al., 2006) was unknown. Astrocytes act as neural stem cells and give rise to new neurons in the subventricular zone and dentate gyrus. Astrocytes in other parts of the central nervous system display little proliferative activity, yet cortical astrocytes gain in vitro neural stem cell properties after injury (Buffo et al., 2008). There are thus three distinct

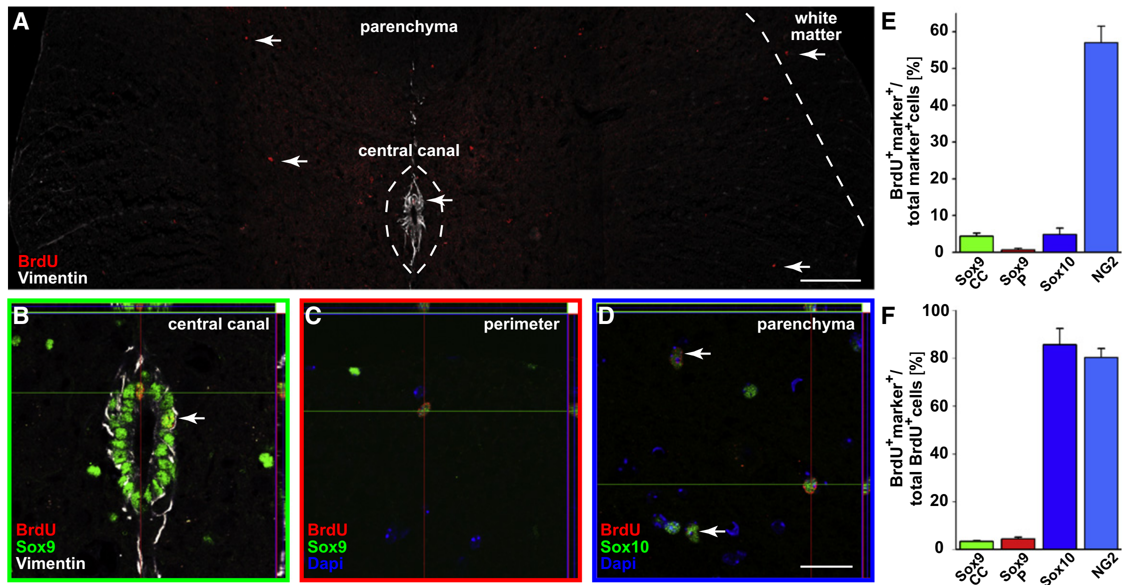

Figure 1. Proliferating Cell Populations in the Adult Spinal Cord

Adult wild-type mice received BrdU in the drinking water for 5 weeks to label proliferating cells.

(A) Arrows point at BrdU$^+$ cells at the central canal, in the parenchyma, and at the perimeter of the spinal cord in an overview image.

(B–D) BrdU is found in Sox9$^+$/Vimentin$^+$ ependymal cells by the central canal (B), in Sox9$^+$ astrocytes at the perimeter of the white matter (C), and in Sox10$^+$ oligodendrocyte progenitors scattered in the parenchyma (D).

(E and F) Quantification of BrdU incorporation relative to all proliferating cells (E) and relative to phenotype (F). Averages and SD from three animals are shown. Scale bars represent 100 μm in (A) and 25 μm in (B)–(D).

cell populations in the adult spinal cord that have been suggested to act as neural stem cells and to contribute to cell turnover. The relationship between these cell types, for example whether they can give rise to each other, if they all have stem cell properties, and their relative contribution to cell turnover in the intact and injured spinal cord, had not been addressed.

We have in parallel performed genetic fate mapping of oligodendrocyte progenitors, ependymal cells, and astrocytes in the adult mouse spinal cord, allowing us to assess the relative roles of these cell populations in cell turnover in the intact and injured spinal cord. We present an integrated view of how differentiated cells, progenitors, and stem cells cooperate in complementary ways to generate new glial cells in the intact and injured adult spinal cord.

RESULTS

Genetic Fate Mapping of the Main Proliferating Cell Populations in the Uninjured Adult Spinal Cord

In order to assess cell turnover in the adult mouse spinal cord, we first characterized which cell types proliferate. BrdU administration via the drinking water for 5 weeks resulted in labeled cells scattered within all areas of the spinal cord (Figure 1A). Ependymal cells, astrocytes, and oligodendrocyte lineage cells together accounted for the vast majority of BrdU-labeled cells (93.4% ± 2.7%, mean ± SD) (Figures 1B–1D). The remaining BrdU-labeled cells are mainly microglia and blood vessel-associated cells (Horner et al., 2000). The proportion of BrdU-labeled cells was similar for Sox10$^+$ oligodendrocyte lineage and ependymal cells (4%–5%) but substantially lower for astrocytes (<1%) (Figure 1E). Oligodendrocyte lineage cells were identified by the expression of Sox10, astrocytes by the expression of Sox9, and ependymal cells by their position and expression of Sox9 (Cahoy et al., 2008; Meletis et al., 2008; Pompolo and Harley, 2001; Rivers et al., 2008). Oligodendrocyte progenitors express NG2 and are also referred to as NG2 cells or polydendrocytes (Nishiyama et al., 2009). The few astrocytes that incorporated BrdU were most often located at the perimeter of the spinal cord white matter (Figure 1C). There was a large variation in proliferation rate within the oligodendroglial lineage, with oligodendrocyte progenitors displaying a high proliferation rate and mature oligodendrocytes being postmitotic (Figure 1E). Oligodendrocyte lineage cells are by far the most numerous of the proliferating cells and represent more than 80% of the BrdU-labeled cells in the adult spinal cord (Figure 1F).

To trace the progeny of these proliferating cell populations, we employed genetic fate mapping with three different transgenic mouse lines expressing tamoxifen-dependent Cre recombinase (CreER) under different cell type-specific promoters. We used mice in which CreER expression was under the control of promoters with specific expression in ependymal cells (FoxJ1), astrocytes (Connexin 30), or oligodendrocyte lineage cells (Olig2). These mice were on R26R Cre-reporter background, which after tamoxifen administration leads to permanent and heritable expression of the reporter gene β-galactosidase (βgal) or yellow fluorescent protein (YFP). The transgenic lines and labeling paradigms are depicted in Figure S1 available online. Tamoxifen was adminstered once daily for 5 days and phenotypic characterization of the recombined cells was performed 5 days later. The mice were 2–5 months old (Table S1).

