Foxp1 Regulates Cortical Radial Migration and Neuronal Morphogenesis in Developing Cerebral Cortex

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Abstract

FOXP1 is a member of FOXP subfamily transcription factors. Mutations in FOXP1 gene have been found in various development-related cognitive disorders. However, little is known about the etiology of these symptoms, and specifically the function of FOXP1 in neuronal development. Here, we report that suppression of Foxp1 expression in mouse cerebral cortex led to a neuronal migration defect, which was rescued by overexpression of Foxp1. Mice with Foxp1 knockdown exhibited ectopic neurons in deep layers of the cortex postnatally. The neuronal differentiation of Foxp1-downregulated cells was normal. However, morphological analysis showed that the neurons with Foxp1 deficiency had an inhibited axonal growth in vitro and a weakened transition from multipolar to bipolar in vivo. Moreover, we found that the expression of Foxp1 modulated the dendritic maturation of neurons at a late postnatal date. Our results demonstrate critical roles of Foxp1 in the radial migration and morphogenesis of cortical neurons during development. This study may shed light on the complex relationship between neuronal development and the related cognitive disorders.

Introduction

Projection neurons in the mammalian cerebral cortex are generated from the progenitor cells in the ventricular and subventricular zone (VZ/SVZ), and then migrate radially toward the cortical plate (CP). Early-born neurons occupy deep cortical layers whereas later-generated neurons take more superficial layers, resulting in an “inside-out” pattern of cortical histogenesis [1]. Neurons arrive at proper positions and acquire their mature morphology in order to establish synaptic connections and form circuits. During the migration, newly-born projection neurons undergo an early morphological transition within the intermediate zone (IZ) [2,3]. The newly-generated neurons first become multipolar in the lower IZ and then change to a bipolar shape when axons start to grow out [4]. The bipolar neurons attach to radial glial cells and
migrate outward from the IZ, reaching their destination in the CP [5,6]. This early polarization process in the IZ is critical for the migration and emergence of proper cortical lamination [7–10]. The migration and morphogenesis of cortical neurons are complex but precisely orchestrated processes, the impairments of which lead to severe brain malformations and neuropsychological disorders in humans, such as schizophrenia, epilepsy, autism, etc. [7,8,10]. The molecular and cellular mechanisms that regulate the development of cortical neurons, however, are not fully understood.

The FOXP1 protein is widely expressed in human and murine tissues and is involved in the regulation of development of multiple tissues and organs, namely heart, lung, esophagus and the immune system [11–15]. Foxp1 is expressed in several regions of the central nervous system including the cerebral cortex, striatum and the spinal cord [13,16,17]. During development, Foxp1 regulates motor neuron migration, midbrain dopamine neuron differentiation and striatum development [18–20]. All these studies provide compelling evidence that Foxp1 may participate in critical phases of brain development. Consequently, malfunctions of Foxp1 have been associated with a spectrum of development-related brain diseases. Point mutations, deletions and other disruptions of the FOXP1 gene have been found in patients with global developmental delay, intellectual disability and autism spectrum disorders [21–27].

In this study, we have explored the role of Foxp1 in mouse cortical development in vivo and found that inhibition of Foxp1 expression in the cerebral cortex resulted in impairments of neuronal migration. Foxp1 knockdown led to defects in the early polarization of neurons and the developmental maturation of neuronal processes. This study suggests that Foxp1 may be a critical determinant regulating neuronal migration and morphogenesis during cortical development.

**Materials and Methods**

**Animals and Ethics Statement**

All animal experiments were approved by the Animal Care Committee of Wenzhou Medical University. The ICR mice used in this study were maintained on a 12-h light/dark cycle in a temperature-controlled room. The morning of the day when a vaginal plug was found was designated as embryonic day (E) 0.5. The day of birth was designated as postnatal day zero (P0). To relieve the pain associated with in utero electroporation, Ibuprofen (oral; 100 mg/kg in water) was provided to the pregnant mothers who had recovered on a heating pad from surgical anesthesia. Pregnant or postnatal mice were euthanized via lethal intraperitoneal injection of pentobarbital (50 mg/kg).

