

# Laminar Patterning in the Developing Neocortex by Temporally Coordinated Fibroblast Growth Factor Signaling

Hiroshi Hasegawa,<sup>1</sup> Shizuko Ashigaki,<sup>1</sup> Masako Takamatsu,<sup>1</sup> Rika Suzuki-Migishima,<sup>2</sup> Norihiko Ohbayashi,<sup>3</sup> Nobuyuki Itoh,<sup>4</sup> Shinji Takada,<sup>3</sup> and Yasuto Tanabe<sup>1</sup>

<sup>1</sup>Neural Organization Research Team, Mitsubishi Kagaku Institute of Life Sciences, Precursory Research for Embryonic Science and Technology, Japan Science and Technology, <sup>2</sup>Mouse Genome Technology Center, Machida, Tokyo 194-8511, Japan, <sup>3</sup>Center for Integrative Bioscience, Okazaki National Research Institutes, Myodaiji-cho, Okazaki 444-8585, Japan, and <sup>4</sup>Department of Genetic Biochemistry, Kyoto University Graduate School of Pharmaceutical Sciences, Yoshida-Shimoadachi, Sakyo, Kyoto 606-8501, Japan

Laminar organization, a fundamental neural architecture in the CNS, is a prominent feature of the neocortex, where the cortical neurons in spatially distinct layers are generated from the common progenitors in a temporally distinct manner during development. Despite many advances in the characterization of the molecular mechanisms of the radial migration of cortical neurons, the way in which the early-late temporal sequence of cortical neuron generation is linked with the deep-superficial spatial sequence of cell body positioning remains obscure. Using *in vivo* electroporation-mediated gene transfer, we show here that the activities mediated by fibroblast growth factor receptors (FGFRs) in cortical progenitors are critical for conferring proper migratory properties on nascent neuronal progeny. Furthermore, we provide supportive evidence that Pea3 subfamily members of Ets (Pea3-Ets) transcription factors mediate the activities of FGFR at the mid to late phase of neocortical development. In addition, using *FGF18* knock-out mice, we demonstrate that FGF18 expressed by early-generated cortical neurons in the cortical plate is critical for the expression of Pea3-Ets transcription factors and that FGF18 is sufficient to induce their expressions. Our results thus imply that a feedback mechanism mediated by FGF signaling is involved in setting up the proper laminar positioning of cortical neurons; FGF18 derived from early-generated cortical neurons acts on the cortical progenitors expressing FGFRs and induces the expression of Pea3-Ets transcription factors that, in turn, confer proper migratory behaviors on nascent cortical progeny during the mid to late stages of neocortical development.

**Key words:** laminar patterning; neocortex; radial migration; feedback control; fibroblast growth factor; Ets transcription factors

## Introduction

In the developing neocortex, hierarchical series of radial and tangential migration of distinct neuronal subtypes culminate in the laminar organization of distinct cortical areas that encode a particular set of brain functions (Anderson et al., 2002; Gupta et al., 2002; Nadarajah and Parnavelas, 2002; Olson and Walsh, 2002). Cortical neurons located in distinct layers are generated from common progenitors in the underlying ventricular zone (VZ) in a temporally precise manner. These cells migrate in a radial direction to comprise the cortical plate (CP) in an “inside-out” manner; the early-generated cortical neurons are located in deep layers, whereas the late-generated cortical neurons migrate beyond the early-generated cortical neurons and are located in up-

per layers. Previous studies using a heterochronic transplantation approach suggested that the specification of laminar identities of cortical neurons is controlled by environmental signals that cortical progenitors encounter at around the time of their final cell division (McConnell and Kaznowski, 1991; McConnell, 1995). These studies led to the suggestion that these environmental factors affect cortical progenitors in a feedback manner to trigger the temporal switch for the specification of distinct laminar identities. In contrast, the mechanism by which distinct radial migratory behaviors and laminar allocation of cortical neurons is controlled is poorly understood (Marin and Rubenstein, 2003). Moreover, the nature of cell-extrinsic signals, the way they act on cortical neurons, and cell-intrinsic programs that in turn control proper migration and cell body allocation remain to be defined.