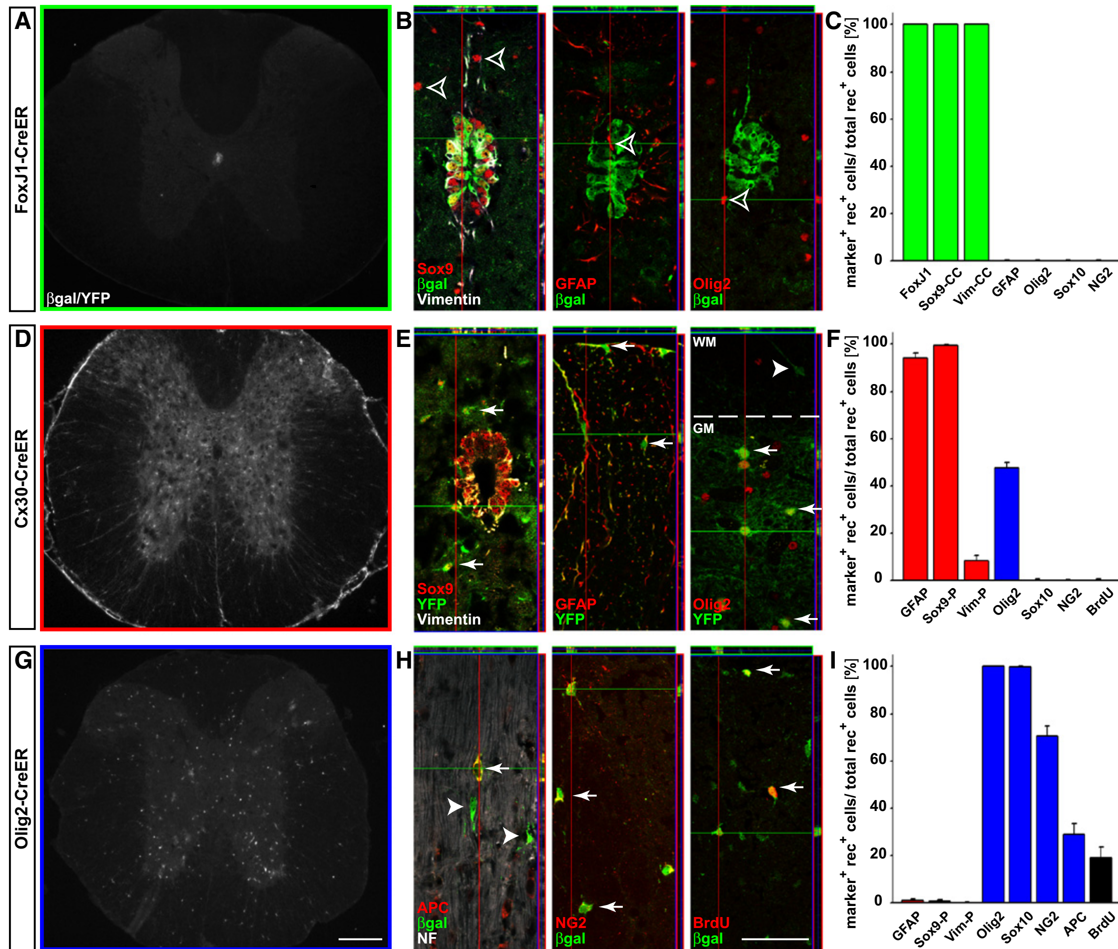

Figure 2. Genetic Labeling of Ependymal Cells, Astrocytes, and Oligodendrocyte Progenitors in the Adult Spinal Cord

(A–C) The FoxJ1-CreER line gives recombination in ependymal cells surrounding the central canal, which all express Sox9, Vimentin, and CreER (FoxJ1 in C), but are negative for GFAP, Olig2, Sox10, and NG2. Arrowheads point to cells that are positive for the indicated marker but are not recombined.

(D–F) The Cx30-CreER line gives recombination in astrocytes that all express Sox9 and GFAP. Recombined gray matter astrocytes express Olig2 and some recombined astrocytes express low levels of Vimentin. Arrows point to recombined cells positive for the marker, and the filled arrowhead points to a recombined white matter cell negative for Olig2.

(G–I) The Olig2-CreER line gives recombination in very few gray matter astrocytes (Sox9$^+$ and GFAP$^+$) and some mature oligodendrocytes (APC$^+$) but mainly target oligodendrocyte progenitors that express NG2 and Sox10 and incorporate BrdU (administered in the drinking water 5 days before sacrifice). Arrows point to recombined cells positive for the marker and filled arrowheads point to recombined cells negative for the marker.

Averages and SD are shown from three animals. Rec$^+$, recombined; CC, central canal; p, parenchyma. Scale bars represent 200 μm in (A), (D), and (G) and 50 μm in (B), (E), and (H).

FoxJ1-CreER transgenic mice allow genetic recombination specifically in ependymal cells in the adult spinal cord (Figures 2A–2C; Meletis et al., 2008). The recombined ependymal cells all have motile cilia and homogenously express, for example, Sox9 and Vimentin (Figures 2A–2C; Meletis et al., 2008). About 80% of ependymal cells are genetically marked with the employed recombination strategy (Meletis et al., 2008). There are GFAP$^+$ astrocyte processes within the ependymal layer and Olig2$^+$ oligodendrocyte progenitors can be found in close vicinity, but ependymal cells express none of these markers and these cells are not recombined in FoxJ1-CreER mice (Figures 2B and 2C; Meletis et al., 2008).

The gap junction protein Connexin 30 (Cx30) is specifically expressed in astrocytes in the nervous system (Kunzelmann et al., 1999; Nagy et al., 1999; Rash et al., 2001). Recombination is restricted to astrocytes in the adult brain of Cx30-CreER transgenic mice (Slezak et al., 2007). In the spinal cord, recombination in Cx30-CreER mice led to numerous labeled cells located throughout the parenchyma (Figures 2D–2F). Almost all recombined cells expressed Sox9 (99.5% ± 0.4%, mean ± SD) and GFAP (93.9% ± 2.4%, mean ± SD), a small subset located in the white matter expressed Vimentin, and the recombined cells were never found in the ependymal layer (Figures 2E and 2F; Figure S2A). Sox9-expressing cells in the parenchyma coexpressed the astrocytic markers GFAP and S100β, but never the oligodendrocyte lineage markers Sox10, NG2, or PDGFRα (Figure S2). The genetic labeling of astrocytes was efficient, with 74.7% ± 2.9% (mean ± SD) of parenchymal Sox9$^+$ cells being recombined.

Based on morphology and marker expression, we could identify three astrocytic subtypes that were recombined in

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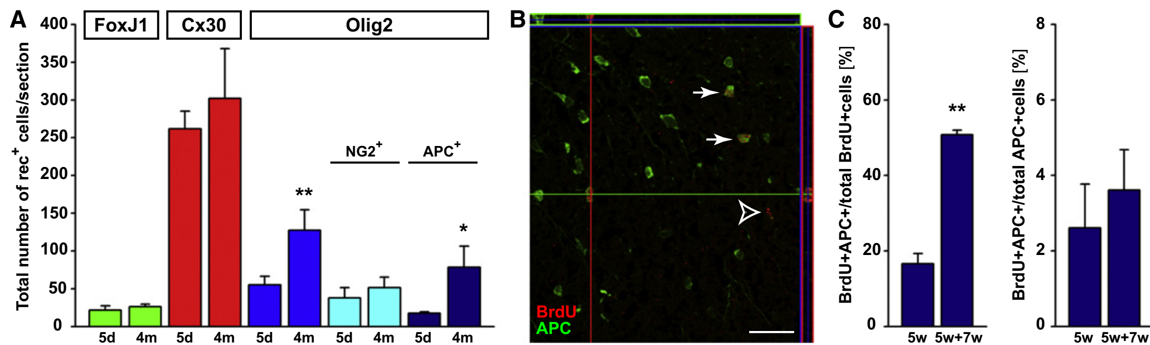


Figure 3. Characterization of the Progeny of Ependymal Cells, Astrocytes, and Oligodendrocyte Progenitors in the Intact Adult Spinal Cord
(A) Adult mice were given tamoxifen daily for 5 days and the total number of recombined cells was assessed after another 5 days or 4 months.
(B and C) Wild-type animals were given BrdU for 5 weeks in the drinking water and analyzed either directly (5w) or after an additional 7 weeks without BrdU (5w+7w). BrdU-labeled APC^+ mature oligodendrocytes (B, arrows point to double-positive cells and the empty arrowhead points to a $BrdU^+$, APC^-) were quantified (C).
Averages and SD are shown from three animals for each experiment. Rec^+, recombined. *$p < 0.05$, **$p < 0.001$, Student's t test. Scale bar represents 25 μm.