**Plasmids**

Mouse Foxp1 expression vector pCMV10-mFoxp1, the short hairpin interfering RNA constructs (pLKO.1_sh_ex13, pLKO.1_sh_ex15) and a scrambled control (pLKO.1_scramble shRNA), were purchased from Addgene and they were the gifts from Benjamin Blencowe and David Sabatini [28,29]. The targeting sequences of Foxp1 shRNA-a and shRNA-b are 5’-GCTAACACTAAACGAAATCTA and 5’-GTGCGAGTAGAGAACGTTAAA, respectively. The scrambled control sequence is 5’-CCTAAGGTTAAGTCGCCCTCG. By blast search, this scramble sequence does not match with any sequences of mouse genes. Both the targeting and the scramble sequences were also cloned into pCAG-miR30 system (Addgene), which is a pri-miRNA based shRNA-expression vector contributed by Connie Cepko [30]. Conditional expression of GFP were performed using plasmid pCAG-ERT2CreERT2 and pCALNL-GFP [30] (Addgene). pCALNL-GFP contains a loxp-neostop-loxp sequence upstream of GFP that can
be induced to express by Cre-mediated DNA recombination. Expression of Cre from pCA-G-ERT2CreERT2 is controlled by Tamoxifen injection.

Mouse Foxp1 was analyzed for potential CRISPR/Cas9 targets in silico by CRISPR gDNA design tool DNA2.0 at https://www.dna20.com/eCommerce/cas9/input. The targeting sequence with the highest predicted on-target score (100) against Foxp1 (TGTATCATTCG TACCTCTTT) plus the NGG was synthesized and subcloned into pX330 vector (Addgene plasmid #42230) [31]. The vector pX330 was taken as the control that expressed Cas9 nuclease without Foxp1 targeting sequence. All constructs were verified by sequencing and purified using EndoFree plasmid maxi kit (Qiagen).

Primary cortical neuron culture

Mouse fetal brains were isolated at embryonic day 14.5 (E14.5) by dissection and placed into chilled (4°C) HBSS solution enriched with 25 mM HEPES (Invitrogen). Cortical tissues from dorsal telencephalon were dissociated with 0.05% trypsin (Invitrogen) for 10 min at 37°C and then triturated with fire-polished glass pipettes. The cell suspension was passed through a cell strainer and resuspended in DMEM (Invitrogen) supplemented with 10% FBS (Gibico). Foxp1 shRNAs were transfected into the primary cortical cells by Nucleofector (Amaxa Biosystems, Cologne, Germany), according to the manufacturer’s protocol. Then, the cells were plated onto poly-ornithine (Sigma, 0.001%) and laminin (Invitrogen, 5 mg/ml)-coated coverslips and cultured in DMEM supplemented with 10% FBS. Following cell attachment, the culture medium was replaced with Neurobasal (Invitrogen) in the presence of 1% penicillin—streptomycin and 2% B27 supplement (Gibico). After culture in vitro for two days, the cells were collected to measure the expression of Foxp1 by quantitative PCR.

N2a Cell Culture and Real-time PCR

Neuroblastoma N2a cells were cultivated in MEM supplemented with 1% glutamine, 10% FBS, and 1% antibiotics. Efficiency of Foxp1 knockdown with shRNAs was tested by transiently transfecting N2a cells with 1μg of shRNAs using Lipofectamine 2000 (Invitrogen). After 48h, mRNA was isolated from independent samples using the RNeasy method (Invitrogen). After conversion into cDNAs using a SuperScript First-Strand cDNA Synthesis kit (Invitrogen), quantitative RT-PCR was performed in a 96-well plate using an ABI 7500HT instrument. The relative expression of Foxp1 was analyzed by the ΔΔCt method (where Ct is the threshold cycle number) using Gapdh as a housekeeping gene.

Western blotting

Proteins from lysed N2a cells were denatured and loaded on sodium dodecyl sulfate polyacrylamide gels. After electrophoresis, the separated proteins were transferred to PVDF membranes, blocked in TBS-T (150 mM NaCl, 10 mM TRIS-HCl pH 7.5, and 0.1% Tween 20) containing 5% (w/v) dry milk, and stained with anti-FOXP1 (1:1000, Abcam) and anti-GAPDH (1:500, Santa Cruz) antibodies. The specific bands were analyzed by immunoblot infrared imaging system (LI-COR Biosciences) after incubation with the corresponding secondary antibodies. The density of each band was measured using NIH ImageJ and subtracted from the nearby background.

