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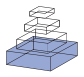

Flexibility of neural stem cells

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Embryonic cortical neural stem cells are self-renewing progenitors that can differentiate into neurons and glia. We generated neurospheres from the developing cerebral cortex using a mouse genetic model that allows for lineage selection and found that the self-renewing neural stem cells are restricted to *Sox2* expressing cells. Under normal conditions, embryonic cortical neurospheres are heterogeneous with regard to *Sox2* expression and contain astrocytes, neural stem cells, and neural progenitor cells sufficiently plastic to give rise to neural crest cells when transplanted into the hindbrain of E1.5 chick and E8 mouse embryos. However, when neurospheres are maintained under lineage selection, such that all cells express *Sox2*, neural stem cells maintain their Pax6$^+$ cortical radial glia identity and exhibit a more restricted fate *in vitro* and after transplantation. These data demonstrate that Sox2 preserves the cortical identity and regulates the plasticity of self-renewing Pax6$^+$ radial glia cells.

Keywords: radial glia, self-renewal, neural stem cell, stem cell niche, *Sox* genes, neurogenesis, gliogenesis, neural crest

INTRODUCTION

Sox genes encode transcriptional regulators with HMG box DNA-binding domains, and are involved in specifying cell fates (Kamachi et al., 2000; Scaffidi and Bianchi, 2001; Wilson and Koopman, 2002; Wegner and Stolt, 2005). *Sox*1, *Sox2*, and *Sox*3 (*Sox*B1 subfamily; Wood and Episkopou, 1999; Kamachi et al., 2000) are expressed broadly within the primitive neuroepithelium during embryogenesis and typically mark uncommitted precursors within the developing (Wood and Episkopou, 1999; Avilion et al., 2003) and adult central nervous system (CNS; Ferri et al., 2004; Brazel et al., 2005).

Gain of function experiments in the chick showed that *SoxB1* factors maintain the neural progenitor (NP) state and inhibits differentiation of spinal cord precursors. In contrast, suppression of SoxB1 function leads to premature cell cycle exit and initiation of neuronal differentiation (Bylund et al., 2003; Graham et al., 2003; Kan et al., 2004; Sandberg et al., 2005). In mice, *SoxB1* loss of function mutations failed to reveal the role of these genes in NSCs since they result in either early lethality (*Sox2*) or no obvious NSC phenotypes (*Sox1* and *Sox3*; Nishiguchi et al., 1998; Avilion et al., 2003; Malas et al., 2003; Rizzoti et al., 2004; Ekonomou et al., 2005). Recent findings suggested that Sox1 maintains cortical NP cells undifferentiated by suppressing cell cycle exit to neurogenesis (Elkouris et al., 2011). *Sox2* hypomorphic mouse mutants exhibit impaired neurogenesis in the adult brain together with neurodegeneration (Ferri et al., 2004). Similarly, conditional ablation of *Sox2* also caused defects in adult neurogenesis, particularly in hippocampal development and NSC maintenance which is *sonic hedgehog (Shh)* dependent (Favaro et al., 2009; Pevny and Nicolis, 2010). However, the precise role of *Sox2* in embryonic NSCs is still elusive.

Cortical NSC can be cultured *ex vivo* as neurospheres which are heterogenous free-floating aggregates consisting of mixed populations of stem, progenitor, and differentiated cells. These cells eventually lose their regional identity in culture (Ellis et al., 2004; Brazel et al., 2005; Ahmed, 2009; Conti and Cattaneo, 2010), which raises important questions about the signals required for their maintenance and differentiation properties *in vitro* and *in vivo*. We used a mouse genetic model ($Sox2^{\beta geo}$) to investigate the molecular properties and the plasticity of a homogeneous population of cortical NSCs obtained by selection for *Sox2* expression (Li et al., 1998; Zhao et al., 2004).

MATERIALS AND METHODS

MICE

Sox2$^{\beta geo/+}$ and *Sox2*$^{\beta geo2/+}$ were maintained and genotyped as previously described (Avilion et al., 2003; Ekonomou et al., 2005). All experiments carried out on mice were approved under the UK Animal (scientific procedures) Act (Project license 80/1949; National Institute for Medical Research), the Animals Act 160/03.05.1991/revised 86/609/EEC/24.11.1986 EU directive for Animal Experimentation (Prot. No. 767/28.02.07; BSRC "Alexander Fleming") and the IACUC animal welfare guidelines and approved protocols and licenses (Stowers Institute for Medical Research).

NEUROSPHERE CULTURES

Neurospheres were derived and maintained as previously described (Zappone et al., 2000; Elkouris et al., 2011). Cortices from *Sox2*$^{\beta geo/+}$ mice at E14.5 were dissected in ice cold DMEM–F12 medium (GIBCO-BRL) supplemented with 2 mM glutamine and antibiotics before they were triturated in DMEM–F12 medium containing 0.6% glucose, 9.6 mg/ml putrescine (SIGMA), 6.3 ng/ml progesterone (SIGMA), 5.2 ng/ml sodium selenite (SIGMA), 25 mg/ml insulin (SIGMA), 100 mg/ml transferrin (SIGMA), 20 ng/ml each of bFGF and EGF (R&D systems), 2 mM glutamine and antibiotics (NSC medium; Zappone et al., 2000). Cell suspensions were plated at clonal density (10^4 cells/ml) in NSC medium and primary neurospheres were grown and maintained in this medium for 3 weeks. When required, G418 was added at a final concentration of 250 mg/ml. β-galactosidase activity was assayed as described (Zappone et al., 2000; Elkouris et al., 2011). At 3 weeks, β-galactosidase$^+$ cells were present as isolated cells (<2% of cells), in coherent patches (2–50% of cells) or dispersed throughout (>50% of cells) in 3–5, 5–7, and approximately 90% of *Sox2*$^{\beta geo/+}$ neurosphere cultures, respectively. For differentiation, single neurospheres were plated on a matrigel support and allowed to differentiate for 10 days. For the FACS analysis, *Sox2*$^{\beta geo/+}$ neurospheres were dissociated and single cells were incubated with CMFDG substrate (Molecular Probes) for 30 min on ice according to the instructions of the manufacturer, before they were subjected to cell sorting for β-galactosidase activity on a MoFlo cell sorter (Cytomation). *Sox2*$^+$ (LacZ$^+$) and *Sox2*$^-$ (LacZ$^-$) cells were cultured as single cell suspension in serial dilutions and allowed to form neurospheres in the absence of G418 selection.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed on differentiated cells fixed with 100% methanol. Whole-mount immunofluorescence was performed on live (RC2 staining) and either methanol or MEMFA (Avilion et al., 2003) fixed neurospheres. On average about 10 neurospheres were used per individual experiment with two to seven replicates. Antibody incubation was generally performed in phosphate buffered saline (PBS) solution containing 1% BSA, 0.15% glycine, and 0.1% Triton-X100 at 4°C overnight or for 1–2 h at room temperature. Images from intact neurospheres with representative staining were recorded on a Leica TCS SP confocal microscope using the TCSNT software.