Cx30-CreER mice (Figures 2D–2F; Figure S2A), one in the white matter and two different in the gray matter. White matter astrocytes expressed Sox9 and high levels of GFAP, but not Olig2. A small number of recombined cells in the gray matter was located very close to the ependymal layer, had a bipolar morphology, expressed Sox9 and high levels of GFAP, and were negative for Olig2 (Figure 2E; Figure S2A). However, the majority of recombined cells in the gray matter had the elaborate morphology of protoplasmatic astrocytes and expressed Sox9 and lower levels of GFAP mostly around the cell body. Surprisingly, we found that this class of gray matter astrocytes was positive for Olig2 (91.3% ± 4.0%, mean ± SD of all gray matter astrocytes) via two different specific antibodies (Figures S2A–S2C). We classified these cells as astrocytes, rather than oligodendrocyte lineage cells, based on the expression of the astrocytic markers GFAP and Sox9, the protoplasmatic astrocyte morphology, and the lack of the oligodendrocyte lineage markers Sox10 and NG2 (Figure 2F; Figure S2A).

Olig2 is expressed in oligodendrocyte progenitors and mature oligodendrocytes in the adult spinal cord (Ohori et al., 2006; Yamamoto et al., 2001), and recombination has been demonstrated in these cell types in Olig2-CreER transgenic mice during development (Masahira et al., 2006; Takebayashi et al., 2002). We found Olig2-CreER recombined cells scattered within both the white and gray matter in the adult spinal cord (Figure 2G). Almost all recombined cells expressed Olig2 and Sox10 and a quarter of them were mature APC^+ oligodendrocytes (Figure 2I). The recombination efficiency was lower in this line than the other two with 4.5% ± 0.1% (mean ± SD) of $Sox10^+$ cells (all maturational stages in the oligodendrocyte lineage) being recombined. Importantly, whereas recombination was inefficient in mature oligodendrocytes, it was more efficient in oligodendrocyte progenitors, with 38% ± 14% (mean ± SD) of $NG2^+$ cells being recombined. About two thirds of recombined cells were proliferating oligodendrocyte progenitors expressing NG2 and 23.5% ± 9.5% (mean ± SD, 3- to 5-month-old mice) of recombined cells had undergone division and were labeled with BrdU after 5 days administration in the drinking water (Figures 2H and 2I; Figure S2D). A very small fraction (0.9% ± 0.7%, mean ± SD) of recombined cells in the Olig2-CreER line were $Olig2^+$ gray matter astrocytes expressing Sox9 and GFAP. This corresponds to <1% of gray matter astrocytes. The very inefficient recombination in $Olig2^+$ astrocytes was fortuitous for the purpose of this study because it allowed us to distinguish the astrocyte and oligodendrocyte lineages in the Olig2-CreER and Cx30-CreER lines (Figure S2E).

Ependymal Cells and Astrocytes Self-Duplicate whereas Oligodendrocyte Progenitors Self-Renew and Generate Mature Oligodendrocytes

To identify the progeny of ependymal cells, astrocytes, and oligodendrocyte lineage cells, the number and phenotype of recombined cells was analyzed 5 days and 4 months after tamoxifen-induced recombination (Figure S1B, Table S1). The total number of recombined cells did not change significantly in the FoxJ1-CreER or Cx30-CreER lines over time (Figure 3A). The phenotype of the recombined cells at 4 months in both these lines was indistinguishable from that at 5 days after recombination (Figure S3), with all cells being ependymal cells in the FoxJ1-CreER line and all cells being astrocytes in the Cx30-CreER line. Thus, ependymal cells and astrocytes do not produce progeny of other fates in the uninjured spinal cord and their number stays constant, indicating that their proliferation serves to compensate for some cell loss to maintain the size of their cell populations.

In contrast, the Olig2-CreER line showed a significant increase in the total number of recombined cells after 4 months. This increase was due to a larger number of labeled mature oligodendrocytes (APC^+) whereas the number of labeled progenitors ($NG2^+$) was largely unaltered (Figure 3A; Figure S3). The finding that the number of oligodendrocyte progenitors stayed constant indicates that these cells both self-renew and produce new mature oligodendrocytes. Mature oligodendrocytes are postmitotic but can be replenished from progenitor cells (Keirstead and Blakemore, 1997). To assess the potential addition of mature oligodendrocytes in an independent way, wild-type mice received BrdU in the drinking water for 5 weeks and were analyzed either at that time (5w) or after another 7 weeks after the end of BrdU administration (5w+7w). There was a significant

increase with time in the proportion of BrdU-labeled mature oligodendrocytes, increasing from 17% ± 2.7% (mean ± SD) after 5 weeks of BrdU administration to 51% ± 1.2% (mean ± SD) of all BrdU-labeled cells 7 weeks later (Figures 3B and 3C). Moreover, direct counts of APC$^+$ cells, independently of BrdU labeling, revealed a significant increase with time in the same animals ($p < 0.05$, Student's t test).

Thus, in the uninjured adult spinal cord ependymal cells and astrocytes proliferate to maintain their population sizes whereas oligodendrocyte progenitors both self-renew and generate an increasing number of mature oligodendrocytes.

In Vitro Neural Stem Cell Potential in the Intact and Injured Spinal Cord Is Confined to Ependymal Cells

We assessed the expression of markers associated with neural stem/progenitor cells in ependymal cells, astrocytes, and oligodendrocyte lineage cells in the adult spinal cord. Sox2 is expressed by the vast majority, if not all, recombined cells in the FoxJ1-CreER and Cx30-CreER lines and in a subset of cells in the Olig2-CreER line. Musashi1 is highest expressed in ependymal cells, and CD133/prominin expression as well as Nestin second intron enhancer activity, both associated with neural stem cells (Carlén et al., 2006; Corti et al., 2007), are exclusive to ependymal cells in the adult spinal cord (Figures S4A–S4E).

The adult spinal cord contains cells with in vitro neural stem cell potential (Johansson et al., 1999; Shihabuddin et al., 1997; Weiss et al., 1996), and genetic fate mapping has established that they are ependymal cells (Meletis et al., 2008). Spinal cord injury results in an increased number of neural stem cells in the rat (Moreno-Manzano et al., 2009; Xu et al., 2006), but their origin was unknown. It is possible, as in the cerebral cortex (Buffo et al., 2008), that injury recruits cells that lack stem cell potential in the uninjured situation to gain such potential.

We first characterized the effect of a transverse dorsal funiculus incision at Th4, not reaching down to the gray matter or central canal, in adult wild-type mice on neurosphere formation. Neurospheres appeared faster, were larger, and 3–4 times more numerous after injury (Figures 4A and 4B). Injury-induced neurospheres displayed self-renewal and multipotency, as shown by the fact that they could be serially passaged and, when exposed to differentiating conditions, generated astrocytes, oligodendrocytes, and neurons at the clonal level (Figure 4A and see further below).