In utero electroporation (IUE)

To study the effects of Foxp1 knockdown or overexpression, we used in utero electroporation (IUE) to reliably deliver plasmid DNA into the somatosensory cortex. In utero electroporation
was performed as described previously [32,33]. Briefly, timed-pregnant female mice were anesthetized by intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg) diluted in sterile 0.9% saline. The abdomen was cleaned with 70% ethanol. A laparotomy of 3cm incision was performed in the low middle abdomen, and the uterus was carefully taken out. Approximately 3μg of indicated plasmid mixed with 1μg of GFP-pCAGGS and 0.025% of Fast-Green was delivered into the lateral ventricles of the embryos. Electric pulses (40 mV for 50 ms) were applied to the brains five times at intervals of 950 ms with an electroporator (BTX, T830). Upon completion of injection and electroporation, the uterus was then placed back into the abdominal cavity, and the abdomen wall and skin were sutured. The pregnant mouse was placed on a heating pad until it became conscious, and the embryos were allowed to develop in utero until when ready for the measurements.

Immunostaining for migration and morphology analysis

Transcardial perfusion with 4% paraformaldehyde (PFA) was performed on the mice for fixation. The brains of E17.5, P0, P2, P4, P7, P14, P30 mice were dissected and post-fixed at 4°C with 4% PFA for 2h to overnight, depending on the size of brains. The brains were dehydrated in 30% sucrose and embedded in OCT compound. Cryosections were cut at 14-μm, or 80-μm thickness with a Cryostat (HM505E, Microm, Germany). Immunostaining was performed with standard protocols: brain sections were incubated overnight with primary antibodies at 4°C and incubated with appropriate fluorescent secondary antibodies for 2h at room temperature. The following primary antibodies were used: Goat anti-GFP (1:1000, Novus Biologicals); Mouse anti-FOXP1 (1:125, Abcam); Rabbit anti-Tbr2 (1:300, Abcam); Rabbit anti-Tbr1 (1:200, Abcam); Mouse anti-Satb2 (1:100, Santa Cruz); Mouse anti-Nestin (1:200, Abcam); Rabbit anti-CDP (1:50, Santa Cruz); Rat anti-Ctip2 (1:500, Abcam); Mouse anti-phospho Histone H3(1:300, Abcam); Mouse anti-β-III-tubulin (1:500, Promega).

Immunofluorescence images were obtained using either Olympus BX41 or Zeiss LSM 710 confocal microscope. The morphology of neurons in the cortex or culture was traced and analyzed using Neurolucida software (MBF Bioscience).

Cell counting and Statistics

Cortical subregions (SVZ/VZ, IZ, and CP) were identified based on cell density using DAPI staining [34]. Brains or slices from at least three independent experiments were processed for each experimental condition. All data were presented as mean ± SEM. Statistical significance was determined using an unpaired Student’s t-test or one-way ANOVA, and differences were considered significant at a level of p<0.05.

Results

Knockdown of Foxp1 delays neuronal radial migration in developing cerebral cortex

Expression of mouse Foxp1 was first detected in the cortical plate at E14.5 [16]. To investigate the potential functions of Foxp1 in corticogenesis, we characterized its expression in embryonic cortices by immunofluorescence. Consistent with the previous study [16], FOXP1 protein was localized in the developing cortical plate but not in the IZ and VZ/SVZ at E16.5 and E18.5 (Fig 1A and 1B). Most Foxp1-expressing cells in the CP co-expressed with Satb2, a marker of callosal projection neurons in the upper layers (Fig 1A and 1B). The results suggest that Foxp1 is expressed by the early post-mitotic neurons in developing cortical plate.
To explore the function of Foxp1 in cortical development, we sought to decrease the endogenous expression of Foxp1 by RNA interference. Two short hairpin RNA constructs designed against Foxp1, and a scrambled shRNA plasmid as a control that does not target any mouse gene were tested in mouse neuroblastoma N2a cells and cultured cortical cells. qRT-PCR

