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The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells

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In the developing cerebral cortex, neurons are born on a predictable schedule. Here we show in mice that the essential timing mechanism is programmed within individual progenitor cells, and its expression depends solely on cell-intrinsic and environmental factors generated within the clonal lineage. Multipotent progenitor cells undergo repeated asymmetric divisions, sequentially generating neurons in their normal *in vivo* order: first preplate cells, including Cajal-Retzius neurons, then deep and finally superficial cortical plate neurons. As each cortical layer arises, stem cells and neuroblasts become restricted from generating earlier-born neuron types. Growth as neurospheres or in co-culture with younger cells did not restore their plasticity. Using short-hairpin RNA (shRNA) to reduce *Foxg1* expression reset the timing of mid- but not late-gestation progenitors, allowing them to remake preplate neurons and then cortical-plate neurons. Our data demonstrate that neural stem cells change neuropotency during development and have a window of plasticity when restrictions can be reversed.

The precise timing of production of different neural cells is essential for generating the final complex central nervous system (CNS) cytoarchitecture. In the cerebral cortex, preplate cells, which include Cajal-Retzius neurons, are born first. The preplate is then separated into marginal and subplate layers by immigrant cortical layer neurons that are born in inside-out order: neurons destined for the deep layers 6 and 5 are born first, followed by those destined for the more superficial layers 4, 3 and 2 (ref. 1). How the timing of CNS neurogenesis is accomplished is not well understood. Studies in invertebrates have shown that individual progenitor cells undergo asymmetric divisions repeatedly and produce particular types of neural progeny at specific points in the lineage trees[2–4]. Understanding these lineage relationships laid the groundwork for uncovering the molecular mechanisms responsible for division pattern and cell fate determination. Similar fundamental studies have not yet been accomplished in vertebrates, however, and we do not know whether related mechanisms that rely on repeated asymmetric cell division and precise division patterns may also be involved in timing vertebrate neural cell generation.

Previous studies on timing CNS neurogenesis indicate that it involves changes in progenitor cell potency. Pioneering transplantation experiments have shown that early cortical progenitors can produce late-born neurons after transplantation into the late germinal zone, indicating that they are plastic and can respond appropriately to older environmental cues[5]. Late cortical progenitor cells, however, are unable to respond to early cues and instead seem to be restricted to late fates[6,7]. These previous studies used entire progenitor cell populations (here we use this term to describe all proliferating precursor cells), including restricted neuroblasts that generate clones of neurons and self-renewing stem cells that produce both neurons and glia. It remains an open question as to whether competency changes occur in both neuroblasts and stem cells. Because stem cells are rare compared to neuroblasts in forebrain germinal zones, their behavior may have been obscured in transplantation studies. If stem cells become restricted, they should make different neuron types depending on the stage of development; if not, they should generate a similar repertoire of neuron subtypes irrespective of developmental stage. This issue has important implications for developing stem cell therapeutics, as biases in neural stem cell potency will influence the utility of various approaches.

The factors responsible for timing neurogenesis remain to be elucidated. It has been suggested that signals from cortical plate outgrowing axons feed back on progenitor cells to extinguish their ability to make earlier-born cells[7]. In that case, one might predict that progenitor cells isolated from their normal environment would show deregulated timing. Previously, we found that isolated cortical stem cells grown in clonal culture show the normal order of neuron then glial production observed *in vivo*[8]. We did not examine the schedule of neuron subtype generation within clones, and there is no *a priori* reason why neuronal timing would also be preserved.

Few *in vivo* studies have been conducted early enough to examine the lineage relationships of the two fundamental cortical neuron populations, preplate and cortical plate cells. One study shows that subplate and cortical plate cells can come from a common progenitor[9], but a common ancestry for Cajal-Retzius and cortical plate cells has not yet been reported. These cells could be pre-fated separately in the early

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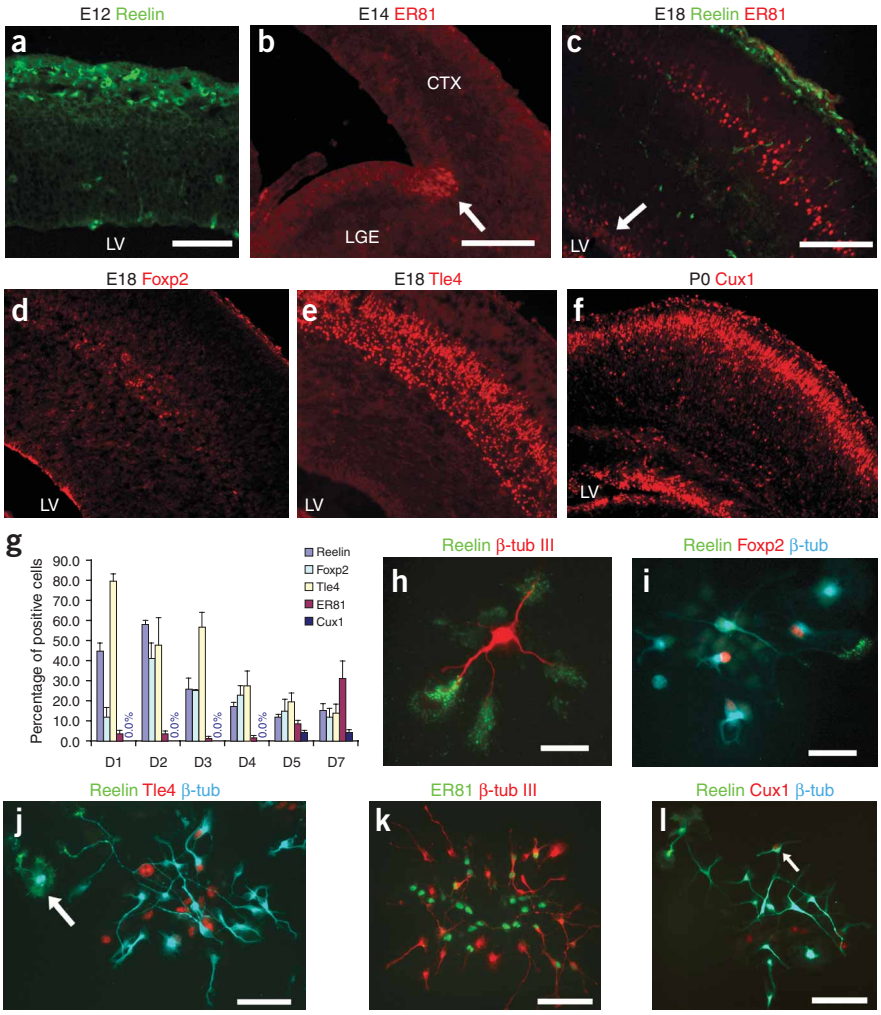

