Impaired Interneuron Development after Foxg1 Disruption

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Abstract

Interneurons play pivotal roles in the modulation of cortical function; however, the mechanisms that control interneuron development remain unclear. This study aimed to explore a new role for Foxg1 in interneuron development. By crossing Foxg1fl/fl mice with a Dlx5/6-Cre line, we determined that conditional disruption of Foxg1 in the subpallium results in defects in interneuron development. In developing interneurons, the expression levels of several receptors, including roundabout-1, Eph receptor A4, and C-X-C motif receptor 4/7, were strongly downregulated, which led to migration defects after Foxg1 ablation. The transcription factors Dlx1/2 and Mash1, which have been reported to be involved in interneuron development, were significantly upregulated at the mRNA levels. Foxg1 mutant cells developed shorter neurites and fewer branches and displayed severe migration defects in vitro. Notably, Prox1, which is a transcription factor that functions as a key regulator in the development of excitatory neurons, was also dramatically upregulated at both the mRNA and protein levels, suggesting that Prox1 is also important for interneuron development. Our work demonstrates that Foxg1 may act as a critical upstream regulator of Dlx1/2, Mash1, and Prox1 to control interneuron development. These findings will further our understanding of the molecular mechanisms of interneuron development.

Key words: Dlx1/2, Foxg1, interneuron, Mash1, Prox1

Introduction

Excitatory and inhibitory neurons function together to establish cortical circuits, and defects in interneuron development often lead to severe neurodevelopmental disorders. However, the mechanisms underlying interneuron development remain unclear. During development, most cortical interneurons are derived from the subpallium and migrate to the cortex in 2 well-defined tangential streams: a superficial stream in the marginal zone and a lower stream in the intermediate and subventricular zones (IZ/SVZ) (Anderson et al. 2001; Jimenez et al. 2002). Several migration guidance cues, such as roundabout-1 (Robo1), Neuropilin1 (Nrp1), and Neuropilin2 (Nrp2), have been identified as crucial receptors that are expressed in immature interneurons in the ventral telencephalon. These receptors respond to chemorepellent signals, such as Slit1 and the class 3 semaphorins Sema3A/3F, which are secreted in the VZ of the ganglionic eminence (GE) and the developing striatum, respectively, to prevent migrating cortical interneurons from entering the developing striatum. The activation of Robo1, Nrp1, and Nrp2 leads to the formation of defined migratory routes (Marin et al. 2001; Andrews et al. 2002).
Ephrins, another group of repulsive signals, have also been reported to guide axon growth and cell migration in the ventral telencephalon (Rudolph et al. 2010). Furthermore, a previous study showed that (C-X-C motif) receptor 4/7 (Cxxr4/7) and their ligand stromal cell-derived factor 1 (SDF1) act as potential chemoattractants in interneuron migration (Wang et al. 2011). With regard to transcriptional control, previous studies have shown that Dlx1/2 and Mash1 are important for the development of cortical interneurons. Double knockout of Dlx1/2 leads to defects in interneuron specification, differentiation, and migration (Anderson et al. 1997). The central role of Dlx1/2 depends on its ability to restrain the neurite outgrowth of interneuron precursors (Cobos et al. 2007). Mash1, a basic helix-loop-helix transcription factor, is reported to be critical for the development of cortical interneurons. Mash1 mutants exhibit a reduction in cortical interneuron differentiation and migration as well as a disruption of the lateral and medial ganglionic eminence (LGE and MGE) (Casarosa et al. 1999; Horton et al. 1999). However, upstream regulation of Dlx1/2 and Mash1 as well as the guidance cues that influence interneuron differentiation and migration remains to be explored.

The Forkhead transcription factor Foxg1 is a candidate gene for West syndrome and Rett syndrome (Roche-Martinez et al. 2011; Striano et al. 2011), which are neurological disorders in which patients suffer from epileptic fits and seizures (Guerini and Parrini 2012), which suggests a potential role for Foxg1 in interneuron development. Previous studies have shown that Foxg1 controls the rate of cell proliferation and excitatory neuron-interneuron development. Previous studies have shown that Foxg1 controls the rate of cell proliferation and excitatory neuron-interneuron development. Previous studies have shown that Foxg1 controls the rate of cell proliferation and excitatory neuron-interneuron development. Previous studies have shown that Foxg1 controls the rate of cell proliferation and excitatory neuron-interneuron development. Previous studies have shown that Foxg1 controls the rate of cell proliferation and excitatory neuron-interneuron development.

Materials and Methods

Generation of Mice with Specific Foxg1 Deletion in the Subpallium

The Foxg1<sup>fl/fl</sup> mice were generated as previously described (Tian et al. 2012). The conditional disruption of Foxg1 was achieved by crossing Dlx5/6-Cre-IRE-EGFP (Stumm et al. 2003) with Foxg1<sup>fl/fl</sup> mice in this study. The Dlx5/6-Cre-IRE-EGFP;Foxg1<sup>fl/fl</sup> mice are referred to as mutants, and the Foxg1<sup>fl/+</sup> mice are referred to as controls. The day of vaginal plug detection was designated as embryonic day 0.5 (E0.5). The day of birth was designated as postnatal day 0. All animals were bred in the animal facility at Southeast University. The gender of the mice was not determined. All experiments were performed according to guidelines approved by Southeast University.