ANTIBODIES

Primary antibodies were: anti-Sox2 (rabbit purified IgG; 1:2500, R. Lovell-Badge); anti-Pax6 (mouse IgG 1:500; Chemicon); anti-nestin (mouse IgG 1:75; DSHB); anti-GFAP, Cy3-linked (mouse IgG 1:100; SIGMA); mouse RC2 (mouse IgM 1:100; DSHB); anti-βIII tubulin (TuJ1; mouse IgG 1:1000; BabCo); anti-*Sox*9 (rabbit purified IgG 4796; 1:1000; a gift from S. Guioli); and anti-Sox10 (mouse IgG 1:10; a gift from D. Anderson). AlexaFluor 488, 594, 555, and 647 chromophores were used with secondary antibodies (Molecular Probes). Photographs were acquired with Leica SP2 (NIMR, London) and SP5 (BFRAA, Athens) confocal microscopes using ALasAF Software (Leica).

NEUROSPHERE TRANSPLANTATIONS AND MOUSE AND CHICK EMBRYO CULTURE

Neurospheres were removed from culture and labeled by incubation in DiI labeling solution (0.05% w/v Dil in 0.3 M sucrose stock, diluted 1:1 in DMEM) for up to 5 min. Neurospheres were then washed multiple times in DMEM (for mouse transplants) or Ringer's solution (for chick embryos). Depending on their size, neurospheres were either dissected into smaller pieces using glass needles for transplantation into mouse embryos or in the case of chick embryos they were transplanted as whole neurospheres at the levels of rhombomere (r) 2 and r4 in the hindbrain.

Mouse embryos were collected from timed pregnant CD1 mice at 8.5 dpc for *in vitro* whole embryo culture. Following dissection of conceptuses from the uterus, the parietal yolk sac was removed leaving the embryo with an intact visceral yolk sac amnion and ectoplacental cone. Post neurosphere transplantation with 0.30 μm glass needles, mouse embryos were cultured in DMEM culture medium supplemented with 50% rat serum, L-glutamine and penicillin/streptomycin (DR50) for 24 h in small glass bottles attached to a rotating drum (BTC engineering, Cambridge) at 37°C with a constant atmosphere of 5% O_2, 5% CO_2, 90% N_2 (Sturm and Tam, 1993).

Fertilized chick eggs were incubated for approximately 36 h at 37°C in a humidified incubator to obtain embryos of the eight somite stage or earlier. Individual eggs were windowed and the embryos were visualized via injection of India Ink (1:10 dilution in Ringer's Solution). The vitelline membrane covering the hindbrain was opened using tungsten needles after which a small slit was made in the midline of the neural tube at the desired axial level in the hindbrain. Post neurosphere transplantation, host chick embryo eggs were resealed with clear tape and returned to a 37°C incubator for either 24–48 h or up to 8–9 days.

ELECTROPORATION AND *IN SITU* HYBRIDIZATION

Chick embryos with eight or less somites were obtained as described above. Control plasmid *pCMV–GFP* was injected alone or together with *pCMV–mouseSox2 or pCMV–mouseSox9* into the cranial neural tube with finely pulled injection needles. Then 0.5 mm gold electrodes (0.5 cm separation) were placed gently on the vitelline membrane on either side of the cranial neural tube and the plasmids were electroporated into the neuroepithelium using the following conditions: 5 pulses of a 25-V, 50 ms wave with a 1-s gap between pulses. After electroporation, host chick embryo eggs were resealed with clear tape and returned to a 37°C incubator for up to 24 h. Electroporated chick embryos were then processed for *in situ* hybridization as previously described (Wilkinson and Nieto, 1993; Wilkinson, 1995) with mouse *Sox2* and chick *Sox10* cRNA probes.

RESULTS

NSC SELF-RENEWAL REQUIRES *Sox2* EXPRESSION

To analyze the properties of *Sox2*$^{\beta geo/+}$ NSC, we cultured neuroepithelial cells from the cerebral cortex of individual E14.5 heterozygous mouse embryos for 3 weeks as primary neurospheres (Zappone et al., 2000) in two distinct populations, either without (*Sox2*$^{\beta geo/+}$) or with G418 selection (*Sox2*$^{\beta geo/+}$-selected; **Figures 1A–E**) without sub-cloning (**Figures 1F–L**). Wild type (*wt*) cells were also used as controls for any *in vitro* effects related to *Sox2* heterozygosity (**Figure 1A**). No significant differences were observed between *wt* and *Sox2*$^{\beta geo/+}$ neurospheres. *Sox2*$^{\beta geo/+}$ neurospheres had mosaic and variable patterns of lacZ activity reflecting *Sox2*-expressing cells, ranging from dispersed single cells (**Figure 1B**), to patches (**Figure 1C**), to a majority of *Sox2*$^{+}$ cells (**Figure 1D**), a heterogeneity similar to adult NSC cultures (Brazel et al., 2005; Machon et al., 2005). In contrast, in *Sox2*$^{\beta geo/+}$-selected neurospheres, a homogene-