Figure 1 Timing of appearance of diverse cortical neurons. (**a**–**f**) Coronal sections of mouse cerebral cortex stained for Reelin (green) and cortical layer markers (red). (**a**) E12, Reelin$^+$ Cajal-Retzius neurons are a single, sub-pial layer of horizontally aligned cells (antibody also stains meninges and blood vessels). (**b**) At E14, ER81$^+$ cells appear in a cluster in the ventral pallium (arrow); perhaps these are early ER81 progenitors. (**c**) At E18, Reelin and ER81 define layers 1 and 5, respectively. ER81$^+$ cells were in the ventricular zone (arrow). (**d**) E18, Foxp2 labels subplate and layer 6 neurons. (**e**) Tle4 labels cortical deep layers, and weakly in the ventricular zone. (**f**) P0, Cux1 in upper layer 2/3 neurons, migrating neurons and ventricular zone cells. LV, lateral ventricle. CTX, cerebral cortex. LGE, lateral ganglionic eminence. (**g**–**k**) E10.5 cortical cells developing in culture express layer-specific markers. (**g**) Layer neurons arise from cultured E10.5 cortical progenitors on a similar time-course to *in vivo*. We counted 300–500 neurons/experiment; error bars = s.e.m. of 3–4 experiments. (**h**) Typical Cajal-Retzius neurons (Reelin, green; β-tubulinIII, red). (**i**) Clone including Reelin$^+$ and Foxp2$^+$ neurons (red nuclear staining) after 4 d.i.v. (**j**) Stem cell clone with Reelin$^+$ and Tle4$^+$ neurons and glial progeny at 5 d.i.v.; one Reelin$^+$ progeny migrated away (arrow). (**k**) ER81$^+$ cells in a 7-d.i.v. E10 clone (ER81, green; β-tubulinIII, red). (**l**) Cux1 appears in an E10.5 cortical cell clone after 7 d.i.v. (arrow, Cux1$^+$ cell). Scale bars: (**a**) 50 μm, (**b**) 100 μm, (**c**–**f**) 200 μm; (**h**) 20 μm; (**i**) 50μm; (**j**–**l**) 100 μm.

neuroepithelium. Furthermore, origins for Cajal-Retzius neurons other than neocortical neuroepithelium have been found in mice, such as the cortical hem[10], the median ganglionic eminence[11] and *Dbx1*-expressing cells in the septum and ventral pallium[12]. Knockout of the winged helix/forkhead transcription factor *Foxg1* stimulates cortical progenitor cells to adopt a Cajal-Retzius fate while suppressing cortical plate fates[13], implying a common progenitor. Hence it is important to determine whether progenitors capable of generating both Cajal-Retzius and cortical plate neurons exist.

Here we have examined the diverse neuronal fates produced from early cortical progenitor cells. Although it is possible to observe a few successive cortical progenitor divisions in slice culture[14–17], we used clonal cultures to follow them more extensively. This way, repeated divisions of identified progenitors can be observed over many days[8,18]. By following their development into clones with time-lapse microscopy, we can reconstruct the complete lineage of each progenitor cell, observing all the cell divisions, differentiation events and cell deaths that occur. This technique allows us to examine lineage relationships of neurons from the earliest stages of cortical development and to determine the information encoded within the starting cells.

Here, we identified common progenitors for preplate Cajal-Retzius cells and cortical plate neurons. We also uncovered a precise timing mechanism for cortical neurogenesis programmed within individual progenitor cells that involves competency changes in both stem cells and neuroblasts. Our data show that cortical progenitor cells undergo repeated rounds of asymmetric cell divisions and provide evidence for characteristic neuronal lineage trees in the developing mammalian forebrain. Finally, by reducing (rather than knocking out) Foxg1 expression, we found evidence for a window of plasticity in which progenitor cells can acquire the characteristic lineages of earlier cells. This study, following previous studies in invertebrates[2–4], brings insight into the fundamental characteristics of vertebrate neuron progenitor cells, the lineage relationships of diverse neuron types and the mechanisms that regulate their production at precise times during development.

RESULTS

Cortical progenitors preserve neurogenic timing *in vitro*

Layer-specific markers enable us to examine the production of different cortical neuron types. Cajal-Retzius cells can be identified by the production of Reelin, a secreted glycoprotein that guides placement of later-born cortical cells[19]. We used antibodies specific to Foxp2, Tle4, ER81 and Cux1 to distinguish different cortical plate neurons. *Foxp2*, a transcription factor of the winged-helix/forkhead family, is expressed in deep-layer neurons, becoming restricted to a subpopulation of layer 6 neurons[20]. *Tle4*, also known as *Grg4*, which belongs to the *groucho*-related gene family, is expressed in deep layers[21,22]. A subpopulation of layer-5 pyramidal neurons is identified by the ets-family transcription factor *ER81* (also known as *Etv1*)[23]. *Cux1* (also known as *Cutl1*) labels upper-layer cortical neurons in embryonic and postnatal stages[24].

Having established that these markers label layer-specific neurons in Swiss-Webster mice *in vivo* in patterns similar to those previously