Immunostaining and in situ Hybridization

The brains were fixed by transcardiac perfusion with PBS followed by 4% paraformaldehyde (PFA) in PBS. The brains were then post-fixed at 4°C overnight, cryoprotected in 30% sucrose, and embedded in OCT. The brains were cryosectioned at 16 μm using a Leica CM 3050S cryostat. Immunostaining was then performed as previously described (Tian et al. 2012). The following antibodies were used in our experiment: rabbit anti-Foxg1 (Abcam, 1:1000); rabbit anti-Prox1 (Millipore, 1:2000); goat anti-Prox1 (R&D, 1:500); rat anti-BrdU (Abcam, 1:1000); goat anti-Calretinin (Chemicon, 1:1000); rabbit anti-Calretinin (Chemicon, 1:1000); mouse anti-Reelin (Chemicon, 1:1000); chicken anti-GFP (Invitrogen, 1:1000); rat anti-Somatostatin (Millipore 1:500); mouse anti-Parvalbumin (Chemicon, 1:1000); rabbit anti-Calbindin (Chemicon, 1:1000); rabbit anti-Nkx2.1 (Epitomics, Abcam, 1:250); mouse anti-COPP-TFII (R&D, 1:1000); rabbit anti-Foxp2 (Abcam, 1:1000); and rat anti-Ctip2 (Abcam, 1:1500). Alexa Fluor 488 goat anti-chicken IgG (Molecular Probes, A11035, 1:500), Alexa Fluor 555 donkey anti-mouse IgG (Molecular Probes, A11050, 1:500), Alexa Fluor 488/633 goat anti-rabbit IgG (Molecular Probes, A11008/A21071, 1:500), Alexa Fluor 555 donkey anti-rabbit IgG (Molecular Probes, A31572, 1:500), Alexa Fluor 546 goat anti-rat IgG (Molecular Probes, A11081, 1:500), and Alexa Fluor 546 rabbit anti-goat IgG (Molecular Probes, A21085, 1:500) were used as secondary antibodies. DAPI was purchased from Sigma–Aldrich (D9545).

In situ hybridization was performed as previously described (Zhao et al. 2006). Information regarding the primers used to generate probes for Gad1, Lhx6, SST, Robo1, Cxxr4, Cxxr7, EphA4, Nrp1, Nrp2, Dilx1/2, Mash1, Notch1, Shh, and Slit1 is listed in Table 1.

BrdU Administration

5-Bromo-2′-deoxyuridine (BrdU; Sigma–Aldrich) was dissolved in physiological saline at a concentration of 10 mg/mL. A single intraperitoneal injection of BrdU (50 mg/kg) was administered to pregnant mice at E13.5 for acute labeling and birthdating analysis. The embryonic brains were harvested 1 h after the injection for acute labeling or at E18.5 for birthdating analysis.

Measurement of Proliferation and Statistical Analyses

To calculate the proliferative cells in the SVZ of the MGE, PH3- and 1 h BrdU-labeled sections were photographed at 20×. The BrdU<sup>+</sup> cells were counted in a 130 × 330 μm field in the SVZ of the MGE in each section. The boundary between the VZ and SVZ was estimated by Dlx5/6-GFP<sup>+</sup> labeling. To determine the number of PH3<sup>+</sup> cells in the SVZ, the reticle was positioned at the VZ/SVZ border. Three MGE sections were sampled and averaged for each case. Three pairs of brains were obtained from 3 different litters for the cell counting. Statistical analysis of data from mutant and control was performed using Student’s t test.

Homotopic Grafting Experiments

E14.5 embryos were removed and decapitated. The GFP (Dlx5/6-Cre-EGFP;Foxg1<sup>fl/fl</sup> and Dlx5/6-Cre-EGFP;Foxg1<sup>fl/fl</sup>-labeled and wild-type embryonic brains were dissected coronally into 250-μm-thick slices. Wild-type slices, including the cortex, MGE, LGE, and area entopedunculare, were placed on porous PETC membranes (Millicell, 0.4 μm pore size). Explants from the SVZ of the mutant MGE of corresponding GFP-labeled slices were homotopically transplanted on wild-type slices. The cultured slices were then incubated in culture medium composed of 70% Neurobasal medium (Invitrogen), 25% Hank’s balanced salt solution (HBSS), 20 mm glucose, 1% glutamine, 0.2% penicillin-streptomycin, and 5% horse serum for 2 days in a standard CO2 incubator. Four pairs of brains were obtained from 3 different litters for the homotopic grafting experiments.
In vitro Neuronal Migration Assays
E14.5 mouse brains were isolated and embedded in 4% low-melt agarose (Sigma–Aldrich), and 250-μm-thick coronal sections were cut on a vibratome. Small pieces of the MGE were dissected from the slices and incubated for 1 h in 1 mL of serum-containing medium (Invitrogen α-MEM with 10% fetal calf serum, glutamine, penicillin, and streptomycin). Explants were then pipetted in a liquid Matrigel solution (BD Biosciences) and rapidly placed on 3.5-cm sterile culture dishes. Matrigel drops containing the explants were allowed to polymerize for 40 min. Serum-free medium was then added, and the explants were cultured for up to 72 h in a standard CO2 incubator. The number of explants is 10 for heterozygous and 9 for mutants. The explants were obtained from 3 different litters for statistical analysis. Statistical analysis of data from mutant and control cultures was performed using Student’s t test.

Primary Cell Cultures
E15.5 mouse brains were used for primary MGE cultures. The brains were removed in ice-cold HBSS (Invitrogen), and the proliferative zones (VZ/SVZ) within the anterior portion of the MGE were isolated. The MGE was dissected in ice-cold HBSS and trypsinized (0.125% trypsin [Invitrogen]), 37°C, 8 min. Serum-containing medium (0.5 mL) was then added, and the solution was centrifuged at 1000 × g for 6 min. The supernatant was then discarded, and the cells were resuspended in serum-containing medium. Five thousand cells were plated per well (24-well plates). The cultures were incubated for 5 days in a standard CO2 incubator.

