



# Sox2 and Pax6 Maintain the Proliferative and Developmental Potential of Gliogenic Neural Stem Cells *In Vitro*

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## KEY WORDS

neural progenitor; radial glia; self-renewal; gliogenesis; SoxB1

## ABSTRACT

Radial-glia-like neural stem (NS) cells may be derived from neural tissues or via differentiation of pluripotent embryonic stem (ES) cells. However, the mechanisms controlling NS cell propagation and differentiation are not yet fully understood. Here we investigated the roles of Sox2 and Pax6, transcription factors widely expressed in central nervous system (CNS) progenitors, in mouse NS cells. Conditional deletion of either *Sox2* or *Pax6* in forebrain-derived NS cells reduced their clonogenicity in a gene dosage-dependent manner. Cells heterozygous for either gene displayed moderate proliferative defects, which may relate to human pathologies attributed to *SOX2* or *PAX6* deficiencies. In the complete absence of *Sox2*, cells exited the cell cycle with concomitant downregulation of neural progenitor markers Nestin and *Blbp*. This occurred despite expression of the close relative *Sox3*. Ablation of *Pax6* also caused major proliferative defects. However, a subpopulation of cells was able to expand continuously without *Pax6*. These *Pax6*-null cells retained progenitor markers but had altered morphology. They exhibited compromised differentiation into astrocytes and oligodendrocytes, highlighting that the role of *Pax6* extends beyond neurogenic competence. Overall these findings indicate that *Sox2* and *Pax6* are both critical for self-renewal of differentiation-competent radial glia-like NS cells. © 2011 Wiley-Liss, Inc.

## INTRODUCTION

Neural progenitors isolated from the developing and adult rodent CNS can be propagated in the presence of epidermal growth factor (EGF) and/or fibroblast growth factor 2 (FGF-2), either as floating neurospheres, or in short-term adherent culture (Palmer et al., 1995; Reynolds and Weiss, 1992; Reynolds et al., 1992; Temple, 1989). We previously extended these pioneering studies and described the long-term expansion in monolayer culture of non-transformed, clonogenic mouse neural stem (NS) cell lines (Conti et al., 2005). The ability to expand NS cells in a controlled tissue culture environment with-

out spontaneous differentiation simplifies the dynamic and multifactorial environment present *in vivo*. This provides a tractable model for dissecting the effects of intrinsic and extrinsic regulators at the cellular and molecular level and elucidating the mechanisms regulating cell fate choice of CNS progenitors. Furthermore, culture-expanded neural stem cells present opportunities for biomedical investigations and may even find direct clinical applications (Conti and Cattaneo, 2010).

NS cells self-renew in response to EGF and FGF-2 and under alternative culture conditions differentiate into neurons, astrocytes, and oligodendrocytes (Glaser et al., 2007). They exhibit bipolar morphology, undergo interkinetic nuclear migration, and express nestin, brain lipid binding protein (BLBP), glutamate-aspartate transporter (GLAST), RC2, and vimentin, thus resembling a radial glia phenotype (Conti et al., 2005; Hartfuss et al., 2001; Pollard et al., 2006b). Here, we set out to explore the functional roles of the transcription factors (TFs) *Sox2* and *Pax6* in NS cell self-renewal and differentiation.

*Sox2* is a high-mobility group (HMG)-box TF present throughout the neuroectoderm from the earliest stages of neural plate formation (Wood and Episkopou, 1999). *Sox2* is expressed in proliferating neural progenitors throughout CNS development and in the two main neu-

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rogenic regions of the adult brain, the subventricular zone of the lateral ventricle and the subgranular zone of the hippocampus (Ellis et al., 2004; Ferri et al., 2004; Suh et al., 2007; Zappone et al., 2000). This expression pattern has suggested a role for Sox2 in regulating the developmental potential of neural progenitors. In humans, heterozygous mutations in *SOX2* are linked to brain abnormalities, epilepsy, and eye defects (Fantes et al., 2003; Sisodiya et al., 2006). However, genetic analyses of its specific functions in developing CNS progenitors have been hampered by the early lethality of *Sox2*-null embryos (Avilion et al., 2003) and later overlap and apparent functional redundancy with close relatives, *Sox1* and *Sox3* (Graham et al., 2003; Miyagi et al., 2008).

The paired box gene *Pax6* is a highly conserved TF involved in the development of endocrine glands, the CNS, and the eye (Callaerts et al., 1997). *Pax6*-mutant rodents display defective neural tube patterning, altered progenitor proliferation, reduced neurogenesis, impaired migration of neuronal precursors, and re-specification of neuronal subtypes, among other defects (Caric et al., 1997; Heins et al., 2002; Kroll and O'Leary, 2005; Quinn et al., 2007; Stoykova et al., 1996). As a multifunctional regulator, the distinct activities of *Pax6* are highly context-dependent (Osumi et al., 2008). Increasing or decreasing *Pax6* levels may affect the properties of distinct populations of neural progenitors in different, sometimes opposing ways (Georgala et al., 2010; Manuel et al., 2007). Analysis of *Pax6* function is therefore complex, but can be simplified by using *in vitro* models (Nikoletopoulou et al., 2007).

In the eye, *Sox2* and *Pax6* interact and cooperatively regulate transcription of the  $\delta$ -crystallin gene and *Sox2* (Inoue et al., 2007; Kamachi et al., 2001). In the present study, we have investigated their function in NS cells by conditional deletions. The results demonstrate that these TFs fulfill distinct but essential roles in maintaining the proliferative capacity and developmental potential of these radial glia-like stem cells in culture.

## MATERIALS AND METHODS

### Cell Culture and Time-Lapse Imaging

Stable *Sox2*<sup>loxP/+</sup>, *Sox2*<sup>Bgeo/loxP</sup>, *Pax6*<sup>loxP/+</sup>, and *Pax6*<sup>loxP/loxP</sup> NS cell lines were established from E14.5 or E16.5 mouse forebrain and maintained as described (Conti et al., 2005; Pollard et al., 2006a). NS expansion medium consisted of RHB basal medium (StemCell Sciences) supplemented with modified N2 (prepared in house; Ying and Smith, 2003) and 10 ng mL<sup>-1</sup> of both recombinant mouse EGF and FGF-2 (PeproTech EC). For passaging, cultures were dissociated into single cells by 1- to 2-min Accutase (Sigma-Aldrich or PAA Laboratories) treatment at 37°C and replated 1:3 to 1:5 every 2–3 days.

To induce astrogial differentiation, 2.5 × 10<sup>4</sup> cells cm<sup>-2</sup> were plated on gelatin-coated plates in NS expansion medium. Cells were allowed to attach and medium was replaced by RHB basal plus N2 and 10 ng mL<sup>-1</sup> of recombinant human bone morphogenetic protein 4

(BMP-4; R&D Systems) or 1% fetal calf serum (PAA Laboratories). Cells were cultured in these conditions for the indicated time, changing the medium every 4 days. For oligodendroglial differentiation, cells were subjected to the protocol described by Glaser et al. (2007), with minor modifications. Cells were plated at 3.75 × 10<sup>4</sup> cells per cm<sup>2</sup> on poly-L-ornithine/laminin-coated plates in NS expansion medium. The following day, medium was switched to RHB basal supplemented with N2, 10 ng mL<sup>-1</sup> of recombinant human platelet-derived growth factor (PDGF)-AA (R&D Systems), 10 ng mL<sup>-1</sup> FGF-2 and 10 μM Forskolin (Sigma-Aldrich). After 4 days of culture, medium was replaced for RHB basal supplemented with N2, 30 ng mL<sup>-1</sup> 3,3,5-triiodothyronine (T3; Sigma-Aldrich) and 200 μM ascorbic acid (Sigma-Aldrich).