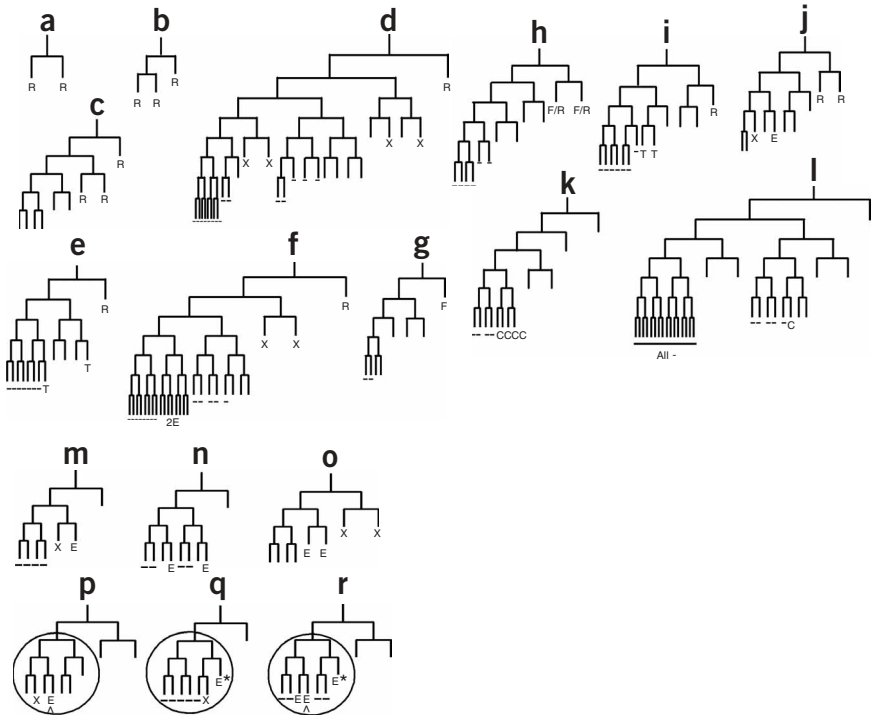

Figure 2 Time-lapse lineage analysis of embryonic mouse cortical progenitor cells. Lineage trees of single E10–E11 cortical progenitor cells reconstructed from long-term time-lapse recordings of clonal growth over 4–5 d.i.v. (**k** and **l** were derived from E11.5–E12 cortical cells after 5 d.i.v.). Lineages were stained for β-tubulinIII to identify neurons and layer-specific markers. (**a**–**d**) Lineage trees of cortical neuroblasts (**a**–**c**) and stem cells (**d**) that generated Reelin$^+$ Cajal-Retzius neurons; note these appeared at the earliest cell divisions. (**e**–**l**) Lineage trees of clones that produced cortical plate neurons and both Cajal-Retzius and cortical plate subtypes. –, cells that were β-tubulinIII$^-$; R, Reelin$^+$; E, ER81$^+$; F, Foxp2$^+$; T, Tle4$^+$; C, Cux1$^+$; X, cell death. All cells not labeled with a minus sign are neurons. (**m**–**r**) Examples of E10-11 lineages obtained after 4-5DIV that included ER81$^+$ neurons. The same division pattern occurs in (**p**–**r**) (circled), and compare with **Figure 2j**. ER81$^+$ neurons appear at equivalent points in the lineages. ER81$^+$ neurons in equivalent tree position indicated with * and ^.

published (**Fig. 1a**–**f**), we examined how layer-specific neurons arose in low-density cultures, starting at E10.5 when the mouse cortical neuroepithelium consists largely of progenitor cells, and few differentiated neurons are present. As early as one day *in vitro* (1 d.i.v.), E10.5 cells made Reelin$^+$ neurons (**Fig. 1g**) that developed their characteristic morphology—large soma and thick processes with punctate, vesicular staining for Reelin, consistent with it being secreted (**Fig. 1h**). Reelin$^+$ neurons were most abundant in the first 2 d.i.v., declining in frequency over time as other neuron types were produced (**Fig. 1g**); their early appearance corresponds with *in vivo* studies showing that most Cajal-Retzius neurons are born at E10.5–E11 in mice[23,25].

E10.5 cells also generated Foxp2$^+$ and Tle4$^+$ deep-layer neurons early in the culture period (**Fig. 1g**,**i**,**j**). In contrast, few ER81$^+$ neurons were detected until 5 d.i.v., though ER81$^+$β-tubulinIII$^-$ progenitor cells were seen earlier, just as we found ER81 staining in the cortical ventricular zone *in vivo* preceding the appearance of ER81$^+$ neurons in layer 5 (**Fig. 1b**,**g**,**k**); these could be progenitors for layer-5 neurons. Cux1 expressing neurons were not detected until 5 d.i.v. (**Fig. 1g**,**l**). Thus, the appearance of layer-specific neurons in culture follows the same order as *in vivo* development: layer 1 neurons first, followed by layer 6, layer 5 and finally, layers 2 and 3 . Moreover, the timing was largely preserved, with neurons of each layer appearing on approximately the equivalent day *in vitro* as their normal day of appearance *in vivo*.

Layer-specific neurons are born in appropriate order

We then tested whether individual cortical progenitor cells can produce both Cajal-Retzius neurons and cortical-layer neurons, and we investigated how this might occur over time. To do this, we followed single progenitor cells as they developed into clones over 4–5 d.i.v., using time-lapse microscopy. After recording, clones were stained for β-tubulinIII to identify neurons and markers to identify layer subtypes. Lineage trees were reconstructed from the video recordings and combined with staining information to map the birth order of neuron subtypes within clones.

We conducted over 40 different experiments and followed the growth of 432 early cortical progenitor cells with time-lapse imaging. The lineage trees of 106 cells were reconstructed; the remainder differentiated, died or produced progeny that moved out of the field so that the complete lineage could not be traced. Neuroblast lineage trees are largely asymmetric, reminiscent of invertebrate neural lineages, as described previously[26]. Stem cells first produce neurons by asymmetric divisions, then they switch to a more proliferative division mode producing glia, which leads to a highly characteristic lineage tree[8].

We first examined the appearance of Cajal-Retzius cells, the earliest neurons to arise *in vivo*. In E10–E11 cortical lineage trees, both from neuroblasts and stem cells, Reelin$^+$ neurons were always generated at the first or second division, when the cell cycle length is typically 12–14 h, and they were never found at late points in the lineages (**Fig. 2a**–**d**). Previously, we observed that a single neuron was often generated at the first asymmetric cell division of E10–E11 cortical neuroblasts[18]; here we found that these are frequently Cajal-Retzius cells (**Fig. 2b**–**f**), which typically move apart from the clone (**Fig. 1j**), perhaps reflecting an endogenous ability to migrate away from germinal cells.

We then examined the appearance of cortical plate neurons. Neurons expressing the deep layer marker Foxp2 always arose at early divisions, sometimes overlapping with Reelin (**Fig. 2g**,**h**). Tle4 was expressed in young neurons generated after Reelin$^+$ neurons (**Fig. 2e**,**i**). ER81$^+$ neurons, which arise later than Reelin$^+$ and Foxp2$^+$ neurons *in vivo*, arose at or after the third cell cycle in E10–11 cortical lineage trees, when the cell cycle length was typically 18–24 h, consistent with *in vivo* evidence for cell cycle lengthening during neurogenesis[27–29]. E10.5 clones often contained both Cajal-Retzius and deep-layer neurons, demonstrating that common progenitors exist in the early cortical epithelium. In these clones, Cajal-Retzius cells were born before deep layer cells (**Fig. 2e**,**f**,**i**,**j**). Cux1$^+$ upper-layer neurons were not detected in E10.5 lineages at 4 d.i.v. (**Fig. 1g**,**l**), but in E11.5–E12 lineages, they were frequent at 3–4 d.i.v. (**Fig. 2k**,**l**), which is equivalent to the normal *in vivo* birthdate of upper-layer cells around E15–16[30]. Both neuroblasts and stem cells showed the normal *in vivo* order of neuron production (**Fig. 2**).