Fig 1. Foxp1 is expressed at CP in mouse embryonic brain and repressed by shRNA constructs. Brain sections (14 μm) obtained from E16.5 (A) and E18.5 (B) mice were immunostained with antibodies against FOXP1 (green) and Satb2 (red). a, High-magnification images of the boxed regions in the left panels. Scale bar, 20μm in A, 50μm in B. (C) and (D), Relative mRNA expression of Foxp1 in N2a cells (C) and in cultured cortical cells (D) transfected with shRNA-Scr or two Foxp1 shRNAs (shRNA-a and shRNA-b). ***p<0.001, one-way ANOVA. (E), GFP-pCAGGS was cotransfected into N2a cells with shRNA-Scr, Foxp1 shRNA-a or shRNA-b. After 48h, cells were collected and analyzed for the expression of FOXP1 and GAPDH by western blot. The expressions of FOXP1 were quantitated as rations of the background-subtracted intensities for FOXP1 to GAPDH. (F), GFP-pCAGGS was electroporated with shRNA-Scr, Foxp1 shRNA-a or shRNA-b into cerebral cortices at E14.5. Coronal sections were obtained from the cerebral cortices at E17.5 and immunostained with anti-GFP (green) and anti-FOXP1 (red). a, b, c, High-magnification images of the boxed regions in the left panels. Arrows indicate GFP-positive neurons. Scale bar, 50μm in F, 20μm in c.
analysis showed that the Foxp1 mRNA level was significantly decreased after transient transfection with the plasmids expressing Foxp1 shRNA-a and shRNA-b as compared to that expressing the control (shRNA-Scr) (Fig 1C and 1D). Western blot analysis revealed that both shRNA-a and shRNA-b dramatically decreased the FOXP1 expression (Fig 1E). To confirm the efficiency of RNAi on the inhibition of FOXP1 in pyramidal neurons, the plasmids were delivered into the lateral cortices of E14.5 embryos by in utero electroporation. Embryonic brains receiving either a Foxp1 shRNA or shRNA-Scr along with the GFP-pCAGGS were harvested three days after the electroporation. Immunofluorescence showed that a sizable percentage of scramble RNA-transfected cells was FOXP1 positive, whereas few cells receiving shRNAs expressed FOXP1 (shRNA-Scr: 32.0%; shRNA-a: 2.6%; shRNA-b: 3.6%) (Fig 1F), suggesting the knockdown of Foxp1 in vivo.

Mutations of FOXP1 have been found in patients with global developmental delay, intellectual disability, and autism spectrum disorders. To explore the functions of Foxp1, we delivered Foxp1 shRNA into the somatosensory cortex, deficits of which have been observed in many developmental neuronal disorders [35]. The developing cortical neurons were transfected at E14.5 with a scramble or Foxp1 shRNAs, and analyzed at E17.5 in the cortical wall that was regionally divided into CP, IZ, and SVZ/VZ (Fig 2A). As shown in Fig 2B, knocking down Foxp1 resulted in accumulation of electroporated cells in IZ (shRNA-Scr: 34.7%; shRNA-a: 58.0%; shRNA-b: 57.3%) and a decrease of the neurons in CP as compared to the control experiments (shRNA-Scr: 42.3%; shRNA-a: 12.7%; shRNA-b: 4.7%). In addition, more GFP-positive cells in SVZ/VZ were found in the brains transfected with Foxp1 shRNAs than that with the control. A significant retention in SVZ/VZ was induced by the potent Foxp1 shRNA-b (shRNA-Scr: 23.0%; shRNA-a: 29.2%; shRNA-b: 37.9%), indicating a migratory delay. To confirm that the migration defect caused by Foxp1 shRNAs is due to Foxp1 deficiency, we performed a rescue experiment by over-expression of a functional Foxp1 (pCMV10-mFoxp1) together with shRNA-b. As shown in Fig 2B, the distribution of neurons transfected with pCMV10-mFoxp1 plus shRNA-b was almost identical to that of the control group (Fig 2B). Introduction of a plasmid overexpressing Foxp1 alone did not induce apparent neuronal migration anomalies since a similar population of GFP-positive cells reached the superficial layers three days after transfection (data not shown). These results indicate that endogenous Foxp1 depletion hindered the radial migration of cortical neurons.