For time-lapse analyses, 6 × 10<sup>3</sup> cells cm<sup>-2</sup> were seeded in 24-well Image Lock plates (Essen Bioscience). Three images per well were acquired every 30 min using an IncuCyte Live Cell imaging system (Essen Instruments). Confluence measurements for each time point were obtained using the IncuCyte cell density detection software. At least two wells per condition were analyzed in each experiment.

### Gene Excision in NS Cells

Conditional mutant NS cells were transiently transfected with a pCAG-Cre-IRES-GFP plasmid using a Nucleofector (Solution V, Programme T-020; Amaxa, Lonza). After 40 h, NS cells expressing GFP were sorted from GFP<sup>-</sup> cells with a MoFlo cell sorter (Beckman Coulter). Dead cells were excluded using ToPro3 (Invitrogen) or DAPI (Sigma-Aldrich). Purified cells were either collected as populations or individually deposited into 96-well plates containing NS expansion medium supplemented with B27 (Gibco). In some experiments, recombinant mouse sonic hedgehog (Shh; R&D systems) was added at 250 ng mL<sup>-1</sup>.

### Flow Cytometry Analysis

NS cells were collected with enzyme-free cell dissociation buffer (Gibco), washed with RHB basal medium, and incubated for 20 min with anti-PDGFR $\alpha$  (CD140a, rat IgG2a APC-conjugated; 1:400; eBioscience) or anti-NG2 (rabbit IgG; 1:1,000; Chemicon). Cells were washed once with medium and, when necessary, labeled with Alexa Fluor-conjugated secondary antibodies (1:1,000, Invitrogen). After one wash with medium, cells were resuspended in RHB basal medium containing 1 ng mL<sup>-1</sup> DAPI and analyzed on a CyAn ADP (DAKO Cytomation).

### Immunocytochemistry

Except for O4 staining, which was performed on live cells, we used standard immunocytochemistry techniques. Cultures were fixed with 4% paraformaldehyde for 10 min at room temperature and washed twice with

PBST (0.1% Triton X-100/PBS). Cells were blocked for at least 30 min in 3% serum/1% BSA/PBST and incubated with the primary antibody in blocking solution overnight at 4°C. After removal of primary antibody, cells were washed thrice with PBST and incubated with secondary antibodies for 45 min at room temperature or overnight at 4°C. Then, cells were washed twice with PBST and nuclei were counterstained with DAPI. Primary antibodies were: GFAP (mouse IgG1 or rabbit IgG; 1:200; Sigma-Aldrich), Ki67 (rabbit IgG; 1:500; LabVision), Nestin (mouse IgG1; 1:10; Developmental Studies Hybridoma Bank), NG2 (rabbit IgG; 1:500; Chemicon), O4 (mouse IgM; 1:400; R&D Systems), Olig2 (rabbit IgG; 1:400; Chemicon), Pax6 (mouse IgG1; 1:40; DSHB), Pdgfra (rat IgG2a; 1:300; BD Pharmingen), Sox2 (mouse IgG2a; 1:50; R&D Systems), Vimentin (mouse IgM; 1:50; DSHB). Species-specific or Ig-subtype-specific Alexa Fluor 488, 555 or 647-conjugated secondary antibodies (1:500; Invitrogen) were used.

For live staining, cells were incubated for 20 min with the primary antibody in culture medium at room temperature. Next, cells were washed twice with medium, fixed with 4% PFA, and stained with the appropriate secondary antibody or processed for an additional antibody staining.

### Immunoblotting

Whole cell lysates were prepared in Laemmeli buffer (2% SDS/10% Glycerol/60 mM Tris pH 6.8/0.1 M DTT), resolved on 4–12% gradient gels (NuPage, Invitrogen), transferred onto nitrocellulose membranes (Hybond, Amersham) and blotted for anti-Sox3 (goat; 1:1,000, R&D Systems) and anti- $\alpha$ -Tubulin (mouse; 1:5,000, Abcam). Species-specific horseradish peroxidase-conjugated secondary antibodies were used (1:5,000, Amersham). Peroxidase activity was visualized using ECL Plus (Amersham).

### RT-PCR and qRT-PCR

Total RNA was extracted using RNeasy Kit (Qiagen) and treated with DNaseI (Qiagen). cDNA was prepared using SuperScript III (Invitrogen). RT-PCR was performed using Taq polymerase (Qiagen). Except for *Actb* which was only amplified for 23 cycles, the following conditions were used: 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 20 s at 72°C, with a final extension at 72°C for 2 min. Primer pairs were designed using Primer3 (Supp. Info. Table 1).

qRT-PCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems), using TaqMan Fast Universal PCR Master mix (Applied Biosystems). Pre-designed TaqMan Gene Expression Assays (Applied Biosystems) or primer pairs and probes from the Universal Probe Library (UPL, Roche Applied Science) were used (Supp. Info. Tables 2 and 3). Expression values were normalized according to *Gapdh* or  $\beta$ -actin levels.

## RESULTS

### NS Cell Lines Carrying Conditional Null Mutations for *Sox2* or *Pax6*

We established NS cell lines from fetal forebrain tissue of mouse embryos carrying a conditional *Sox2*<sup>loxP</sup> allele (Favarro et al., 2009) and a *Sox2*<sup>βgeo</sup> null allele (Zappone et al., 2000), or bearing one or both *Pax6* alleles floxed (Simpson et al., 2009). These cultures were indistinguishable from previously described mouse NS cells (Conti et al., 2005; Pollard et al., 2006b). They uniformly expressed Nestin, Vimentin, Sox2, and Olig2, and lacked differentiated cells expressing GFAP (glial fibrillary acidic protein) or TuJ1 ( $\beta$ -III-tubulin). *Pax6* was expressed rather homogeneously in all NS cells, but at relatively low levels (Supp. Info. Figs. 1A,B and 2A,C). RT-PCR analyses revealed the presence of the two main isoforms of *Pax6*, canonical *Pax6* and *Pax6(5a)* (Supp. Info. Fig. 1B). Transcripts of *Sox1* were not detected, as previously reported for wild-type NS cells (Conti et al., 2005) (Supp. Info. Fig. 1B). Presence of *Sox3* protein was substantiated by immunoblotting (Supp. Info. Fig. 1C). Upon exposure to differentiation conditions all the NS cell lines generated GFAP<sup>+</sup> astrocytes, O4<sup>+</sup> oligodendrocytes and, to a lesser extent, TuJ1<sup>+</sup> immature neuronal cells (Supp. Info. Figs. 1D and 2D).

To achieve gene deletion, conditional mutant NS cells were transiently transfected with a CAG-Cre-IRES-GFP expression vector, sorted for GFP expression by flow cytometry and replated in the presence of EGF and FGF-2 (Fig. 1A). qRT-PCR analyses 7 days after *Cre* transfection confirmed virtually complete loss of *Sox2* or *Pax6* transcripts in the *Sox2*<sup>βgeo/loxP</sup> GFP<sup>+</sup> and *Pax6*<sup>loxP/loxP</sup> GFP<sup>+</sup> NS cell populations, respectively (Figs. 1C and 2B).