Members of Ets family transcription factors have been implicated in the control of development of a wide variety of tissues (Wasylyk et al., 1998). They hold a winged-helix-turn-helix structure similar to that of hepatocyte nuclear factor 3/forkhead transcription factors and are critical downstream nuclear targets of signal transduction cascades. In the developing spinal cord, three Pea3 subfamily members of Ets (Pea3-Ets) transcription factors, Erm, Er81, and Pea3, are expressed by restricted groups of cells (Lin et al., 1998). The expression of Pea3-Ets transcription factors in these cells is induced by cell-extrinsic signals derived

Received Dec. 28, 2003; revised Aug. 23, 2004; accepted Aug. 23, 2004.

This work was supported by grants from Precursory Research for Embryonic Science and Technology of the Japan Science and Technology. We thank T. Saito (Kyoto University) for his kind advice on the electroporation techniques, H. Yamazaki (Kyoto University) for his kind advice on the *in situ* hybridization techniques, J. Miyazaki (Osaka University) for *pCAGGS*, H. Ohuchi (Tokushima University) for *FGF8*, and Y. Arimatsu (Mitsubishi Kagaku Institute of Life Sciences) for anti-βIII tubulin antibody. We are grateful to T. M. Jessell (Columbia University) for his initial encouragement of this work.

Correspondence should be addressed to either of the following: Yasuto Tanabe, 11 Minami-Ooya, Machida, Tokyo, 194-8511, Japan, E-mail: ytanabe@libra.lis.m-kagaku.co.jp; or Shinji Takada, 5-1 Higashiyama, Myodaiji-cho, Okazaki, 444-8585, Japan, E-mail: stakada@nibb.ac.jp.

DOI:10.1523/JNEUROSCI.3070-04.2004

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from the periphery and is critical for the control of proper cell body positioning and axonal trajectory patterns (Lin et al., 1998; Arber et al., 2000; Haase et al., 2002; Livet et al., 2002; Helmbacher et al., 2003; Patel et al., 2003). These observations led us to examine whether Pea3-Ets transcription factors expressed in the developing neocortex (Chotteau-Lelievre et al., 1997) might be involved in the control of characteristic laminar framework formation.

We show here that Pea3-Ets transcription factors are expressed by cortical progenitors at the mid to late stages of neocortical development and appear to be critical for conferring proper migratory behaviors on nascent neuronal progeny. The expression of Pea3-Ets transcription factors depends on the activation of FGFRs in cortical progenitors and is controlled by FGF18 signals derived from early-generated cortical neurons in the CP. Our studies imply that temporally coordinated FGF signaling regulates distinct migratory behaviors and laminar allocation of cortical neurons at the mid to late stages of neocortical development and thereby underlie the laminar patterning of developing neocortex.

## Materials and Methods

**Animals.** ICR Jcl mice (CLEA Japan, Tokyo, Japan) were mated to obtain offspring in-house in an environmentally controlled room according to the Guidelines for Use of Laboratory Animals (Japan Neuroscience Society) and the Animal Care and Use Committee (Mitsubishi Kagaku Institute of Life Sciences). Noon of the date on which the vaginal plug was detected in the morning was designated as embryonic day 0.5 (E0.5). Embryos of *FGF18* knock-out mice were obtained as described previously (Ohbayashi et al., 2002).

**In situ hybridization and immunohistochemistry.** Embryos were fixed by cardiac perfusion with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.3, and postfixed in the same buffer overnight at 4°C. They were then placed in PBS with 25–30% sucrose for at least 24 hr before embedding in OCT compound (Sakura, Tokyo). Frozen sections were cut with a cryostat (MICROM HM5000M, Carl Zeiss, Jena, Germany) at 10  $\mu$ m and mounted onto MAS-coated glass slides (Matsunami, Osaka, Japan).