Image Analysis and Quantification of Neurite Growth
Quantification of neurite growth was performed in GFP/GABA-expressing immature interneurons in primary cultures. We defined a systematic series of fields of view in the culture area and randomly chose 1–5 cells in each field for imaging. We sampled 30 interneurons for each genotype. Only neurons with unambiguous extension of neurites from the cell soma were included in the analysis. This was repeated for four separate experiments, resulting in a total of 120 neurons for mutant and control cultures. For each neuron, the number of neurites (filopodia longer than 5 μm were considered branch tips), the length of the longest neurite, the total length of all neurites, and average neurite length was recorded. Four pairs of brains were obtained from 3 different litters for statistical analysis. Statistical analysis of data from mutant and control cultures was performed using Student’s t test.

Quantitative Real-Time PCR
The total RNA from the control and mutant GE at E14.5 was isolated using the RNeasy Plus mini kit for RNA isolation (Qiagen, 74104) according to the manufacturer’s instructions; each sample was reverse transcribed using Multiscribe reverse transcriptase (Fermentas, EP044). The quantitative real-time PCR reactions were performed using SYBR Green fluorescent master mix (Roche, 4913914001) on an ABI StepOne plus Real-time PCR System (Applied Biosystems). The sequence of the primers is listed in Table 2. The samples were run in triplicate and contained 1×SYBR Green master mix, 10 μM of each primer, and RNase-free water for a final volume of 20 μL. Samples without cDNAs were run for each reaction as a negative control. We subjected the samples to an initial denaturation cycle of 95°C for 10 min, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 1 min. Finally, one melting curve cycle of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s was performed. Relative gene expression was compared between samples after the expression was normalized to the expression of the most reliable endogenous gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH). Three pairs of brains were obtained from 3 different litters for the quantitative real-time PCR analysis. Statistical analysis of data from mutant and control cultures was performed using Student’s t test.

Table 1 Primers used for probe synthesis for real-time PCR

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<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Length</th>
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<td>Cxcr4</td>
<td>CCGGAATTCACATCTGTGACGGCCTTACC</td>
<td>ACGGGTCGACGCTGGAGTGAAGCTGAGATTTC</td>
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<tr>
<td>Cxcr7</td>
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<td>EphA4</td>
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<td>Gad1</td>
<td>CCGGAATTCGCGCAAACTGTGCTTCCT</td>
<td>ACGGGTCGACTGCGATGATTTCTCTTGG</td>
<td>1000</td>
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<tr>
<td>Dlx1</td>
<td>ACGGGTGAAGAACTGCTACGACCCGACTT</td>
<td>CCAAGTTGGAACAGAACGACAGACGACTACGC</td>
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<tr>
<td>Dlx2</td>
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<td>ACGGGTCAAGAAGGAGGAGGAGGAGGATG</td>
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<tr>
<td>Notch1</td>
<td>TGCTGTGTGCTCTGTGAGA</td>
<td>GTGATGTCTCAGACTACCCAGG</td>
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<tr>
<td>Shh</td>
<td>CCGGAATTCATAATGATGGGCACTCAAA</td>
<td>AAAACTGCAAAAGGTGCCGTATCA</td>
<td>595</td>
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<td>Mash1</td>
<td>CCGGAATTCGTGCTCTGCCGTCTCCTAC</td>
<td>AAAACTGCAACTCCGGCTGCTCTATG</td>
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<tr>
<td>Nrp1</td>
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<td>Nrp2</td>
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<td>Robo1</td>
<td>ACGGGTACACCCCTTACAGACACGCCAAGA</td>
<td>CCGGAATTCGTGCTCTGCCGTCTCTGCCTTTC</td>
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<td>SST</td>
<td>CCGGAATTCGCGCTACGGCAGGCTGCCGC</td>
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<td>Sema 3A</td>
<td>ACGGGTACCCCAACATTTT</td>
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<td>Sema 3F</td>
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<tr>
<td>Slit1</td>
<td>CTCTGTGTGTCGACAGGCTTACAGT</td>
<td>GGCAATGGCATCTGAGAGATGACTA</td>
<td>726</td>
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Table 2 Primers for quantitative real-time PCR

<table>
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<tr>
<th>Gene</th>
<th>Primers</th>
<th>Tm</th>
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| Prox1 | F: 5'-AAGTGCCAGTGGATGACCTGAGTGA-3  
R: 5'-CGGAGGCGATGTTTTTGGATTTTGGT-3 | 60°C |
| Mash1 | F: 5'-CACACACACACGACGACACTAT-3  
R: 5'-ACAGAGATGGTGAGGAGA-3 | 60°C |
| Dlx1  | F: 5'-AGCGGTCTCCTGGCAAGATCTAAAT-3  
R: 5'-AAACAGCTCCGCATGACCCACAG-3 | 60°C |
| Dlx2  | F: 5'-AAAGAACGCTCGGAAAACACGACAC-3  
R: 5'-TCTCTGTGACCTGTGGATCAGCAGT-3 | 60°C |
Microscopy and Image Analysis

The tissue sections were viewed on a confocal microscope (FV1000; Olympus), and the images were collected and analyzed with FV10-ASW image analysis software. The images were optimized for size, color, and contrast using Illustrator (Adobe).

Statistical Analysis

Student’s t tests were performed to compare specific brain regions. More than 3 pairs of brains were obtained from 3 different litters for statistical analysis. The gender of the mice was not determined. Statistical tests were performed with GraphPad 5.0. Differences were considered significant at P < 0.05.

