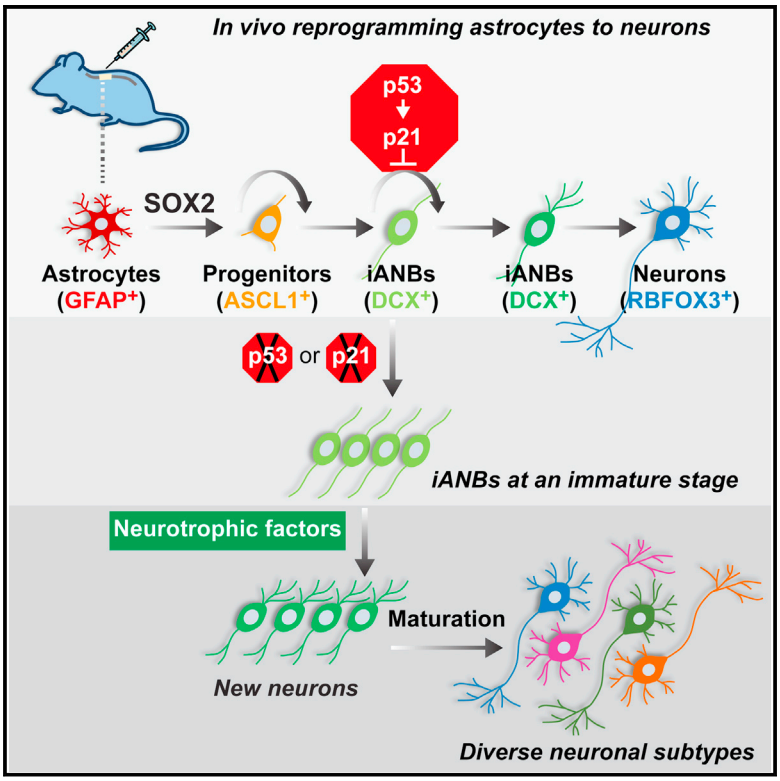
Resource

Cell Reports

The p53 Pathway Controls SOX2-Mediated Reprogramming in the Adult Mouse Spinal Cord

Graphical Abstract



Highlights

- In vivo screens examine reprogramming in the adult mouse spinal cord
- The p53 pathway controls cell-cycle exit of induced neuroblasts from astrocytes
- A neurotrophic milieu is required for efficient maturation of induced neuroblasts
- Induced neuroblasts mature into diverse but predominantly glutamatergic neurons

Authors

Lei-Lei Wang, Zhida Su, Wenjiao Tai, Yuhua Zou, Xiao-Ming Xu, Chun-Li Zhang

Correspondence

suzhida@smmu.edu.cn (Z.S.), chun-li.zhang@utsouthwestern.edu (C.-L.Z.)

In Brief

Through a series of in vivo screens, Wang et al. find that the p53-p21 pathway regulates reprogramming of resident astrocytes to mature neurons in the adult mouse spinal cord. Their results suggest a possible molecular and cellular basis for future regeneration-based therapy after spinal cord injury.

Wang et al., 2016, Cell Reports *17*, 891–903
October 11, 2016
http://dx.doi.org/10.1016/j.celrep.2016.09.038

CellPress

Cell Reports
Resource

The p53 Pathway Controls SOX2-Mediated Reprogramming in the Adult Mouse Spinal Cord

Lei-Lei Wang,[1,2] Zhida Su,[1,3,*] Wenjiao Tai,[1,2] Yuhua Zou,[1,2] Xiao-Ming Xu,[4] and Chun-Li Zhang[1,2,5,*]
[1]Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA
[2]Hamon Center for Regenerative Science and Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA
[3]Institute of Neuroscience and Key Laboratory of Molecular Neurobiology of Ministry of Education, Second Military Medical University, Shanghai 200433, China
[4]Spinal Cord and Brain Injury Research Group, Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, IN 46202, USA
[5]Lead Contact
*Correspondence: suzhida@smmu.edu.cn (Z.S.), chun-li.zhang@utsouthwestern.edu (C.-L.Z.)
http://dx.doi.org/10.1016/j.celrep.2016.09.038

SUMMARY

Although the adult mammalian spinal cord lacks intrinsic neurogenic capacity, glial cells can be reprogrammed in vivo to generate neurons after spinal cord injury (SCI). How this reprogramming process is molecularly regulated, however, is not clear. Through a series of in vivo screens, we show here that the p53-dependent pathway constitutes a critical checkpoint for SOX2-mediated reprogramming of resident glial cells in the adult mouse spinal cord. While it has no effect on the reprogramming efficiency, the p53 pathway promotes cell-cycle exit of SOX2-induced adult neuroblasts (iANBs). As such, silencing of either *p53* or *p21* markedly boosts the overall production of iANBs. A neurotrophic milieu supported by BDNF and NOG can robustly enhance maturation of these iANBs into diverse but predominantly glutamatergic neurons. Together, these findings have uncovered critical molecular and cellular checkpoints that may be manipulated to boost neuron regeneration after SCI.

INTRODUCTION

Severe morbidity and mortality are commonly associated with spinal cord injury (SCI). Human patients who survive SCI frequently live with paralysis and extremely reduced quality of life and productivity. The financial and emotional burdens to patients and their caregivers are enormous. There is currently no effective cure. This is largely because SCI often results in a permanent loss of neurons and the disruption of neural circuits that are critical for normal motor, sensory, and autonomic functions (Bradbury and McMahon, 2006; Rossignol et al., 2007; Thuret et al., 2006).

Unlike discrete regions of the adult brain in which new neurons are constantly born (Jessberger and Gage, 2014; Kempermann et al., 2015; Lim and Alvarez-Buylla, 2016), the adult mammalian spinal cord exhibits minimal regenerative capacity under physiological and pathological conditions (Chi et al., 2006; Horky et al., 2006; Horner et al., 2000; Shechter et al., 2007; Vessal et al., 2007; Yang et al., 2006). Proliferative cells in the intact adult spinal cord exclusively give rise to glial cells (Horner et al., 2000). In response to SCI, astrocytes, ependymal cells, NG2-glia, pericytes, fibroblasts, and microglia become activated, proliferate, migrate, and generate scar-forming glial cells surrounding the damaged regions (Fitch and Silver, 2008; Göritz et al., 2011; Horky et al., 2006; Johansson et al., 1999; Ohori et al., 2006; Sellers et al., 2009; Sofroniew, 2009). Injury-induced neurogenesis in the adult spinal cord is extremely rare and new neurons are not detectable in most cases (Horky et al., 2006; Johansson et al., 1999; Su et al., 2014). However, neurosphere-forming cells can be isolated from the adult spinal cord and show multi-lineage differentiation potential in vitro (Ohori et al., 2006; Shihabuddin et al., 2000), suggesting that the fate of certain resident non-neuronal cells may be manipulated for neurogenesis in vivo. Indeed, growth factor treatment and ectopic expression of NEUROG2 can stimulate endogenous neural progenitors to produce neurons after SCI (Ohori et al., 2006). We and others also have shown that resident glial cells, such as astrocytes and NG2 glia, can be in vivo reprogrammed to neurons in the adult brain and spinal cord (Guo et al., 2014b; Heinrich et al., 2014; Islam et al., 2015; Liu et al., 2015; Niu et al., 2013, 2015; Su et al., 2014; Torper et al., 2013, 2015).

In contrast to a direct reprogramming process controlled by ectopic expression of NEUROD1 or other neurogenic factors (Guo et al., 2014b; Liu et al., 2015; Torper et al., 2013, 2015), SOX2-mediated in vivo reprogramming of brain astrocytes is a multistep process that passes through $ASCL1^+$ progenitors and DCX^+ neuroblasts before transitioning into mature neurons (Niu et al., 2015). Both ASCL1 and the orphan nuclear receptor TLX are critically involved in this process (Islam et al., 2015; Niu et al., 2015). Importantly, SOX2-induced adult neuroblasts (iANBs) can be similarly detected in the injured adult spinal cord, even though the total number of iANBs and iANB-derived mature neurons was low (Su et al., 2014). To understand the reprogramming process and improve the production of iANBs in the adult spinal cord, we modified several procedures and screened a series of transcription factors and signaling molecules. We found that the p53 pathway is a critical checkpoint

that controls cell-cycle exit of iANBs. Downregulation of this pathway leads to a significant increase in iANBs, which can mature into both excitatory and inhibitory neurons in the presence of neurotrophic factors in the injured adult spinal cord.

RESULTS

The p53 Pathway Restricts the Generation of Spinal iANBs

In contrast to robust generation of iANBs and adult-born neurons in the striatum (Niu et al., 2013), SOX2-mediated reprogramming in the adult spinal cord was less efficient, with only about 200 DCX^+ cells detected surrounding the injected area at 4–8 weeks post-injection (wpi) of virus (Su et al., 2014). We then conducted a series of in vivo screens for factors that might potentiate SOX2 reprogramming ability. Most of these screens, including conditional deletion of *Pten* or ectopic expression of *Pax6*, *Neurog2*, *Ascl1*, *Sox11*, *Tlx*, *Pou5f1*, *Olig2*, *Ptf1a*, *Sox1*, *Sox3*, *Brn2*, *NeuroD1*, *Fgf2*, *miR-9/9**, or *miR-124*, failed to significantly enhance the appearance of iANBs.