To exclude possible off-targeting effects of vector-driven shRNA, we employed two different strategies to inhibit the Foxp1 specifically. First, the expression of targeting sequences from primiRNA undergoes more natural microRNA processing and does not produce any off-target effect [36]. Therefore, the targeting and scramble sequences were embedded into the murine miR-30 using pCAG-miR30 vector system. The miR30-based shRNA expression system was introduced into the brain by IUE at E14.5. At E17.5, a reproducible migration defect was observed in Foxp1 miR30-shRNA-b group by comparison with the control (miR30-ScrRNA) (Fig 2C). The miR30-shRNA-b transfected cortex had a decreased population of the cells in CP when compared with the scrambled control (miR30-ScrRNA: 41.6%; miR30-shRNA-b: 11.3%). Correspondingly, more neurons were stalled in the IZ when Foxp1 was inhibited (miR30-ScrRNA: 29.2%; miR30-shRNA-b: 53.5%) (Fig 2D), indicating a migratory delay. Next, to further prove the specificity, the fourth exon of mouse Foxp1 was targeted for disruption by CRISPR/Cas9-mediated genetic editing. pX330-gFoxp1 together with GFP-pCAGGS were electroporated into E14.5 brains. A previous study had showed the effectiveness of pX330 in driving gene knockdown in post-mitotic neurons following IUE [37]. Compared to the control (CP: 40.6%; IZ: 31.9%), only 15.6% of pX330-gFoxp1 transfected neurons entered the CP while 51.7% remained in the IZ (Fig 2E and 2F). Both miR30-shRNA and pX330-gFoxp1 inhibited the expression of FOXP1 in the E17.5 neurons as demonstrated by the loss of
Fig 2. Suppression of Foxp1 inhibits neuronal migration. (A), E14.5 mouse embryos electroporated with a GFP expression plasmid along with shRNA-Scr, Foxp1 shRNA-a or shRNA-b together with pCMV10-mFoxp1 were allowed to develop until E17.5. Coronal sections (14 μm) of E17.5 brains were immunostained with an antibody against GFP (green). Nuclei were stained with DAPI (blue). Short black lines indicate the borders between CP, IZ, SVZ/VZ. Scale bar, 50 μm. (B), Statistical analysis of the percentages of GFP-positive cells.
colocalization of GFP and FOXP1 immunofluorescence in the CP (Fig 2Ca–b and 2Ea–b).

Taken together, by targeting to two different regions of the gene at RNA and genomic levels, these results reveal that the migration defect was unlikely due to off-target effects but caused by the specific down-regulation of Foxp1. This conclusion was further supported by preliminary studies on the expression of upper layer marker Cux1 in the mice with a brain-specific deletion of Foxp1 [20]. In contrast to the wild-type where most Cux1+ cells appeared only in the layer II-IV, many Cux1+ cells were present in the deep layer V and VI of the knockout mice during the early postnatal development (Data not shown).

To investigate the long-term effects of knocking down Foxp1 on neuronal migration, we electroporated shRNA-b into VZ cells at E14.5 and determined the positions of GFP-positive cells at different time points during postnatal development (Fig 3A and 3B). At P2, a time point by which cortical neurons have almost completed radial migration in normal condition; most control plasmid transfected neurons migrated to the cortical layer II-IV (96.0%). By contrast, only 37.2% of shRNA-b transfected neurons were in layer II-IV, whereas a large number of GFP-positive neurons ectopically located in layer V-VI (41.1%) and the white matter (WM) (21.8%) (Fig 3C). Thus, the defect in migration of Foxp1 knockdown cells was not temporary but persisted after six days of electroporation. Moreover, the aberrant positioning of shRNA-b transfected neurons in the CP were also observed at P4, P7, and P14, suggesting that the migratory defects cannot be attributed to a developmental delay (Fig 3D–3F). Together, these data imply that the disruption of Foxp1 has a significant impact on the neuronal placement in the cerebral cortex.