To determine the cell type of origin of injury-induced neurospheres, we established neurosphere cultures from FoxJ1-CreER, Cx30-CreER, and Olig2-CreER mice on R26R-YFP background (Figure 4C). Recombination was induced by five daily tamoxifen injections. After 5 additional days, to ensure that tamoxifen had been eliminated and no further recombination could take place (Meletis et al., 2008; Robinson et al., 1991), cultures were either established directly or animals were subjected to a spinal cord injury and cultures initiated 3 days after the injury (Figure S1C). Efficient recombination in each animal was verified histologically in a spinal cord segment adjacent to the lesion (Figure 4C). Approximately 80% of neurospheres from FoxJ1-CreER mice were recombined, both in cultures deriving from uninjured and injured animals, establishing that most neurospheres derive from ependymal cells in both situations. Recombination is never complete, which results in an underestimation of the actual contribution. The recombination rate in ependymal cells and in neurospheres from FoxJ1-CreER mice is similar, suggesting that close to all neurospheres derive from ependymal cells. This is further supported by the finding that when ependymal cells were isolated by flow cytometry, no neurosphere-initiating activity was found in the nonependymal fraction (Meletis et al., 2008). In contrast, we only found one single recombined neurosphere in each condition in cultures from the Cx30-CreER line and not a single recombined neurosphere in any condition in cultures from the Olig2-CreER line even if the primary cultures were kept for a long time (Figures 4D and 4E).

Restricted progenitor cells have been shown to give rise to primary neurospheres with limited self-renewal and/or differentiation potential (Reynolds and Rietze, 2005; Wu et al., 2005). To assess self-renewal capacity and multipotency at the clonal level, single recombined neurospheres were collected and passaged. The two Cx30-CreER-derived recombined neurospheres did not generate secondary neurospheres, whereas FoxJ1-CreER-derived recombined neurospheres expressed markers associated with neural stem cells and displayed self-renewal during serial passages as well as multipotency as they gave rise to the three main neural cell types after induction of differentiation (Figures S4F–S4I). Thus, ependymal cells constitute the main neural stem cell population in the spinal cord and their neurosphere-forming potential is enhanced after injury, whereas astrocytes and oligodendrocyte progenitors do not demonstrate in vitro stem cell properties, at least under the standard conditions employed here.

Ependymal Cells, Astrocytes, and Oligodendrocyte Progenitors Produce Progeny that Occupy Complementary Domains after Spinal Cord Injury

To characterize the in vivo response of ependymal cells, astrocytes, and oligodendrocyte progenitors to spinal cord injury, an incision in the dorsal funiculus was made in adult FoxJ1-CreER, Cx30-CreER, and Olig2-CreER mice 5 days after completion of tamoxifen administration (Figure S1D, Table S1). The 5 day clearing period allows elimination of tamoxifen, precluding potentially altered promoter activity in response to injury to result in recombination in other cell types (Meletis et al., 2008; Robinson et al., 1991). Control animals not receiving tamoxifen did not show recombination after injury (data not shown).

Quantification of the total number of recombined cells in the three transgenic lines showed a significant increase at the injury site compared to an adjacent segment (Figure 5A; Figure S5A, Table S1). The number of recombined cells at the injury increased 4- to 5-fold in the FoxJ1-CreER line and approximately 2-fold in the Cx30-CreER and Olig2-CreER lines. This increase was established already at 2 weeks after the injury and was maintained at almost the same level after 4 months (Figure 5A). Approximately 80% of FoxJ1-CreER, Cx30-CreER, and Olig2-CreER recombined cells at the injury were labeled with BrdU administered the first 5 days postinjury (Figure 5A; Figure S1D).

The distribution of recombined cells was altered by the injury (Figures 5B and 5C; Figures S5B–S5D). Ependymal cells are normally exclusively situated by the central canal, but after injury recombined ependymal cell progeny in FoxJ1-CreER mice were also found in the core of the scar tissue forming at the

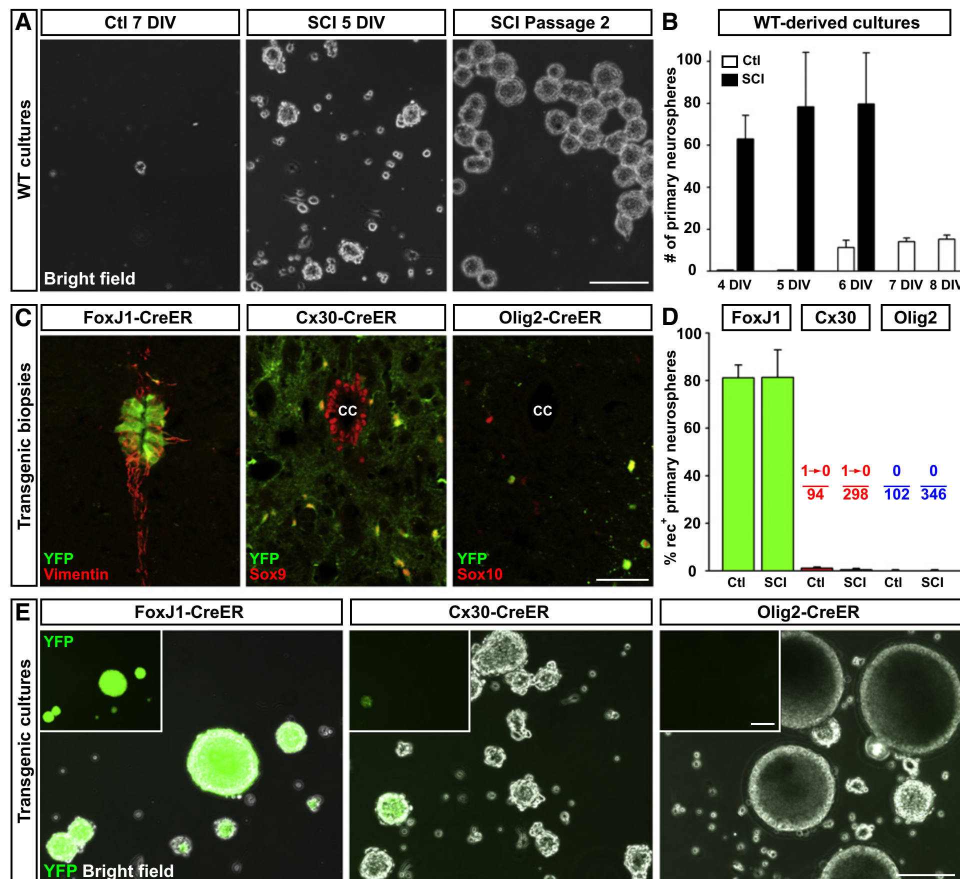


Figure 4. Spinal Cord Injury Increases the Number of Ependymal Lineage Cells with In Vitro Stem Cell Activity

(A and B) Neurosphere cultures were established from the intact adult spinal cord from wild-type (WT) control mice (Ctl) or 3 days after injury (SCI). Neurospheres appear earlier and are more numerous after injury.

(C–E) Transgenic mice received tamoxifen for 5 days and, after a 5 day clearing time, neurosphere cultures were established either directly or mice underwent a spinal cord injury and cultures were established 3 days later.

(C) A biopsy was obtained from each spinal cord and analyzed by histology to ensure efficient recombination.

(D and E) Only ependymal cells (FoxJ1-CreER) give rise to self-renewing neurospheres both from uninjured animals and after spinal cord injury. The number of primary neurospheres and the number of those that could be passaged (→) to give rise to secondary neurospheres is given above the total number of primary neurospheres obtained from the Cx30-CreER and Olig2-CreER lines. One recombined neurosphere was obtained from the Cx30-CreER line in each condition, but which could not be further passaged (see Figure S4). Olig2-CreER-derived cultures were kept for 2 weeks to ensure that slowly growing neurospheres were not missed.