Conditional removal of *p53*, on the other hand, greatly enhanced the number of SOX2-induced iANBs, with about 1,500 DCX^+ cells detected at 5 or 6 wpi (Figures S1A and S1B). Very interestingly, the largest increase came from optimization of several procedures, such as improvements on SOX2 virus quality and injection methods. These optimizations led to the detection of ~10,000 DCX^+ cells in the SOX2-alone-injected spinal region at 4 wpi (Figure S1C). Even under this optimized condition, additional genetic deletion of *p53* continued to result in a roughly 100% increase in the number of iANBs (Figures 1A and 1B). This increase also was observed when *p53* expression was transiently downregulated by RNA polymerase II- and human GFAP promoter-dependent expression of *p53* small hairpin RNA (shRNA) (Figures 1C and S2A–S2D).

p21 is a major downstream target of p53. Its expression in astrocytes is also dependent on p53 (Figure S2E). To examine the role of p21 in the reprogramming process, we injected *SOX2*-expressing virus into the spinal cord of adult *p21* knockout (KO) mice. When examined at 4 wpi and compared to the wild-type controls, the number of DCX^+ cells was nearly doubled in the *p21* KO mice (Figures 1D and 1E). Together, these results demonstrated a critical role of the p53-p21 pathway in controlling the SOX2-mediated generation of iANBs.

The p53 Pathway Promotes Cell Cycle Exit of Spinal iANBs

In response to stresses or insults, p53 activation induces cell-cycle arrest or apoptosis to safeguard cells from undergoing uncontrolled proliferation (Lane and Levine, 2010). We examined iANB apoptosis by staining for activated caspase 3, and we did not find a significant difference in the spinal cord with or without p53 downregulation by shRNA or in *p21* KO mice when compared to wild-type controls (Figures S3A and S3B). To further examine cell apoptosis, we performed terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assays. A few $TUNEL^+$ cells were detected in spinal cords injected with the lentivirus-expressing SOX2 and the control shRNA or shRNA-p53, although none of them were DCX^+ (Figures S3C and S3D). A small but significant increase of $TUNEL^+$ cells was observed in the group with shRNA-p53 only at 3 wpi, but not at 4 wpi, suggesting that downregulation of p53 did not enhance cell survival.

During adult neurogenesis, DCX^+ neuroblasts consist of both early proliferating and later non-proliferating cells in the endogenous neurogenic niches (Lugert et al., 2012). A fraction of iANBs also was shown to be proliferative in the adult brain. We examined iANB proliferation in the adult spinal cord by staining for KI67, a nuclear protein present in all phases of the cell cycle but absent in quiescent cells (Scholzen and Gerdes, 2000). A time course analysis showed that proliferation of SOX2-dependent iANBs was significantly decreased from 20% at 1 wpi to 5% at 4 wpi in the wild-type spinal cord (Figure 2A). When *p53* was conditionally deleted by Cre expression or downregulated through shRNAs, a roughly 3-fold increase in proliferating iANBs was observed when compared to controls at 4 wpi (Figures 2B–2D).

Similarly, immunohistochemistry analysis showed that *p21* deletion resulted in a dramatic increase in proliferating iANBs, from 5% in wild-type mice to nearly 30% in *p21* KO mice (Figures 2E and 2F). To further characterize cell proliferation, we treated mice with bromodeoxyuridine (BrdU)-containing drinking water for 3 days starting at 3 wpi (Figure 2G). Over 90% of iANBs were labeled by BrdU in adult *p21* KO mice when examined at 4 wpi (Figure 2H). Cell-cycle exit of iANBs was determined by quantifying the fraction of $KI67^+BrdU^+DCX^+$ cells among the total $BrdU^+DCX^+$ cells (Figure 2I). The *p21* KO mice showed a 22.5% increase in iANBs remaining in cell cycle compared to wild-type controls (Figure 2J). Together these data show that the p53-p21 pathway tightly regulates iANB production by restricting proliferation and cell-cycle exit.

Reprogramming Efficiency Is Not Affected by the p53 Pathway

The p53 pathway was shown to be a roadblock during reprogramming of somatic cells to pluripotency. Its silencing significantly increases the reprogramming efficiency of fibroblasts to pluripotent stem cells (Banito et al., 2009; Hanna et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009). We examined whether the p53 pathway has a similar effect on SOX2-mediated in vivo reprogramming efficiency, which may contribute to the overall production of iANBs.

Our previous genetic lineage mapping showed that SOX2 initially reprograms astrocytes to $ASCL1^+$ progenitors, which further differentiate into DCX^+ iANBs and mature neurons (Niu et al., 2015). Therefore, the induction of $ASCL1^+$ progenitors can be used to estimate the in vivo reprogramming efficiency. *SOX2* virus was injected into the spinal cord of adult wild-type or *p21* KO mice, which were examined at 3 wpi. $ASCL1^+$ cells were robustly induced; however, no difference in their number was detected between *p21* KO and wild-type control mice (Figures 3A and 3B). KI67 staining and quantification also failed to reveal any significant difference in progenitor proliferation between these groups (Figure 3C).

$ASCL1^+$ progenitors gradually transited to early DCX^+ neuroblasts, which expressed both markers (Figure 3D). Quantification

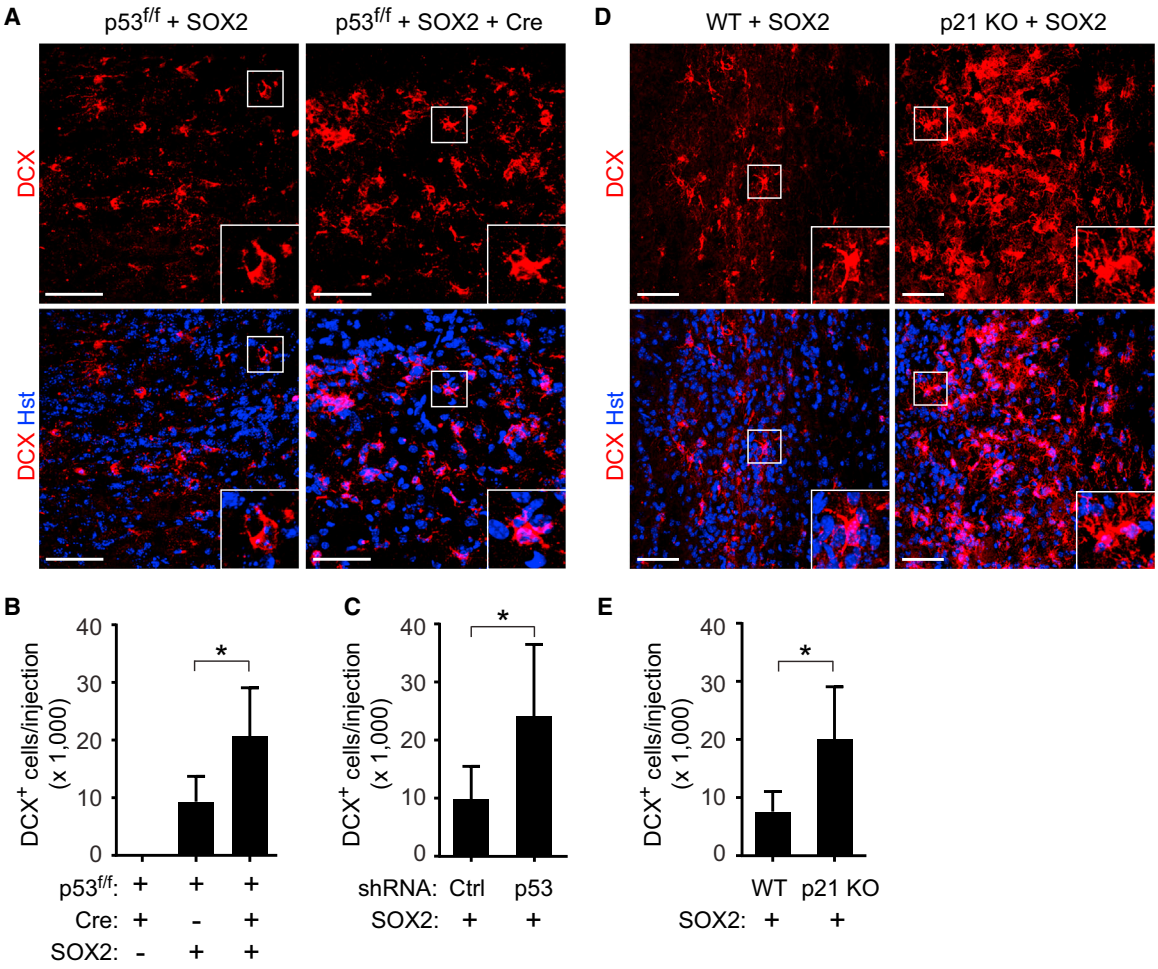


Figure 1. The p53 Pathway Impedes iANB Production In Vivo

(A and B) Conditional deletion of *p53* promotes SOX2-dependent iANBs in the adult spinal cord. iANBs were determined by DCX staining at 4 wpi (mean ± SD; n = 4 mice per group; *p = 0.05 by Student's t test). Nuclei were counterstained with Hoechst 33342 (Hst). wpi, weeks post-virus injection. Scale bar, 50 μm.

(C) The shRNA-mediated knockdown of p53 expression enhances spinal iANBs. Cells were quantified at 4 wpi (mean ± SD; n = 6 mice per group; *p = 0.027 by Student's t test). An shRNA against firefly luciferase was used as a control (Ctrl).

(D and E) The number of iANBs is increased in *p21*-null spinal cords. DCX^+ iANBs were analyzed at 4 wpi (mean ± SD; n = 4 mice per group; *p = 0.042 by Student's t test). Scale bar, 50 μm.

See also Figures S1 and S2.

of this $ASCL1^+DCX^+$ subgroup among all progenitors did not reveal any influence by *p21* deletion (Figure 3E). In contrast, the ratio of $ASCL1^+DCX^+$ cells to total DCX^+ cells was much lower in the *p21* KO group (Figure 3F), consistent with a dramatic increase in DCX^+ iANBs caused by silencing *p21* (Figure 1E). Together these results suggest that the p53 pathway does not have a major impact on the induction of $ASCL1^+$ progenitors, their proliferation, or their transition to early DCX^+ iANBs.