### Inactivation of *Sox2* Impairs NS Cell Maintenance

*Sox2* ablation in NS cells led to dramatic loss of proliferative capacity. To monitor this effect, *Sox2*<sup>βgeo/loxP</sup> and *Sox2*<sup>loxP/+</sup> NS cells transfected with *Cre*-GFP were sorted for GFP expression, plated at equivalent cell densities and continuously imaged for 1 week. Confluence measurements showed that the *Sox2*<sup>βgeo/loxP</sup> GFP<sup>+</sup> NS cells barely expanded over this period (Fig. 1B). Moreover, those cells that did expand appeared to be contaminant non-excised cells, because antibody staining performed at the end of the culture period revealed an occasional cluster of *Sox2* positive cells (not shown). Interestingly, *Sox2* heterozygous NS cells (*Sox2*<sup>βgeo/loxP</sup> GFP<sup>-</sup> and *Sox2*<sup>loxP/+</sup> GFP<sup>+</sup>) showed moderately reduced proliferation when compared with non-excised cells (*Sox2*<sup>loxP/+</sup> GFP<sup>-</sup>).

Consistent with impaired expansion, immunocytochemical analyses at day 7 post-transfection showed loss of immunoreactivity for the proliferation marker Ki67 in *Sox2*-null cultures (Fig. 1D,E). *Sox2*-deleted cells also exhibited diminished Nestin immunostaining and reduced transcript levels for *Blbp* (*Fabp7*), a marker of

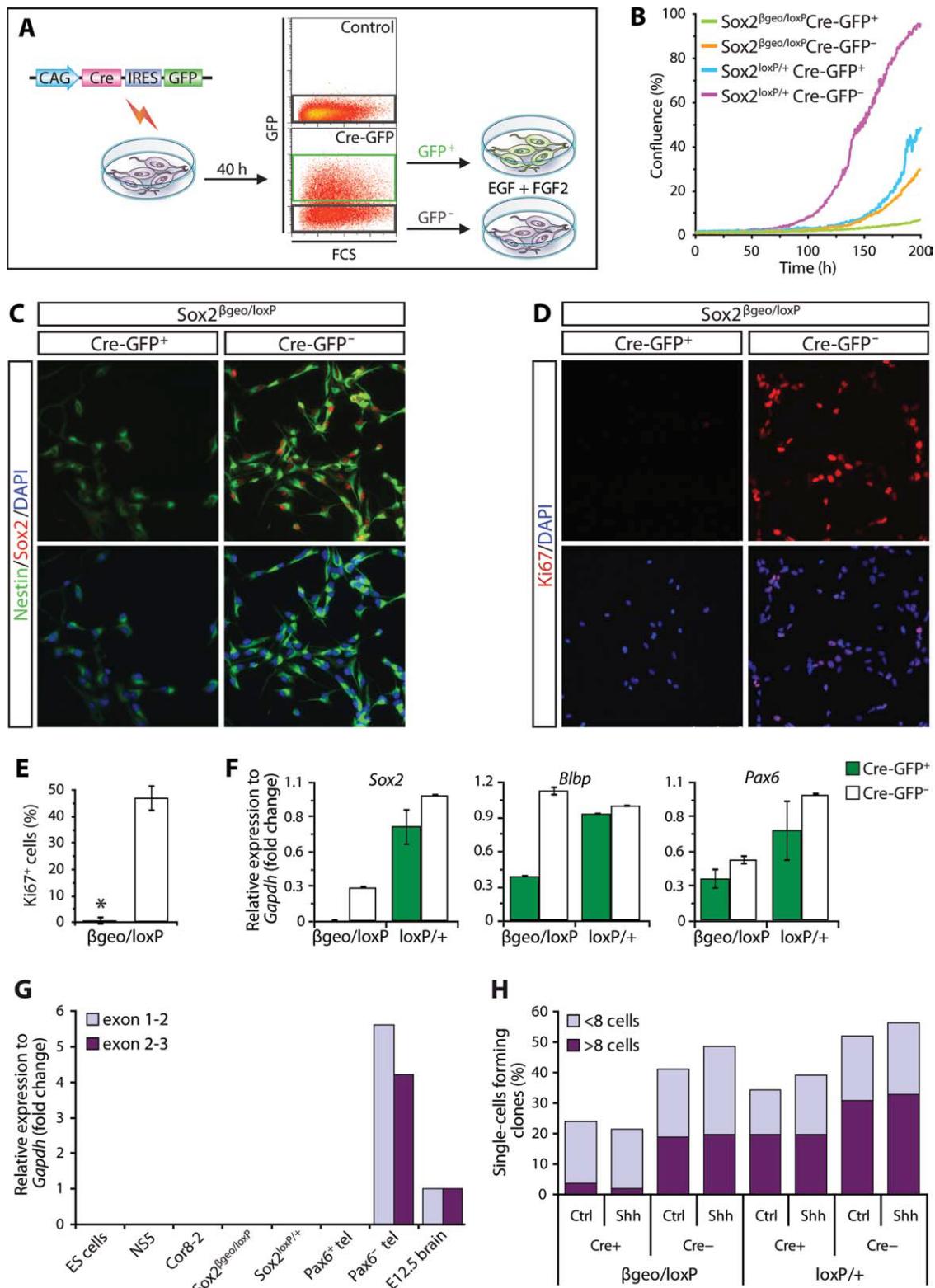


Fig. 1. Deletion of Sox2 impairs NS cell maintenance. (A) Strategy for conditional gene deletion in NS cells. FCS, forward scatter. (B) Confluence estimates obtained by time-lapse imaging from Day 2 post-transfection show impaired NS cell proliferation after Sox2 deletion. (C, D) Immunostaining analyses show reduced intensity of Nestin staining and loss of Sox2 and Ki67 immunoreactivity one week after Sox2-deletion. (E) Quantitation of Ki67<sup>+</sup> cells in Sox2<sup>βgeo/loxP</sup> Cre-GFP<sup>+</sup> and Cre-GFP<sup>-</sup> cultures at day 7 post-transfection. Bars represent mean  $\pm$  SD. \*Student's one-tailed *t* test,  $P = 1.07 \times 10^{-6}$ . (F) qRT-PCR analyses for

Sox2, Blbp and Pax6 one week after Cre transfection.  $Sox2^{loxP/+}$  Cre-GFP<sup>-</sup> = 1. (G) qRT-PCR for Shh. Plot shows data obtained with two different intron-spanning primer/probe sets. E14.5 telencephalic (Tel) tissue from a PAX6-GFP reporter mouse (Tyas et al., 2006) was used as control. E12.5 brain = 1. (H) Histogram showing number and size of clones obtained from individual Cre-transfected  $Sox2^{\betageo/loxP}$  or  $Sox2^{loxP/+}$  NS cells cultured for 15 days with or without 250 ng mL<sup>-1</sup> Shh. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