**Foxp1 knockdown does not affect fate determination, proliferation and differentiation of cortical neurons**

To examine whether the shRNA-electroporated cells blocked in the IZ adopt a correct neuronal fate, we stained the brain sections at E17.5 with antibody against β-III-tubulin (a neuronal marker). Foxp1 knockdown cells expressed β-III-tubulin normally as those cells receiving the control shRNA (Fig 4A), suggesting the stalled cells in the IZ already exited from the last mitosis and became neurons. Consistently, only a small proportion of transfected cells remained to be mitotic, as recognized by immunostaining of phosphor-Histone, pH3. The ratios of pH3 and GFP double positive cells in the total GFP-positive population were 2.7% in the control and 2.9% in the shRNA-b transfected group (Fig 4B and 4C). This indicates that Foxp1 knockdown did not alter the mitotic activity of cells in the VZ and SVZ. To analyze the specification of neuronal progenitors, the brain sections were examined for the expression of Tbr2 (intermediate/basal progenitor marker) three days following IUE. The percentage of the electroporated cells labeled with Tbr2 in the SVZ/VZ did not have a significant difference between the control and Foxp1 knockdown group (shRNA-Scr: 29.1%; shRNA-b: 24.7%) (Fig 4D and 4E). Further, to exclude the possible contribution of cell death to the abnormal migration, we examined the brain sections with an apoptotic marker, the cleaved caspase-3(CC3). No significant change
Fig 3. Effects of Foxp1 knockdown on the placement of cortical neurons. (A), (B) E14.5 embryos were electroporated with the control or Foxp1 shRNA-b, together with GFP-pCAGGS, and analyzed at P2, P4, P7, and P14. Scale bar, 50 μm. (C), (D), (E), (F) Histograms showed the percentage of transfected cells in different regions of the cerebral cortex at P2, P4, P7, and P14. *p<0.05 and ***p<0.001 for comparisons between shRNA-b and the corresponding control, Student’s t-test.

doi:10.1371/journal.pone.0127671.g003
was detected in the proportions of the apoptotic cells. There was an average of 0–2 apoptotic cells per section in both control and Foxp1 knockdown brains (data not shown).

To determine whether this perturbation of neuronal localization was induced by aberrant differentiation of cortical progenitors, we examined the expression of cell type-specific markers (Cux1, a marker of layer II-IV neurons; Satb2, a marker of callosal projection neurons in the upper layers; Ctip2, a marker of layer V pyramidal neurons; Tbr1, a marker of layer VI neurons) in GFP-positive neurons at P2. As illustrated in Fig 5A and 5B, most Foxp1 knockdown
cells in the CP correctly expressed Satb2 and Cux1, similar to those in the control group. The GFP-positive neurons stalled in the deep layers weakly expressed Satb2 and Cux1 but not Ctip2 and Tbr1, suggesting that they were not the typical neurons in layer V and VI (Fig 5C and 5D). These data demonstrate that Foxp1-downregulated cells share the molecular characteristics of the upper layer cortical neurons, despite their aberrant positions.

**Foxp1 knockdown disrupts early neurite development**

To investigate the possible mechanisms underlying the migration defect, we first examined the morphology changes of neurite outgrowth in vitro. Cortical neurons were isolated one day after in utero electroporation at E14.5 with GFP-pCAGGS together with either the control shRNA or Foxp1 shRNA-b. These neurons were maintained in culture for 4 days and then fixed. The morphology of individual GFP-positive neurons was imaged by confocal microscopy and analyzed using Neurolucida. The majority of GFP-positive neurons in the control shRNA group had a typical polarity: a single, long axon and multiple shorter dendrites (Fig 6A).
Quantitative analysis revealed that the complexity and the total length of dendrites did not differ between Foxp1 knockdown and the control neurons (Fig 6C). However, Foxp1 knockdown led to a remarkable reduction in the total length of axons as compared with the control (shRNA-Scr: 400.5 μm; shRNA-b: 210.8 μm) (Fig 6B). Indeed, on transfection of Foxp1 shRNA-b constructs, the length of axons in a few of the GFP-labeled neurons was virtually equivalent to that of the dendrites. These data demonstrate an essential role of Foxp1 in regulating axonal elongation and neuronal polarity in vitro.

The early neuronal transition from multipolar to bipolar morphology in the IZ is a critical point of migration control and a vulnerable target for disruption of neocortical development [3,38,39]. To confirm the importance of Foxp1 in cortical neuronal morphogenesis in vivo, we determined the percentages of transfected neurons with bipolar or multipolar processes for both control and Foxp1 shRNA-b group (Fig 7A). We found that those cells reaching CP in both groups acquired uniform bipolar morphology at E17.5. However, the percentage of bipolar cells in the IZ was substantial lower in the Foxp1 knockdown mice (36.9%) than in the
control (63.0%), indicating that Foxp1 depletion compromises the polarization process that normally occurs for the newborn neurons in the IZ. The reduction of bipolar neurons was accompanied by an increase in the multipolar cells (Fig 7C). These results are consistent with our findings from the neuronal culture in vitro (Fig 6). A reduced capability in the axon outgrowth will likely hinder the bipolar formation in vivo. It is possible that disruption of multipolar to bipolar transition may contribute to the radial migration defects caused by knockdown of Foxp1 gene in the embryonic cortex.