Results

Impaired Cortical Interneuron Differentiation and Tangential Migration after Foxg1 Disruption

To evaluate the function of Foxg1 in the development of cortical interneurons, we first examined the expression of Foxg1 during interneuron development. Foxg1 was expressed in the subpallium at early developmental stages and was co-localized with Reelin, CR, SST, and PV in interneurons at P20 (Fig. 1A–E), which indicates a potential role for Foxg1 in interneuron development. Next, we conditionally disrupted Foxg1 in the subpallium by crossing Foxg1fl/fl mice with the Dlx5/6-Cre-IRE-EGFP line, in which GFP is expressed in the majority of interneurons (Stenman et al. 2003). As expected, Foxg1 was effectively ablated in the SVZ and mantle zone but not in the VZ of the GE (Fig. 1F,G). The Dlx5/6-Cre-IRE-EGFP mice died at birth, so all of the observations were performed embryonically. Because the majority of the interneurons could be visualized as GFP-expressing cells, we first observed interneuron migration and differentiation by tracing the GFP+ cells. At E13.5, which corresponds to intense tangential migration of cortical interneurons, 2 tangential migratory streams had appeared in the Dlx5/6-Cre-IRE-EGFP; Foxg1fl/fl brains (Fig. 2A,A'). In contrast, very few GFP+ cells migrated across the dorsal–ventral boundary; however, in the mutants, GFP+ cells had accumulated in the mantle zone and SVZ of the GE (Fig. 2B,B').

In the controls, more GFP+ cells had reached the dorsal cortex and had invaded the cortical plate at E18.5 (Fig. 2C). However, in the mutants, the superficial migratory route had completely disappeared, and the IZ/SVZ migratory stream was only partially formed (Fig. 2D).

To further examine the migration defect, a BrdU birthdating assay was performed. BrdU was administered at E13.5, and the brains were harvested at E18.5. Numerous BrdU+ cells were

Figure 1. Foxg1 is expressed in the GE and in developing interneurons. (A–D') Foxg1 (red, arrows) was expressed in Reelin+ (A,A'), CR+ (B,B'), SST+ (C,C'), and PV+ (D,D') interneurons at P20. A–D' are higher power images of the boxed areas in A–D. (E) In the controls, Foxg1 was expressed in the subpallium at E14.5. (F,F') In the mutants, the expression of Foxg1 was abolished in the GFP+ area at E14.5. Scale bars: 50 μm in A–D'; and 200 μm in E,F, and F'.

4 Cerebral Cortex
Disruption of Foxg1 impairs the tangential migration of cortical interneurons. (A–B') At E13.5, 2 tangential migratory streams were evident in the controls (A, A', arrows); however, these streams were not present in the mutants (B, B', arrows). A' and B' are higher power images of the boxed areas in A and B. (C, D) At E18.5, more GFP+ cells reached the dorsal cortex and invaded the cortical plate in the controls (C). In contrast, the superficial migratory route was completely absent (arrows), and the IZ/SVZ migratory stream (arrowheads) was only partially formed in the mutants (D). (E, F) BrdU was administered at E13.5, and the brains were harvested at E18.5. In the controls, numerous BrdU+/GFP+ cells were observed in the mantle zone of the GE (asterisk) (E); however, in the mutants, most BrdU+/GFP+ cells accumulated in the area close to the SVZ (dashed line) rather than migrating to the mantle zone of the GE (asterisk) (F). A large number of Gad1+ (G, G') and Calbindin+ (H, H') cells were observed in the control cortex at E18.5. However, Gad1+ (H, H', arrows) and Calbindin+ (H', J') cells accumulated at the subpallium rather than migrating to the cortex in the mutants. (K–L', O) The numbers of BrdU+ cells in the SVZ of the MGE were decreased in the mutants (L, L', O) compared with the controls (K, K', P = 0.0084). (M, N, P) The numbers of PH3-labeled M-phase cells were reduced in the SVZ of the MGE in the mutants (P = 0.0061). Dashed line demarcates the VZ/SVZ border. A'–L' are higher power images of the boxed areas in A–L. Scale bars: 50 μm in A, B, K, L, M, and N; and 200 μm in A,B, and C–F.
observed in the mantle zone of the GE (Fig. 2E); however, in the mutants, most of the BrdU+ cells accumulated in the area close to the SVZ rather than migrating to the mantle zone (Fig. 2F). We also performed in situ hybridization at E18.5 to detect Gad1, which is expressed in all GABAergic interneurons (Tamamaki et al. 2003). Only a few Gad1+ cells were detected in the IZ/SVZ migratory stream, consistent with the GFP labeling (Fig. 2G–H). When the brains were immunostained with an antibody against Calbindin (CB), which is expressed in immature interneurons, a large number of CB+ cells were observed in the control cortex and hippocampus at E18.5; however, only sporadic CB+ cells were detected in the mutant dorsal cortex, and most of the cells had accumulated at the subpallium (Fig. 2I–J), further demonstrating the severe defects in interneuron migration.

**Cortical Interneurons Derived from Both the MGE and the CGE are Affected**

It has been reported that most cortical interneurons are derived from the MGE and caudal ganglionic eminence (CGE). We next investigated whether the interneurons that originate from these 2 sources were similarly affected. Previous studies have shown that most MGE-derived interneurons are Lhx6+ and SST+ (Xu et al. 2004; Liodis et al. 2007) and that COUP-TFI and Calretinin (CR) label subgroups of the CGE-derived interneurons (Xu et al. 2004; Cai et al. 2013). At E18.5, numerous Lhx6+ and SST+ interneurons were detected in the dorsal telencephalon, but very few Lhx6+ and SST+ interneurons were observed in the corresponding region in the mutants (Fig. 3A–D). In the mutant subpallium, Lhx6+ staining was predominately localized to the MGE, and the migratory stream stopped at the dorsal–ventral interface.