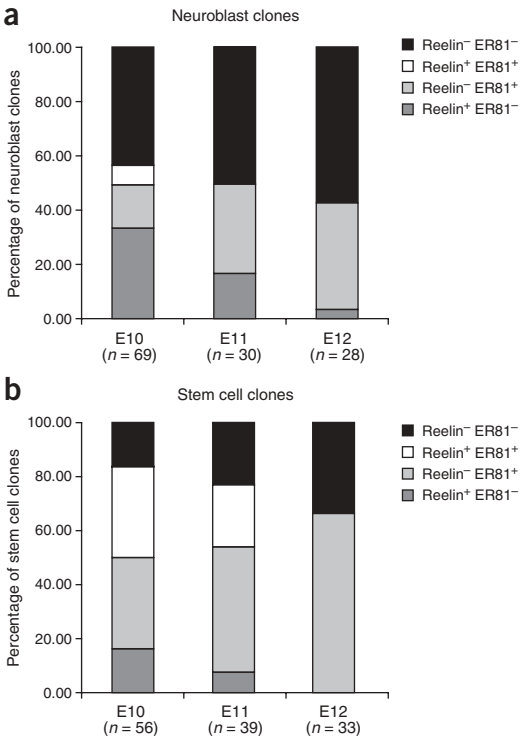

Figure 3 Cortical neuroblasts and stem cells from different embryonic stages show progressive restriction in neuropotency. Single dissociated E10, E11 and E12 cortical cells grown as clones for 4 d.i.v. then triple-stained for β-tubulinIII, Reelin and ER81. Number of clones analyzed (pooled from 2 or 3 experiments) shown in parentheses under each age. The percentage of clones containing Reelin$^+$ Cajal-Retzius neurons decreases with stage, as does the percentage of clones generating both Reelin$^+$ Cajal-Retzius neurons and ER81$^+$ neurons, while clones generating ER81$^+$ cells increase in incidence.

Among all the cortical progenitors observed with time-lapse imaging, we did not find cells that underwent a series of symmetric divisions to produce more than four neurons of one layer, agreeing with *in vivo* lineage studies showing that homogenous cortical clones are small[9,31–33]. These smaller clones may originate from divisions of cortical neuroblasts occurring in the SVZ[14–16]. Some terminal neuroblast divisions were asymmetric, resulting for example in an ER81$^+$ and an ER81$^-$ cell (**Fig. 2j**); the latter could be a different layer-5 neuron type, or a neuron destined for a different layer.

Invertebrate progenitor cells undergo highly stereotyped division patterns to produce diverse neurons[2–4]. Remarkably, we found that mouse cortical lineages contained some repeated neurogenic division patterns (**Fig. 2p–r**).

Stem cells and neuroblasts restrict neuropotency

Given that E10–11 cortical progenitor cells generate neuron subtypes in order, we asked whether this is accompanied by an intrinsic restriction in their ability to generate earlier fates. Cortical progenitor cells were isolated from E10, E11 and E12 mouse embryos, grown into clones for 4 d.i.v., then stained for Reelin, ER81 and β-tubulinIII. Clones containing solely neurons were identified as neuroblast clones, and clones containing both neurons and glia were designated as stem cell clones, as in previous studies[8,18]. As development proceeded, the percentage of clones generating Cajal-Retzius neurons declined, whereas the percentage generating ER81$^+$ neurons increased (**Fig. 3**). By E12, few cortical neuroblasts and no stem cells produced clones containing Cajal-Retzius neurons. In contrast, 67% of clones contained ER81$^+$ neurons, which was approximately half of the neurons made. Both neuroblasts and stem cells showed a similar restriction in potential (**Fig. 3a**,**b**). Importantly, this was also observed when stem cells were cultured as neurospheres rather than as adherent clones. All the neurospheres examined from E10–11 cortical progenitor cells ($n = 75$ from three experiments) generated neurons, on average 8.16% of the neurosphere cells were neurons, however none were Cajal-Retzius neurons, whereas 12% were ER81$^+$ (**Supplementary Fig. 1** online, **Table 1**). E17–18 cortical neurospheres ($n = 42$) made only 0.08% neurons, and none were Cajal-Retzius or ER81$^+$ neurons (**Table 1**). Thus, early stem cells can generate a wider range of neuron subtypes than later stem cells, and common progenitors are more prevalent at early stages.

Table 1 Restriction in neuropotency of neurosphere progenitor cells

Differentiated NS clones (mean ± s.e.m., $n = 3$ expts)	E10.5–E11.5 (% of total 75)	E14–E15 (% of total 68)	E17–E18 (% of total 42)
Clones that contained neurons	100 ± 0	46.24 ± 13.77	15.13 ± 2.09
Neurons in each clone	8.16 ± 0.19	0.87 ± 0.68	0.08 ± 0.01
Neurons that were Reelin$^+$	0	0	0
Neurons that were ER81$^+$	12.00 ± 0.67	0	0

To determine whether restriction proceeds *in vitro*, we grew E10 cortical progenitor cells as adherent clones for 5 d.i.v. and then removed them by trypsinization and replated at clonal density. The subcloned cells were no longer able to make Cajal-Retzius neurons. Furthermore, when E12 cortical cells were subcloned at 4 d.i.v., secondary clones contained few if any neurons, consistent with previous studies showing reduced neurogenic potential as cortical progenitor cells develop[8,34,35]. Hence, restriction in neurogenic potential does not depend on environmental factors specific to the developing cerebral cortex *in vivo*, but can be established from factors made within clones *in vitro*.

Potency is not changed by early-stage environments *in vitro*

To examine cortical stem cell plasticity, we used a co-culture paradigm in which cells from two cortical stages (E10.5, E12.5 or E17.5) were cultured in compartments separated by a membrane that permits medium exchange but does not allow cells to intermingle. E10.5 cortical progenitor cells co-cultured with E12.5 cortical cells did not change their production of Cajal-Retzius neurons, and although they generated more ER81$^+$ neurons than those cultured with E10.5 feeders, the difference was not significant (**Table 2**). We then examined whether older progenitors would change their output when exposed to factors from younger cells. E12.5 neuroblasts and stem cells cultured with E10.5 cortical cells did not reinstate production of Reelin$^+$ neurons. Similarly, E17.5 progenitor cells did not increase neurogenesis nor produce clones containing Reelin$^+$ or ER81$^+$ neurons when exposed to factors from earlier cells (**Table 2**). To find out whether cell-cell contact was necessary, we plated E12.5 cortical progenitor cells derived from a green fluorescent protein (GFP)-expressing transgenic mouse together with E10.5 wild-type cortical cultures. E12.5 stem cells did not make Cajal-Retzius neurons, even when contacting E10.5 cells, and the percentage of ER81$^+$ neurons was unchanged (**Table 3**).