Spinal iANBs Are Halted at an Immature Stage

Further maturation of iANBs is important for functional integration within a neuronal network. As SOX2-mediated in vivo reprogramming passes through a proliferative stage, we used BrdU in drinking water to trace the fate of iANBs (Figure 4A). Mature neurons were identified by staining for RBFOX3 (also known as NEUN), a marker tightly associated with neuron differentiation and maturation (Mullen et al., 1992). When examined at 8 wpi, $BrdU^+RBFOX3^+$ cells were observed in *SOX2* virus-injected spinal regions of both wild-type and *p21* KO mice (Figures 4B and 4C); however, quantification failed to show any increase by silencing *p21* expression.

A separate cohort of mice also was treated with valproic acid (VPA) in drinking water for 4 weeks to promote neuronal maturation (Figure 4A). Consistent with our previous results (Niu et al., 2013; Su et al., 2014), VPA treatment resulted in nearly 6,000 $BrdU^+RBFOX3^+$ cells in *SOX2* virus-injected spinal regions of wild-type mice; however, this number of cells was not observed in *p21* KO mice (Figure 4C).

Such a low number of mature neurons in *p21* KO mice might be due to a loss of iANBs during the maturation process.

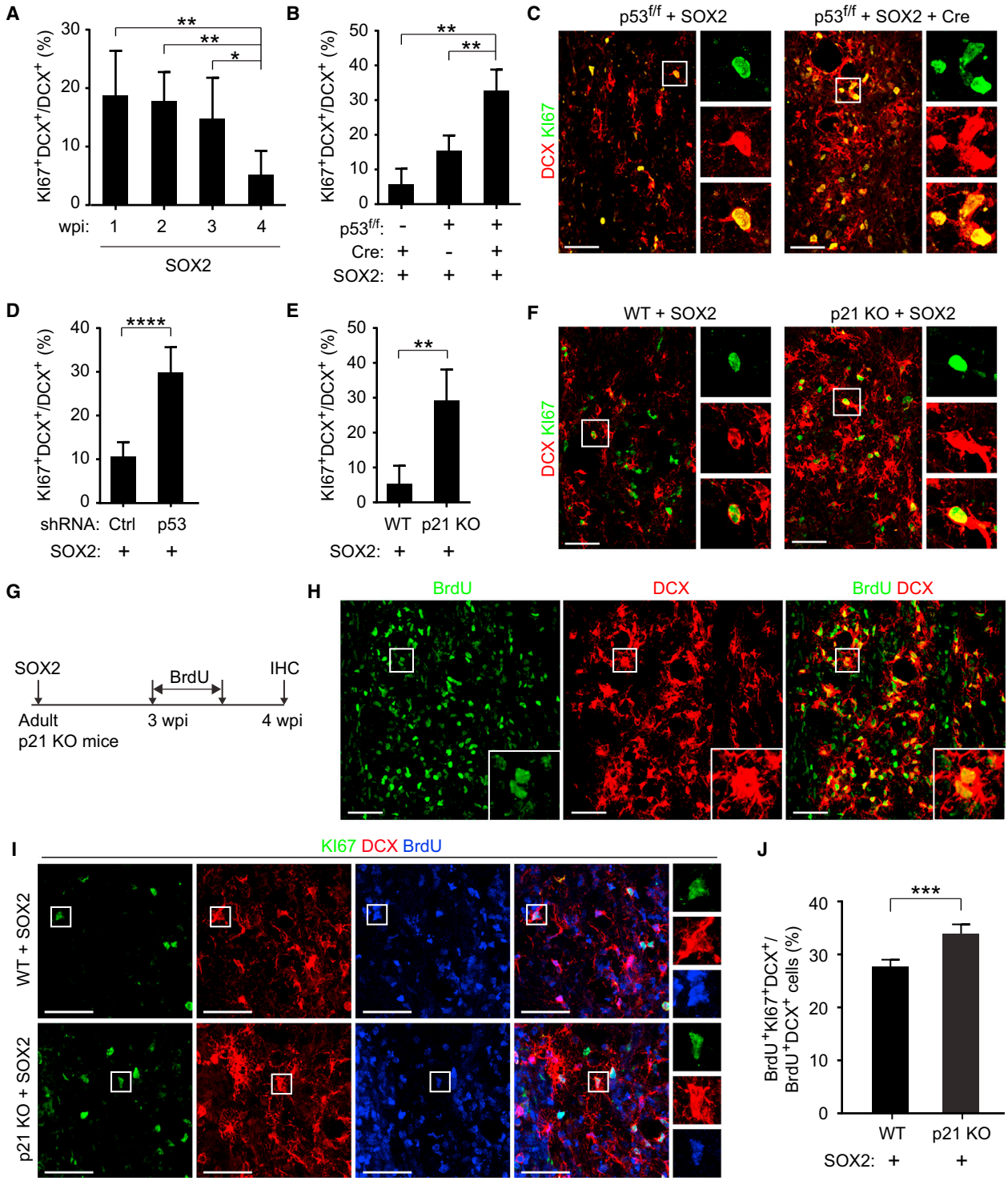


Figure 2. The p53 Pathway Promotes Cell Cycle Exit of iANBs

(A) The ratio of proliferating iANBs decreases with time (mean ± SD; n = 5 mice per group; *p = 0.03 and **p < 0.01 by Student's t test).

(B) Conditional *p53* deletion leads to an increased ratio of proliferating iANBs. Cells were quantified in the virus-injected spinal regions at 4 wpi (mean ± SD; n = 4 mice per group; **p < 0.01 by Student's t test).

(C) Immunohistochemistry shows the influence of *p53* status on iANB proliferation at 4 wpi. Scale bar, 50 μm.

(D) Knockdown of *p53* expression increases the ratio of proliferating iANBs when examined at 4 wpi (mean ± SD; n = 4–6 mice per group; ****p < 0.0001 by Student's t test; Ctrl, control shRNA).

(legend continued on next page)

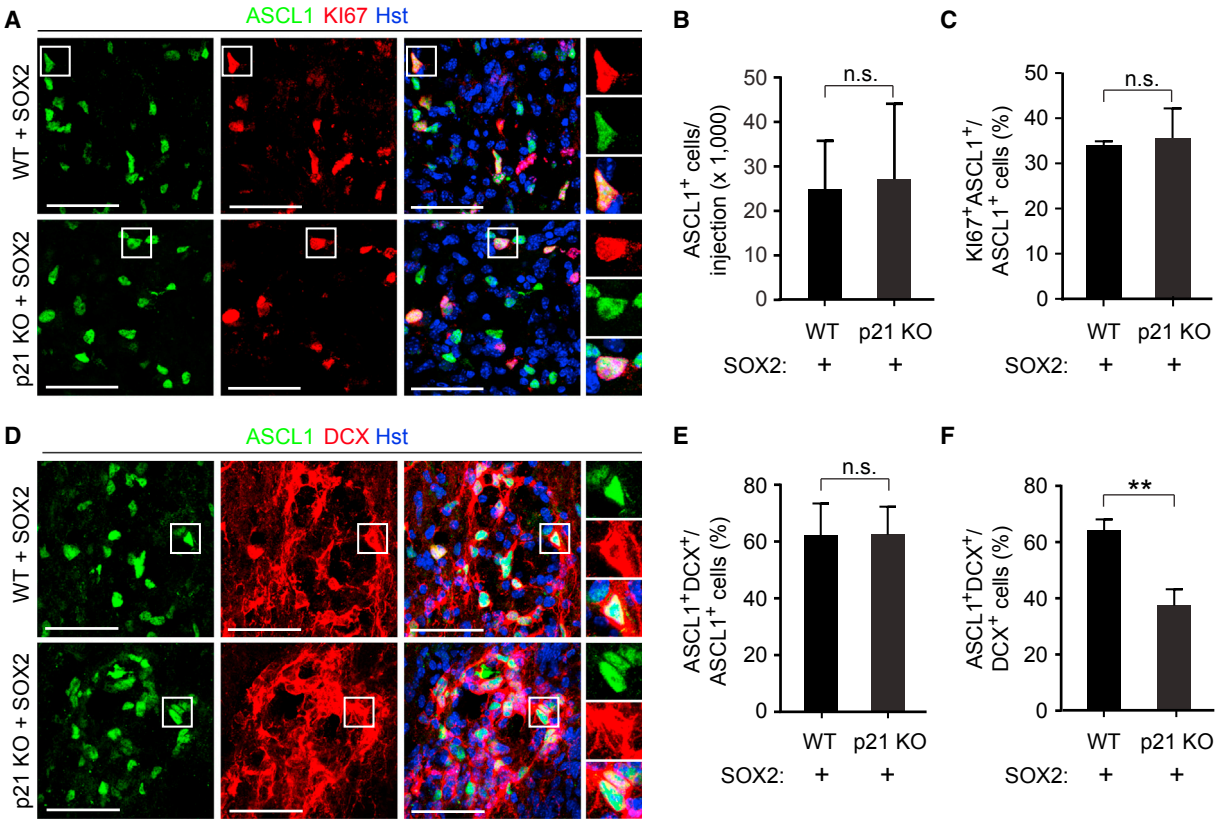


Figure 3. The p53 Pathway Specifically Targets Later Reprogramming Steps

(A) Immunohistochemical analysis of SOX2-induced ASCL1$^+$ progenitors at 3 wpi. Cell proliferation was analyzed by KI67 staining. Scale bar, 50 μm.

(B) *p21* status has no effect on the number of SOX2-induced ASCL1$^+$ progenitors (mean ± SD; n = 3 mice per group; n.s., not significant by Student's t test).

(C) *p21* status has no effect on proliferation of ASCL1$^+$ progenitors (mean ± SD; n = 3 mice per group; n.s., not significant by Student's t test).

(D) Immunohistochemical analysis of induced ASCL1$^+$ progenitors transitioning to DCX$^+$ neuroblasts is shown. Scale bar, 50 μm.

(E) *p21* status has no effect on the transition of progenitors to early ASCL1$^+$DCX$^+$ neuroblasts (mean ± SD; n = 3 mice per group; n.s., not significant by Student's t test).