**In situ hybridization** was performed essentially as described (Ishibashi et al., 1995), with minor modifications. Sections were first immersed in PBT (PBS containing 0.1% of Tween 20), treated with 1  $\mu$ g/ml proteinase K in PBT at 37°C for 7 min, and washed three times with PBT. They were subsequently fixed with 4% PFA in PBS at room temperature for 30 min and again washed three times with PBT. Hybridization was performed overnight at 65°C with either digoxigenin-labeled or <sup>33</sup>P-labeled/<sup>35</sup>S-labeled RNA probes. Hybridized sections were washed two times with buffer A [50% formamide, 5  $\times$  SSC (150 mM NaCl, 15 mM sodium citrate, pH 4.5) and 1% SDS at 65°C for 30 min and then washed three times with buffer B (50% formamide, 2  $\times$  SSC) at 65°C for 30 min. For sections treated with digoxigenin-labeled RNA probes, sections were immersed in Tris-buffered saline (TBS), pH 7.0, and TBS containing 0.1% Tween 20 (TBST), treated with 0.5% Blocking Reagent (DIG Nucleic Acid Detection Kit; Roche, Mannheim, Germany), and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (diluted to 1:1000; Roche) in TBST containing 0.5% Blocking Reagent at 4°C overnight. After washing three times with TBST containing 2 mM levamisol (Sigma, St. Louis, MO), they were treated with NTMT solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, 0.1% Tween 20, and 2 mM levamisol). The coloring reaction was performed in the NTMT solution containing 35 mg/ml nitro blue tetrazolium (Roche), 17.5 mg/ml 5-bromo-4-chloro-3-iodyl phosphate (Roche), and 2 mM levamisol. For sections treated with <sup>33</sup>P-labeled or <sup>35</sup>S-labeled RNA probes, hybridized sections were washed in the same condition as described for the sections hybridized with digoxigenin-labeled RNA probes and then immersed in TBS. Sections were dehydrated in a series of ethanol solutions and exposed by dipping the slides in emulsion according to the manufacturer's protocol (Autoradiography Emulsion Type NTB-3; Kodak, New Haven,

CT). The dipped sections were kept at 4°C for 2–3 weeks and developed with HI-RENDOL/HI-RENFIX (Fuji Film, Tokyo, Japan).

The prepared RNA probes were *Erm* nucleotides 1385–2054 (Chotteau-Lelievre et al., 1997), *Er81* nucleotides 1704–2854 (Brown and McKnight, 1992), *Pea3* nucleotides 1491–2380 (Xin et al., 1992), *FGFR3* nucleotides 3099–4070 (Ornitz and Leder, 1992), *FGF18* nucleotides 403–922 (Hu et al., 1998), and *FGF17* nucleotides 163–683 (Xu et al., 1999). RNA probes for *FGFR1–3c* were prepared as described previously (Ohbayashi et al., 2002).

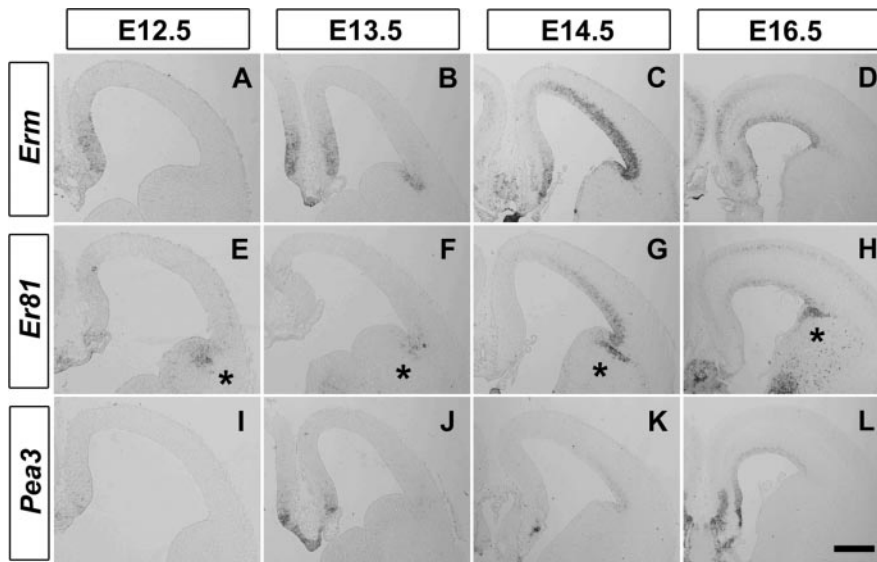
Immunohistochemical staining was performed as described previously (Takiguchi-Hayashi et al., 2004). The primary antibodies (Abs) were polyclonal rabbit anti- $\beta$ III tubulin Ab (kindly provided by Dr. Arimatsu, Mitsubishi Kagaku Institute of Life Sciences) at 1:1000 and polyclonal rabbit anti-MAP2 Ab (Chemicon, Temecula, CA) at 1:200. The secondary Ab used was cyanine3 (Cy3)-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) at 1:200.