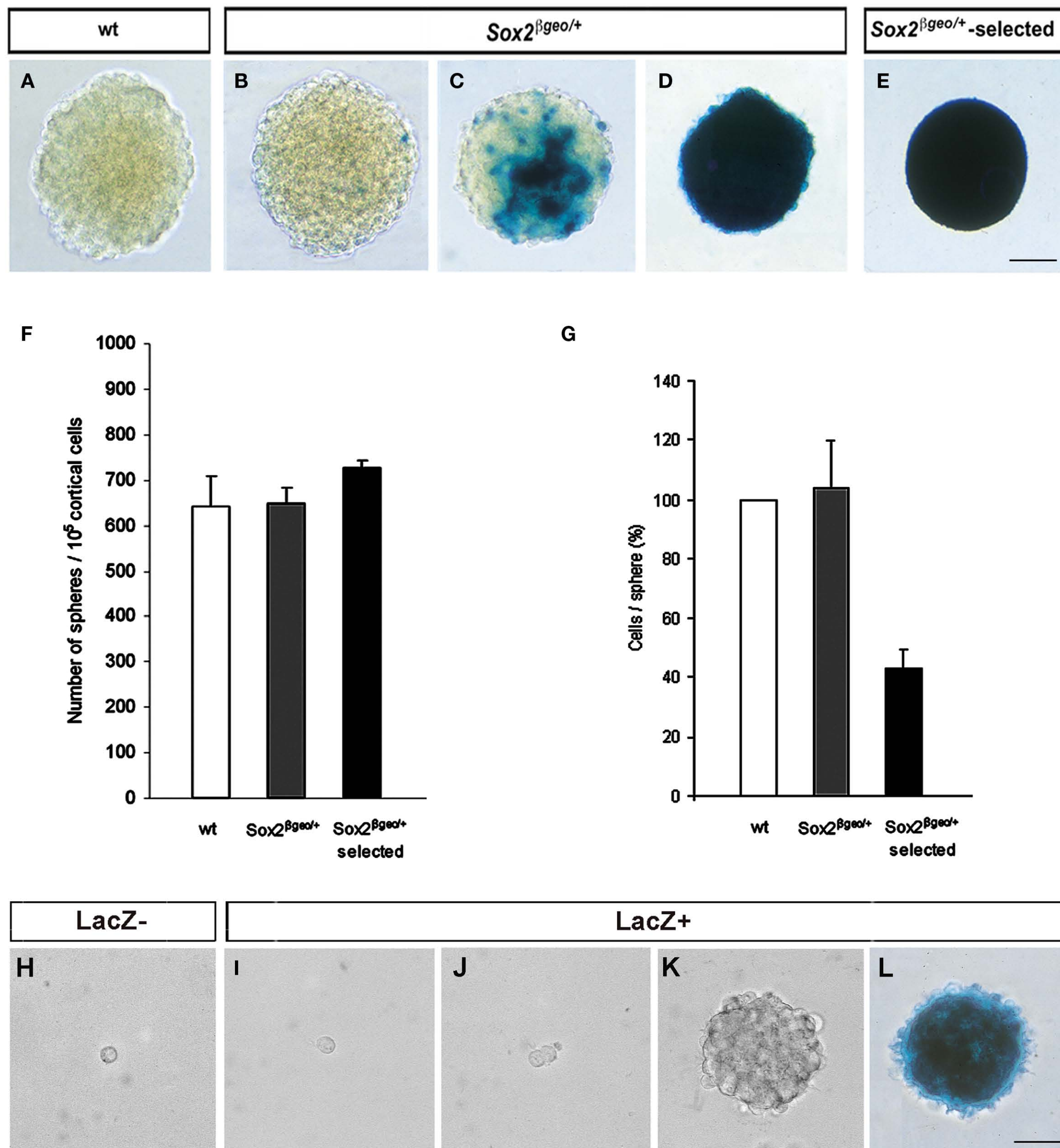

FIGURE 1 | Cortical neurospheres contain stochastic numbers of *Sox2*-expressing self-renewing cells. β-galactosidase activity in *wt* **(A)**, *Sox2*$^{\beta geo/+}$ **(B–D)**, and *Sox2*$^{\beta geo/+}$-selected **(E)** neurospheres cultured for 3 weeks. Cortical neuroepithelial cells from E14.5 *Sox2*$^{\beta geo/+}$ embryos were grown either without [*Sox2*$^{\beta geo/+}$, **(B–D)**] or with G418 selection [*Sox2*$^{\beta geo/+}$-selected, **(E)**]. *Wt* littermates were used as a control [**(A)**; scale bar = 50 μm]. Numbers of neurosphere-forming cells were similar for *wt*, *Sox2*$^{\beta geo/+}$, and *Sox2*$^{\beta geo/+}$-selected cells **(F)**, but the size of *Sox2*$^{\beta geo/+}$-selected cell pool was significantly smaller **(G)**. The data are presented as the mean ± SD **(H, I)**. *Sox2*$^{\beta geo/+}$ neurospheres were dissociated and fractionated into *Sox2*$^{\beta geo/+}$-expressing [**(I)**, LacZ^{+}] and non-expressing cells [**(H)**, LacZ^{-}] using FACS. Each cell population was cultured at clonal density (10^4 cells/ml) and allowed to proliferate **(J)** without selective pressure. Only *Sox2*$^{\beta geo/+}$-expressing cells generated neurospheres **(K)** expressing variable levels of β-galactosidase activity [**(L)**; scale bar = 50 μm].

ous population of *Sox2*⁺ cells was obtained (**Figure 1E**, *Sox2*$^{\beta geo/+}$-selected). These neurospheres were smaller and contained 40% of the number of cells present in *wt* and *Sox2*$^{\beta geo/+}$ neurospheres. Since the original numbers of neurosphere-forming cells were similar for *wt*, *Sox2*$^{\beta geo/+}$, and *Sox2*$^{\beta geo/+}$-selected cultures (**Figure 1F**), the cells that failed to express *Sox2* were eliminated by selection.

To determine which cells were capable of self-renewal, we tested the ability of *Sox2*$^{-}$ and *Sox2*$^{+}$ cells to give rise to secondary neurospheres (**Figures 1H–L**). FACS sorting for β-galactosidase expression separated the two cell populations present in dissociated unselected *Sox2*$^{\beta geo/+}$ neurospheres. When each fraction was cultured at clonal density in serial dilutions, no neurospheres were produced from the *Sox2*$^{-}$ cells, which did not survive beyond 12–24 h in culture (**Figure 1H**). Only *Sox2*$^{+}$ cells divided (**Figures 1I,J**) and gave rise to secondary neurospheres (**Figure 1K**). On average, about 1% of the cells exhibited the ability to generate secondary neurospheres. As observed for the primary neurospheres (**Figures 1B–D**), the majority of cells within the secondary neurospheres expressed *Sox2* (**Figure 1L**). Thus, self-renewing NSCs are restricted to *Sox2*-expressing cells.

NSCs REQUIRE *Sox2* EXPRESSION TO MAINTAIN THEIR Pax6^{+} RG IDENTITY IN NEUROSPHERE CULTURES.

Radial glia (RG) comprise the predominant form of NP cells in the E14.5 cortex (60–70%; Gotz et al., 2002; Gotz, 2003; Malatesta et al., 2003; Gotz and Barde, 2005). These cells exhibit self-renewal properties and multipotency (Campbell and Gotz, 2002; Gotz et al., 2002; Gotz, 2003; Gotz and Barde, 2005) and express the nestin-linked epitope RC2 (Malatesta et al., 2003; Mori et al., 2005; **Figure 2A**). RC2^{+} cells co-express Sox2 in the proliferating zones of the cortex and in cell spreads (**Figure 2A** and inset photo). Mitogens can change the character of cells in culture and as a result neurosphere assays may not reflect the endogenous progenitor state and fate of the cortical environment *in vivo* ((Brazel et al., 2005; Jensen and Parmar, 2006; Conti and Cattaneo, 2010) We, therefore, examined the ability of cortical *Sox2*$^{+}$ cells to maintain their *in vivo* identity in neurosphere cultures.