Averages and SD are shown from three animals for each condition (B, D). DIV, days in vitro; rec$^+$, recombined. Scale bars represent 200 μm in (A), (E) and 100 μm in (C).

lesion. A smaller number of ependymal cell-derived cells were found scattered in the spared parenchyma (Figures 5B and 5C; Figures S5B and S5C; Meletis et al., 2008). Astrocytic progeny in spinal cord-injured Cx30-CreER mice also accumulated at the lesion site, but in a complementary domain to the ependymal-derived cells. Whereas ependymal progeny mainly occupied the center of the lesion site, new astrocyte-derived cells were most numerous in the perimeter of the forming scar tissue (Figures 5B and 5C; Figures S5B and S5C). Oligodendrocyte progenitor progeny was increased throughout the injured segment, with cells present at the injury site but in similar numbers also in areas of the segment that had not been directly affected by the lesion (Figures 5B and 5C; Figures S5B and S5C). All three lines gave rise to progeny within the glial scar, but only recombined cells in the FoxJ1-CreER and Cx30-CreER lines expressed nestin (Figure S5D), which in this situation marks scar-forming astrocytes (Frisén et al., 1995). These results demonstrate that ependymal cells, astrocytes, and oligodendrocyte progenitors produce progeny after injury, which persists in complementary domains long term after injury.

Phenotype of New Cells in the Injured Spinal Cord

In order to assess the phenotype of the progeny of ependymal cells, astrocytes, and oligodendrocyte progenitors, we characterized the marker profile of recombined cells 2 weeks and 4 months after injury in FoxJ1-CreER, Cx30-CreER, and Olig2-CreER mice. No apparent difference in the number of recombined cells was observed in segments adjacent to the injury compared to uninjured animals (Figures 2 and 6A; Figures S3 and S6A), indicating that the reaction to injury is largely restricted to the lesion site.

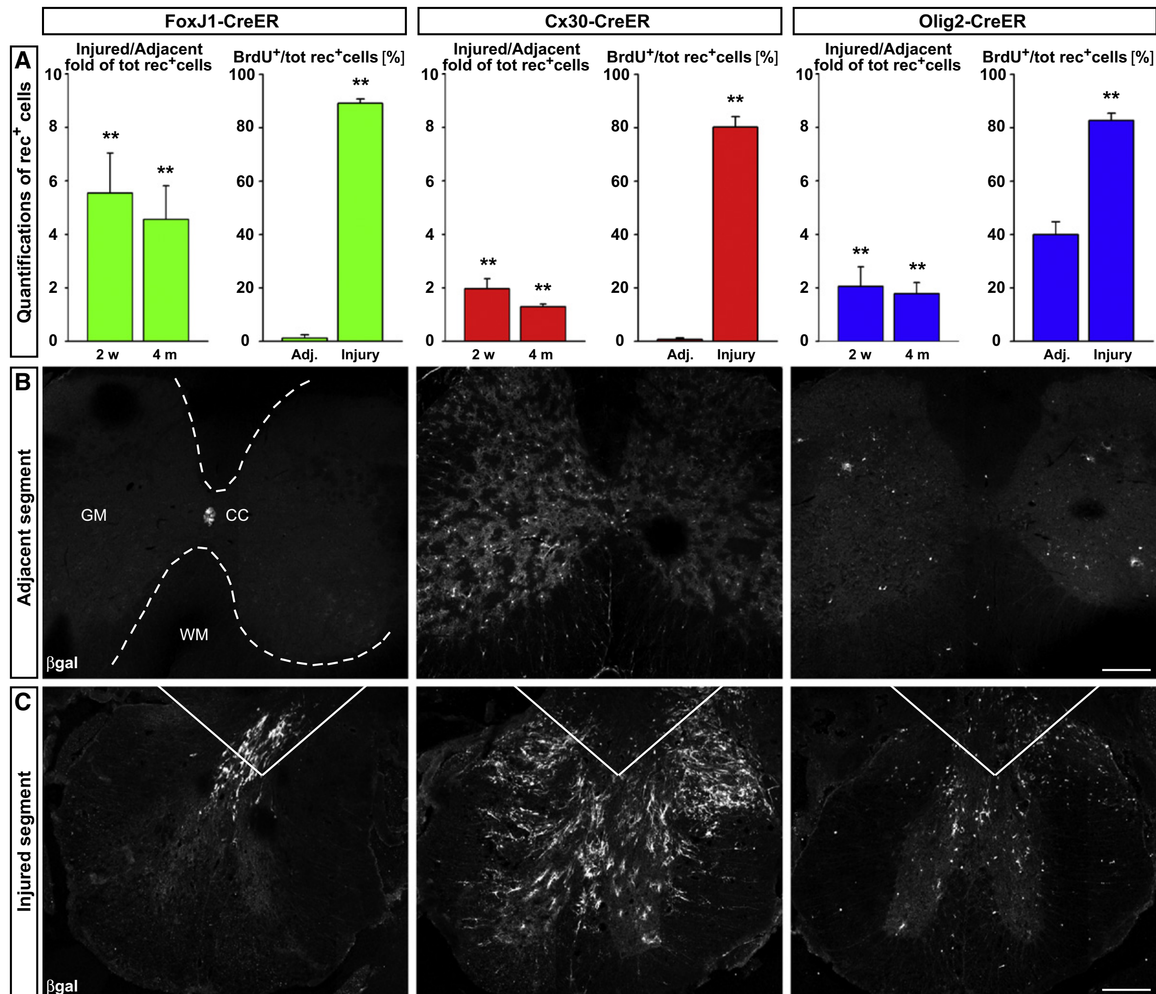


Figure 5. Ependymal Cell, Astrocyte, and Oligodendrocyte Progenitor Progeny Occupy Complementary Domains after Injury

Adult mice were given tamoxifen daily for 5 days and, after a 5 day clearing period, were subjected to a dorsal funiculus incision and analyzed 2 weeks or 4 months later.

(A) Quantification of the total number of recombined cells shows an increase at the injury over an adjacent segment in all transgenic lines (left graphs). The majority of recombined cells have incorporated BrdU administered the first 5 days after the injury (right panels). Averages and SD are shown from three animals per transgenic line for each time point.

(B and C) Overview coronal photographs show recombined cells (βgal$^+$) after injury, where a marked distinction is observed between the recombination pattern in a segment adjacent to the lesion (B) and at the injured segment (C). The Cx30-CreER recombined cells are less profusely branched after injury, and the βgal is therefore more concentrated and appears brighter. The broken line outlines the gray matter in (B) and the white lines indicate the site of injury in (C).

GM, gray matter; WM, white matter; CC, central canal. **$p < 0.001$. Scale bars represent 200 μm.

There was an approximate 75% reduction in the number of NeuN$^+$ neurons in the injured segment at both 2 weeks and 4 months after the injury (Figure S6B). We did not detect recombined neurons in any of the lines nor BrdU-labeled neurons after injury (data not shown), suggesting that new neurons are not generated after this type of spinal cord injury.

Recombined cells located outside the ependymal layer in FoxJ1-CreER mice had lost their ependymal phenotype and downregulated FoxJ1-CreER transgene expression (Figures 6B–6D). The vast majority of these cells were Sox9$^+$ astrocytes and a subpopulation of them was also GFAP$^+$. Four months after the injury, a small number of ependymal cell-derived recombined cells were Olig2$^+$ and APC$^+$ mature oligodendrocytes (Figures 6B and 6C; Meletis et al., 2008). FoxJ1-CreER recombined cells at the injury site appear to derive only from ependymal cells and not by circulating cells, as indicated by the fact that blood and bone marrow cells are not recombined in these animals and recombined cells at the lesion site did not express the hematopoietic marker CD45 (Figures S6C and S6D).

All Cx30-CreER recombined cells maintained astrocytic features, expressing Sox9 and high levels of GFAP. The phenotype of gray matter astrocytes was altered by injury, with few expressing Olig2 and now displaying a less ramified and more elongated morphology (Figures 6B, 6C, and 6E).