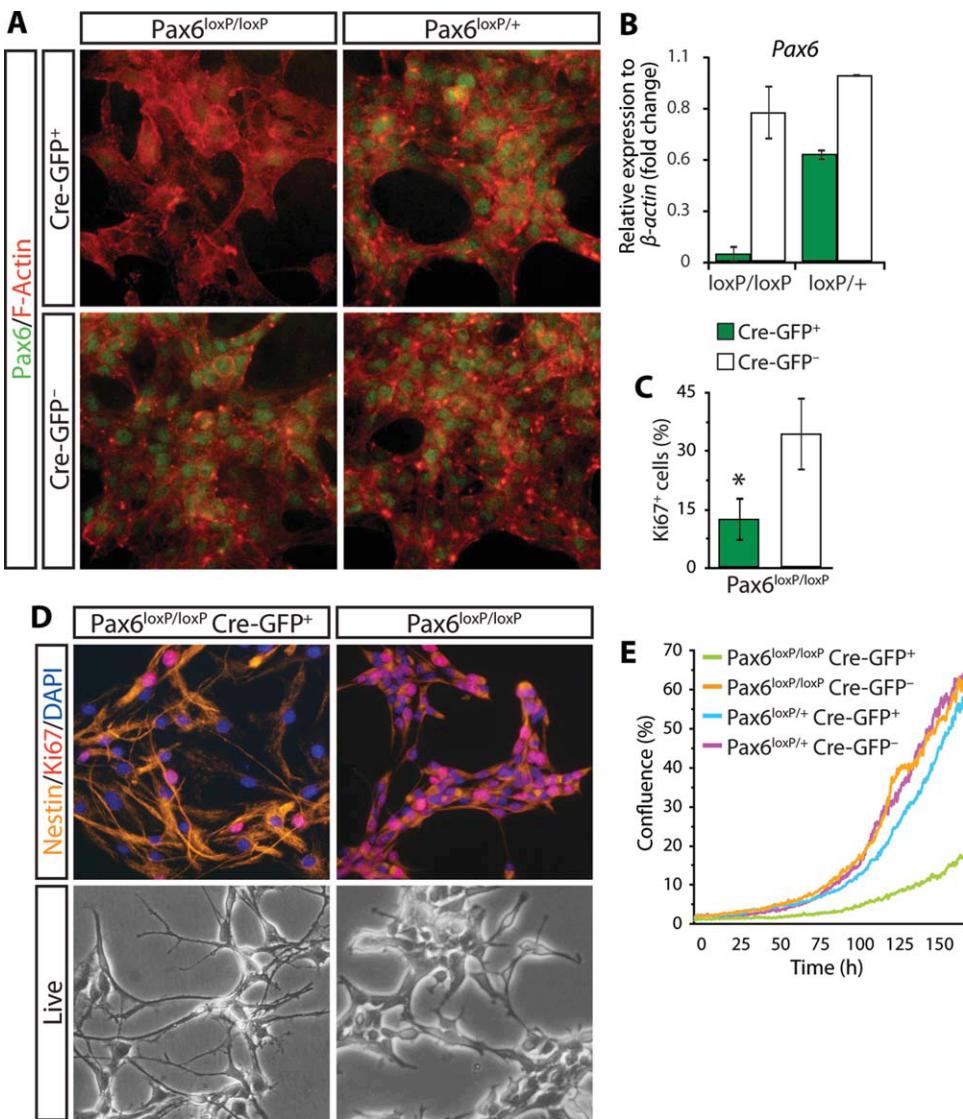


Fig. 2. Conditional inactivation of Pax6 in NS cells. (A) Fluorescence images showing loss of Pax6 immunoreactivity in *Pax6*<sup>loxP/loxP</sup> Cre-GFP<sup>+</sup> NS cells 1 week after *Cre* transfection. (B) qRT-PCR analysis for *Pax6* at day 7 post-transfection. *Pax6*<sup>loxP/+</sup> Cre-GFP<sup>-</sup> = 1. (C, D) Pax6-deficient cultures display reduced proportion of Ki67<sup>+</sup> cells and

abnormal NS cell morphology. Bars represent mean  $\pm$  SD, \*Student's one-tailed *t* test,  $P = 3.2 \times 10^{-5}$ . (E) Time-lapse analyses initiated 48 h after *Cre* transfection indicate a reduction in the proliferation rate of Pax6-deleted cells. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

multipotent radial glia. Furthermore, *Pax6* expression decreased with reduction in *Sox2* (Fig. 1C,F).

*Sox2* has been shown to maintain the proliferative capacity of postnatal hippocampal progenitors, at least in part, by regulating *Shh* expression (Favaro et al., 2009). We therefore evaluated whether this mechanism also operates in NS cells. qRT-PCR analyses showed that under expansion conditions, NS cells do not express *Shh*. Furthermore, in either parental or *Sox2*-deleted cells, addition of exogenous *Shh* had no effect on colony forming ability (Fig. 1G,H). These findings indicate that *Sox2* effector genes may differ in distinct populations of CNS progenitors.

We conclude that *Sox2* activity is required for maintaining NS cells in an undifferentiated and proliferative state. This finding also indicates that *Sox3*, which is

expressed in NS cells at levels comparable to those in the fetal cortex (Supp. Info. Fig. 1B,C), may not be competent to replace all functions of *Sox2*. Alternatively, a combined threshold dosage of *Sox2* plus *Sox3* may be necessary.

### Pax6-Deficient NS Cells Exhibit Proliferative Defects and Altered Morphology

*Pax6* immunoreactivity was lost in the *Pax6*<sup>loxP/loxP</sup> GFP<sup>+</sup> NS cell population 7 days after *Cre-GFP* transfection (Fig. 2A). While wild-type cells displayed an elongated bipolar morphology, Pax6-deficient cells showed an unusual multipolar morphology, with several long branch-like extensions and numerous small processes

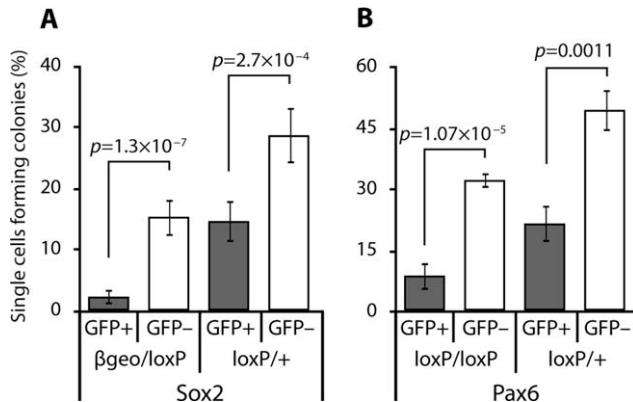


Fig. 3. The dosage of Sox2 and Pax6 determines the clonogenic capacity of NS cells. Plots show percentage of single cells giving rise to clones containing  $\geq 20$  cells 15 days after (A) Sox2 or (B) Pax6 deletion. Bars represent mean  $\pm$  SD;  $P$  values are for Student's one-tailed  $t$  tests.

(Fig. 2D). No obvious phenotype was detected in heterozygote cells ( $Pax6^{loxP/+}$  GFP $^+$ ). Pax6-deficient NS cells exhibited impaired proliferation, as demonstrated by a reduced percentage of cells immunoreactive for Ki67 when compared with non-excised cells ( $Pax6^{loxP/loxP}$  GFP $^+$   $12.60\% \pm 5.67$  SD,  $Pax6^{loxP/loxP}$  GFP $^-$   $34.48\% \pm 9.48$  SD; Student's  $t$  test,  $P = 3.28 \times 10^{-5}$ ) (Fig. 2C,D).

To gain detail into the dynamics of the cellular changes after excising Pax6, we carried out time-lapse analyses. This revealed that in addition to the morphological changes, Pax6-deficient NS cells display reduced motility (Supp. Info. Movie 1). Moreover, cell density estimates confirmed that homozygous inactivation of Pax6 strongly reduces NS cell growth rate (Fig. 2E).