(F) *p21* restricts overall production of later neuroblasts (mean ± SD; n = 3 mice per group; **p = 0.003 by Student's t test).

Immunohistochemistry was conducted to determine whether iANBs were detectable at 8 wpi. Unexpectedly, a significantly larger number of DCX$^+$ iANBs was still observed in mice with *p21* deletion (Figures 4D and 4E). Continuous VPA treatment in drinking water showed a minimal effect on the number of iANBs. Together these results indicate that iANBs, though robustly generated, are halted at an immature state after silencing the p53-p21 pathway.

A Neurotrophic Milieu Promotes the Maturation of Spinal iANBs

During neural development, neurotrophic factors are critically important for neuronal survival and maturation. A neurotrophic milieu suitable for neuron maturation may not persist in the adult spinal cord. By using a lentiviral delivery approach, we conducted a candidate screen for factors that might enhance the maturation of SOX2-induced spinal iANBs. These included GDNF, FGF2, BDNF, and NOG. Once again, continuous BrdU treatments in drinking water were employed to trace adult-born neurons, which were examined at 8 wpi (Figure 5A).

The presence of either GDNF or FGF2 increased the number of BrdU$^+$RBFOX3$^+$ cells to roughly 6,000 in injected regions of the *p21* KO spinal cord, while these two factors had minimal effect in the wild-type background (Figure 5B). On the other hand, wild-type mice responded to treatment with BDNF-NOG and showed about 7,000 BrdU$^+$RBFOX3$^+$ cells surrounding the *SOX2* virus-injected spinal regions. The *p21* KO mice had the most striking response to BDNF-NOG, with nearly 30,000

(E) The ratio of proliferating iANBs is enhanced in *p21* KO mice when examined at 4 wpi (mean ± SD; n = 4 mice per group; **p = 0.004 by Student's t test).

(F) Confocal images show proliferating iANBs in wild-type (WT) or *p21* KO mice. Scale bar, 50 μm.

(G) An experimental scheme to analyze cell proliferation and cell-cycle exit is shown. IHC, immunohistochemistry.

(H) Confocal images show iANBs traced by BrdU incorporation in *p21* KO mice. Scale bar, 50 μm.

(I) Confocal images show proliferating BrdU-traced iANBs at 4 wpi. Scale bar, 50 μm.

(J) More iANBs remain in cell-cycle in the spinal cord of adult *p21* KO mice (mean ± SD; n = 4 mice per group; ***p = 0.001 by Student's t test).

See also Figure S3.

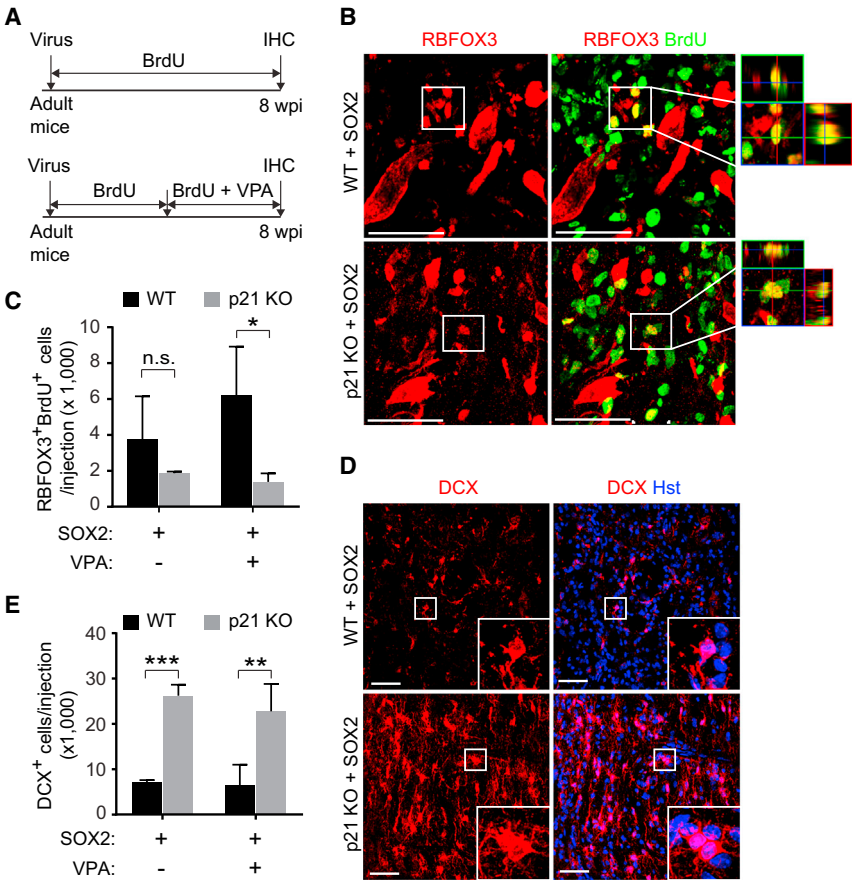


Figure 4. iANBs Are Arrested at an Immature Stage

(A) Experimental schemes for the analysis of reprogrammed spinal neurons. Both BrdU and valproic acid (VPA) were supplied in drinking water.

(B) Confocal images show new neurons with expression of RBFOX3, a marker for neuronal maturation. Scale bar, 50 μm.

(C) Reprogrammed neurons rarely become mature in *p21* KO mice (mean ± SD; n = 3–4 mice per group; n.s., not significant; *p = 0.03 by Student's t test).

(D) Immunohistochemical analysis of iANBs at 8 wpi is shown. Scale bar, 50 μm.

(E) *p21* deletion results in iANBs remaining at an immature stage (mean ± SD; n = 3–4 mice per group; **p = 0.006 and ***p = 0.0002 by Student's t test).

$BrdU^+RBFOX3^+$ cells detected in the penumbra of injected regions (Figures 5B and 5C). In addition to in the gray matter, these cells were also robustly detectable in the white matter where the somas of endogenous neurons are not normally present (Figure 5C). The effect of BDNF-NOG on iANB maturation also was confirmed in wild-type mice with shRNA-mediated knockdown of *p53* (Figure 5D). Of note, $BrdU^+RBFOX3^+$ cells were undetectable in control mice when *SOX2*-expressing virus was not injected.

Treatments with BDNF-NOG also promoted $BrdU^+$ new neurons to express MAP2, another well-established marker for mature neurons (Dehmelt and Halpain, 2005) (Figure 5E). Staining for SYN1, a presynaptic vesicle-associated protein, showed punctate patterns in the processes and somas of adult-born neurons, indicating synaptic connections with local neurons (Figure 5F). Many of these new neurons could be detected in the white matter, with potential to readily form circuits with ascending and descending axons.

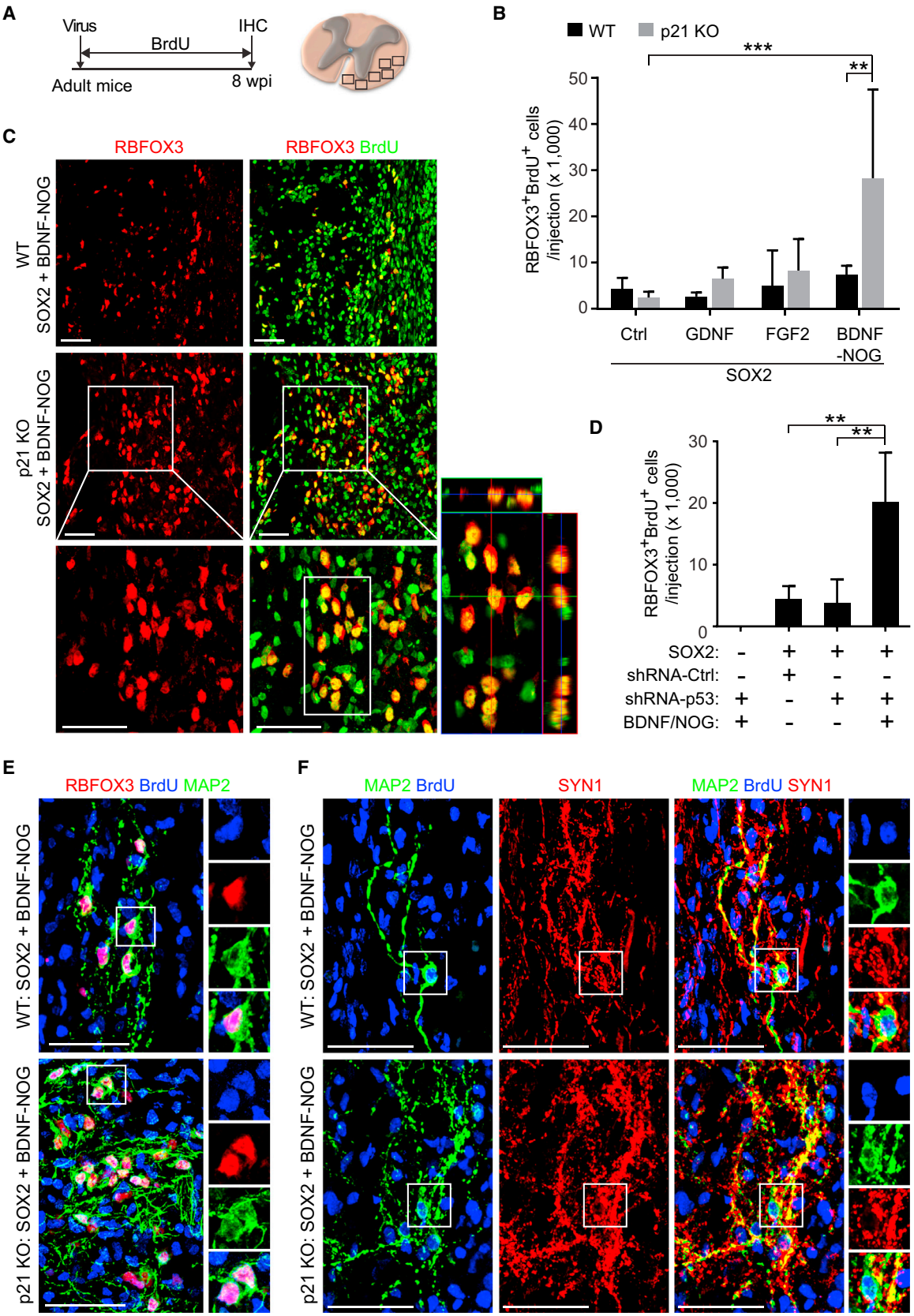
Spinal iANBs Mature into Diverse Neuronal Subtypes