Knocking down Foxp1 alters dendritic morphology of mature cortical neurons

To further confirm the effects of Foxp1 knockdown on neuronal morphology, dendrite formation was examined in mature cortical pyramidal neurons. To improve the separation of single neurons for morphological analysis, we utilized a conditional Cre/loxP-mediated expression
system to induce the relatively sparse distribution of transfected cells that express GFP. The number and density of GFP-labeled cells were controlled by the dose and exposure time of tamoxifen. pCAG-CreERT2Cre and pCALNL-GFP (1:1 mixture) were co-transfected into the brains at E15.5, along with either Foxp1 shRNA-b or shRNA-Scr, by in utero electroporation. Electroporation at E15.5 allows for transfection of only external pyramidal neurons in layer II–III without labeling the spiny cells in layer IV, thus simplifying the morphological comparison between the two groups. The expression of GFP was initiated at P22 by intraperitoneal injection of tamoxifen. Brains of P30 mice were collected and processed. As shown in Fig 8A, part of the Foxp1 shRNA-b transfected neurons had abnormal placement within the laminated cortex while others reached the upper layers normally. The neurons in layer II–III assumed the characteristic pyramidal morphology in both Foxp1-knockdown and control group (Fig 8B) and were taken for morphological analysis using Neurolucida. Quantitative analysis found that loss of Foxp1 significantly increased the number of dendritic segments and total length of the apical dendrites (Fig 8D and 8E), whereas the average segment length of the apical dendrites was decreased (Fig 8F). In contrast, the number of dendritic segments and the average segment length of basal dendrites were not altered although the total dendrite length was reduced in the Foxp1 suppression neurons (Fig 8D–8F). Thus, this set of data suggests that Foxp1 gene may be a determinant of growth and branching of apical dendrites of the cortical pyramidal neurons.

Interestingly, the Foxp1 shRNA-b transfected neurons stalled in layer V had a remarkably different morphology as those pyramidal neurons normally in the deep layers. The normal layer V neurons were labeled by transfection with pCAG-CreERT2Cre and pCALNL-GFP at E13.5, and induced at P22 by tamoxifen. Examination of dendritic pattern at P30 demonstrated that ectopic neurons with Foxp1 knockdown exhibited marked shorter apical and basal dendrites when compared with the normal layer V neurons (Fig 8G). In addition, mean segment length of the dendrites was significantly reduced (Fig 8H). These data suggest that the ectopic neurons may have acquired a morphology with certain similarities to the upper layer pyramidal neurons, which are shorter and smaller than those of layer V.

Discussion

In this study, we have identified Foxp1 as an essential transcription factor required for appropriate migration of cortical neurons. The migration defects observed at E17.5 persisted in the Foxp1 knockdown neurons after birth, leading to inappropriate placement of electroporated cells in the cerebral cortex. Foxp1 knockdown did not alter the neuronal identities. The apparent neuronal heterotopias at postnatal days due to Foxp1 deficiency were similar to the phenotypes resulting from the suppression of Doublecortin, Robo4, cKit, Dpy19L1 or PHF6 in the cerebral cortex by in utero RNA interference [40–44]. In addition, Foxp1 is involved in the regulation of early axonal elongation as observed in the neurons cultured in vitro after gene transfection in utero. The advantage of this experimental scheme is that a group of pyramidal neurons were selectively labeled and analyzed in cultures. Consistent with the in vitro observation, we have discovered that Foxp1 has an impact on multipolar-bipolar shape conversion in vivo. Since the multipolar stage is a critical point of migration control [3], the migratory defect caused by Foxp1 knockdown may result from a weakened bipolar formation of early neurons. It is well known that the mammalian cerebral cortex is the region of the brain responsible for cognitive function, sensory perception, voluntary motor control, and consciousness. The coordinated migration of neurons is essential for functional and architectural formation of the mammalian cerebral cortex. The migration errors of cortical neurons may lead to cognitive impairments and increase susceptibility to major psychiatric and neurological disorders [45–47]. A de novo heterozygous deletion of FOXP1 has been found in a patient with severe speech
Fig 8. Knockdown of Foxp1 in pyramidal neurons leads to abnormal dendritic development at P30. (A), Coronal sections (80 μm) from Foxp1 knockdown and the control mice. Scale bar: 50μm. (B), Representative images and Neurolucida tracings of GFP-positive, layer II-III neurons from the control (n = 18) and Foxp1 knockdown (n = 17) mice at E15.5. Scale bar: 50μm. (C), Representative images and Neurolucida tracing of GFP-positive neurons stalled in layer V (n = 15) from shRNA-b transfected mice at E15.5 or from the control (n = 11) transfected mice at E13.5. (D), (E), (F), Quantitative and statistical analysis of dendritic...
delay, delayed motor development, and epileptiform discharges [22]. Based on the findings from this study, it is likely that the deletion of FOXP1 may interfere with the normal localization of cortical neurons. Such kind of neuronal heterotopias may bring about the incorrect activity of cortical circuits and contribute to the pathogenesis of epileptiform discharges [48,49]. Future studies are needed to determine the electrophysiological properties and connectivity of cortical neurons with haploinsufficiency of functional Foxp1.