#### The Dose of Sox2 and Pax6 Determines the Clonogenic Potential of NS Cells

To evaluate more stringently the effect of the loss of Sox2 or Pax6, we quantified colony formation ability. *Cre-GFP* transfected  $Sox2^{loxP/+}$ ,  $Sox2^{\beta\text{geo}/loxP}$ ,  $Pax6^{loxP/+}$ , and  $Pax6^{loxP/loxP}$  NS cells were sorted for GFP expression and deposited as single cells in 96-well plates. The plates were examined 15 days later and any clone consisting of 20 or more cells was scored as a colony. We found that the clonogenic capacity of each pool of NS cells correlated with the dosage of Sox2 (Fig. 3A). Heterozygote cells produced half the number of colonies compared with non-excised NS cells ( $Sox2^{loxP/+}$  GFP $^+$   $14.7\% \pm 3.3$  SD,  $Sox2^{loxP/+}$  GFP $^-$   $28.8\% \pm 4.5$  SD; Student's  $t$  test,  $P = 2.7 \times 10^{-4}$ ). Sox2-null NS cells generated about six times less colonies than heterozygote cells ( $Sox2^{\beta\text{geo}/loxP}$  GFP $^+$   $2.4\% \pm 1.2$  SD,  $Sox2^{\beta\text{geo}/loxP}$  GFP $^-$   $15.5\% \pm 3.1$  SD; Student's  $t$  test,  $P = 1.3 \times 10^{-7}$ ). Importantly, those rare Sox2-deficient colonies could not be expanded further, confirming that Sox2 is essential for NS cell self-renewal.

*Pax6* heterozygosity modestly reduced NS cell clonogenicity ( $Pax6^{loxP/+}$  GFP $^+$   $21.61\% \pm 4.4$  SD,  $Pax6^{loxP/+}$  GFP $^-$   $49.47\% \pm 5.1$  SD; Student's  $t$  test,  $P = 1.1 \times 10^{-3}$ ). Homozygous deletion of *Pax6* had a more pronounced effect, although not as strong as *Sox2* deletion ( $Pax6^{loxP/loxP}$  GFP $^+$   $8.85\% \pm 3.4$  SD,  $Pax6^{loxP/loxP}$  GFP $^-$   $32.29\% \pm 1.9$  SD; Student's  $t$  test,  $P = 1.07 \times 10^{-5}$ ) (Fig. 3B).

#### A Subpopulation of NS Cells Expands Without Pax6

In contrast to the situation with *Sox2*, NS cell propagation was not completely suppressed by deletion of *Pax6* (Fig. 3B). Of 14 *Pax6*-deleted ( $Pax6^{loxP/loxP}$  Cre-GFP $^+$ ) clones that were replated, 9 were capable of long-term expansion. These cells retained a euploid chromosome count. qRT-PCR analyses confirmed that all the isolated clonal lines were indeed *Pax6*-null ( $Pax6^{\Delta/\Delta}$ ) (Fig. 4A). Similar to wild-type NS cells, under self-renewing conditions these  $Pax6^{\Delta/\Delta}$  NS cells expressed Nestin, *Sox2*, and Olig2, as assessed by immunostaining (Fig. 4C). At  $\sim 70\%$  confluence, no significant differences in the proportion of Ki67 $^+$  cells were found between expandable  $Pax6^{\Delta/\Delta}$  and control  $Pax6^{loxP/loxP}$  NS cell cultures (ANOVA,  $P = 0.09$ ) (Fig. 4B). Nevertheless, in each  $Pax6^{\Delta/\Delta}$  NS cell line a substantial proportion of the cells displayed multi-branched processes (Fig. 4C). This altered morphology suggested an underlying difference in the NS cell state.

#### *Pax6*-Null NS Cells Show Defective Gliogenesis

The role of *Pax6* during lineage-commitment and postmitotic differentiation of neural progenitors has been extensively studied in the context of neurogenesis (Osumi et al., 2008). However, upon completion of neurogenesis, a significant proportion of radial glial cells, astrocyte precursors, and differentiated astrocytes maintain *Pax6* in both the embryonic and postnatal CNS (Hochstim et al., 2008; Ogawa et al., 2005; Sakurai and Osumi, 2008). Hence, it seems likely that *Pax6* may still be active during gliogenesis, at least in some regions of the CNS. In fact, it has been shown that *Pax6* plays a role in astrocyte maturation (Sakurai and Osumi, 2008). The isolation of self-renewing  $Pax6^{\Delta/\Delta}$  NS cells provided the opportunity to evaluate if the gliogenic differentiation potential of NS cells was altered in the absence of *Pax6*.

We first examined astrogli differentiation of  $Pax6^{\Delta/\Delta}$  NS cells. Upon exposure to BMP-4 (Gross et al., 1996) for 10 days both  $Pax6^{\Delta/\Delta}$  and  $Pax6^{loxP/loxP}$  NS cells acquired a flattened morphology and became mitotically inactive, as indicated by loss of Ki67 immunoreactivity (Fig. 4E). In control  $Pax6^{loxP/loxP}$  cell cultures the vast majority of cells activated expression of GFAP. In contrast,  $Pax6^{\Delta/\Delta}$  cell cultures displayed heterogeneous expression of GFAP, with a significant number of nega-