Primary neurospheres from *Sox2*$^{\beta geo/+}$ embryos were cultured with or without selection (**Figures 2B–E**) and characterized for neural markers (**Figures 2C,Di–xv**). To exclude the possibility that some of the cellular phenotypes could arise from *Sox2* heterozygosity, neurospheres produced from *wt* littermates were also analyzed. *Wt* and *Sox2*$^{\beta geo/+}$ neurospheres were heterogeneous with respect to Sox2^{+} and nestin^{+} cells (**Figures 2Ci,ii,iv,v**). In contrast, *Sox2*$^{\beta geo/+}$-selected neurospheres comprised of Sox2^{+} nestin^{+} cells (**Figures 2Ciii,vi**) and showed suppressed astrogenesis as evidenced by the reduction in numbers of GFAP^{+} cells and in levels of GFAP staining (**Figure 2Dxviii**) when compared to control neurosphere populations (**Figures 2Dxvi,xvii**). *Wt* and *Sox2*$^{\beta geo/+}$ neurospheres contained high numbers of Sox9^{+} cells (**Figures 2Dxiii,xiv**) and as Sox9 is implicated in the change of competence of neuroepithelial cells to generate glial lineages (Wegner and Stolt, 2005), the high

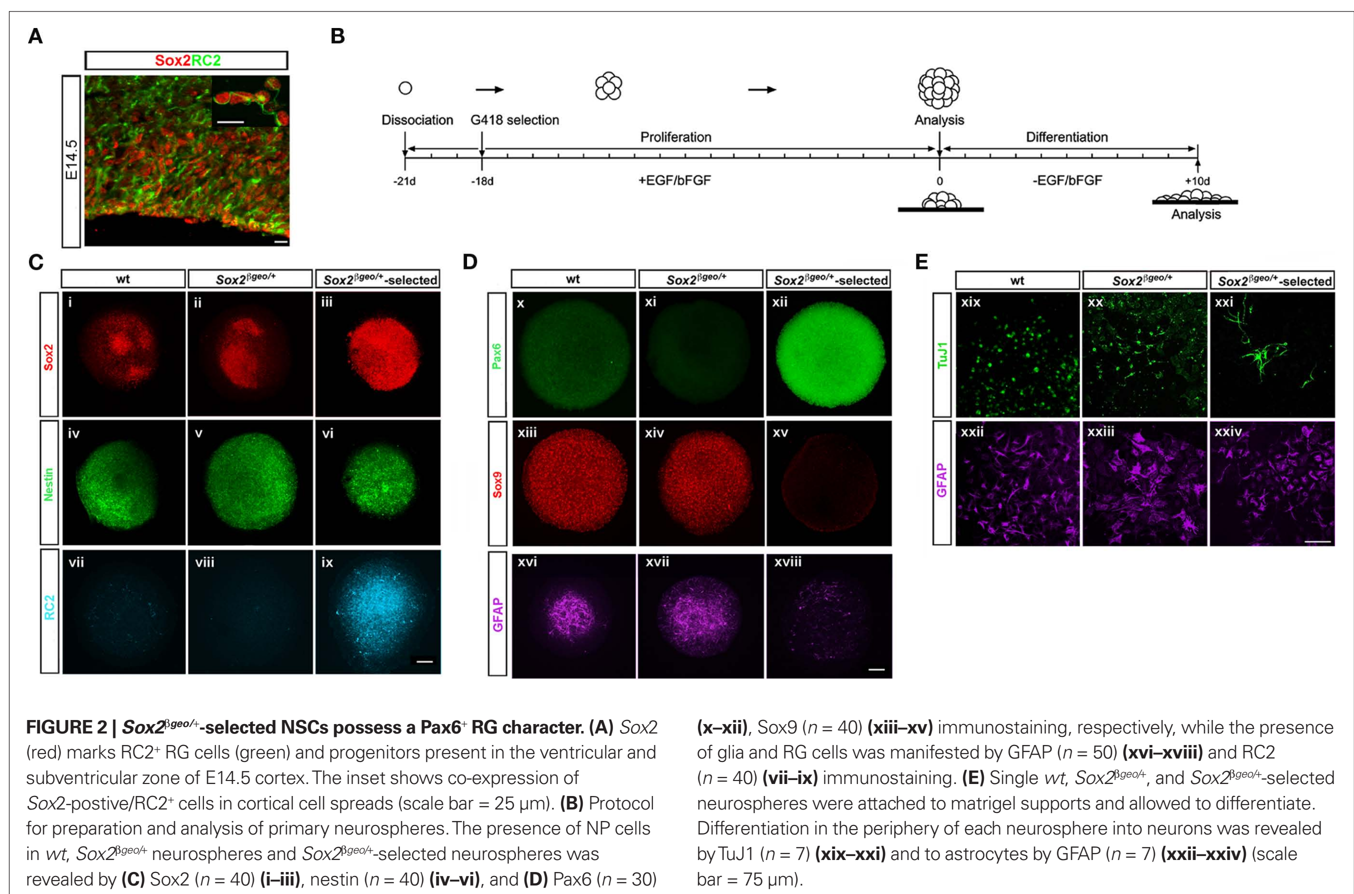

FIGURE 2 | *Sox2*$^{\beta geo/+}$-selected NSCs possess a Pax6^{+} RG character. (A) *Sox2* (red) marks RC2^{+} RG cells (green) and progenitors present in the ventricular and subventricular zone of E14.5 cortex. The inset shows co-expression of *Sox2*-postive/RC2^{+} cells in cortical cell spreads (scale bar = 25 μm). **(B)** Protocol for preparation and analysis of primary neurospheres. The presence of NP cells in *wt*, *Sox2*$^{\beta geo/+}$ neurospheres and *Sox2*$^{\beta geo/+}$-selected neurospheres was revealed by **(C)** Sox2 (*n* = 40) **(i–iii)**, nestin (*n* = 40) **(iv–vi)**, and **(D)** Pax6 (*n* = 30) **(x–xii)**, Sox9 (*n* = 40) **(xiii–xv)** immunostaining, respectively, while the presence of glia and RG cells was manifested by GFAP (*n* = 50) **(xvi–xviii)** and RC2 (*n* = 40) **(vii–ix)** immunostaining. **(E)** Single *wt*, *Sox2*$^{\beta geo/+}$, and *Sox2*$^{\beta geo/+}$-selected neurospheres were attached to matrigel supports and allowed to differentiate. Differentiation in the periphery of each neurosphere into neurons was revealed by TuJ1 (*n* = 7) **(xix–xxi)** and to astrocytes by GFAP (*n* = 7) **(xxii–xxiv)** (scale bar = 75 μm).