Foxg1 knockdown resets the timing program

These data indicate that the fate of CNS progenitors is not easily changed by environmental factors, suggesting that cell-intrinsic factors

Table 2 Cortical progenitor cells from later embryonic stages do not revert to making earlier stage neurons when cocultured with E10.5 cells

Cocultures (mean ± s.e.m., $n = 3$ expts)	E10.5/ E10.5 feeder	E12.5/ E12.5 feeder	E12.5/ E10.5 feeder	E17.5/ E10.5 feeder
Neurons/stem cell clones	52.52% ± 5.63%	8.58% ± 4.80%	5.89% ± 2.03%	0
Reelin$^+$ neurons/stem cell clones	11.03% ± 2.73%	0% ± 0%	0% ± 0%	0
ER81$^+$ neurons/stem cell clones	31.11% ± 8.71%	25.21% ± 12.85%	37.35% ± 10.72%	0

may be more critical for timing. Previous studies show that knockout of *Foxg1*, a factor required for forebrain development[36], stimulates cortical progenitors to adopt a Reelin$^+$, Cajal-Retzius fate, while cortical plate fates are suppressed[13]. Removing *Foxg1* respecifies the regional identity of cortical precursors to a medial fate, a change consistent with abundant Cajal-Retzius neuron production[37]. This occurs at E13, when few Reelin$^+$ cells are normally generated, which suggests that Foxg1 suppresses this fate[13]. Given that we identified common progenitors for Cajal-Retzius and cortical plate neurons, we examined how their production of neuron subtypes is altered when Foxg1 levels are reduced.

We generated four lentiviral constructs with different shRNA targeting sequences to the *Foxg1* gene. Two (F-2, F-4) specifically knocked down *Foxg1*mRNA in cortical progenitor cells to approximately 50% of the control vector levels, whereas F-1 and F-3 and a different shRNA (URS) to an unrelated gene did not affect *Foxg1* mRNA levels (**Fig. 4a**). E12 cortical cells were plated at clonal density, transduced with experimental or control vectors, then grown into clones for 4–7 d.i.v., before staining for Reelin and cortical layer markers. Consistent with their knockdown of *Foxg1* mRNA levels, F-2 or F-4 transduction significantly enhanced Reelin$^+$ neuron production, while constructs with shRNA sequences that did not reduce *Foxg1* mRNA levels (F-1, F-3, URS lentiviruses) did not alter Reelin$^+$ neuron generation compared to control empty vector (**Supplementary Fig. 2** online). We selected the F-4 lentivirus to use in further experiments.

shRNA*Foxg1*-treated E12 cortical cells generated smaller clones that contained more neurons, and more Reelin$^+$ neurons, than control vector and mock controls (**Fig. 4b–e**). Importantly, shRNA*Foxg1*-treated E12 clones included Foxp2$^+$, ER81$^+$ and Tle4$^+$ neurons, which were born after Reelin$^+$ neurons were generated (**Fig. 4f,g**). Thus Foxg1 reduction, unlike *Foxg1* knockout[13], does not repress production of cortical plate neuron fates. In fact, there was an increase in Tle4$^+$ neurons (control 15.91 ± 7.32%, shRNA*Foxg1* 49.28 ± 6.75%) (**Fig. 4g**) and in Foxp2$^+$ neurons (not shown).

Whereas neuron generation was enhanced, glial cell generation was impaired in shRNA*Foxg1*-treated clones compared to controls, which might reflect either reduced cell division so that clones cannot progress to making glial fates, or reduced competence to make glia. Consistent with this observation, there was a decline in *Olig2* expression after shRNA*Foxg1* treatment (**Supplementary Fig. 3** online).

We examined whether lineage trees were altered after Foxg1 knockdown, for example whether overproduction of Cajal-Retzius neurons occurred along with increased symmetric divisions. Time-lapse analysis revealed that shRNA*Foxg1*-treated E12 cells still underwent the repeated asymmetric divisions typical of cortical progenitor cells. However, they generated Reelin$^+$ neurons at points in the E12 lineage trees when they would not normally be observed (**Fig. 5a–e**). There were more binucleate neurons, including binucleate Reelin$^+$ neurons, in shRNA*Foxg1* treated cultures compared to control cultures (**Fig. 5**), consistent with Foxg1 reduction disrupting normal cell division. Notably, some shRNA*Foxg1*-treated E12 lineages (for example, **Fig. 5d,e**) were similar to E10 lineages, with the first division producing a single Cajal-Retzius neuron, followed by repeated asymmetric divisions generating other types of neurons (3/17 shRNA*Foxg1*-treated lineages, compared to 0/22 control E12 lineages). We conclude that knockdown of Foxg1 resets the cortical neurogenesis timing mechanism, allowing late cortical progenitors to acquire the division pattern and competence of early progenitors, producing layer-1 and then cortical plate fates. Importantly, we did not observe more Cajal-Retzius neuron production from E15 cells after Foxg1 reduction (not shown), indicating that this plasticity is limited to a narrow time window.

DISCUSSION

In this study we examined how early mouse cerebral cortical progenitor cells generate neurons over time. Our data provide direct evidence for a timing mechanism encoded within progenitor cells that involves distinctive division patterns, and we have shown that both stem cells and neuroblasts change their ability to generate neuron subtypes during development.

We found that single progenitor cells derived from pre-neurogenic stage mouse cerebral cortex can generate both Cajal-Retzius and cortical-layer neurons. This argues against the possibility that Cajal-Retzius neurons, which are developmentally transient and disappear postnatally, have a distinct origin from neurons in other layers. Both stem cells and neuroblasts can be common progenitors for preplate and cortical neurons, rather than stem cells generating a subset of cortical neuron types.

Isolated progenitor cells produce neurons in the normal *in vivo* order, even when grown at clonal density in a highly simplified environment. As neurogenesis proceeds, later-stage progenitors become restricted from generating earlier fates, and this restriction occurs in culture as it does *in vivo*. Progenitor cells first lose the ability to make Cajal-Retzius neurons, then deep layer cortical neurons, and so on, demonstrating that restriction is gradual and does not just govern the preplate to cortical plate transition. Together, these data show that the information for timing neurogenesis and the accompanying restriction in neuropotency is programmed within progenitor cells and does not require extrinsic factors from outside the lineage to be expressed. A similar lineage-intrinsic timing mechanism has been demonstrated in *Drosophila melanogaster*, where isolated neuroblasts show normal changes in the sequence of neuroblast transcription factor expression *in vitro*[38,39]. Moreover, advancement through some of the stages occurs normally in G2-arrested *D. melanogaster* neuroblasts, showing that extrinsic factors are unnecessary for these steps. In our case, we did not rule out environmental factors playing a role in timing, but we can conclude that any

Table 3 E12.5 cortical progenitor cells do not make Reelin$^+$ neurons when cultured in contact with E10.5 cells

Contact coculture (mean ± s.e.m., $n = 3$ expts)	E10.5 alone	E10.5 (w/E12.5 GFP)	E12.5GFP (with E10.5)	E12.5GFP alone
Reelin$^+$ neurons/ stem cell clones	9.05% ± 1.12%	7.78% ± 0.93%	0% ± 0%	0% ± 0%
ER81$^+$ neurons/ stem cell clones	41.02% ± 2.13%	48.84% ± 0.79%	42.64% ± 2.42%	31.38% ± 7.43%

environmental signals necessary for orderly neurogenesis are made sufficiently within developing clones.