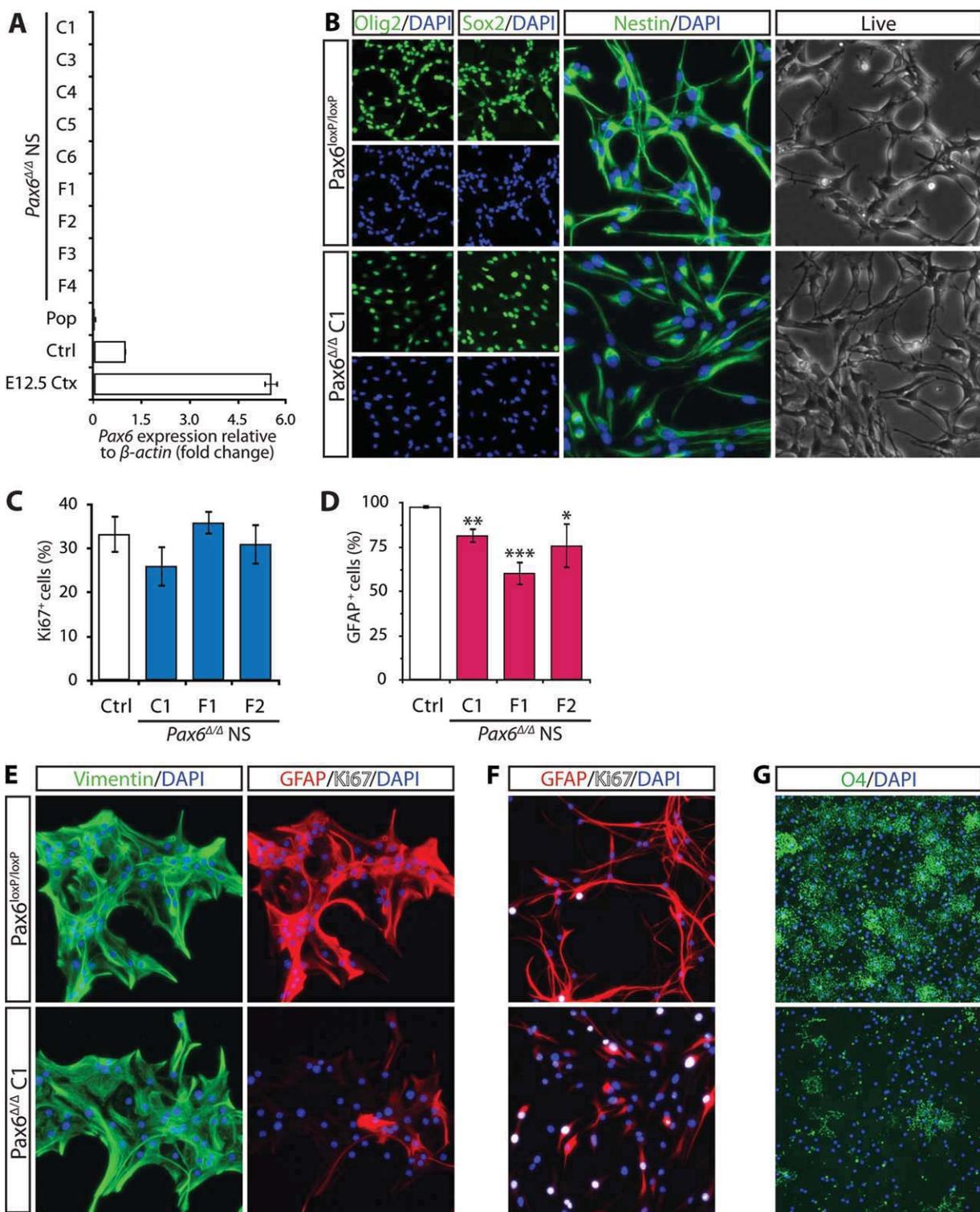


Fig. 4. Characterisation of *Pax6*<sup>Δ/Δ</sup> NS cells. (A) qRT-PCR using intron-spanning primers amplifying Exons 5–6 confirms excision of *Pax6* paired domain in single-cell derived *Pax6*<sup>Δ/Δ</sup> NS cell lines. Acutely excised population (Pop), *Pax6*<sup>loxP/loxP</sup> NS cells (Ctrl) and E12.5 cortical (Ctx) tissue are shown as controls. Ctrl = 1. (B) Antibody staining for Sox2, Olig2, and Nestin and phase-contrast images of *Pax6*<sup>loxP/loxP</sup> and *Pax6*<sup>Δ/Δ</sup> NS cells in expansion. (C) Histogram showing similar proportions of proliferating Ki67<sup>+</sup> cells in *Pax6*<sup>loxP/loxP</sup> and *Pax6*<sup>Δ/Δ</sup> NS cells expanding in EGF and FGF-2. (D, E) After 10 of BMP treatment, a sig-

nificant proportion of cells in the *Pax6*<sup>Δ/Δ</sup> cell cultures fails to activate GFAP expression (Student's one tailed *t* test; \**P* = 8.9 × 10<sup>-4</sup>, \*\**P* = 2.2 × 10<sup>-6</sup>, \*\*\**P* = 2.5 × 10<sup>-8</sup>). (F) Immunostaining for GFAP and Ki67 on NS-derived astrocytes treated with EGF and FGF-2 for 5 days. (G) Antibody staining for O4 after 8 days of culture in conditions favoring oligodendroglial differentiation. Note that *Pax6*<sup>Δ/Δ</sup> NS cells generate less O4<sup>+</sup> oligodendrocytes than controls. Bars in C and D represent mean ± SD. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

tive cells despite the dramatically altered morphology (Fig. 4D,E). Comparable results were obtained after serum-induced differentiation (not shown). Re-addition of EGF and FGF-2 for 5 days after 10–12 days of culture in BMP or serum, resulted in a substantial proportion of cells in the *Pax6*<sup>Δ/Δ</sup> cultures re-acquiring expression of Ki67. In contrast, only a minor population of cells re-entered the cell cycle in control cultures (Fig. 4F).

When cultured under conditions favoring oligodendroglial differentiation (Glaser et al., 2007) (Fig. 5A) in all cases *Pax6*<sup>Δ/Δ</sup> NS cells generated fewer O4<sup>+</sup> oligodendrocytes than control *Pax6*<sup>loxP/loxP</sup> cultures (Fig. 4G). Both the astroglial and the oligodendroglial differentiation deficient phenotypes were observed in six independent *Pax6*<sup>Δ/Δ</sup> NS cell lines.

During the differentiation process (Fig. 5A), we observed an increased incidence of pyknotic nuclei and overt cell death in all mutant cell lines after growth factor withdrawal, leading to a reduction in the total number of cells by the end of the differentiation period. To assess whether cell death may be attributable to aberrant glial differentiation, we analyzed expression of *Pax6* and lineage-specific markers by qRT-PCR at various time points. This revealed that in control *Pax6*<sup>loxP/loxP</sup> cultures *Pax6* expression increased throughout the course of differentiation (Fig. 5B). Transcripts of the radial glia/astrocyte lineage marker *Glast* (*Slc1a3*) (Shibata et al., 1997) showed an increment in all NS cell lines upon induction of differentiation, although levels were appreciably lower in *Pax6*<sup>Δ/Δ</sup> cells in comparison to control cultures at all time points examined (Fig. 5C). By Day 4, control cells had activated expression of the astroglial marker *S100b* (Hyden and McEwen, 1966) and the oligodendroglial marker *Sox10* (Kuhlbrodt et al., 1998). Levels of both genes increased more than six-fold by Day 8 (Fig. 5B). Consistent with the deficient glial differentiation of *Pax6*<sup>Δ/Δ</sup> cells, all *Pax6*-null cell cultures showed substantially lower induction of *S100b* and negligible levels of *Sox10* transcripts at any time point (Fig. 5B).

We then examined expression of the oligodendrocyte precursor cell (OPC) markers NG2 (*Cspg4*) (Nishiyama et al., 1996) and PDGFR $\alpha$  (Pringle and Richardson, 1993). Unexpectedly, we found that prior to induction of differentiation all NS cell lines examined showed heterogeneous levels of expression of NG2. However, flow cytometry analyses demonstrated that the proportion of NG2<sup>high</sup> cells was markedly lower in all *Pax6*<sup>Δ/Δ</sup> NS cell lines, relative to control cells, as shown by a negative shift in the distribution of the population (Fig. 5D). Negligible PDGFR $\alpha$  immunoreactivity was detected in these conditions (Fig. 5E).

After switching culture conditions to FGF-2, PDGF, and Forskolin, which promote generation and proliferation of OPCs (Bogler et al., 1990; Raible and McMorris, 1990), immunostaining and flow cytometry analyses showed an increasing population of PDGFR $\alpha$ <sup>+</sup> cells from Day 2 in control cultures (Fig. 5E). In contrast, in *Pax6*<sup>Δ/Δ</sup> cultures this population was considerably smaller, or even absent (Fig. 5E,F). Further examination by qRT-PCR showed that the increasing number of

PDGFR $\alpha$ <sup>+</sup> cells in control cultures correlated with a dramatic increase in *Pdgfra* transcripts, in contrast with the low levels of *Pdgfra* expression in *Pax6*<sup>Δ/Δ</sup> cell cultures (Fig. 5G). Expression of NG2 also increased considerably in control cells from Day 2 of differentiation, whereas in *Pax6*<sup>Δ/Δ</sup> cells changes in expression were less noticeable (Fig. 5G). These results suggest that the deficient generation of oligodendrocytes from *Pax6*<sup>Δ/Δ</sup> NS cells might be attributable to a failure during the specification and/or development of OPCs. Taken together these data identify an unexpected role of Pax6 in gliogenic competence in addition to its well known role in neurogenesis.

### Sox2 is Dispensable for Astroglial Differentiation of NS Cells

Finally, we examined whether Sox2 might also be involved in glial differentiation of NS cells. Since self-renewing NS cells are not recovered after homozygous inactivation of Sox2 (Figs. 1 and 3A), *Sox2*<sup>βgeo/loxP</sup> NS cells were transfected with *Cre-GFP*, sorted for GFP expression, and directly replated in the presence of BMP. Immunostaining analyses performed 10 days later, revealed the presence of GFAP<sup>+</sup> cells with characteristic astrocyte morphology and Sox2<sup>-</sup> nuclei in the GFP<sup>+</sup> cell cultures (Fig. 6). This experiment does not exclude the possibility that low levels of Sox2 that might persist transiently after excision may be required for initial commitment into the astroglial pathway. However, the observation strongly suggests that Sox2 is not required to execute the astroglial differentiation programme.