number of astrocytes could be due to the presence of Sox9⁺ cells. Sox9⁺ levels were severely reduced in *Sox2*$^{βgeo/+}$-selected neurosphere cells (**Figure 2Dxv**). Given the clonal origin of the neurospheres (Zappone et al., 2000), and the fact that these arise from the Sox2⁺ fraction (**Figures 1K,L**), it is most likely that the Sox9⁺ cells are descendents of the Sox2⁺ cells (Gotz and Barde, 2005). *Wt* and *Sox2*$^{βgeo/+}$ neurospheres contained few RC2⁺ cells (**Figures 2Cvii,viii**). In contrast, we found that the *Sox2*$^{βgeo/+}$-selected neurospheres were comprised of RC2⁺ cells (**Figure 2Cix**). Moreover, these RC2⁺ cells maintain their cortical identity by expressing high levels of Pax6 protein (**Figure 3Dxii**), when compared to *wt* and *Sox2*$^{βgeo/+}$ neurospheres (**Figures 2Dx,xi**). Collectively, these data demonstrated that cortical NSCs depend on homogeneous *Sox2* expression to maintain their Pax6⁺ RG identity *ex vivo*.

Radial glia cells differentiate into neurons and glial cells later in development (Gotz et al., 1998; Malatesta et al., 2000; Campbell and Gotz, 2002; Gotz et al., 2002; Gotz, 2003; Kriegstein and Gotz, 2003; Malatesta et al., 2003; Gotz and Barde, 2005). To assess the differentiation potential of *Sox2*$^{βgeo/+}$-selected neurospheres, we allowed matrigel-attached single neurospheres to grow in the absence of selection and growth factors for 10 days (**Figure 2E**). GFAP⁺ astrocytes (**Figures 2Exix-xxi**) and TuJ1⁺ neurons (**Figures 2Exxii–xxiv**) were present in all neurosphere cultures. However, in *wt* and *Sox2*$^{βgeo/+}$ neurospheres TuJ1⁺ neurons had very short axons (**Figures 2Exix,xx**), while in *Sox2*$^{βgeo/+}$-selected neurospheres TuJ1⁺ cells had extended axons as typical cortical neurons were present. Our data implies that cells within *Sox2*$^{βgeo/+}$ and *Sox2*$^{βgeo/+}$-selected neurospheres have indeed distinct developmental and differentiation potential.

Sox2 RESTRICTS THE GENERATION OF CRANIAL NCCs FROM CORTICAL NSCs *IN VIVO*

Embryonic stem cell-derived Pax6⁺ RG cells can not readily revert to a more primitive type of progenitor and as a result appear to be developmentally restricted (Gotz and Barde, 2005). To assess whether primary *Sox2*$^{βgeo/+}$-selected Pax6⁺ RG cells behave in a similar manner, we performed heterotopic and heterochronic transplantations into the hindbrains of *in vitro* cultured mouse and *in ovo* cultured chick embryos (**Figures 3A,B**). Transplantations were performed at E8.5 (five somite stage) in mouse embryos and at E1.5 (eight somites) in chick embryos to ensure that the grafted neurospheres were incorporated into the neuroectoderm prior to the earliest waves of NCC formation and migration (Trainor, 2000, 2005; Trainor and Krumlauf, 2000; Basch et al., 2006). *Wt*, *Sox2*$^{βgeo/+}$, and *Sox2*$^{βgeo/+}$-selected neurospheres transplanted into either r2 or r4 of the hindbrain of both chick (**Figures 3C–H**) and mouse (**Figures 3I–Q**) embryos were all incorporated into the neural plate as a cohort of cells with little mixing or intermingling with their immediate neighbors. Remarkably, both *wt* and *Sox2*$^{βgeo/+}$ neurospheres exhibited consistent abilities to generate migrating NCCs as