To facilitate subtype analysis of astrocyte-converted neurons, we created the *mGfap-Cre*;*Thy1-STOP-YFP* (GcTy) mice (Figure 6A). The *mGfap-Cre* transgenic line 77.6 exhibits astrocyte-restricted Cre recombinase expression under the mouse *Gfap* promoter in the brain and spinal cord (Gregorian et al., 2009; Su et al., 2014). In the *Thy1-STOP-YFP* reporter line, yellow fluorescent protein (YFP) expression is under the neuron-specific regulator elements of the mouse *Thy1* promoter after Cre-mediated removal of the STOP cassette (Buffelli et al., 2003). The GcTy mice showed weak but restricted YFP expression in spinal astrocytes (Figure S4). Nonetheless, very few sparsely distributed YFP^+ cells (<0.01%) were also $RBFOX3^+$ found in the dorsal horns, but not ventral regions (Figure S4); we therefore limited our analysis to the ventral spinal cord to exclude any potential contamination from endogenous neurons. Reprogramming factors were mainly delivered into the ventral spinal cord of adult GcTy mice. Immunohistochemistry was performed at 8–24 wpi.

In GcTy mice, SOX2-reprogrammed DCX^+ iANBs were robustly labeled by YFP, confirming an astrocyte-origin (Figure 6B). Clustered YFP^+ cells expressing markers for mature neurons, such as MAP2 and RBFOX3, could be detected in both the white matter and gray matter (Figures S5A and S5B). These YFP^+ cells were not observed in control virus-injected GcTy mice. Over 99.9% of the YFP-traced neurons also robustly expressed SYN1 with a punctate pattern (Figure S5C), indicating synaptic connections with local neurons. An extensive survey of neuronal subtypes showed astrocyte-converted neurons were diverse, including $GAD6^+$ and $GABA^+$ GABAergic neurons (Figures 6C and S5D), $GLYT2^+$ glycinergic neurons (Figure 6D), $5\text{-}HT^+$ serotonergic neurons (Figure 6E), $VGLUT2^+$ glutamatergic neurons (Figure 6F), $CALB^+$ neurons (Figure 6G), and cholinergic neurons (Figure 6H). Quantification showed that the predominant subtype was glutamatergic, which represented over 80% of the reprogrammed neurons, while the other subtypes were below 20% or rarely observed (Figure 6I).

Robust Generation of New Neurons after Contusion Injury

We next examined whether new neurons can be reprogrammed from reactive astrocytes after contusion injury, which is the most



(legend on next page)

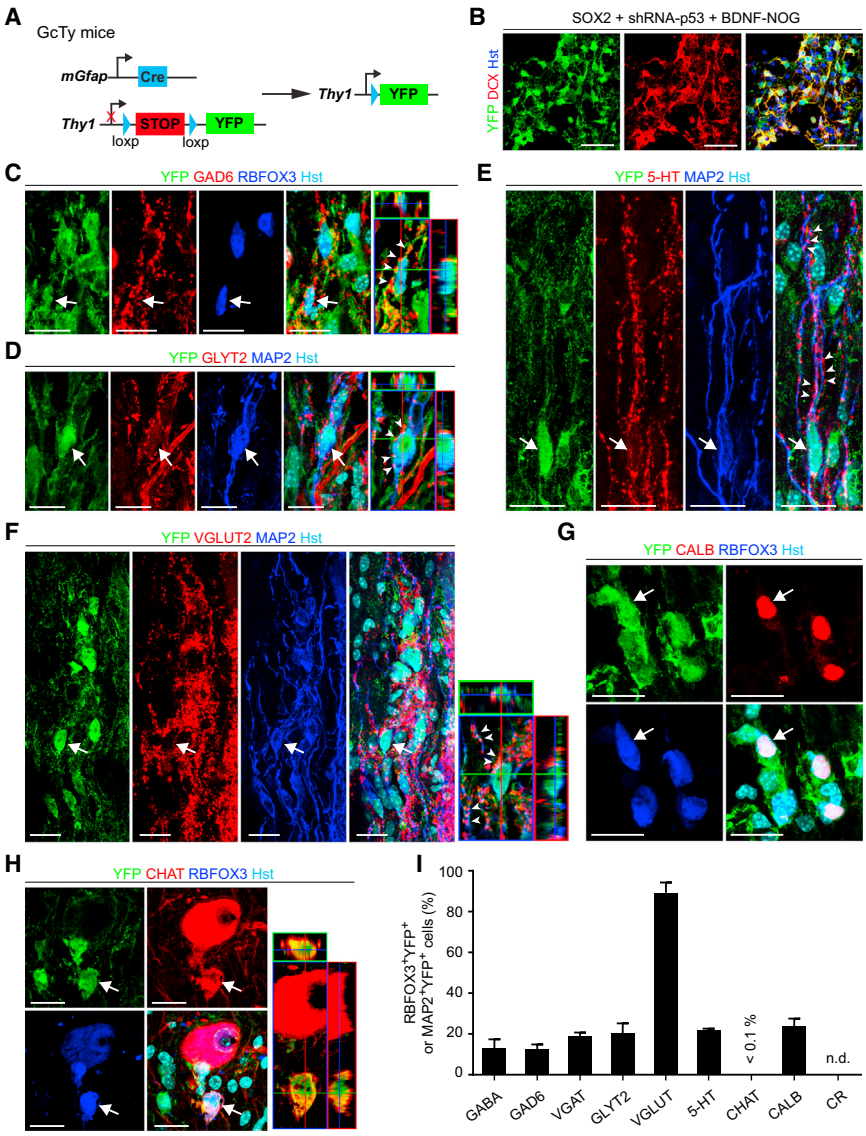


Figure 6. iANBs Mature into Diverse Neuronal Subtypes

(A) A genetic approach to trace astrocyte-converted neurons is shown.

(B) Robust labeling of SOX2-dependent iANBs with the genetic reporter YFP is shown. Scale bar, 50 μm.

(C–H) Confocal images of astrocyte-converted neurons with expression of the indicated markers. A typical cell is marked by an arrow, whereas arrowheads indicate punctate patterns of marker staining. Scale bar, 20 μm.

(I) Quantification of subtypes of astrocyte-converted neurons is shown (mean ± SD; n = 3 mice per group; n.d., not detected).

See also Figures S4 and S5.

clinically relevant SCI type. Astrocytes were genetically traced in *mGfap-Cre*;*Rosa-tdT* mice, in which the expression of tdTomato (tdT) is under the control of a *CAG* promoter after Cre-mediated deletion of a STOP cassette (Madisen et al., 2010). In vivo reprogramming factors were delivered through virus into the penumbra of the injury site immediately after contusion injury (Figures 7A and 7B). Astrocyte-converted mature neurons were examined by RBFOX3 staining and tdT fluorescence at 8 wpi. In control virus-injected mice with contusion SCI, RBFOX3$^+$ neurons were detected in the gray matter and none were labeled with tdT, confirming the glial specificity of the lineage-tracing reporter even under severe injury conditions (Figures 7C and 7D). In sharp contrast, nearly 6,000 tdT$^+$RBFOX3$^+$ cells were detected surrounding the lesion core in mice injected with the reprogramming factors (Figures 7C and 7D). The somas of many newly reprogrammed neurons were in the white matter, a feature not observed for endogenous neurons.

DISCUSSION

A lack of neurogenesis in the adult spinal cord may contribute to the failure of functional recovery after SCI. We now provide a molecular and cellular roadmap for robust generation of new spinal neurons after a mild or severe contusion SCI. This entails removing an inhibitory checkpoint during reprogramming of resident glial cells to mature neurons in vivo (Figure 7E).

Figure 5. Maturation of iANBs Requires a Neurotrophic Milieu

(A) A diagram to show experimental design. Viruses encoding SOX2 and neurotrophic factors were injected into the spinal cord of adult mice. IHC, immunohistochemistry.

(B) Neuronal maturation is greatly enhanced by neurotrophic factors (mean ± SD; n = 3–5 mice per group; $F(1,26) = 5.180$ and $p = 0.031$ for genotype effect, $F(3,26) = 6.325$ and $p = 0.002$ for treatment effect, $F(3,26) = 3.532$ and $p = 0.029$ for genotype-treatment interactions by two-way ANOVA; **p = 0.0092 and ***p = 0.0008 by post hoc Tukey's multiple comparisons test). Ctrl, empty virus as a control.

(C) Confocal images show reprogrammed cells expressing a marker for mature neurons at 8 wpi. Scale bar, 50 μm.

(D) *p53* knockdown promotes generation of SOX2-induced neurons under a neurotrophic milieu (mean ± SD; n = 5 mice per group; **$p < 0.004$ by Student's t test).

(E) SOX2-induced neurons express multiple markers for mature neurons. Confocal images were taken from the virus-injected white matter. Scale bar, 50 μm.

(F) SOX2-induced neurons exhibit robust expression of the synaptic protein SYN1. Confocal images were taken from the virus-injected white matter. Scale bar, 50 μm.