The fact that neuron subtypes arise *in vitro* on approximately the equivalent day to their time of appearance *in vivo*, even from isolated progenitor cells growing under stringent conditions, is quite remarkable. Cell cycle length *in vitro* was similar to that reported *in vivo*, including the lengthening observed as development proceeds[27–29], and this likely underlies the congruence. Cell cycle control is clearly finely regulated within individual progenitors as they undergo successive divisions, and it will be interesting to determine whether it is involved in cell fate determination.

We found that progenitor cells generate diverse neuronal subtypes by undergoing repeated asymmetric cell divisions, which was originally predicted from retroviral labeling of neuronal clones *in vivo*[9,40]. This division mode would allow progenitors to repeatedly traverse cell cycle stages when they can be influenced by regulatory and feedback mechanisms that determine cell fates[5]. Importantly, we found that mouse cortical neuronal lineages include evidence of repeated division patterns, as previously described for invertebrates. Moreover, after *Foxg1* reduction, some E12 cortical progenitors acquired lineage trees typical of E10 progenitors. These observations suggest that vertebrate CNS development may be much more regimented and dependent on precise division patterns than previously appreciated.

The molecular mechanisms underlying timing of neurogenesis remain to be elucidated. Asymmetric cortical cell divisions involve the Numb adaptor protein[41,42], but Numb segregation alone is unlikely to account for the sequential generation of different neuron subtypes, which in *Drosophila* depends on sequential expression of transcription factors starting with hunchback, then Kruppel, pdm1 and castor[38]. In vertebrates, an equivalent sequence has not yet been found. However,

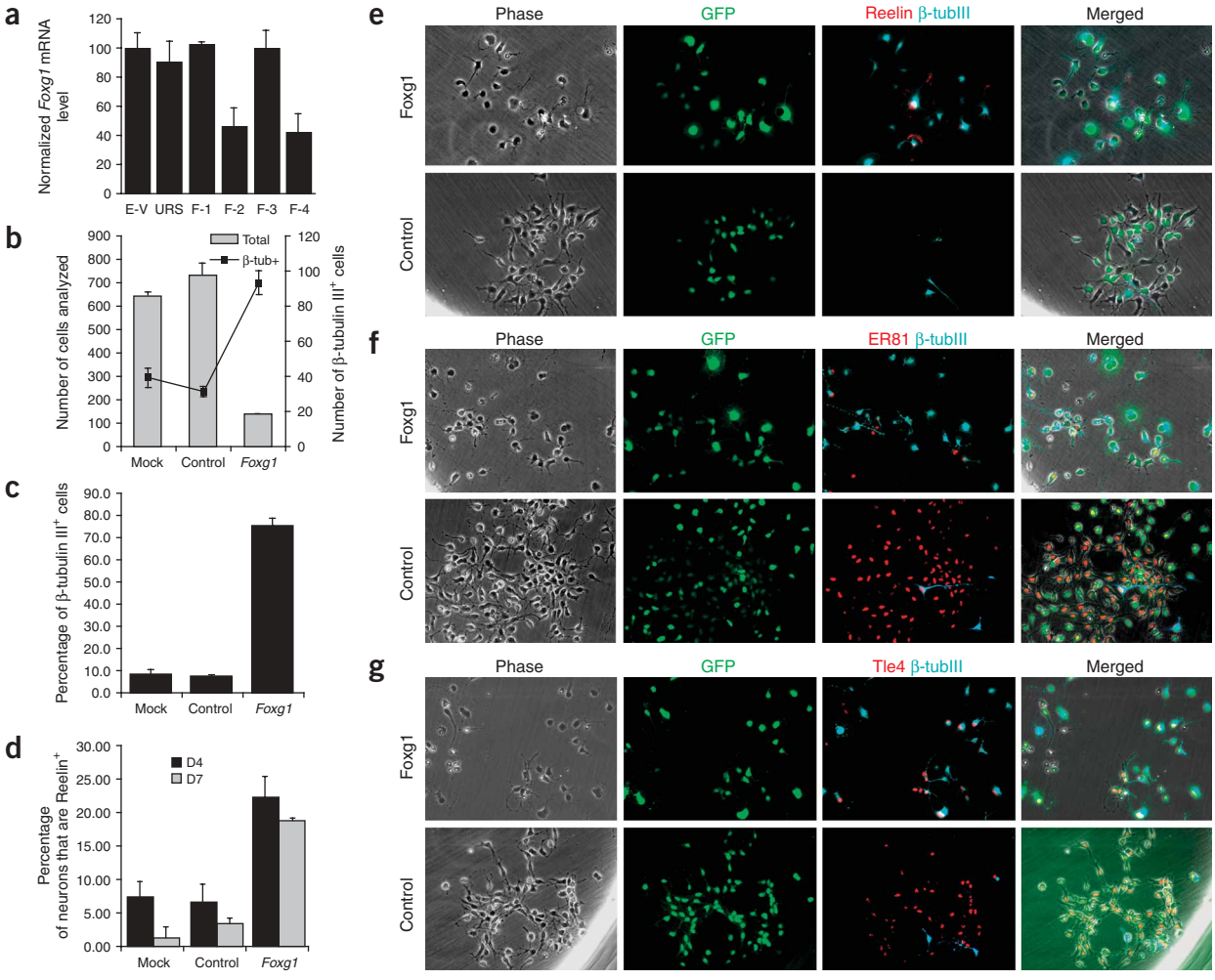

Figure 4 shRNA*Foxg1* treatment of E12 cortical cells enhances neurogenesis but impairs gliogenesis. (**a**) Quantification of *Foxg1* mRNA levels by real-time RT-PCR, normalized to *GAPDH* levels. *Foxg1* mRNA was reduced by more than 50% 48 h after transduction of shRNA*Foxg1* with F-2 and F-4 lentiviruses in cortical cells, whereas lentiviruses with shRNA*Foxg1* F-1, F-3 or shRNA against an unrelated gene (URS) did not change *Foxg1* mRNA level significantly compared to empty vector (E-V) lentivirus. (**b**) Total cell number and total β-tubulin III$^+$ cells after 7 d.i.v. (ANOVA, total cell number: $P = 1.15 \times 10^{-5}$; total neuron number: $P = 0.00035$). (**c**) The percentage of β-tubulinIII$^+$ cells was significantly increased after shRNA*Foxg1* lentiviral transduction compared to control groups (ANOVA, $P = 6.76 \times 10^{-7}$). (**d**) Production of Reelin$^+$ neurons was significantly increased after shRNA*Foxg1* lentiviral transduction compared to control groups at both 4 d.i.v. and 7 d.i.v. (ANOVA test, D4, $P = 0.010003$; D7, $P = 0.0002036$). In **a**–**d**, error bars represent s.e.m. ($n = 3$ experiments). (**e**–**g**) Clonal growth and layer-specific marker expression after shRNA*Foxg1* treatment compared to control vector treatment at 7 d.i.v. Mock, without lentiviral transduction. Cells were transduced with either control lentiviral vector (control) or with lentiviral shRNA*Foxg1* vector (Foxg1).