The phenotype of Olig2-CreER recombined cells was not affected by injury, where the majority of cells expressed either the progenitor marker NG2 or the mature oligodendrocyte marker APC and the myelin-associated marker MBP (Figures 6B and 6C; Figures S6E and S6F). Thus, the increased proliferation after injury of both astrocytes and oligodendrocyte

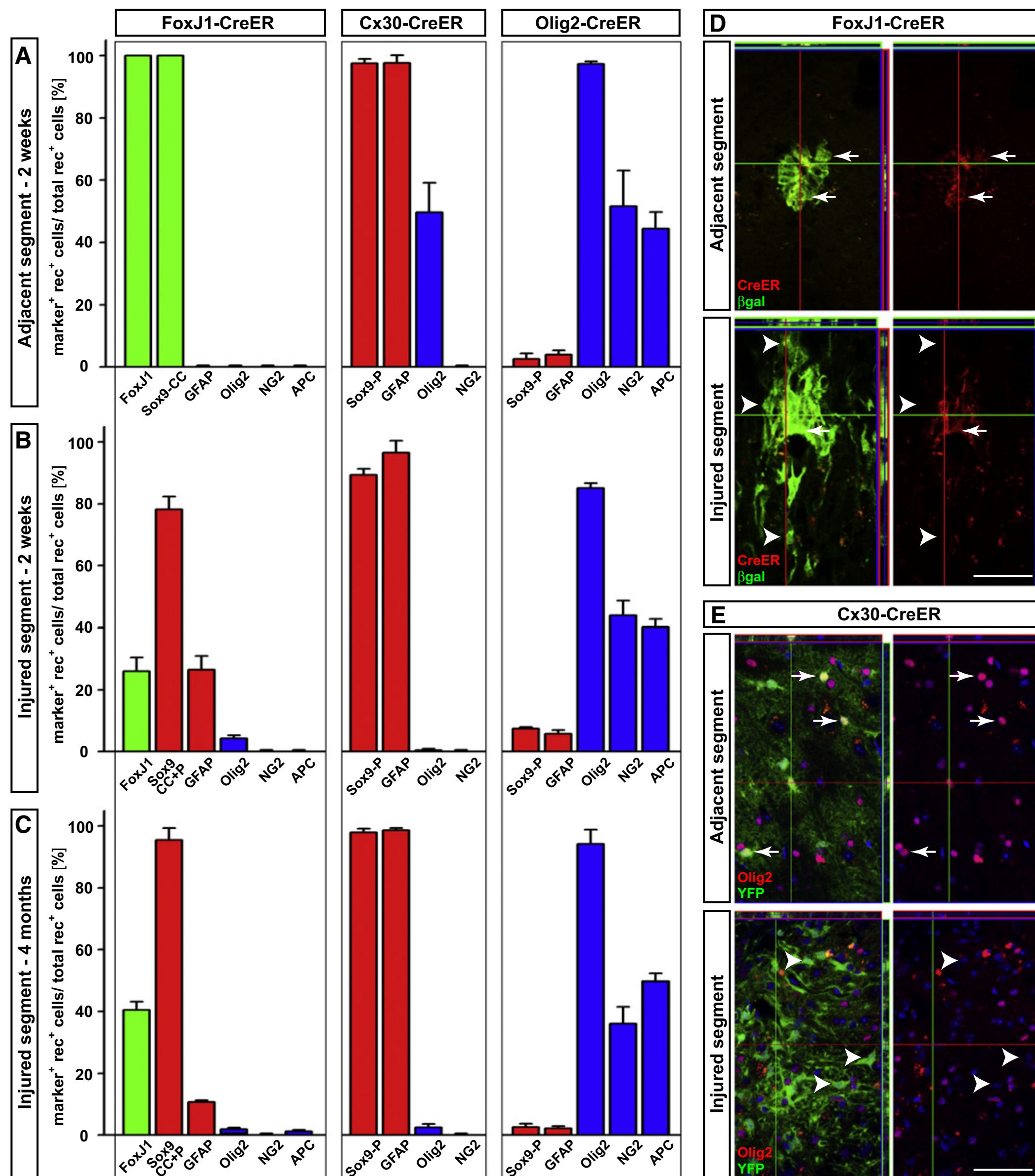


Figure 6. Phenotypic Characterization of New Glial Cells in the Injured Spinal Cord

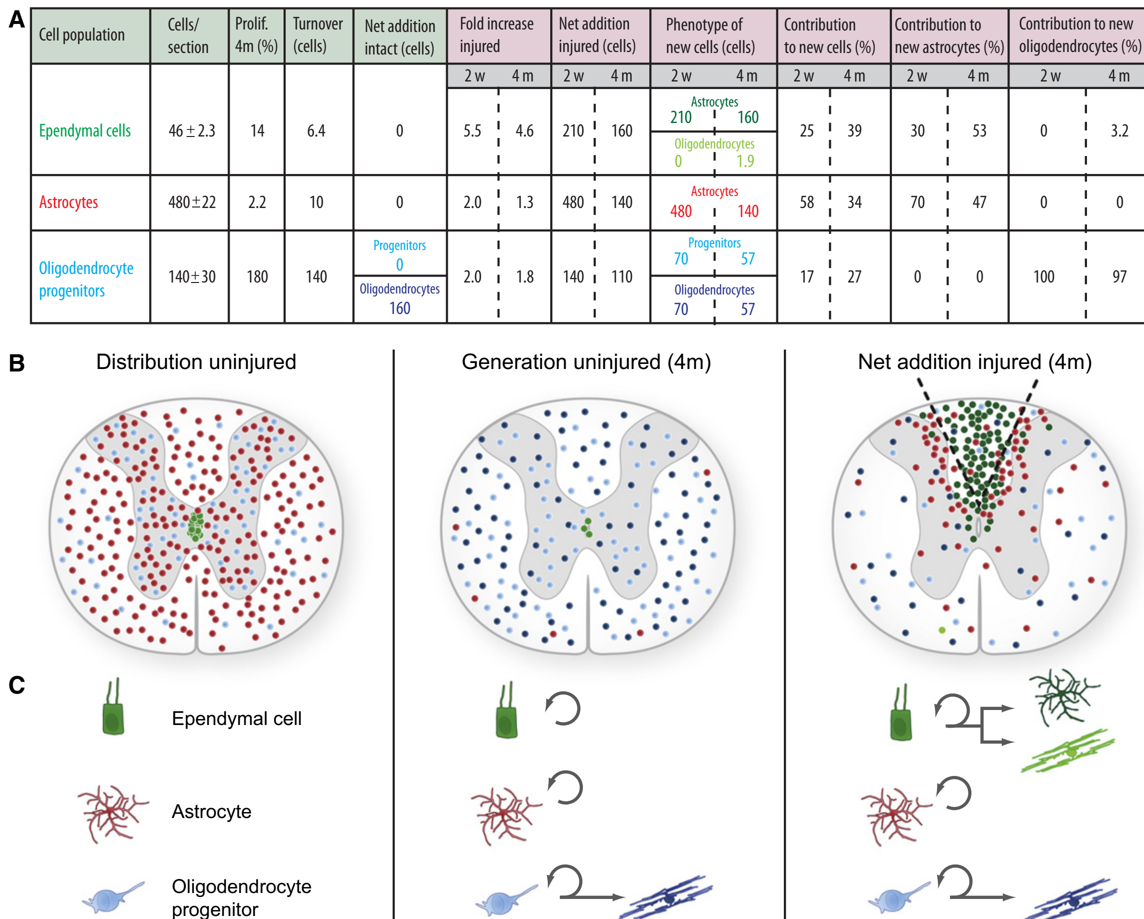
(A–C) Quantification of recombined (rec$^+$) cells according to phenotypic markers in a segment adjacent to the lesion (A) and in the injured segment 2 weeks (B) or 4 months (C) after the injury. FoxJ1 gene expression was assessed immunohistochemically with antibodies against Cre. Averages with SD are shown from three animals per transgenic line for each time point.