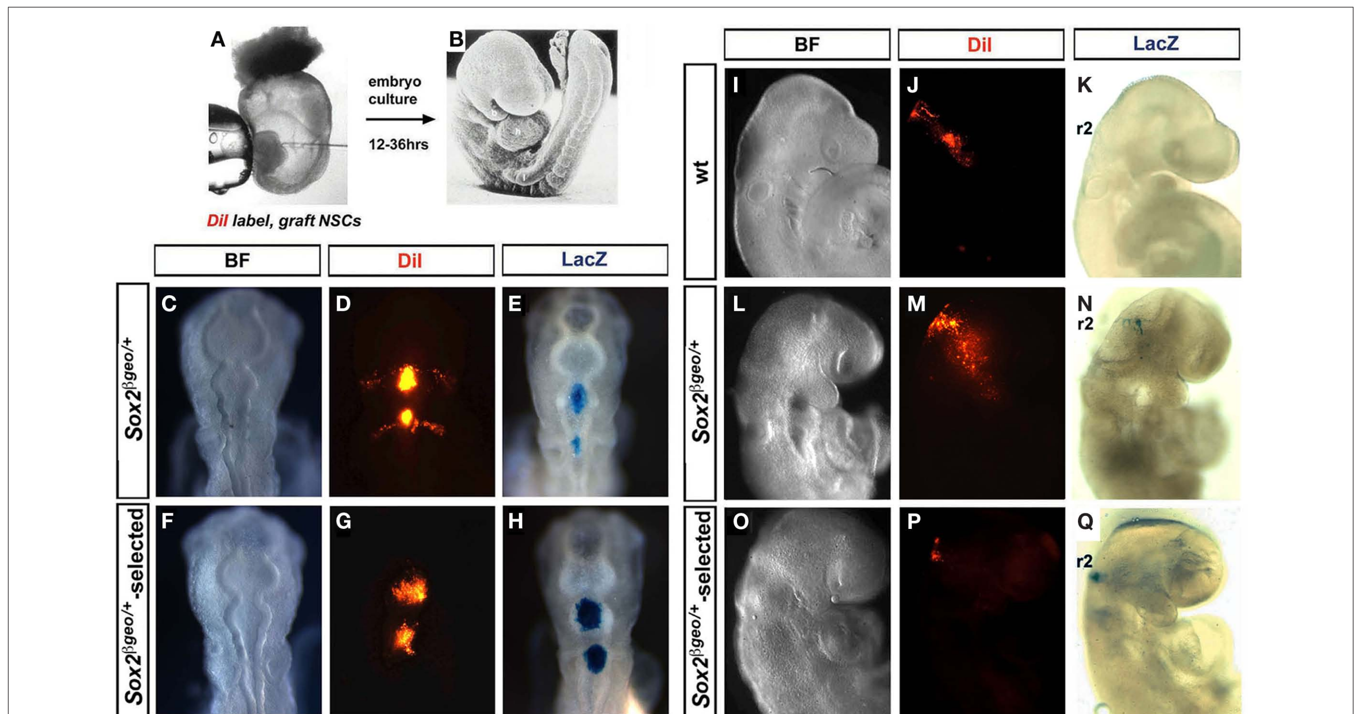

FIGURE 3 | *Sox2* restricts NSC commitment to a cranial NCC fate. Neurospheres were transplanted into the hindbrains of mouse and chick embryos **(A,B)** which were then cultured for up to 36 h. Transplantation of *Sox2*$^{βgeo/+}$ **(C–E)** and *Sox2*$^{βgeo/+}$-selected **(F–H)** neurospheres into r2 and r4 of E1.5 chick embryos. Transplantation of *wt* **(I–K)**, *Sox2*$^{βgeo/+}$ **(L–N)**, and *Sox2*$^{βgeo/+}$-selected **(O–Q)** neurospheres into r2 and r4 of E8.5 mouse embryos. Incorporation into the neural tube, the potential generation of NCCs and the identity of the transplanted cells were determined by DiI labeling **(D,G,J,M,P)** and β-galactosidase reporter staining **(E,H,K,N,Q)**. Compared to *wt* **(J–K)** and *Sox2*$^{βgeo/+}$ **(C–E, L–N)** neurospheres, which readily generated NCCs, *Sox2*$^{βgeo/+}$-selected neurospheres **(F–H, O–Q)** exhibited a decreased ability to generate migrating NCCs.

evidenced by DiI lineage tracing (**Figures 3C–E,I–N**). Neurospheres transplanted into r2 in mouse and chick embryos generated substantial numbers of NCCs that colonized the proximo-distal extent of the first branchial arch (**Figures 3D,J,M**). Similarly, neurospheres transplanted into r4 in mouse and chick embryos generated substantial numbers of NCCs that colonized the proximo-distal extent of the second branchial arch (**Figure 3D**). The *wt* and *Sox2*$^{\beta geo/+}$ neurosphere cells respond appropriately to the NCC-inducing signals and migrate ventro-laterally until the first and second pharyngeal arches (**Figures 3D,J,M**).

The migration pathways adopted by DiI labeled cells derived from the transplanted neurospheres indicated that these cells might be NCCs. To rule out the possibility that the DiI$^+$ cells were simply moving passively and being carried along by endogenous NCCs, we used *Sox10,* a well-known marker of migrating NCCs, to assess the NCC character of the neurosphere-derived cells in the cranial mesenchyme (**Figures A1A–C** in Appendix). We observed three distinct populations of cells: Sox10$^+$ endogenous migrating neural cells (green) lateral to the neural tube and underlying the surface ectoderm, Sox10$^+$DiI$^+$ cells (orange) and Sox10$^-$DiI$^+$ (red) derived from DiI labeled neurospheres transplanted into the hindbrain (**Figure A1C** in Appendix). The identification of both Sox10$^+$DiI$^+$ (neural derivatives) and Sox10$^-$DiI$^+$ (mesenchymal derivatives) reflects the endogenous patterning of NCCs during normal embryonic development. Irrespective of whether neurospheres were transplanted into r2 or r4 of the hindbrains of mouse and chick embryos, we identified DiI$^+$Tuj1$^+$ labeled cells located proximally within the branchial arches and in close proximity to the neural tube which contributed to the formation of the trigeminal (**Figures A1D–F** in Appendix) and facial ganglia (**Figures A1G–I** in Appendix) in both species. In contrast, DiI$^+$Tuj1$^-$ NCCs extensively populated the distal regions of the first and second branchial arches (**Figures A1D–I** in Appendix). This suggested that migrating cells derived from transplanted neurospheres indeed possess NCC properties. Given the appropriate embryonic niche and exposure to signals therein, E14.5 cortical wt and *Sox2*$^{\beta geo/+}$ NSCs could generate cranial NCCs and their typical neural derivatives.

However, when *Sox2*$^{\beta geo/+}$-selected neurospheres were similarly transplanted, we observed the complete absence of any NCC generation (**Figures 3F–H,O–Q**). This suggested that *Sox2* expression inhibits the generation of NSC-derived cranial NCCs. To test this notion, we overexpressed mouse *Sox2* in the neuroepithelium of E1.5 chick embryos (**Figure A2** in Appendix). Whereas control GFP plasmid was continually expressed in migrating NCCs (**Figures A2A,B** in Appendix), neuroepithelial cells overexpressing *Sox2* (**Figures A2C–G** in Appendix), failed to delaminate and migrate, remaining within the neural tube. NCC-specific *Sox10* staining (**Figures A2H,I,J** in Appendix) revealed significantly fewer migrating NCCs on the *Sox2* electroporated side and cranial ganglia reduced in size when compared to the control side (**Figures A2I,J** in Appendix). We concluded that *Sox2* inhibited the generation of NCCs by restricting NSC differentiation. To test if transition from a Sox2$^+$; Sox9$^-$ state to a *Sox2*$^-$; *Sox9*$^+$ state was required for NSC differentiation into NCCs, we overexpressed mouse *Sox9* in E1.5 chick cranial neural tubes and observed enhanced NCC generation and consequently enlarged cranial ganglia (**Figures A2K–O** in Appendix). Therefore, as NSCs differentiate into NCCs, there is a clear switch in *Sox* expression state with *Sox2* inactivated in the NCC progenitors, whereas *Sox9* and then *Sox10* activated in newly migrating NCCs (Melton et al., 2004).

DISCUSSION

Cortical neurospheres are heterogeneous free-floating aggregates consisting of mixed populations of NSC, NP, and differentiated cells that eventually lose their regional identity in culture. Here, we describe, for the first time, a unique way of generating homogenous neurospheres with spatio-temporal identity that resembles the *in vivo* profile of proliferating cells in the embryonic cortex. Our results confirm and significantly extend previous findings by showing that *Sox2* is not only important for NSC self-renewal (Zappone et al., 2000; Ferri et al., 2004; Favaro et al., 2009; Pevny and Nicolis, 2010), but also for maintaining the cortical Pax6$^+$ identity and properties of RG cells *ex vivo*. Our results also show that maintenance of *Sox2* expression prevents the NSC progression into committed NPs and differentiated cells. This is particularly evident in the *Sox2*$^{\beta geo/+}$-selected neurospheres, where the absence of Sox2$^-$Sox9$^+$ cells prevents progression to lineage-committed NPs and when these selected neurospheres are transplanted they cannot respond readily to local signals to differentiate unless they downregulate *Sox2*. This may reflect the normal progression of NSCs in the embryo (Pevny and Placzek, 2005) and highlights the balance required between a *Sox2*-mediated intrinsic program versus one dictated by the surrounding extrinsic signals considered to constitute the stem cell niche environment. The differences in properties between homogenous and heterogeneous neurospheres implies that in heterogeneous neurospheres, which consist of a mixed population of Sox2$^+$/Sox9$^-$ NSC and Sox2$^-$/Sox9$^+$ NPs that are capable of generating NCCs upon back-transplantation into avian and mouse embryonic hindbrains, that it is the progenitor cells that impact significantly on the patterning of NSCs. Hence, not only is Sox2 important for the cellular memory of cortical NSCs, but also as a cell intrinsic regulator of NSC plasticity.