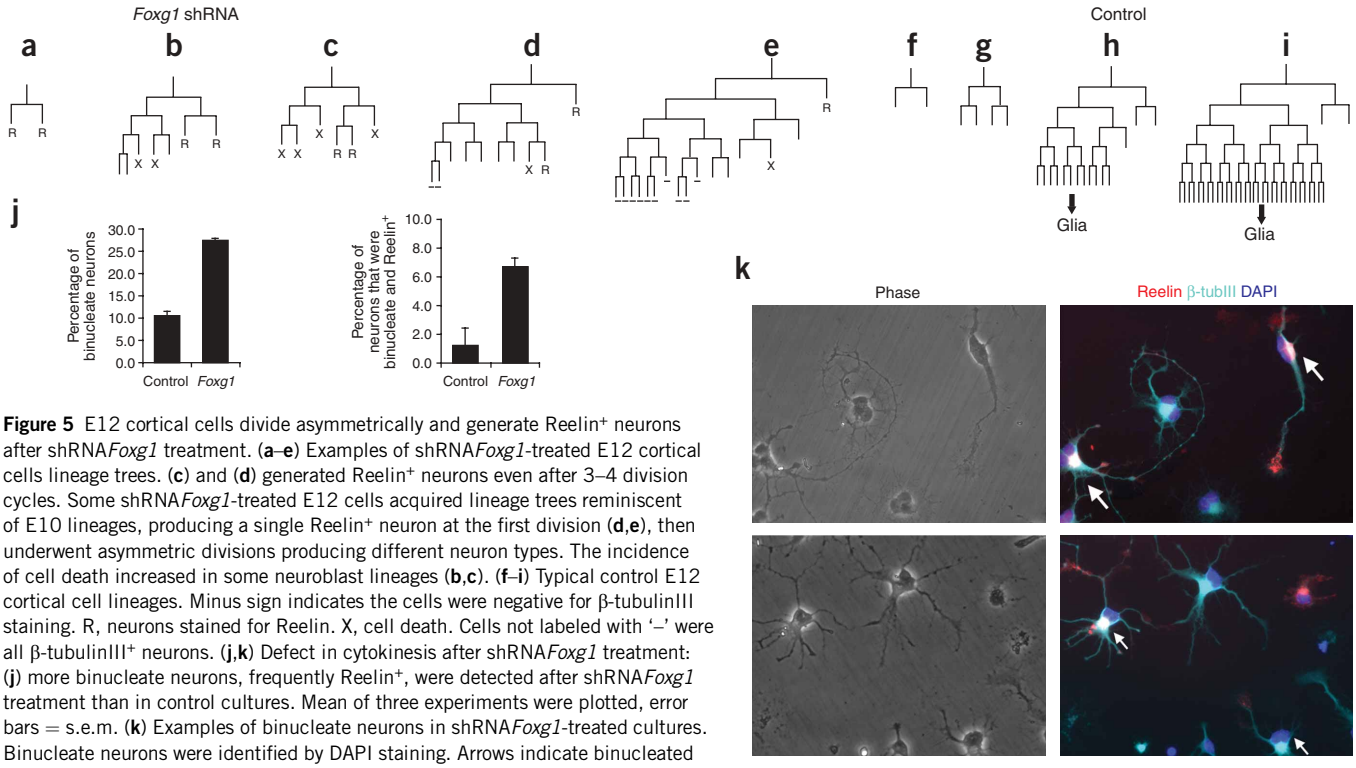

Figure 5 E12 cortical cells divide asymmetrically and generate Reelin$^+$ neurons after shRNA*Foxg1* treatment. (**a**–**e**) Examples of shRNA*Foxg1*-treated E12 cortical cells lineage trees. (**c**) and (**d**) generated Reelin$^+$ neurons even after 3–4 division cycles. Some shRNA*Foxg1*-treated E12 cells acquired lineage trees reminiscent of E10 lineages, producing a single Reelin$^+$ neuron at the first division (**d**,**e**), then underwent asymmetric divisions producing different neuron types. The incidence of cell death increased in some neuroblast lineages (**b**,**c**). (**f**–**i**) Typical control E12 cortical cell lineages. Minus sign indicates the cells were negative for β-tubulinIII staining. R, neurons stained for Reelin. X, cell death. Cells not labeled with '–' were all β-tubulinIII$^+$ neurons. (**j**,**k**) Defect in cytokinesis after shRNA*Foxg1* treatment: (**j**) more binucleate neurons, frequently Reelin$^+$, were detected after shRNA*Foxg1* treatment than in control cultures. Mean of three experiments were plotted, error bars = s.e.m. (**k**) Examples of binucleate neurons in shRNA*Foxg1*-treated cultures. Binucleate neurons were identified by DAPI staining. Arrows indicate binucleated Reelin$^+$ neurons (red, or white when overlapped with other colors).

Foxg1 may have a role in timing neurogenesis. Whereas knockout of *Foxg1* respecifies cortical progenitors to a medial fate and enhances Cajal-Retzius neuron generation[13,37], knockdown of *Foxg1* can reset the timing process so that midgestation cortical progenitors that would not normally generate Cajal-Retzius neurons can now do so, and importantly they then produce later cortical neurons, including Foxp2$^+$ cells, which are not present in hippocampus[20]. Thus, dosage of Foxg1 could be important in regulating cortical neurogenesis, both spatially and temporally.

We noted that some of the shRNA*Foxg1*-treated cells had difficulty completing cytokinesis, which was correlated with an increase in binucleate Cajal-Retzius cells, and some had a slower cell cycle as described in the *Foxg1*$^{-/-}$ mouse[43]. In *D. melanogaster*, progress from the firstborn neuroblasts expressing hunchback to the next neuroblasts in the sequence, which express Kruppel, depends on cytokinesis, while subsequent steps do not[39]. It is possible that a parallel process underlies progression from the firstborn neurons in mouse cortex, the preplate cells, to later cortical plate neurons. Thus Foxg1 knockdown, by impairing cytokinesis, might delay the generation of daughter cells that feedback on progenitors to inhibit Cajal-Retzius neuron generation, or prevent the normal asymmetric partitioning of transcriptional regulators during mitosis.