The long-term effect of Foxp1-knockdown on morphology of mature neurons was evaluated here by a conditional inducible system. Cre-loxp inducible expression system in combination with in utero electroporation allow us to minimize the number of cells expressing GFP at specific time points so that the morphology of transfected neurons could be better visualized and analyzed. Since axons become tangled in the corpus callosum and were truncated after the tissue sectioning, we cannot measure the length of individual axons. However, the morphology of dendritic trees in the mature neurons was successively quantitated. It was found that neurons in layer II-III with Foxp1 knockdown have an increase in the branching orders and a decrease in the segment length of the apical dendrites (Fig 8). The disturbance of dendrite architecture in the upper layers may lead to the pathology of mental retardation and cognition-affecting neurological diseases [50–52]. Haploinsufficiency of FOXP1, either from an intragenic deletion, N-terminal deletion, a premature translational stop, a reading frame shift or other protein altering mutations has been found in cohorts of patients with intellectual disability and autism spectrum disorders [23–25,27]. It is possible that the aberrant dendritic structure from the loss of Foxp1 may contribute to the etiology of these developmental disorders.

The mechanisms of action by Foxp1 remain to be determined. Interestingly, FOXP1 is co-localized with its closely related family member FOXP2 in several brain regions of the bird, mouse, and human brain [16,53]. Clinical studies illustrate that disruptions of FOXP1 and FOXP2 in human could have overlapping phenotypes including the neurodevelopment disorders [21]. FOXP1 protein interacts with FOXP2 and FOXP4 to form a homo-, or hetero-dimer, which controls transcription of multiple downstream target genes [54,55]. Although many target genes of Foxp2 have been identified through high-through analysis of promoter occupancy [56,57], the precise target mediating the neuronal function of Foxp1 in the cerebral cortex is not clear. Foxp2 regulates a gene network implicated in neurite outgrowth in the developing brain [58]. This gene network may provide a clue to delineate the Foxp1 targets that modulate the morphogenesis of cortical neurons. In addition, a recent study found that Foxp1 stimulates angiogenesis by repressing semaphorin 5B (Sema5B) [59]. Since Sema5B is an inhibitory guidance cue for neuronal axons and is expressed in the cerebral cortex [60,61], this finding presents a possibility that Sema5B may be a target gene of Foxp1 for regulating the cortical development. It will be interesting to determine whether Foxp1 represses the expression of Sema5B in the cortical neurons.

In sum, we have delineated Foxp1 as a molecular determinant of radial neuronal migration and morphogenesis. These results not only shed light on the development of the cerebral cortex; they might also represent an interesting entry point for dissecting the molecular mechanisms underlying certain neurodevelopmental disorders such as intellectual disability, autism, and language impairment.
Author Contributions

Conceived and designed the experiments: JGC. Performed the experiments: XL JX HF XMT. Analyzed the data: LLL YX HTC. Contributed reagents/materials/analysis tools: HF JQ. Wrote the paper: XL GAR JGC.

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