Cranial NCCs are a transient migratory population that exhibit a significant degree of plasticity and differentiation fates, particularly in their numerous cell and tissues contributions in the vertebrate head (Trainor and Krumlauf, 2000). NCCs are induced to form transiently at the junction between the neuroepithelium and adjacent ectoderm by the specific interplay of distinct signals from these tissues (Selleck and Bronner-Fraser, 1995) between E8.5 and E9.5 of embryonic development in mouse and between E1.5 and E2.5 in chick. These cells migrate in distinct segregated streams from the neural tube into the adjacent pharyngeal arches (Tam, 1998). Given the transient nature of NCC induction and migration from primitive neuroepithelium, which is prior to the major period or neuroepithelial maturation, it was considered highly unlikely that cranial neural crest precursor cells would persist in or could be generated from the cortex of E14.5 embryos. Our data however suggests this is indeed the case and interestingly, neural crest precursor cells have recently been shown to persist even throughout adult life in mouse whisker hair follicles (Sieber-Blum et al., 2004).

A key issue in stem cell biology is how distinct derivatives are generated from stem cell populations by a balance between cell intrinsic and cell extrinsic cues. Similar issues have been the

focus of the NCC field However, the transition of a Sox2+; Sox9− neural stem cell into a Sox2−; Sox9+ differentiating cell appears to be generally conserved throughout embryonic neurogenesis. Not only is this mechanism important during cortical neurogenesis at E14.5 as we have shown, but a similar transition appears to occur during the transformation of neural stem cells into neural crest cells at E8.5 (Trainor and Krumlauf, 2001; Cheung and Briscoe, 2003). In support of this idea, it has been suggested that down-regulation of *Sox2* in concert with *Sox9* (Cheung and Briscoe, 2003; Cheung et al., 2005) up-regulation is required as part of a complex mechanism to generate neural crest cells from neural stem cells in avian embryos (Wakamatsu et al., 2004). Furthermore, *Sox9* was shown to antagonize *Sox2*, and more importantly *Sox9* was also demonstrated to be required for trunk NCC formation (Wakamatsu et al., 2004; Wegner and Stolt, 2005). Interestingly, this reflects the properties of $Sox2^{\beta geo/+}$ neurospheres and implies that neural stem cells within the cortex may retain and endogenous capacity to generate neural crest cells if provided with the appropriate environment.

The generation of NCCs from wild type and $Sox2^{\beta geo/+}$ neurospheres was surprising and significant since the transplanted cells were derived from the cortex of E14.5 embryos, which is nearly a week of gestation beyond the transient window of endogenous NCC formation and migration. This indicates the importance of Sox2–Sox9 signaling mechanisms in the regulation of neural stem cell maintenance and differentiation (Scott et al., 2010), but it also highlights their reiterative use throughout multiple stages of embryonic neurogenesis. Moreover, our results provocatively imply that the developmental segregation of the CNS and neural crest may be reversible even over extended periods of time. In support of this idea, migrating chick NCCs in an E2.5 embryo have been transplanted back into the ventral neural tube of younger E1.5 chick embryos. In doing so the NCCs were able to generate ventral motor neuron cell types, which are not typical NCC derivatives (Ruffins et al., 1998) lending further weight to the inherent plasticity of NCCs and the reversibility between NCCs and neuroepithelium. Lastly the differences in properties between homogenous and heterogenous neurospheres imply that in heterogeneous neurospheres, the progenitor cells impact significantly on the patterning of NSCs.

Irrespective of whether neurospheres are grown in the presence or absence of selective pressure for *Sox2* expression, both populations contain Sox2+ NSCs. The failure of homogeneous Sox2+ neurospheres to generate NCCs implies that *Sox2*+ NSCs are difficult to reprogram when cultured in the absence of their niche provided by their associated progenitors or differentiated cells. This also implies that the *Sox2*+ NSC-derived *Sox2*− NP cells could be the ones that predominantly give rise to NCCs in response to environmental signals provided by the local environment in the graft site of the embryo. Overall, our results highlight the flexibility of NSCs and the balance required between a *Sox2*-mediated intrinsic program to preserve the cellular memory of cortical NSCs and another one to regulate NSC plasticity, dictated by their niche.