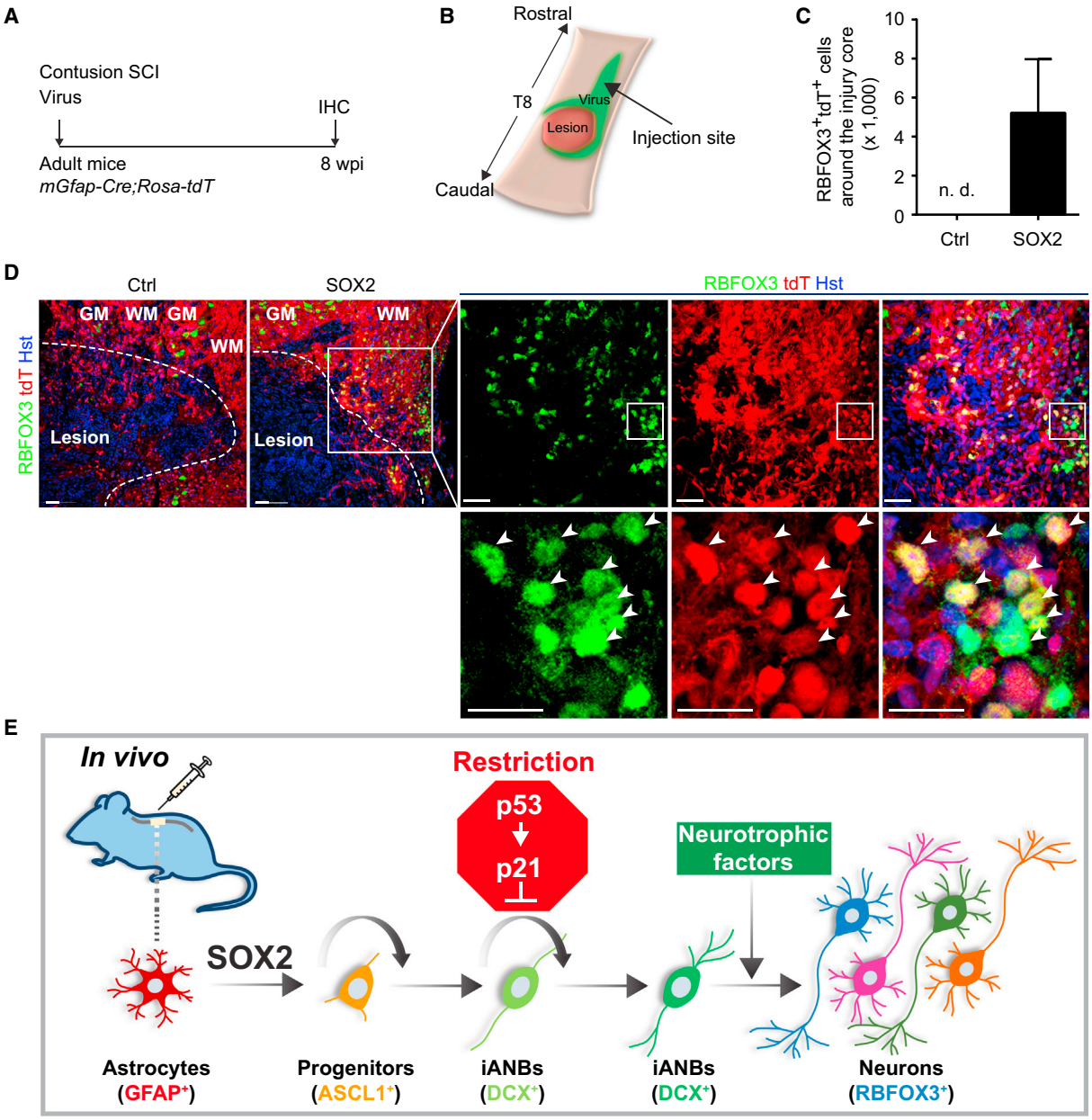


Figure 7. Robust Induction of New Neurons after Contusion Injury

(A) An experimental scheme to examine astrocyte-converted neurons in vivo is shown.

(B) A diagram for contusion injury and virus injection is shown.

(C) Quantification of astrocyte-converted neurons surrounding the lesion site is shown (mean ± SD; n = 3 mice per group; n.d., not detected; Ctrl, control virus).

(D) Confocal images of astrocyte-converted neurons surrounding the lesion core. Enlarged views of the boxed regions also are shown. Arrowheads show typical astrocyte-converted neurons. Scale bar, 20 μm.

(E) A molecular and cellular roadmap from resident astrocytes to mature neurons in the adult spinal cord. The p53-p21 pathway constitutes a checkpoint restricting expansion of iANBs.

The ectopic expression of SOX2 is sufficient to reprogram resident glial cells to neurons in the adult CNS (Heinrich et al., 2014; Niu et al., 2013, 2015; Su et al., 2014). Nonetheless, the number of reprogrammed neurons was low in the adult spinal cord (Su et al., 2014). This urged a better understanding of the reprogramming process and an improved strategy for the robust induction of new spinal neurons essential to regeneration after SCI. Among a score of factors examined in vivo, the p53-p21 pathway was uniquely revealed to be an essential checkpoint for SOX2-mediated reprogramming of spinal astrocytes in vivo. Silencing this

pathway, either through genetic deletion or transient knockdown, led to a significant increase in iANB generation, which can further give rise to mature neurons in a neurotrophic milieu. Although enhanced iANB production may elevate the risk of tumorigenesis, we failed to detect any spinal tumors 15 months after *p53* was conditionally silenced. Consistent with our previous results showing the number of iANBs is greatly reduced in a time-dependent manner (Niu et al., 2013), iANBs were rarely observed when examined around 6 months post-virus injection in the adult spinal cord. Additionally, transient inhibition of the p53 pathway may be achieved by the small molecule inhibitor Pifithrin-α (Komarov et al., 1999) or by AAV-mediated expression of shRNAs.

The p53 pathway is activated by reprogramming factors and acts as a roadblock to reprogramming somatic cells to pluripotency. Silencing this pathway in culture can significantly increase reprogramming efficiency for both the formation of induced pluripotent stem cells and transdifferentiation of fibroblasts to induced neurons (Banito et al., 2009; Guo et al., 2014a; Hanna et al., 2009; Hong et al., 2009; Jiang et al., 2015; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009; Zhao et al., 2008). In contrast, using the induction of $ASCL1^+$ progenitors to estimate reprogramming efficiency, we showed that silencing the p53-p21 pathway has little effect on the early steps of SOX2-mediated in vivo reprogramming. Both the induction and proliferation of $ASCL1^+$ progenitors were not significantly affected by *p21* deletion. These results may not be unexpected, as we and others previously have shown that p21 expression in neural stem/progenitor cells is normally suppressed by robust expression of the orphan nuclear receptor TLX (Li et al., 2012; Niu et al., 2011; Sun et al., 2007). Accompanying naturally diminished TLX expression in DCX^+ neuroblasts (Li et al., 2012), p21 expression is increased in these cells and in newly developing neurons (Pechnick et al., 2008). Consistent with a positive role in cell-cycle exit, *p21* deletion leads to greatly increased proliferation of both endogenous and SOX2-induced neuroblasts (Pechnick et al., 2008; this study). Together these data indicate that SOX2-mediated in vivo reprogramming passes through a multistep cellular process, which resembles that of endogenous adult neurogenesis and is subject to a tight molecular regulation.

A permissive microenvironment, also known as neurogenic niche, is critical for neuronal survival and maturation during adult neurogenesis in the lateral ventricle and dentate gyrus (Jessberger and Gage, 2014; Kempermann et al., 2015; Lim and Alvarez-Buylla, 2016). The niche consists of not only cellular contexts and extracellular matrix but also secreted humoral factors. Our results indicate that an appropriate niche is also critical for further differentiation of iANBs into mature neurons in the otherwise non-neurogenic adult spinal cord. Although silencing the p53 pathway creates a large population of iANBs, only a fraction can become mature under normal or injured conditions. Our further in vivo screens revealed that locally secreted BDNF and NOG constitute important niche factors supporting robust generation of newly mature spinal neurons from iANBs. These induced neurons are often in clusters and can be established in both the white matter and gray matter. Such broader and clustered distribution may provide more opportunities for the induced neurons to form connections between themselves, with preexisting local neurons, and with descending and ascending spinal axons. Although a detailed electrophysiological analysis is needed for confirmation, the robust detection of punctate SYN1 expression in induced neurons is indicative of their ability to form synaptic connections.

The local microenvironment and/or intrinsic properties of the originating resident astrocytes also are instructive for the formation of region-specific neuronal subtypes from astrocyte-converted iANBs. iANBs in the adult spinal cord do not generate CR^+ GABAergic interneurons, which are the major subtype of induced neurons in the adult striatum (Niu et al., 2015). In contrast, $VGLUT2^+$ glutamatergic neurons are the predominant subtype of induced neurons in the adult spinal cord. Endogenous $VGLUT2^+$ neurons are distributed throughout the spinal gray matter (Llewellyn-Smith et al., 2007). They recently were shown to be essential components of the locomotor circuitry and to play an essential role for proper organization of the spinal locomotor network (Borgius et al., 2014). As such, it is conceivable that the iANB-generated VGLUT2 excitatory neurons may well be capable of forming relay circuits with ascending and descending motor pathways, which are frequently disrupted by SCI (Abematsu et al., 2010; Courtine et al., 2008). Such relays, if confirmed, may contribute to functional motor recovery after SCI. In addition to glutamatergic neurons, 10%–20% of the induced neurons are GABAergic, glycinergic, or serotonergic. These neuronal subtypes are thought to be essential in coordinating muscle activation during locomotion (Nishimaru and Kakizaki, 2009).

Our ability to successfully produce a large population of long-lived and diverse subtypes of new neurons in the adult spinal cord provides a cellular basis for regeneration-based therapy for SCI. Compared to cell transplantation-based approaches, in vivo reprogrammed neurons from resident glial cells are immune homologous to the host. Therefore, the reprogrammed neurons will not require immune suppression for survival and integration into the local neuronal network. Future studies are warranted to investigate their biological function after SCI.