These data have important implications for therapeutic application of stem cells, as they show that CNS stem cell populations derived from different developmental stages differ in their ability to make neuron subtypes. This extends previous findings of restriction in potential of CNS progenitor populations in cortex and retina[38], showing that the process also occurs in CNS stem cell populations, as described for enteric neural crest stem cells[44]. Hence, CNS stem cells do not always self-renew with perfect fidelity in terms of developmental potential: unlike adult stem cell populations whose main function is homeostasis, embryonic stem cell populations change potential as they produce diverse cells during development. Why neurogenesis involves restriction in potency is not clear; perhaps sequential specification of diverse neuron types involves changes in gene expression or chromatin arrangement that preclude reversal to an earlier fate, outside of a small time window. This study focuses our attention on the need to elucidate how temporal restrictions in neuropotency occur in individual progenitor cells in order to understand the timing of CNS development and to allow controlled production of different types of neurons from stem cells for therapeutic purposes.

METHODS

Animals. Animal use was overseen by the Animal Resources Facility at Albany Medical College, which is licensed by the US Department of Agriculture and the New York State Department of Health, Division of Laboratories and Research and accredited by the American Association for the Accreditation of Laboratory Animal Care.

Cortical cell culture. For the adherent culture, cerebral cortices of timed pregnant Swiss Webster mouse embryos were dissociated and cultured as described previously[18,45] in serum-free DMEM medium with 10 ng/ml FGF2 (Invitrogen) in PLL-coated Terasaki or six-well plates. For clonal analysis, 10–20 cells/well were plated in Terasaki microwells and clonal development monitored by daily microscopic inspection.

For the neurosphere culture, single dissociated cortical cells were cultured in serum-free medium with 20 ng/ml FGF2 in uncoated six-well plates for 7 d.i.v.[45]. For cell fate assessment, neurospheres were collected, transferred to PLL-coated plates to adhere, and mitogen was removed to stimulate differentiation; the cells were fixed and immunostained 7 d later.

Co-culture of different stage cortical cells: E10.5, E12.5 or E17.5 cells were plated at clonal density into PLL-coated six-well plates in serum-free medium. A Costar transwell insert (pore size 0.4 μm, which cells cannot migrate through[45]) was placed in the well. A high density of cortical cells from the same or different stage was plated inside the insert.

Immunostaining. Cryostat sections and cell cultures were immunostained as described previously[45]. The primary antibodies we used were as follows: monoclonal anti-Reelin (T. Miyata, Nagoya University Graduate School of Medicine, Nagoya, Japan; Chemicon), 1:400; rabbit anti-ER81 (S. Arber, University of Basel, Basel, Switzerland; T. Jessell and S. Morton, Columbia University, New York), 1:2000; Rabbit polyclonals specific to Foxp2 (E. Morrisey, University of Pennsylvania, Philadelphia), Tle4 (S. Stifani, McGill University, Montreal), Cux1 (J. Cunningham and C. Walsh, Harvard Medical School, Boston), 1:5,000. β-tubulinIII, mouse IgG2b (Sigma), 1:600. Immunoreactivity was visualized using Alexa fluor-conjugated secondary antibodies (Molecular Probes, 1:800). For triple labeling of Reelin, β-tubulinIII and other markers, Alexa 488 goat anti-mouse IgG was used for Reelin, Alexa 647 goat anti-mouse isotype-specific IgG2b for β-tubulinIII and Alexa 546 goat anti-rabbit IgG for rabbit polyclonals. Phase and fluorescent images were acquired using a Zeiss Axiovert 200 inverted microscope and a Zeiss AxioCam MRm digital camera with AxioVision 4 software. Figure images were assembled using Adobe Photoshop.

Time-lapse video recording and cell lineage reconstruction. Timelapse video recording was carried out as described[29]. Digital images were recorded using customized software (B. Lewis and B. Roysam, Rensselaer Polytechnic Institute, Troy, New York) and movies were generated using Image Video Machine software (Dandans Digital Media). Stem cell lineages were defined by their characteristic division pattern[11], producing first neurons via asymmetric cell divisions then changing to a more proliferative division mode to produce glia. We found that lineages with this division pattern that included eight or more β-tubulinIII$^-$ cells at the end of the lineage (at 4–5 d.i.v.) emanate from stem cells and include both neuronal and glial progeny: the late-born, β-tubulinIII$^-$ progenitor cells at the end of the lineage tree consistently express glial-associated markers such as NG2, and generate oligodendrocytes and astrocytes.

***Foxg1* shRNA knockdown.** The shRNA*Foxg1* cassette was cloned into a self-inactivating lentiviral vector derived from *FUGW*[46] (N. Ivanova and I.R. Lemischka, unpublished data). The cassette, consisting of a 5′ cloning site, the shRNA 19mer, a short loop sequence of TTCAAGAGA, the reverse complement of the shRNA, a polIII transcriptional terminator and a 3′ cloning site, is expressed from the human H1 polIII promoter. Lentiviral vectors were produced by cotransfecting the shRNA cassette, HIV-1 packaging vector Delta 8.9, and VSVG envelope glycoprotein into 293 fibroblasts. Concentrated viruses were titered on 293 fibroblasts by serial dilution, and infectivity was determined at 48 h by fluorescent activated cell sorting for GFP expression.

For viral transduction, lentiviral vectors at a multiplicity of infection (MOI) of ten were added to dissociated cortical cells before plating into Terasaki wells at a density of 40 cells/well. After 48 h, GFP expression was detected by fluorescence microscopy. Lentiviral vectors containing different shRNA*Foxg1* constructs or shRNA to an unrelated gene (URS), *Spry1*, were introduced into E12 cortical cells and *Foxg1* mRNA levels were measured 48 h after transduction. E12 cells were typically transduced at 95–100% efficiency. Clone size and numbers of neurons and glia were not significantly different in mock-transduced versus control vector–transduced clones (not shown), demonstrating that the vector itself does not influence clonal development. The efficiency of *Foxg1* knock-down was determined by quantitative real-time RT-PCR. A lentiviral vector without shRNA*Foxg1* was used as control, and a mock transduction without vectors was conducted.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

Q.S., working with technician Y.W., designed and performed all the experiments, collected and analyzed the data, prepared the figures and contributed to manuscript preparation. J.T.D. and N.B.I. under I.R.L.'s direction, generated the shRNA*Foxg1* lentiviral vectors, and with C.A.F. and T.N.P. tested the lentiviral vector controls. J.T.D. carried out quantitative real-time RT-PCR for *Foxg1* and *Olig2*. S.S. and E.E.M. provided antibodies to Tle4 and Foxp2, respectively. S.T. initiated and headed the project and helped with data interpretation and manuscript preparation.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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