## DISCUSSION

This study has revealed essential and non-redundant roles for Sox2 and Pax6 in mouse NS cells. Sox2 is essential for maintaining proliferative capacity and progenitor identity, even though NS cells express the closely related Sox1 factor Sox3. Pax6 is expressed only at low levels in NS cells, yet appears to be indispensable as a multifunctional regulator, preserving normal NS cell proliferation potential and morphology and also mediating gliogenic differentiation capacity.

Mouse NS cell lines display many hallmarks of radial glia, including expression of Pax6, BLBP, RC2, Vimentin, and GLAST (Conti et al., 2005; Pollard et al., 2006b). In this study we found that NS cells also show heterogeneous expression of NG2 (Fig. 5D), which is considered a marker of oligodendroglial progenitors (Nishiyama et al., 1996). Lineage-tracing analyses have revealed that radial glial cells act as neural progenitors throughout the CNS, giving rise first to neurons and, later, to glia (Anthony et al., 2004; Casper and McCarthy, 2006). When expanded *in vitro* in the presence of FGF-2 radial glial cells partially lose regional identity and acquire markers that would not normally be co-expressed *in vivo* (Hack et al., 2004; Pollard et al.,

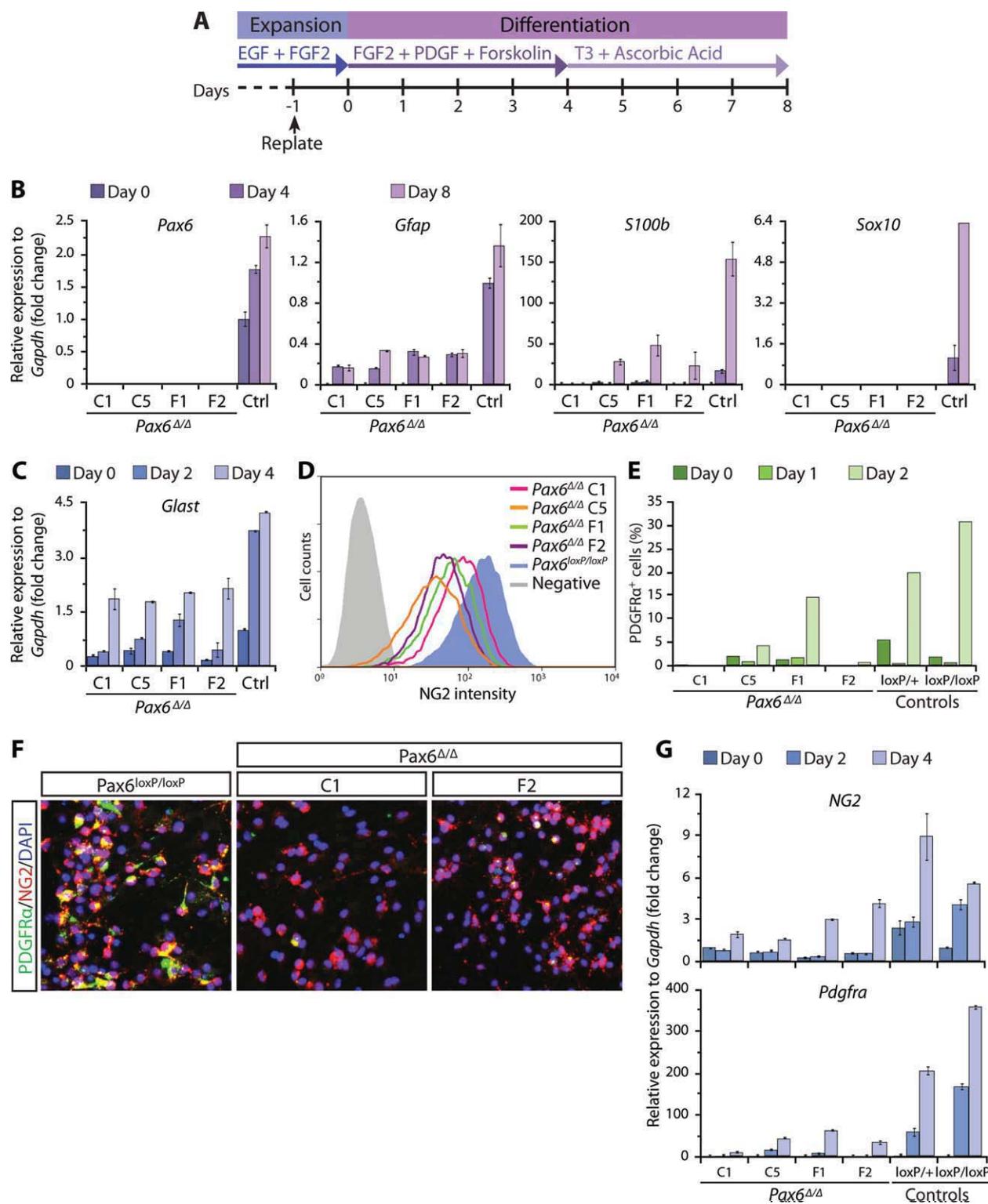


Fig. 5. Pax6 deficiency affects gliogenesis from NS cells. (A) Trilineage differentiation protocol for NS cells. (B, C) qRT-PCR analyses for *Pax6*, *Gfap*, *S100b*, *Sox10*, and *Glast* in *Pax6*<sup>Δ/Δ</sup> NS cells in expansion and differentiation conditions. *Pax6*<sup>loxP/loxP</sup> cell cultures are shown as controls (Ctrl). Ctrl Day 0 = 1, except for *Gfap* and *Sox10*, where Ctrl Day 4 = 1. (D) Flow cytometry analysis for NG2 in NS cells expanding

in EGF and FGF-2 (Day 0). (E) Flow cytometry analyses for PDGFRα in self-renewing and differentiating NS cells. (F) Immunostaining for NG2 and PDGFRα at Day 4 of differentiation. (G) qRT-PCR for *NG2* (*Cspg4*) and *Pdgfra* in expanding and differentiating *Pax6*<sup>Δ/Δ</sup> cells. *Pax6*<sup>loxP/loxP</sup> Day 0 = 1. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

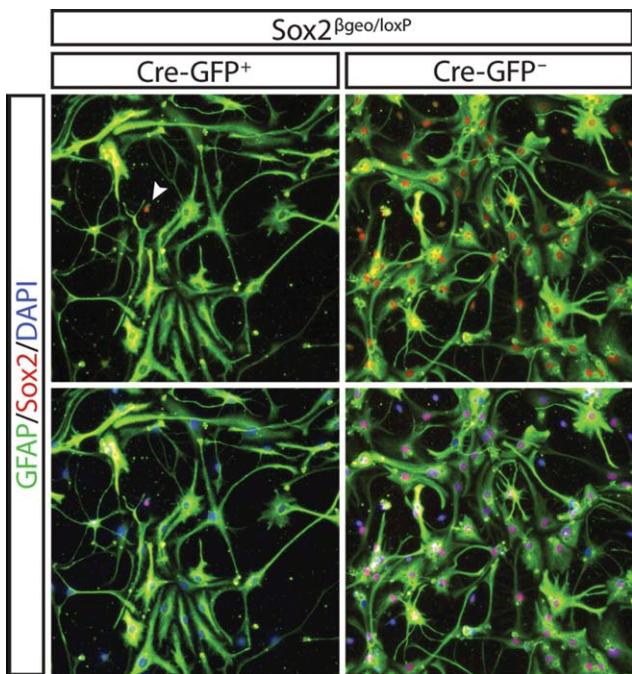


Fig. 6. Sox2 is not essential for astrogli differentiation of NS cells. Immunostaining for GFAP and Sox2 on *Sox2*-ablated NS cells cultured in the presence of BMP for 10 days. Note that, except for a few contaminant non-excised cells (arrowhead), the majority of the *Sox2*<sup>βgeo/loxP</sup> Cre-GFP<sup>+</sup> cells are not Sox2 immunoreactive, yet express GFAP and exhibit astrocyte morphology. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