(D and E) The two most pronounced changes observed after injury are the loss of ependymal cell-specific marker from FoxJ1-CreER-derived progeny leaving the central canal (D) and the loss of Olig2 expression and morphological change of the gray matter astrocytes from the Cx30-CreER line (E). Arrows point to recombined cells positive for the marker and filled arrowheads point to recombined cells negative for the marker. Scale bars represent 50 μm in (D) and (E).

progenitors leads to the generation of new cells restricted to their own lineage, whereas ependymal cells display an altered differentiation repertoire after injury.

The Relative Contribution of Different Cellular Sources to Glial Cell Generation in the Intact and Injured Adult Spinal Cord

By analyzing the dynamics of the three main proliferating neural cell populations in parallel in the intact and injured spinal cord, it is possible to estimate the relative contribution of the different cell types to cell renewal (Figure 7). The dominating proliferative population in the intact spinal cord is oligodendrocyte progenitors, which continuously give rise to new mature oligodendrocytes of which there is an increasing number over time in the adult. In response to injury, there is a substantial net increase in progeny of ependymal cells, astrocytes, and oligodendrocyte lineage cells. Two weeks after the injury, astrocytes had produced the largest number of new cells (58% of new cells) followed by ependymal cells (25%) and oligodendrocyte progenitors (17%). By 4 months after the injury, there was a reduction



A

Cell population	Cells/ section	Prolif. 4m (%)	Turnover (cells)	Net addition intact (cells)	Fold increase injured		Net addition injured (cells)		Phenotype of new cells (cells)		Contribution to new cells (%)		Contribution to new astrocytes (%)		Contribution to new oligodendrocytes (%)	
					2 w	4 m	2 w	4 m	2 w	4 m	2 w	4 m	2 w	4 m	2 w	4 m
Ependymal cells	46 ± 2.3	14	6.4	0	5.5	4.6	210	160	Astrocytes 210 Oligodendrocytes 0	Astrocytes 160 Oligodendrocytes 1.9	25	39	30	53	0	3.2
Astrocytes	480±22	2.2	10	0	2.0	1.3	480	140	Astrocytes 480	Astrocytes 140	58	34	70	47	0	0
Oligodendrocyte progenitors	140±30	180	140	Progenitors 0 Oligodendrocytes 160	2.0	1.8	140	110	Progenitors 70 Oligodendrocytes 70	Progenitors 57 Oligodendrocytes 57	17	27	0	0	100	97

Figure 7. The Relative Contribution of Ependymal Cells, Astrocytes, and Oligodendrocyte Progenitors to New Glial Cells in the Adult Spinal Cord

(A) The number of ependymal cells ($Sox9^+$ cells by the central canal), astrocytes ($Sox9^+$ cells in the parenchyma), and oligodendrocyte progenitors ($NG2^+$ cells with branched morphology) were counted in 20 μm cryostat sections. The proliferation at 4 months was extrapolated from 5 weeks of BrdU administration. The net cell addition in the intact spinal cord after 4 months was obtained by the difference in the total number of recombined cells 5 days and 4 months after recombination and compensated for the recombination frequency. The increase in the number of recombined cells at the injury site was used to estimate the net addition of cells 2 weeks and 4 months after injury.

(B) The distribution of ependymal cells (green), astrocytes (red), and oligodendrocyte progenitors (blue) and their progeny are shown at 50% of their estimated number. Note that the right panel depicts the net addition of new cells after injury, and that the addition of the left and right panels gives the full picture of all cells present after the lesion.

(C) Schematic depiction of the fate of proliferating ependymal cells, astrocytes, and oligodendrocyte progenitors in the intact and injured spinal cord.

in the number of progeny from all three cell types, which was most pronounced for astrocytes. At this time the largest number of new glial cells derived from ependymal cells (39%), followed by astrocytes (34%) and oligodendrocytes (27%). Thus, oligodendrocyte progenitors go from being the completely dominating source of new cells in the intact spinal cord to being outnumbered by both astrocytes and ependymal cells after injury. This is due not to a reduced production of progeny by oligodendrocyte lineage cells, which is doubled, but to a larger increase in the production of progeny by ependymal cells and astrocytes.

The astrocytes contributing to the scar that forms at the injury derive both from ependymal cells and from preexisting astrocytes, with astrocytes in the core of the scar deriving from ependymal cells and those at the periphery of the scar deriving from duplication of preexisting astrocytes. Two weeks after the injury, more than two thirds of the new astrocytes in a lesioned segment arise by astrocyte duplication, whereas slightly more than half of the new astrocytes derive from ependymal cells 4 months after the injury. Nearly all ependymal cell-derived astrocytes were located in the center of the scar tissue, whereas astrocyte duplication occurs much more widespread (Figure 7). The vast majority (97%) of new mature oligodendrocytes in an injured segment derive from oligodendrocyte progenitors and ependymal cells contribute 3.2% of new oligodendrocytes 4 months after an injury.

DISCUSSION

We have assessed the role of the three main proliferating neural cell populations in the intact and injured spinal cord in cell

renewal. We found that ependymal cell and astrocyte proliferation is restricted to self-duplication to maintain their populations in the uninjured spinal cord, whereas oligodendrocyte progenitors self-renew and give rise to an increasing number of mature oligodendrocytes. Injury recruits ependymal cells, astrocytes, and oligodendrocyte progenitors to generate progeny in complementary domains during scar formation and remyelination. Only ependymal cells show multilineage potential and stem cell activity is contained within the ependymal cell population in both the intact and injured spinal cord. We provide an integrated view of how several cell types have distinct and complementary roles in homeostasis and in the response to injury.

Stem, Progenitor, and Differentiated Cells Make New Glial Cells after Injury

There is a large difference in the proliferative activity of astrocytes, ependymal cells, and oligodendrocyte progenitors in the uninjured spinal cord. Ependymal cells incorporate BrdU approximately ten times more frequently than do astrocytes, and oligodendrocyte progenitors incorporate BrdU approximately ten times more frequently than do ependymal cells (Figure 1E). In contrast to the relative quiescence of ependymal cells and astrocytes in the uninjured situation, both populations produce a larger number of progeny than do the oligodendrocyte progenitors after injury. This is in spite of a doubling of the production of progeny by oligodendrocyte progenitors.

Astrocytes in the adult spinal cord give rise to new cells of the same fate, although to very different extents in the intact or injured situation, and appear as differentiated cells with a latent but extensive self-duplication capacity. Neural stem cells in the adult lateral ventricle wall, which give rise to olfactory bulb interneurons, have astrocytic features (Zhao et al., 2008), and the majority of neurospheres from this region are recombined in Cx30-CreER mice (our unpublished data). We did not find evidence for in vitro neural stem cell properties or in vivo multilineage potential of adult spinal cord astrocytes. As in the spinal cord, cortical astrocytes do not generate cells of other fates in the intact or injured situation. However, cortical astrocytes can give rise to neurospheres after injury (Buffo et al., 2008). There is thus considerable heterogeneity with regard to stem cell properties between astrocytes in different parts of the adult central nervous system.