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APPENDIX

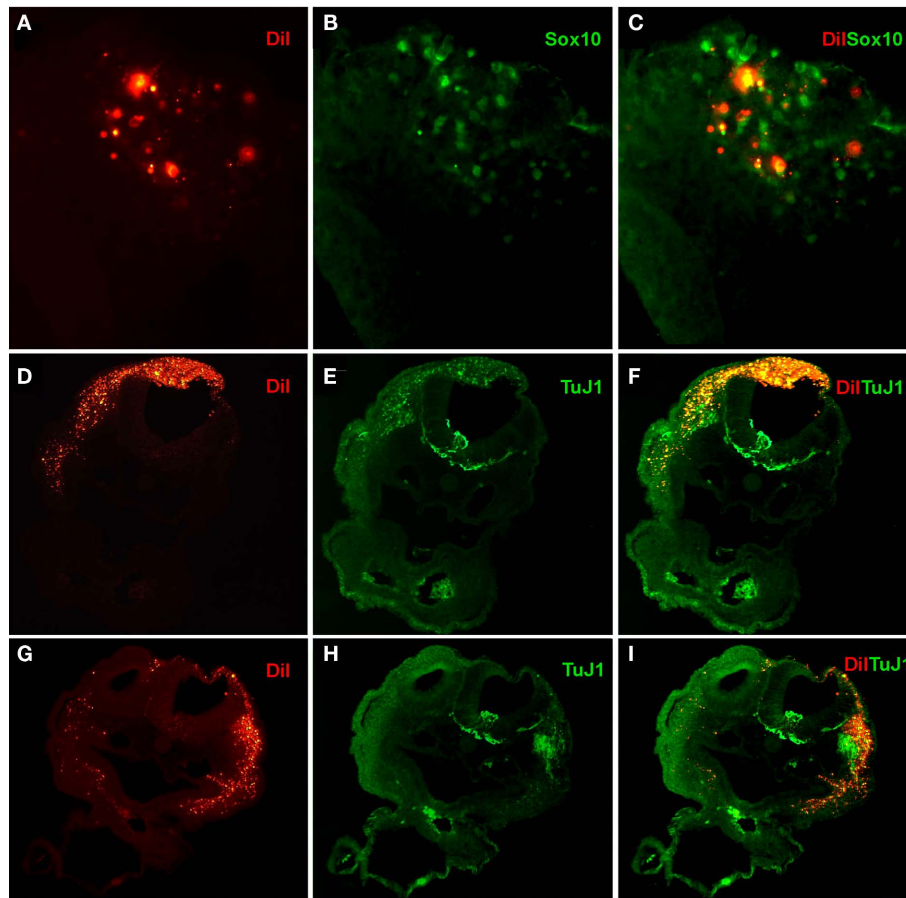

FIGURE A1 | Differentiation of NSC-derived cranial NCCs. Transplanted DiI labeled *Sox2*$^{\beta geo/+}$ neurospheres give rise to migrating NCCs **(A)** as evidenced by DiI labeling in cryosections of host embryos cultured for 24 h *in ovo*. *Sox*10 immunostaining **(B)** not only labels endogenous migrating NCCs but also many DiI labeled migrating cells derived from the transplanted neurospheres, confirming their NCC identity **(C)**. Neurospheres transplanted into r2 and r4 give rise to migrating NCCs as evidenced by DiI labeling **(D,G)** which colonize the entire proximo-distal extent of the first and second branchial arches respectively **(D,G)**. Tuj1 immunostaining **(E,H)** revealed the neurogenic potential of neurosphere-derived NCCs by demonstrating that a subpopulation of the proximally located cells contribute to the trigeminal **(D–F)** and facial **(G–I)** ganglia respectively.

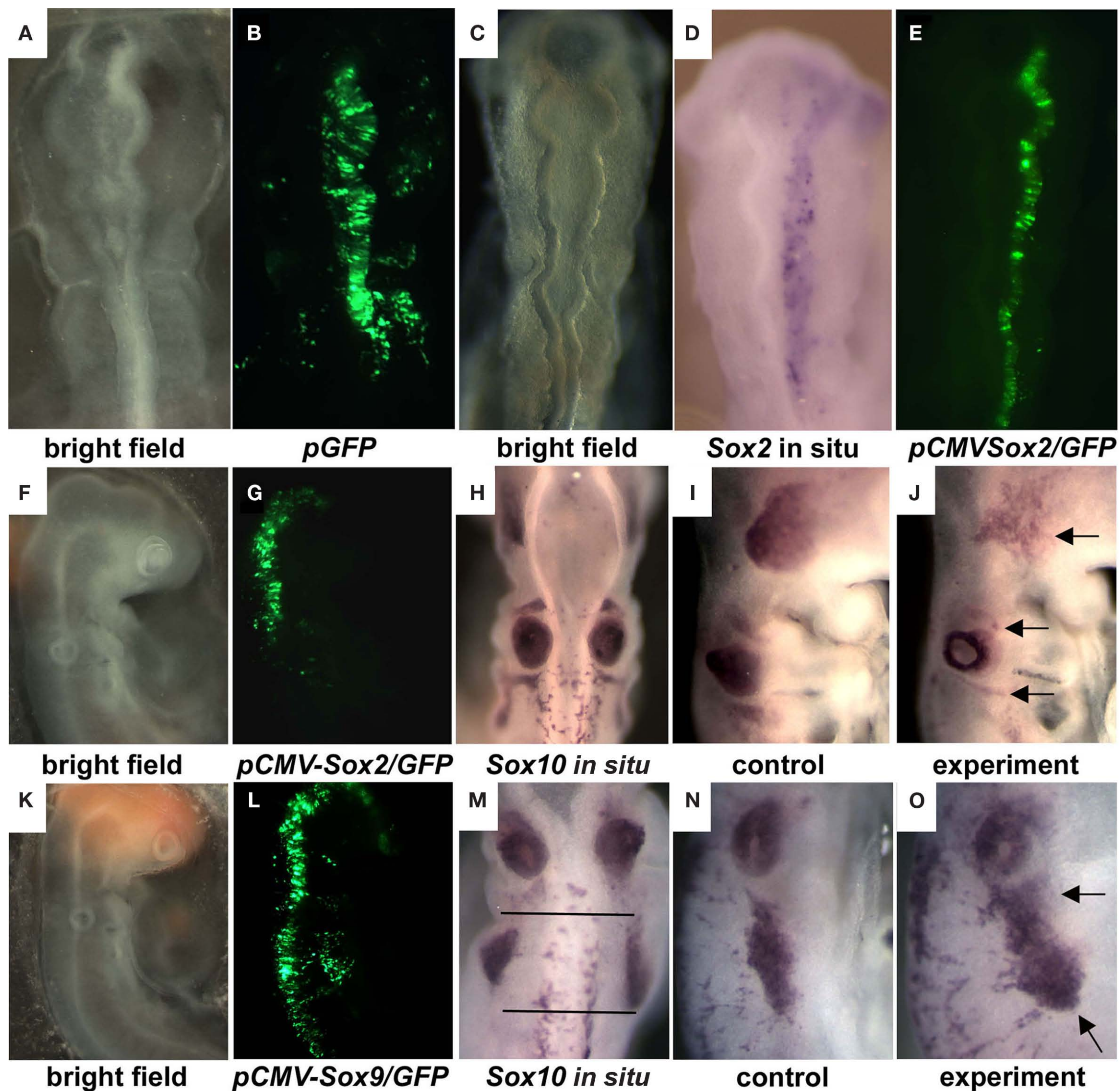

FIGURE A2 | *Sox2* restricts neuroepithelial differentiation into NCCs. Electroporation of a control pCMV–GFP plasmid into the cranial neural tube of a 1.5-day chick embryo **(A)** labels one side of the neural tube and its derived migrating NCCs **(B)**. In contrast, electroporation of pCMV–*Sox2*/pCMV–GFP into the cranial neural tube of a 1.5 day chick embryo **(C)** leads to the overexpression of *Sox2* **(D)** and labeling of one side of the neural tube but these labeled cells are unable to give rise to migrating NCCs **(E)**. Overexpressing pCMV–*Sox2*/pCMV–GFP in the neural tube of chick embryos **(F–J)** restricts the generation of NCCs on the electroporated side [arrows in **(J)**] as evidenced by dorsal **(H)** and lateral views **(I,J)** of *Sox*10 *in situ* hybridization staining of migrating NCCs after 24 h *in ovo* culture. In contrast, overexpression of *Sox*9 on one side of the cranial neural tube by electroporation of pCMV–*Sox*9/pCMV–GFP **(K–O)** enhances the production of NCCs, as evidenced by dorsal **(M)** and lateral views **(N,O)** of *Sox10 in situ* hybridization staining of migrating NCCs after 24 h *in ovo* culture.