EXPERIMENTAL PROCEDURES

Animals

Wild-type C57BL/6J mice and the following mutant mouse lines were obtained from The Jackson Laboratory: *Pten^{flox}* (stock 004597) (Groszer et al., 2001), *p53^{flox}* (stock 008462) (Marino et al., 2000), *p21* KO (stock 003263) (Brugarolas et al., 1995), *mGfap-Cre* line 77.6 (stock 024098) (Gregorian et al., 2009), *Thy1-STOP-YFP* (stock 005630) (Buffelli et al., 2003), and *Rosa-tdT* (stock 007914) (Madisen et al., 2010). Unless otherwise stated, both male and female mice at 8 weeks and older were used for all experiments. All mice were housed under a controlled temperature and a 12-hr light/dark cycle, with free access to water and food, in an animal facility at University of Texas (UT) Southwestern. The sample sizes were empirically determined. The experiments were not randomized and the researchers were not blinded to the allocation of animals during experiments and outcome assessment. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee at UT Southwestern.

Virus Preparation and Intraspinal Injection

Lentivirus was used to deliver *SOX2*, *GFP-T2A-Cre*, *Bdnf*, *Nog*, *shRNA*, and the other listed genes into the adult spinal cord, as previously described (Niu

et al., 2013; Su et al., 2014). Gene expression was under the control of a modified human *GFAP* promoter, which was shown to mainly target astrocytes in the mouse spinal cord (Su et al., 2014). Briefly, the third-generation replication-deficient lentivirus was generated by transient transfections of HEK293T cells with lentiviral vectors together with the packaging plasmids (pMDL, VSV-G, and pREV). The virus-containing culture supernatants were collected and concentrated by centrifugation. Viral titers were estimated by quantifying GFP-expressing cells or by immunofluorescence staining of virus-transduced U251 glioma cells at 72 hr after viral infection. Using a 5-μl Hamilton syringe and a 34-gauge, 18-degree-beveled needle (Hamilton), 1.5 μl lentivirus (0.5–1 × 10^9 pfu/mL) was manually injected into the spinal parenchyma at each of the two locations 2 mm apart at the T8 level. The injection rate was maintained at 0.3 μL/min, and 1 min was taken to slowly withdraw the needle upon completion of the injection.

BrdU and VPA Administration

When appropriate, mice were administered BrdU (B5002, Sigma; 0.5 g/L) and/or VPA (P4543, Sigma; 4 g/L) in drinking water for the indicated duration.

Contusion Spinal Cord Injury

Adult mice were anesthetized and a laminectomy was performed at the T7–9 segments. The exposed spinal cord was subjected to a contusion injury that was introduced by using the IH impactor (Precision Systems and Instrumentation) with a 1-mm tip and a force of 60 kdyn.

Immunohistochemistry

Mice were euthanized and perfused with intracardial injection of 4% (w/v) paraformaldehyde in PBS. Spinal cords were isolated and post-fixed overnight with 4% (w/v) paraformaldehyde at 4°C. After cryoprotection with 30% sucrose in PBS for 48 hr at 4°C, transverse sections or 1.5-cm longitudinal sections of spinal cords spanning the injection/injury sites were collected on a cryostat (Leica) set at 20-μm thickness. The primary antibodies used for immunofluorescence are listed in Table S1. When mouse primary antibodies were used, tissue sections were pretreated to block non-specific staining. Specifically, the sections were incubated with 50% formamide in 1× saline sodium citrate (SSC) buffer for 1.5 hr at 65°C. For BrdU staining, the sections were pretreated with 2 N HCl for 30 min at 37°C. The corresponding secondary antibodies conjugated with Alexa Fluor 488, 555, or 647 dye (Jackson ImmunoResearch Laboratories) were applied at 1:2,000 dilution for indirect fluorescence. Nuclei were counterstained with Hoechst 33342 (Hst). Images were captured and examined by using a Zeiss LSM 700 confocal microscope.

TUNEL Assay

TUNEL assay was performed on cryostat sections by using the R&D Systems TACS TdT In Situ Apoptosis Detection Kit (Fisher Scientific). TUNEL-positive controls were set up with TACS-Nuclease provided in the kit. All TUNEL-positive cells surrounding the injection area were counted on each section.

Statistical Analysis

The quantification data were expressed as mean ± SD from four to six mice. Statistical analysis was performed by ANOVA and Tukey's post hoc multiple comparisons or the unpaired Student's t tests where appropriate. Differences were considered statistically significant at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.038.

AUTHOR CONTRIBUTIONS

L.-L.W., Z.S., and C.-L.Z. conceived and designed the experiments. L.-L.W., Z.S., and Y.Z. performed the experiments. W.T. and X.-M.X. provided critical reagents and technical inputs. L.-L.W. and C.-L.Z. analyzed data and prepared the manuscript. All authors reviewed and approved the manuscript.

ACKNOWLEDGMENTS

We thank members of the C.-L.Z. laboratory for discussions and reagents. We also thank Jane Johnson (UT Southwestern) for providing the ASCL1 antibody and Michael Brenner (University of Alabama at Birmingham) for providing the *hGFAP* promoter. C-.L.Z. is a W.W. Caruth, Jr. Scholar in Biomedical Research. This work was supported by the Welch Foundation Award (I-1724), Texas Institute for Brain Injury and Repair, the Decherd Foundation, the Mobility Foundation, NIH Grants (NS070981, NS088095, NS092616, 1DP2OD006484, and NS093502 to C.-L.Z.), and the National Key Research and Development Program of China (2016YFA0100802 to Z.S.).

Received: May 5, 2016
Revised: June 6, 2016
Accepted: September 13, 2016
Published: October 11, 2016

REFERENCES

Abematsu, M., Tsujimura, K., Yamano, M., Saito, M., Kohno, K., Kohyama, J., Namihira, M., Komiya, S., and Nakashima, K. (2010). Neurons derived from transplanted neural stem cells restore disrupted neuronal circuitry in a mouse model of spinal cord injury. J. Clin. Invest. *120*, 3255–3266.

Banito, A., Rashid, S.T., Acosta, J.C., Li, S., Pereira, C.F., Geti, I., Pinho, S., Silva, J.C., Azuara, V., Walsh, M., et al. (2009). Senescence impairs successful reprogramming to pluripotent stem cells. Genes Dev. *23*, 2134–2139.

Borgius, L., Nishimaru, H., Caldeira, V., Kunugise, Y., Löw, P., Reig, R., Itohara, S., Iwasato, T., and Kiehn, O. (2014). Spinal glutamatergic neurons defined by EphA4 signaling are essential components of normal locomotor circuits. J. Neurosci. *34*, 3841–3853.

Bradbury, E.J., and McMahon, S.B. (2006). Spinal cord repair strategies: why do they work? Nat. Rev. Neurosci. *7*, 644–653.

Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks, T., and Hannon, G.J. (1995). Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature *377*, 552–557.

Buffelli, M., Burgess, R.W., Feng, G., Lobe, C.G., Lichtman, J.W., and Sanes, J.R. (2003). Genetic evidence that relative synaptic efficacy biases the outcome of synaptic competition. Nature *424*, 430–434.

Chi, L., Ke, Y., Luo, C., Li, B., Gozal, D., Kalyanaraman, B., and Liu, R. (2006). Motor neuron degeneration promotes neural progenitor cell proliferation, migration, and neurogenesis in the spinal cords of amyotrophic lateral sclerosis mice. Stem Cells *24*, 34–43.

Courtine, G., Song, B., Roy, R.R., Zhong, H., Herrmann, J.E., Ao, Y., Qi, J., Edgerton, V.R., and Sofroniew, M.V. (2008). Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury. Nat. Med. *14*, 69–74.

Dehmelt, L., and Halpain, S. (2005). The MAP2/Tau family of microtubule-associated proteins. Genome Biol. *6*, 204.

Fitch, M.T., and Silver, J. (2008). CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. Exp. Neurol. *209*, 294–301.

Göritz, C., Dias, D.O., Tomilin, N., Barbacid, M., Shupliakov, O., and Frisén, J. (2011). A pericyte origin of spinal cord scar tissue. Science *333*, 238–242.

Gregorian, C., Nakashima, J., Le Belle, J., Ohab, J., Kim, R., Liu, A., Smith, K.B., Groszer, M., Garcia, A.D., Sofroniew, M.V., et al. (2009). Pten deletion in adult neural stem/progenitor cells enhances constitutive neurogenesis. J. Neurosci. *29*, 1874–1886.

Groszer, M., Erickson, R., Scripture-Adams, D.D., Lesche, R., Trumpp, A., Zack, J.A., Kornblum, H.I., Liu, X., and Wu, H. (2001). Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. Science *294*, 2186–2189.

Guo, S., Zi, X., Schulz, V.P., Cheng, J., Zhong, M., Koochaki, S.H., Megyola, C.M., Pan, X., Heydari, K., Weissman, S.M., et al. (2014a). Nonstochastic reprogramming from a privileged somatic cell state. Cell *156*, 649–662.

Guo, Z., Zhang, L., Wu, Z., Chen, Y., Wang, F., and Chen, G. (2014b). In vivo direct reprogramming of reactive glial cells into functional neurons after brain injury and in an Alzheimer's disease model. Cell Stem Cell *14*, 188–202.

Hanna, J., Saha, K., Pando, B., van Zon, J., Lengner, C.J., Creyghton, M.P., van Oudenaarden, A., and Jaenisch, R. (2009). Direct cell reprogramming is a stochastic process amenable to acceleration. Nature *462*, 595–601.

Heinrich, C., Bergami, M., Gascón, S., Lepier, A., Viganò, F., Dimou, L., Sutor, B., Berninger, B., and Götz, M. (2014). Sox2-mediated conversion of NG2 glia into induced neurons in the injured adult cerebral cortex. Stem Cell Reports *3*, 1000–1014.