Figure 3. The tangential migration of both MGE- and CGE-derived cortical interneurons is disrupted. (A–F) At E18.5, numerous Lhx6+ (A, A’), SST+ (C, C’), and COUP-TFI+ (E, E’) interneurons were detected in the cortex in the controls. Very few Lhx6+ (B, B’), SST+ (D, D’), and COUP-TFI+ (F, F’) interneurons were observed in the cortex in the mutants; however, they accumulated as clusters in the subpallium (B, D, F). (E, G–H) In the mutants, most of the COUP-TFI+ (F, arrowheads) and CR+ (G–H) cells accumulated at lower positions within the subpallium compared with the MGE-derived Lhx6+ (B) and SST+ (C) cells. A’–H’ are higher power images of the boxed areas in A–H. Scale bars: 200 μm.
boundary (Fig. 3B). Meanwhile, a large number of SST⁺ interneurons accumulated around the region corresponding to the future striatum below the dorsal–ventral boundary (Fig. 3D). However, no accumulation was observed in the control GE (Fig. 3A,C). Regarding the distribution of the CGE-derived interneurons, there were only a few COUP-TFI⁺ and CR⁺ cells in the mutant cortical plate at E18.5, as expected (Fig. 3E–F, G,H). Interestingly, most COUP-TFI⁺ and CR⁺ cells accumulated at the lower-lateral positions of the GE, which contrasted with localization of the MGE-derived Lhx6⁺ and SST⁺ cells at the upper-medial positions in the mutants (Fig. 3F,H,H'), suggesting that the place of origin may influence the region of interneuron accumulation.

Previous studies have shown reduced proliferation in Foxg1-ablated progenitor cells (Xuan et al. 1995; Hanashima et al. 2004; Tian et al. 2012). Therefore, we then detected whether ablation of Foxg1 affects proliferation in the MGE. At E13.5, the number of BrdU-labeled cells in the SVZ of the mutant MGE was significantly reduced compared with that in controls (Fig. 2K–L, O). This result was further confirmed by immunostaining for PH3, which labels M-phase cells (Fig. 2M,N,P). Our data suggest that a reduction in the size of the proliferating progenitor pool in the SVZ of the MGE may also contribute to the decreased number of interneurons in mutants.

**Migration Guidance Cues are Strongly Downregulated**

Repulsive and attractive signaling molecules reportedly act together to guide cortical interneuron migration from the subpallium to the cortex. Semaphorins, such as Sema3A and Sema3F, are normally expressed in the striatum. These molecules guide the cortical interneurons that migrate into the neocortex and prevent them from entering the striatum by causing repulsive responses in Nrp1- and Nrp2-positive interneuron processes (Marin et al. 2001). At E13.5, we determined that the expression levels of Sema3A and Sema3F were severely decreased in the mutant striatum compared with the control striatum (Fig. 4A–B'). However, the expression level of their receptors, Nrp1 and Nrp2, in migrating interneurons did not seem to be obviously
The compartmentalization of the ventral telencephalon was affected by Foxg1 ablation. (A–B') In the controls, Ctip2 and Foxp2 were highly expressed in the mantle zone and moderately expressed in the SVZ of the striatum at E18.5 (A, B), but they were dramatically reduced in the mutants (A', B'). (C–C') The expression of EphB1, which was found specifically in the striatum in the controls (C), nearly disappeared in the mutants (C', arrow). (D, D') At E18.5, Nkx2.1 was strongly expressed in the pallidum of the controls (D), but it was ectopically expressed in the upper portion of the subpallium in the mutants (D') (E–G) At E14.5, Ctip2, Foxp2, and EphB1 were highly expressed in the mantle zone of the developing striatum in the controls (E–G), however, the expression levels were decreased in the mutants (E'–G', asterisk). (H, H') At E14.5, Nkx2.1+ cells in the pallidum were clearly detected in the controls (H), however, the pallidum was disrupted in the mutants (H', asterisk). (I, I') At E12.5, Mash1 was expressed in the ventral telencephalon, and its expression was not changed in the mutants. (J, J') At E12.5, the expression of Nkx2.1, which marks the MGE, was not affected in the mutants. Scale bars: 200 μm.
changed (Fig. 4C–D). Consistent with our observation of the downregulation of Sem3A/F, in the mutants, an augmented number of CB+ interneurons invaded the region corresponding to the future striatum at E14.5 (Fig. 4E,E'). Robo1, the receptor for the chemorepulsive ligand Slit1, is typically expressed in early-born immature interneurons in the mantle zone and regulates the migration of interneurons to the cortex along the route around the striatum (Andrews et al. 2006; Andrews et al. 2008). Here, Robo1 was absent in the mutants at E13.5 (Fig. 4G,G'). However, no significant change was detected in the expression of Slit1 in the VZ of the GE (Fig. 4F,F').

Abnormal Compartmentalization of the Ventral Telencephalon after FoxG1 Deletion

To further investigate whether ablation of FoxG1 in the subpallial domain impairs ventral telencephalon morphogenesis, we first examined the expression of Ctip2 and Foxp2, which are primarily expressed in differentiated cells of the striatal primordium (Takahashi et al. 2003; Arlotta et al. 2008). In the controls, the expression of Ctip2 and Foxp2 was low in the SVZ and higher in the mantle zone at E18.5 (Fig. 5A,B). However, in the absence of FoxG1, this expression pattern was disrupted, and only low levels of Ctip2 and Foxp2 expression were detected in the ventral telencephalon (Fig. 5A',B'), suggesting that the striatum was not fully differentiated. EphB1, which is another molecule that is specifically expressed in the striatum, was also absent in the mutants, with the exception of a small patch of expression (Fig. 5C,C'). In addition to the striatum, the architecture of the pallidum was also severely disrupted, as shown by Nkx2.1 immunostaining. Strong Nkx2.1 staining was specifically detected in the pallidum; however, in the mutants at E18.5, Nkx2.1+ cells were ectopically located in the upper area of the subpallium (Fig. 5D,D').