2008). These changes may reflect partial activation of an oligodendroglial pathway, indicated by expression of Olig2, Nkx2.2 and, in a proportion of cells, of NG2 (Chandran et al., 2003; Kessaris et al., 2004). In NS cell cultures, continuous stimulation with EGF and FGF-2 prevents further differentiation, stabilizing these cells as a late fetal radial glia-like cell with nascent properties of a pre-OPC. Consistent with this, we find that in expanded mouse NS cells the capacity for neuronal differentiation is often attenuated while they readily differentiate into GFAP<sup>+</sup> astrocytes in response to BMP or serum treatment, or reinitiate the oligodendroglial programme upon treatment with PDGF, FGF-2 and Forskolin.

Preliminary characterization of NS cells heterozygous for *Sox2* or *Pax6* indicated that in terms of marker expression and differentiation potential these cells appeared similar wild-type cells. However, further examination demonstrated that their self-renewal ability was partially compromised (Figs. 1B and 3), indicating NS cell sensitivity to *Sox2* and *Pax6* gene dosages. These dosage requirements are especially interesting when considering that in humans heterozygous mutations for *SOX2* or *PAX6* are associated with severe eye malformations and distinct brain defects, among other abnormalities (Fantes et al., 2003; Sisodiya et al., 2001, 2006).

Complete ablation of *Sox2* in NS cells led to rapid loss of their proliferative and clonogenic potential, as well as to downregulation of radial glial cell markers (Figs. 1

and 3A). Previously, *in vivo* disruption of *Sox2* activity in chick embryonic neural progenitors was found to result in a similar premature exit from the cell cycle and downregulation of progenitor markers (Graham et al., 2003). However, this was achieved by means of a chimeric repressor form (*HMG-Engrailed*), which is expected to affect all *SoxB1* factors. *In vivo* deletions of *Sox2* in the mouse CNS have resulted in only moderate reduction in the number of mitotically active progenitor cells and attenuated neurogenic potential during fetal development (Miyagi et al., 2008). Postnatally, however, hippocampal progenitors and neurogenesis were severely affected (Favaro et al., 2009). It is speculated that at early stages of development, the effects of the loss of *Sox2* are masked by the compensatory activities of *Sox1* and *Sox3*, whose expression domains extensively overlap with that of *Sox2* (Wood and Episkopou, 1999). NS cells do not express *Sox1*, but they have appreciable levels of *Sox3*, yet exhibit a strong phenotype upon *Sox2* deletion. Thus, there may be a non-redundant function of *Sox2* which has not been detected in the limited series of temporal conditional deletions described to date, or is compensated through a non-cell autonomous mechanism *in vivo*. Alternatively, NS cells may have a threshold requirement for total amount of *SoxB1* proteins. In that context it is also possible that *Sox2* may contribute directly to the expression of *Sox3*. Overall, however, it is evident that *Sox2* and *SoxB1* proteins have a central function in sustaining the proliferative and developmental capacities of CNS progenitors both *in vivo* and *in vitro*.

In the postnatal hippocampus *Sox2* mediates its activity through a non cell-autonomous mechanism involving activation of *Shh* (Favaro et al., 2009). In this study, we neither detected *Shh* expression, nor effects, in NS cell cultures (Fig. 1F), highlighting stage and region-specific distinctions in *Sox2* functions (Pevny and Nicolis 2009). *In vivo* ablation of *Sox2* at different developmental time points will clarify the relationship between *Sox2* requirements in NS cells and *in vivo* action.

Culture of fetal cortical progenitors in the form of NS cells is accompanied by a considerable decline in *Pax6* expression levels (Supp. Info. Fig. 2B,C), as also observed in neurosphere cultures (Hack et al., 2004). However, we have shown that these low levels of *Pax6* expression remain necessary to maintain the proliferative capacity and bipolar morphology of NS cells (Figs. 2 and 3B). Acute inactivation of *Pax6* in NS cells led to a drastic reduction in the proportion of proliferating cells and resulted in acquisition of a multipolar morphology by those cells that persisted. Intriguingly, these alterations phenocopy aspects of *Pax6* loss-of-function in cortical radial glia *in vivo*. First, analyses of mouse chimeras composed of wild-type and *Pax6*-null (*Pax6*<sup>Sey/Sey</sup>) cells revealed that cortical progenitors lacking *Pax6* exit the cell cycle precociously and in abnormally high proportions, leading to an early depletion of the neural progenitor pool (Quinn et al., 2007). Second, it has been shown that in primary cultures of dissociated mouse cortices, wild-type RC2<sup>+</sup> radial glia display an elongated bipolar

morphology. In contrast, most of the *Pax6*-null RC2<sup>+</sup> cells show multi-branched morphology (Gotz et al., 1998). Thus, despite continuous *ex vivo* expansion and low *Pax6* expression levels, NS cells reflect some key features of radial glial cells.

NS cells lacking *Pax6* exhibit defective gliogenesis (Figs. 4D,E,G and 5). Upon induction of astroglial differentiation, *Pax6*-null NS cells exited the cell cycle and acquired a flattened morphology but a significant proportion of the cells failed to activate GFAP expression. When induced to differentiate into oligodendroglia, *Pax6*<sup>Δ/Δ</sup> NS cells generated abnormally low numbers of PDGFR $\alpha^+$  OPCs, and subsequently few O4<sup>+</sup> oligodendrocytes. The defective differentiation of *Pax6*-null NS cells into glial lineages may either reflect a requirement for *Pax6* during gliogenesis, or an alteration of progenitor properties which precludes an appropriate response to inductive signals. It has been reported that in astrocytes derived from *Pax6*<sup>Sey/Sey</sup> mice the proportion of cells expressing GFAP is significantly reduced (Sakurai and Osumi, 2008). These authors proposed that *Pax6* is required for astrocyte maturation. However, in agreement with the role of *Pax6* as a neurogenic determinant in radial glial cells (Heins et al., 2002), overexpression of *Pax6* in NS cells triggered a neurogenic programme (Supp. Info. Fig. 3). Thus, in addition to controlling NS cell proliferation/maintenance, *Pax6* allows progression into a gliogenic fate and, at higher levels, activates neurogenesis.

The different aspects of *Pax6* activity appear to be mediated by dosage and developmental stage-specific interactions with TF partners and/or modified occupancy of different promoter elements. In such a scenario, self-renewal or cell fate specification may require a critical threshold of *Pax6* to activate a specific transcriptional programme and probably also to repress alternative fates. This type of quantitative modulation of transcriptional activity has been shown to operate in ES cells and hematopoietic stem cells (Laslo et al., 2006; Niwa et al., 2000), and may be a widespread mechanism to control stem cell fates. Recently, it was reported that in the developing mouse cortex *Pax6* directly regulates distinct groups of genes involved in the control of self-renewal, basal progenitor development and neurogenesis, and that increasing *Pax6* levels drives the system towards neurogenesis at the expense of self-renewal (Sansom et al., 2009). Thus, *Pax6* appears to be a pivot for transitions between gene regulatory networks in neural progenitors. Our findings suggest that NS cells provide a tractable system in which to investigate and manipulate these transitions.

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