Hong, H., Takahashi, K., Ichisaka, T., Aoi, T., Kanagawa, O., Nakagawa, M., Okita, K., and Yamanaka, S. (2009). Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature *460*, 1132–1135.

Horky, L.L., Galimi, F., Gage, F.H., and Horner, P.J. (2006). Fate of endogenous stem/progenitor cells following spinal cord injury. J. Comp. Neurol. *498*, 525–538.

Horner, P.J., Power, A.E., Kempermann, G., Kuhn, H.G., Palmer, T.D., Winkler, J., Thal, L.J., and Gage, F.H. (2000). Proliferation and differentiation of progenitor cells throughout the intact adult rat spinal cord. J. Neurosci. *20*, 2218–2228.

Islam, M.M., Smith, D.K., Niu, W., Fang, S., Iqbal, N., Sun, G., Shi, Y., and Zhang, C.L. (2015). Enhancer analysis unveils genetic interactions between TLX and SOX2 in neural stem cells and in vivo reprogramming. Stem Cell Reports *5*, 805–815.

Jessberger, S., and Gage, F.H. (2014). Adult neurogenesis: bridging the gap between mice and humans. Trends Cell Biol. *24*, 558–563.

Jiang, H., Xu, Z., Zhong, P., Ren, Y., Liang, G., Schilling, H.A., Hu, Z., Zhang, Y., Wang, X., Chen, S., et al. (2015). Cell cycle and p53 gate the direct conversion of human fibroblasts to dopaminergic neurons. Nat. Commun. *6*, 10100.

Johansson, C.B., Momma, S., Clarke, D.L., Risling, M., Lendahl, U., and Frisén, J. (1999). Identification of a neural stem cell in the adult mammalian central nervous system. Cell *96*, 25–34.

Kawamura, T., Suzuki, J., Wang, Y.V., Menendez, S., Morera, L.B., Raya, A., Wahl, G.M., and Izpisúa Belmonte, J.C. (2009). Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature *460*, 1140–1144.

Kempermann, G., Song, H., and Gage, F.H. (2015). Neurogenesis in the adult hippocampus. Cold Spring Harb. Perspect. Biol. *7*, a018812.

Komarov, P.G., Komarova, E.A., Kondratov, R.V., Christov-Tselkov, K., Coon, J.S., Chernov, M.V., and Gudkov, A.V. (1999). A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science *285*, 1733–1737.

Lane, D., and Levine, A. (2010). p53 Research: the past thirty years and the next thirty years. Cold Spring Harb. Perspect. Biol. *2*, a000893.

Li, H., Collado, M., Villasante, A., Strati, K., Ortega, S., Cañamero, M., Blasco, M.A., and Serrano, M. (2009). The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature *460*, 1136–1139.

Li, S., Sun, G., Murai, K., Ye, P., and Shi, Y. (2012). Characterization of TLX expression in neural stem cells and progenitor cells in adult brains. PLoS ONE *7*, e43324.

Lim, D.A., and Alvarez-Buylla, A. (2016). The adult ventricular–subventricular zone (V-SVZ) and olfactory bulb (OB) neurogenesis. Cold Spring Harb. Perspect. Biol. http://dx.doi.org/10.1101/cshperspect.a018820

Liu, Y., Miao, Q., Yuan, J., Han, S., Zhang, P., Li, S., Rao, Z., Zhao, W., Ye, Q., Geng, J., et al. (2015). Ascl1 converts dorsal midbrain astrocytes into functional neurons in vivo. J. Neurosci. *35*, 9336–9355.

Llewellyn-Smith, I.J., Martin, C.L., Fenwick, N.M., Dicarlo, S.E., Lujan, H.L., and Schreihofer, A.M. (2007). VGLUT1 and VGLUT2 innervation in autonomic regions of intact and transected rat spinal cord. J. Comp. Neurol. *503*, 741–767.

Lugert, S., Vogt, M., Tchorz, J.S., Müller, M., Giachino, C., and Taylor, V. (2012). Homeostatic neurogenesis in the adult hippocampus does not involve amplification of Ascl1(high) intermediate progenitors. Nat. Commun. *3*, 670.

Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zariwala, H.A., Gu, H., Ng, L.L., Palmiter, R.D., Hawrylycz, M.J., Jones, A.R., et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat. Neurosci. *13*, 133–140.

Marino, S., Vooijs, M., van Der Gulden, H., Jonkers, J., and Berns, A. (2000). Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. Genes Dev. *14*, 994–1004.

Marión, R.M., Strati, K., Li, H., Murga, M., Blanco, R., Ortega, S., Fernandez-Capetillo, O., Serrano, M., and Blasco, M.A. (2009). A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. Nature *460*, 1149–1153.

Mullen, R.J., Buck, C.R., and Smith, A.M. (1992). NeuN, a neuronal specific nuclear protein in vertebrates. Development *116*, 201–211.

Nishimaru, H., and Kakizaki, M. (2009). The role of inhibitory neurotransmission in locomotor circuits of the developing mammalian spinal cord. Acta Physiol. (Oxf.) *197*, 83–97.

Niu, W., Zou, Y., Shen, C., and Zhang, C.L. (2011). Activation of postnatal neural stem cells requires nuclear receptor TLX. J. Neurosci. *31*, 13816–13828.

Niu, W., Zang, T., Zou, Y., Fang, S., Smith, D.K., Bachoo, R., and Zhang, C.L. (2013). In vivo reprogramming of astrocytes to neuroblasts in the adult brain. Nat. Cell Biol. *15*, 1164–1175.

Niu, W., Zang, T., Smith, D.K., Vue, T.Y., Zou, Y., Bachoo, R., Johnson, J.E., and Zhang, C.L. (2015). SOX2 reprograms resident astrocytes into neural progenitors in the adult brain. Stem Cell Reports *4*, 780–794.

Ohori, Y., Yamamoto, S., Nagao, M., Sugimori, M., Yamamoto, N., Nakamura, K., and Nakafuku, M. (2006). Growth factor treatment and genetic manipulation stimulate neurogenesis and oligodendrogenesis by endogenous neural progenitors in the injured adult spinal cord. J. Neurosci. *26*, 11948–11960.

Pechnick, R.N., Zonis, S., Wawrowsky, K., Pourmorady, J., and Chesnokova, V. (2008). p21Cip1 restricts neuronal proliferation in the subgranular zone of the dentate gyrus of the hippocampus. Proc. Natl. Acad. Sci. USA *105*, 1358–1363.

Rossignol, S., Schwab, M., Schwartz, M., and Fehlings, M.G. (2007). Spinal cord injury: time to move? J. Neurosci. *27*, 11782–11792.

Scholzen, T., and Gerdes, J. (2000). The Ki-67 protein: from the known and the unknown. J. Cell. Physiol. *182*, 311–322.

Sellers, D.L., Maris, D.O., and Horner, P.J. (2009). Postinjury niches induce temporal shifts in progenitor fates to direct lesion repair after spinal cord injury. J. Neurosci. *29*, 6722–6733.

Shechter, R., Ziv, Y., and Schwartz, M. (2007). New GABAergic interneurons supported by myelin-specific T cells are formed in intact adult spinal cord. Stem Cells *25*, 2277–2282.

Shihabuddin, L.S., Horner, P.J., Ray, J., and Gage, F.H. (2000). Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. J. Neurosci. *20*, 8727–8735.

Sofroniew, M.V. (2009). Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci. *32*, 638–647.

Su, Z., Niu, W., Liu, M.L., Zou, Y., and Zhang, C.L. (2014). In vivo conversion of astrocytes to neurons in the injured adult spinal cord. Nat. Commun. *5*, 3338.

Sun, G., Yu, R.T., Evans, R.M., and Shi, Y. (2007). Orphan nuclear receptor TLX recruits histone deacetylases to repress transcription and regulate neural stem cell proliferation. Proc. Natl. Acad. Sci. USA *104*, 15282–15287.

Thuret, S., Moon, L.D., and Gage, F.H. (2006). Therapeutic interventions after spinal cord injury. Nat. Rev. Neurosci. *7*, 628–643.

Torper, O., Pfisterer, U., Wolf, D.A., Pereira, M., Lau, S., Jakobsson, J., Björklund, A., Grealish, S., and Parmar, M. (2013). Generation of induced neurons via direct conversion in vivo. Proc. Natl. Acad. Sci. USA *110*, 7038–7043.

Torper, O., Ottosson, D.R., Pereira, M., Lau, S., Cardoso, T., Grealish, S., and Parmar, M. (2015). In vivo reprogramming of striatal NG2 glia into functional neurons that integrate into local host circuitry. Cell Rep. *12*, 474–481.

Utikal, J., Polo, J.M., Stadtfeld, M., Maherali, N., Kulalert, W., Walsh, R.M., Khalil, A., Rheinwald, J.G., and Hochedlinger, K. (2009). Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. Nature *460*, 1145–1148.

Vessal, M., Aycock, A., Garton, M.T., Ciferri, M., and Darian-Smith, C. (2007). Adult neurogenesis in primate and rodent spinal cord: comparing a cervical dorsal rhizotomy with a dorsal column transection. Eur. J. Neurosci. *26*, 2777–2794.

Yang, H., Lu, P., McKay, H.M., Bernot, T., Keirstead, H., Steward, O., Gage, F.H., Edgerton, V.R., and Tuszynski, M.H. (2006). Endogenous neurogenesis replaces oligodendrocytes and astrocytes after primate spinal cord injury. J. Neurosci. *26*, 2157–2166.

Zhao, Y., Yin, X., Qin, H., Zhu, F., Liu, H., Yang, W., Zhang, Q., Xiang, C., Hou, P., Song, Z., et al. (2008). Two supporting factors greatly improve the efficiency of human iPSC generation. Cell Stem Cell *3*, 475–479.