To further investigate pattern formation in the striatum and the pallidum, we next examined the development of these structures at the early stage of E14.5. In the controls, Ctip2, Foxp2, and EphB1 were strongly expressed in the mantle zone but not in the VZ/SVZ of the developing striatum (Fig. 5E,E'). However, only a low level of expression of Ctip2, Foxp2, and EphB1 was detected in Interneuron Development Yang et al. | 9 of several repulsive ligands suggested that compartmentalization of the ventral telencephalon may also have been affected.

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Figure 6. A cell-autonomous migration defect of FoxG1-ablated cells, as demonstrated by grafting experiments. (A) Schematic illustration of the grafting experiments. (B,F') Homotopic transplantation of a GFP+ SVZ-derived MGE microexplant to a wild-type slice. Arrows illustrate the GFP-expressing microexplant. (C–E) Microexplants of the GFP-expressing SVZ of the MGE of Dlx5/6-Cre-EGFP;Foxg1+/− and Dlx5/6-Cre-EGFP;Foxg1−/− mice were homotopically transplanted on wild-type slices at E14.5. A large number of GFP+ cells (C–E) were observed in the cortex in controls, whereas very few FoxG1-ablated GFP+ cells (C–E) were detected in the IZ/SVZ of the cortex after 2 days in vitro. D,D',E,E' are higher power images of the boxed areas in C,C',D, D'. Scale bars: 200 μm in B–D'; and 50 μm in E,E'.
in the mutants (Fig. S5E–G), indicating that the differentiation of striatal cells was severely affected. The expression pattern of Nkx2.1 was also disrupted at E14.5. In the controls, Nkx2.1 was strongly expressed in the pallidum primordium; however, in the mutants, the expression of Nkx2.1 was absent from the pallidum primordium, and many Nkx2.1\(^{\text{+}}\) cells were found scattered in the upper region of the ventral telencephalon (Fig. S5H). Next, we assessed whether the lack of Foxg1 affected early regionalization of the ventral telencephalon. In situ hybridization for Mash1 and immunostaining for Nkx2.1 were performed at E12.5, and no differences were observed between controls and mutants (Fig. S5I–J). These data suggest that ablation of Foxg1 disrupted later but not early compartmentalization of the ventral telencephalon and that this effect was likely partially responsible for the substantial changes in the expression of tangential migration ligands, such as Sema3A and Sema3F, as well as for the defects in interneuronal migration.

The Effects of Foxg1 in Interneuron Migration Are Cell-autonomous

Because the migration defects of cortical interneurons may be a combined consequence of the differentiation defects and the disruption of the compartmentalization of the ventral telencephalon, to directly investigate the cell-autonomous function of Foxg1 in the development of cortical interneurons, we performed homotopic grafting experiments in organotypic brain slices at E14.5, as previously described (Zimmer et al. 2008; Zimmer et al. 2010). Explants from the SVZ of the mutant MGE of corresponding GFP-labeled slices were homotypically transplanted on wild-type slices (Fig. 6A,B). Two days later, a large number of GFP\(^{+}\) cells were detected in the cortex in controls (Fig. 6C–E). However, the number of Foxg1-ablated GFP\(^{+}\) cells invading the cortex was significantly decreased; only very few Foxg1-ablated GFP\(^{+}\) cells migrated to the IZ/SVZ of the cortex (Fig. 6C–E). Thus, these results clearly suggest that Foxg1 has a cell-autonomous function in the migration of cortical interneurons.

To further investigate the migratory deficiency, MGE explants from E14.5 Dlx5/6-Cre-EGFP;Foxg1\(^{fl/fl}\) and Dlx5/6-Cre-EGFP;Foxg1\(^{lox/lox}\) mice were isolated and cultured for 72 h in a three-dimensional collagen matrix (Fig. 7A), as previously described (Colombo et al. 2007). Heterozygous GFP\(^{+}\) cells profusely invaded the surrounding collagen and migrated considerable distances after 72 h (Fig. 7C). In mutants, GFP\(^{+}\) cells were also observed to invade the surrounding collagen from the explants but showed a severe reduction in migration (Fig. 7D). We measured the migration distance from the edge of the explants to the front of the migrating GFP\(^{+}\) cells. Quantitative analysis demonstrated that the migration distance of the Foxg1-ablated cells was ~36% lower than that of the control cells (Fig. 7B). These observations suggest that the defective tangential migration of cortical interneurons observed in the mutants can also be attributed to an intrinsic motility deficit.

These observations showed that the lack of Foxg1 led to a cell-autonomous defect in interneuron migration. To further characterize the development of interneuron neurites, we analyzed neurite outgrowth in primary cultures of interneurons isolated from the E15.5 MGE. The number of neurites, total neurite length, the average neurite length, and the length of the longest neurite of each GFP\(^{+}/\text{GABA}\(^{+}\) cell were measured after 5 days in culture (Fig. 8A–E). The number of neurites, total neurite length, and the average neurite length had significantly decreased in the mutants compared with the corresponding values in the GFP\(^{+}/\text{GABA}\(^{+}\) control cells (Fig. 8A–E), although the average length of the longest neurite was similar in the mutants and controls (Fig. 8F). Thus, the primary cell culture experiment demonstrated that the mutant interneurons developed shorter and fewer neurites, which could have led to the tangential migration defects.