Previous work suggested that adult spinal cord oligodendrocyte progenitors generate astrocytes (Horky et al., 2006; Ohori et al., 2006; Sellers et al., 2009; Zhu et al., 2008a, 2008b), but our genetic lineage analysis does not support this. Our results rather indicate that spinal cord oligodendrocyte progenitors are similar to those in the forebrain, which recently have been demonstrated by similar genetic fate mapping strategies to give rise to new oligodendrocytes but not astrocytes (Dimou et al., 2008; Rivers et al., 2008). We failed to find evidence for multilineage potential of oligodendrocyte progenitor cells, so they are, in spite of their extensive self-renewal capacity, better defined as progenitor cells than stem cells.

The limited proliferation of ependymal cells in the uninjured situation, together with the large increase in their production of progeny after injury, describes a quiescent stem cell population that can be recruited on demand after injury. Forebrain ependymal cells lining the lateral ventricles similarly only after injury give rise to other cell types (Carlén et al., 2009). Although the production of progeny by stem cells is increased after injury in many tissues, it is unusual that a stem cell population does not partake in any cell replacement in the uninjured situation. However, several previous studies support that the response of a tissue to an injury may differ from simply modulating the activity of the stem or progenitor cells that maintain homeostasis in the intact tissue (Dor et al., 2004; Dor and Melton, 2008; Ito et al., 2005; Levy et al., 2005; Nakagawa et al., 2007; Simon and Frisén, 2007; Xu et al., 2008).

Remyelination and Scar Formation in the Injured Spinal Cord

Spinal cord injury results in rapid loss of oligodendrocytes (Crowe et al., 1997; Grossman et al., 2001; Lytle and Wrathall, 2007), leaving many axons partly denuded and unable to efficiently propagate impulses. In partial spinal cord lesions, which are most common in humans, a substantial part of the neurological dysfunction is thought to be secondary to the loss of oligodendrocytes (McDonald and Belegu, 2006). Demyelination also results in rearrangement of axonal ion channels, which further may impair propagation of action potentials (Nashmi and Fehlings, 2001). Demyelinated axons are vulnerable to degeneration, and rapid remyelination is important not only to regain conductance but also to avoid further neurodegeneration (Franklin and Ffrench-Constant, 2008). Both oligodendrocyte progenitors and ependymal cells have previously been demonstrated to generate myelinating oligodendrocytes (Dimou et al., 2008; Meletis et al., 2008; Rivers et al., 2008). We show here that the vast majority of new oligodendrocytes in the injured spinal cord derive from oligodendrocyte progenitors and a smaller fraction from ependymal cells. The beneficial effects of transplantation of myelinating cells after spinal cord injury in animal models (Franklin and Ffrench-Constant, 2008) indicate that the replacement of oligodendrocytes from endogenous cells is incomplete or too slow to be fully efficient.

Astrocytes are the dominating glial cells in the scar that forms at the injury, and chondrotin sulfate proteoglycans in the scar inhibit axonal regrowth, indicating that astrocytes prevent axonal regeneration (Busch and Silver, 2007; Silver and Miller, 2004). However, astrocytes shield the intact tissue from further damage and limit the infiltration of inflammatory cells and further demyelination (Faulkner et al., 2004; Okada et al., 2006). Thus, the large number of astrocytes at the injury site appears important to limit and restrain the inflammatory response, but this may be at the expense of axonal regrowth. We demonstrate two different cellular origins of new astrocytes after spinal cord injury, preexisting astrocytes and ependymal cells. These new astrocytes differ with regard to phenotype and distribution. Most ependymal cell-derived astrocytes are GFAP negative but express other markers and ultrastructural characteristics of astrocytes (Meletis et al., 2008). The ependymal cell-derived astrocytes form the core of the glial scar and the astrocytes at the periphery of the scar arise by astrocyte duplication.

Potential Strategies to Modulate Endogenous Neural Stem and Progenitor Cells for Spinal Cord Repair

Transplantation of in vitro expanded spinal cord-derived neural stem cells promotes the recovery after spinal cord injury in

experimental models (Hofstetter et al., 2005). The presence of resident cells in the adult spinal cord capable to promote functional recovery is encouraging for the prospect of developing pharmacological strategies to modulate endogenous spinal cord stem cells in situ.

A goal for strategies aiming at promoting repair by endogenous spinal cord stem cells is to promote the generation of oligodendrocytes. Although ependymal cells generate considerably fewer oligodendrocytes than the oligodendrocyte progenitors after spinal cord injury, they give rise to a larger total number of progeny. Most ependymal progeny become astrocytes and contribute to scar formation. It appears attractive to direct the differentiation of ependymal cell progeny from the astrocytic to the oligodendrocyte lineage. This could potentially result in both reduced scar formation and improved remyelination. That ependymal cell-derived oligodendrocytes indeed may promote functional recovery is supported by the fact that ependymal cells are the origin of the in vitro expanded neural stem cells that improve function after transplantation in animal models of spinal cord injury, and that this effect was augmented when such cells were manipulated to promote oligodendrocyte differentiation (Hofstetter et al., 2005). The rapidly increasing knowledge on cell fate specification during embryogenesis and in adult lineages will aid in the development of experimental strategies to modulate endogenous stem and progenitor cells for spinal cord repair.

EXPERIMENTAL PROCEDURES

Transgenic Mice

FoxJ1-CreER (Meletis et al., 2008), Cx30-CreER (Slezak et al., 2007), and Olig2-CreER (Takebayashi et al., 2002) mice on R26R-βgalactosidase or yellow fluorescent protein Cre-reporter background (Soriano, 1999; Srinivas et al., 2001) backcrossed to C57/Bl6 were studied in adult stages (Table S1). Heterozygous Olig2-CreER mice, which appear normal (Masahira et al., 2006; Takebayashi et al., 2002), were used. Recombination was induced by 5 daily intraperitoneal injections of 2 mg tamoxifen (Sigma, 20 mg/ml in corn oil).

Immunohistochemistry

Mice were perfused transcardially with PBS followed by 4% formaldehyde in PBS and spinal cords were postfixed overnight at 4°C and then cryoprotected in 30% sucrose. All antibodies used are specified in the Supplemental Information. Cell nuclei were visualized with DAPI (1 mg/mL, Sigma). Control sections were stained with secondary antibody alone. Images show one focal plane and the Z-projection of stacks.

Neural Stem Cell Cultures

Spinal cord cells were dissociated and neurosphere cultures were established as described (Meletis et al., 2008). All cells isolated from one spinal cord were plated in 10 cm cultures dishes. Single recombined neurospheres were picked manually, dissociated, and plated in one well of a 96-well plate. Clonally derived secondary neurospheres were collected and split into two: one half was used for continuous passaging and subsequent neural stem cell differentiation whereas the other half was used for plating in differentiating conditions (poly-D-lysine-coated slides with growth factor free medium supplemented by 1% FCS). After 2 weeks in differentiation condition, immunocytochemistry was performed as described above with antibodies described in the Supplemental Information.

Spinal Cord Injury and BrdU Labeling

Mice were anesthetized with 2.5% Avertin and the dorsal funiculus was cut transversely without reaching the gray matter or central canal at Th4 and was extended rostrally with microsurgical scissors to span one segment (Frisén et al., 1993; Meletis et al., 2008).

BrdU (1 mg/ml and 1% sucrose, exchanged every 3 days and kept in dark) was administered in the drinking water or administered twice by intraperitoneal injections (12 mg/ml, 100 μl per injection) at 6 hr interval, followed by BrdU administration in the drinking water (5 days).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at doi:10.1016/j.stem.2010.07.014.

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