The Transcription Factors Dlx1/2, Mash1, and Prox1 are Dramatically Upregulated after Foxg1 Deletion

When searching for downstream targets for Foxg1, we examined several genes that have been reported to be important for the development of cortical interneurons and the ventral telencephalon, including Dlx1/2, Mash1, Notch1, and Shh. Dlx1/2 have been reported to control interneuron differentiation, to regulate interneuron migration (Anderson et al. 1997; Wonders and Anderson 2005) and to control ventral telencephalic patterning and differentiation (Long et al. 2009). We first examined the mRNA level of Dlx1/2 in the GE at E14.5 using in situ hybridization. As we speculated, the transcription level of Dlx1/2 was clearly increased, and the expression area was expanded in the E14.5 mutant GE (Fig. 9A–B). Quantitative real-time PCR indicated an approximately 2.4-fold and 5.5-fold increase in the transcription levels of Dlx1 and Dlx2, respectively (Fig. 9C–D). Similarly, Mash1, which is the only neural bHLH gene known to be expressed in a subset of VZ cells and most SVZ cells in the ventral telencephalon, is required for the specification of neuronal precursors, controls the timing of their production, and regulates interneuron migration (Casarosa et al. 1999; Horton et al. 1999; Long et al. 2009). We found that in the GE of the mutant mice at E14.5, the region of Mash1 expression was significantly expanded in the SVZ and the mantle zone where Foxg1 was ablated, but it was not changed in the VZ where Foxg1 was expressed (Fig. 9C,C). Quantitative real-time PCR further confirmed that the
transcription of Mash1 was upregulated by approximately 2-fold (Fig. 9). In addition to their function in interneuron differentiation, Mash1 and Dlx1/2 have also been reported to control ventral telencephalic patterning and the differentiation of GE derivatives (Long et al. 2009). Thus, it is tempting to speculate that in addition to the impaired interneuron migration, the abnormal compartmentalization of the ventral telencephalon after Foxg1 ablation may also result from upregulated Dlx1/2 and Mash1 expression. In addition to Dlx1/2 and Mash1, Notch1 and Shh are also essential for the development of the ventral telencephalon (Yun et al. 2002; Xu et al. 2005). However, similar expression levels of Notch1 and Shh were observed in both the controls and the mutants (Fig. 9 E–F).

Surprisingly, we also found that the transcription factor Prox1, which is considered a key regulator of excitatory neuron development, was dramatically upregulated in the GE following Foxg1 deletion. At E14.5, the expression level of Prox1 was low in the ventral telencephalon of the controls; however, it was significantly upregulated in the mutants (Fig. 9D, D’). Quantitative real-time PCR indicated an approximately 3-fold increase in the transcription level of Prox1 (Fig. 9J). The dramatically increased expression level of Prox1 in the mutant ventral telencephalon strongly suggests that Prox1 may be involved in the development of interneurons as well.

Prox1 may be Important for the Development of CGE-derived Cortical Interneurons

We next examined Prox1 expression during interneuron development. At E18.5, with the exception of a low level of Prox1 expression in the ventral telencephalon, which was similar to that observed at E14.5 (Fig. 10A), a large number of Prox1+ cells were detected throughout the cortex in the controls. A dense band of Prox1+ cells was located in the deep migratory route (Fig. 10A).

In contrast, at E18.5 in the mutant mice, no Prox1+ cells were observed in the cortex, except for the observation of a small number of Prox1+ cells in the deep migratory route (Fig. 10B); meanwhile, a large number of Prox1+ cells had accumulated in the ventral telencephalon (Fig. 10B). We further determined that in the control cortex at P20, nearly all Prox1+ cells were GABA+ (Fig. 10C). When we double immunostained for Prox1 and GFP in our Dlx5/6-Cre-IRESCGFPP;Foxg1fl/+ heterozygotes, nearly all (∼91%) of Prox1+ cells in the cortex were GFP+ at E18.5 (Fig. 10D, I). These findings demonstrate that these Prox1+ cells were developing interneurons, which is consistent with a recent report (Rubin and Kessaris 2013). We further examined the Prox1+ cells in the cortex over postnatal development, from P2 to P20, and found that the Prox1+ cells preferentially occupied superficial cortical layers (Fig. 10E–H), suggesting they might be CGE-derived interneurons.
To clarify the subtypes of Prox1+ interneurons, we performed immunostaining and found that few Prox1+ cells co-expressed PV or SST (Fig. 10J,K), demonstrating that the Prox1+ cells were not MGE-derived interneurons. In contrast, Prox1 was found to be expressed in large numbers of COUP-TFII-, VIP-, CR-, and Reelin-positive interneurons (Fig. 10L–O), which were previously shown to be mainly derived from the CGE. Our data indicate that Prox1, which has previously been considered a key regulator of excitatory neuron development, may also be crucial for the development of interneurons, especially those that are derived from the CGE. Thus, based on our results, Foxg1 functions as a key upstream regulator of Mash1, Dlx1/2, and Prox1 in the control of interneuron development.

**Discussion**

Herein, we report a new role for Foxg1 as a critical upstream regulator of the development of cortical interneurons. Conditional inactivation of Foxg1 in the subpallium led to significant upregulation of transcription factors such as Dlx1/2, Mash1, and Prox1. Prox1, which is known to be a crucial regulator of excitatory neuron development, may also be important for interneuron development. Foxg1 plays a cell-autonomous role in regulating the development of cortical interneurons.

Previous studies have shown that the transcription factors Dlx1/2 and Mash1 have essential roles in the specification and differentiation of GE progenitors. In Dlx1/2-deficient brains,
tangential migration of cortical interneurons from the subpallial SVZ to the cortex is nearly abolished (Anderson et al. 1997; Wonders and Anderson 2005). Dlx1/2 reportedly control the differentiation and tangential migration of cortical interneurons, and the loss of Dlx1/2 leads to overgrowth of interneuron axons and dendrites (Cobos et al. 2007). In our mutants, upregulation of Dlx1/2 after Foxg1 deletion resulted in migrating interneurons with shorter and fewer neurites, consistent with previous studies. Mash1, which is highly expressed in ventral progenitors, can induce interneuron differentiation, and in Mash1 mutants, the

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**Figure 10.** Prox1 is expressed in CGE-derived cortical interneurons. (A–B′) Prox1+ cells were scattered in the control cortex (A, A′, arrows) at E18.5; however, there were only a few Prox1+ cells located in the cortical SVZ in the mutants (B, B′, arrows). (A′, B′) Higher power images of the boxed areas in A and B. (C) At P20, nearly all of the scattered Prox1+ cells in the cortex were GABA+ (arrows), suggesting that these cells were interneurons. (D, I) Nearly all of the Prox1+ cells in the cortex of the Dlx5/6-Cre-IREs-EGFP; Foxg1fl/fl heterozygotes were GFP+ at E18.5 (D, arrows), which further confirmed that the Prox1+ cells were interneurons. (E–H) During postnatal development (P2, P6, P10, and P20), Prox1+ cells preferentially occupied superficial cortical layers. (J, K) Few Prox1+ cells co-expressed PV (J) or SST (K) at P20. (L–O) Prox1 was expressed in a large number of COUP-TFII- (L), VIP- (M), CR- (N), and Reelin-positive (O) interneurons. Scale bars: 200 μm.
population of migratory cortical interneurons is greatly reduced (Casarosa et al. 1999; Horton et al. 1999). Furthermore, a gain-of-function study has shown that ectopic expression of Mash1 in cortical progenitors can induce the expression of Dlx1/2, suggesting that Dlx genes are downstream targets of Mash1 (Fode et al. 2000). Recently, Mash1 has been reported to directly activate Dlx1/2 by binding to an E-box in the I12b intergenic enhancer of the Dlx1/2 bigene cluster (Poitras et al. 2007). However, the upstream regulator of Mash1 and Dlx1/2 has not been identified. The findings of our study suggest that Foxg1 is a key repressor of Mash1 and Dlx1/2 and an upstream determinant of cortical interneuron development.

Notably, we demonstrated that the transcription factor Prox1, which is considered a key regulator in the development of excitatory neurons, was dramatically upregulated in the GE in our mutants. In the embryonic vertebrate central nervous system, Prox1 is expressed in the SVZ, where it controls cell cycle exit and regulates early excitatory neuronal differentiation (Elkouris et al. 2011). However, its function in the development of cortical interneurons has rarely been examined. In this study, we report that Prox1 was expressed in a portion of developing cortical interneurons and that the expression level of Prox1 is dramatically increased after Foxg1 deletion, suggesting an important role of Prox1 for the development of CGE-derived interneurons. Recently, it has been reported Prox1 is required for the development of CGE-derived interneurons, which is consistent with our findings (Miyoshi et al. 2015). Interestingly, previous studies have reported that Mash1 also functions upstream of Prox1 and activates its expression (Torii et al. 1999; Misra et al. 2008). Whether the increased expression of Prox1 is the consequence of the upregulation of Mash1 following Foxg1 ablation or Foxg1 directly represses Prox1 needs to be further explored.

Previous studies have shown that both chemoattractants, signaling through the Cxcr4/7 receptors, and chemorepellents, mediated through the Robo1 and EphA4 receptors, are required for proper interneuron migration and their final laminar distributions in the cortex (Marin et al. 2001; Andrews et al. 2008; Rudolph et al. 2010; Wang et al. 2011). In this study, the expression of both types of receptors in developing interneurons was significantly downregulated in our mutants, indicating that interneuronal differentiation was abnormal. Additionally, the decreased striatal expression of the Sema3A and Sema3F ligands may be a consequence of both the disruption of the compartmentalization of the ventral telencephalon and the cell-autonomous role of Foxg1 in the development of striatal neurons. Mash1 and Dlx1/2 not only play key roles in interneuron development but also are important for regionalization of the ventral telencephalon. Disruption of Dlx1/2 or Mash1 leads to abnormal development of the MGE and LGE (Horton et al. 1999; Long et al. 2009). Similarly, the ventral telencephalon has been reported to be lost in conventional Foxg1 knockouts (Xuan et al. 1995; Martynoga et al. 2005). In our mutants, the compartmentalization of the ventral telencephalon occurred in the absence of early regionalization defects was affected as well. In addition to the role of Foxg1, the upregulation Dlx1/2 and Mash1 may also contribute to the abnormal compartmentalization of the ventral telencephalon. Thus, it is quite reasonable that the migration defects of cortical interneurons may be a combined consequence of differentiation defects and disruption of the compartmentalization of the ventral telencephalon. Therefore, both cell-autonomous and noncell-autonomous defects, which are not mutually exclusive, may account for the impaired interneuron migration. Further studies are needed to identify the interaction between Foxg1 and these transcription factors.

Foxg1 has been identified as a candidate gene in Rett syndrome (Roche-Martinez et al. 2011) and West syndrome (Striano et al. 2011). Both Rett syndrome and West syndrome are neurodevelopmental disorders that are associated with the occurrence of...
epilepsy and mental disorders (Kato 2006; Pintaudi et al. 2010; Guerrini and Parrini 2012). In this study, we determined that Foxg1 is required for the differentiation and tangential migration of interneurons. In our mutants, interneurons could not migrate to the cortex; this may cause an imbalance between excitation and inhibition in cortical circuits and could lead to the occurrence of epilepsy and other interneuron-related symptoms. Although the precise mechanisms that underlie Rett syndrome and West syndrome remain unclear, our work informs the mechanisms of Foxg1-related diseases.

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Authors’ Contributions

Y.Y., W.S., and C.Z. designed the research; Y.Y., W.S., and Y.N. performed the immunostaining and in situ hybridization; Y.Y. performed the in vitro neuronal migration assay, the primary cell culture and quantification of neurite growth, and the homotopic grafting experiments; Y.Y., W.S., and C.Z. analyzed the data; Y.S. contributed to unpublished reagents; Z.Y. contributed to analytic grafting experiments; Y.Y., W.S., and C.Z. analyzed the data; Y.S. contributed to unpublished reagents; Z.Y. contributed to analytic tools; and Y.Y., W.S., and C.Z. wrote the paper.

References