**DNA constructs.** A CAG promoter-driven *EYFP* (enhanced yellow fluorescent protein) expression vector was prepared by subcloning *EYFP* (Invitrogen, San Diego, CA) into a *pCAGGS* vector (kindly provided by Dr. J. Miyazaki, Osaka University, Japan) (Niwa et al., 1991). A CAG promoter-driven *FGF18* expression vector was prepared by subcloning *FGF18* into a *pCAGGS* vector. The dominant-negative (DN) *Erm*, *Er81*, and *Pea3* constructs were prepared by isolating the Ets domain of mouse *Erm* (corresponding to amino acids 364–449), *Er81* (corresponding to amino acids 407–493), and *Pea3* (corresponding to amino acids 331–416), respectively, by a PCR-based approach, and subcloning into *pCAGGS* vectors. The *FGFR3c- $\Delta$ C* terminus construct and *FGFR3c- $\Delta$ TM* construct were prepared by PCR-based cloning of the *FGFR3c* coding region (Ornitz and Leder, 1992) containing the extracellular and transmembrane domain (mouse *FGFR3c* amino acids 1–384) and *FGFR3* coding region containing only the extracellular domain (mouse *FGFR3c* amino acids 1–347), respectively, and subcloning into *pCAGGS* vectors.

**In vivo electroporation.** *In vivo* electroporation was performed as described previously (Saito and Nakatsuji, 2001). Briefly, pregnant mice were deeply anesthetized with sodium pentobarbital (Nembutal, Abbott, North Chicago, IL) at 50 mg/kg body weight. An incision was made in the abdomen of a pregnant mouse and then the uterus was exposed. The uterine wall was cut, and the embryos covered with extra-embryonic membranes were exposed. DNA solutions (5 mg/ml in PBS with 0.016% trypan blue to permit visualization of injected solution in the embryos) were injected into the lateral ventricle of E12.5 or E13.5 telencephalon with a glass micropipette under a microscope. Electroporation was performed using tweezer-type electrodes with 5-mm-diameter discs (CUY650P5; Nepa Gene Co., Chiba, Japan). Electronic pulses of 27 V for E12.5 embryos and 30 V for E13.5 embryos were charged five times for 50 msec at 950 msec intervals using a square-pulse electroporator (CUY21EDIT; Nepa Gene Co.). After the procedure, the embryos were placed back into the abdominal cavity of the pregnant mouse. The pregnant mice were maintained for several days, and the embryos at E14.5–16.5 were subjected to the analyses. The *EYFP* construct was used to follow the gene-incorporated descendants and adjusted to the same concentration (1 mg/ml) among the experiments.

To monitor the fluorescence of *EYFP*, embryos were permitted to develop *in vivo* for 48–72 hr and fixed by cardiac perfusion with 4% PFA in PB, and then the whole brains were isolated. The isolated whole brains were postfixed and equilibrated with sucrose as described above. Cryostat coronal sections were made at 20  $\mu$ m. Axiovert 200M camera (Carl Zeiss) and Olympus BX61 with a CCD camera DP50 (Olympus, Tokyo, Japan) were used to obtain fluorescent images. Images were processed using Picture Publisher (Micrografix) and Photoshop (Adobe Systems, San Jose, CA) software.

**Dual luciferase reporter assay.** Dual luciferase reporter assay was performed according to the manufacturer's protocol (Promega, Madison, WI). *pGL3*-promoter vector (Promega) was used to construct the Pea3-Ets transcription factor-responsive reporter, in which five repeats of an optimal Pea3 binding site juxtaposed to *SV40* promoter were incorporated as a synthetic promoter, as described previously (Bojovic and Hassell, 2001). *pCAGGS* vector (Niwa et al., 1991) was used to construct



**Figure 1.** Restricted expression of *Pea3-Ets* transcription factors in the developing neocortex. Coronal sections of E12.5 (A, E, I), E13.5 (B, F, J), E14.5 (C, G, K), and E16.5 (D, H, L) cerebral cortices show patterns of expression of *Erm* (A–D), *Er81* (E–H), and *Pea3* (I–L), determined by *in situ* hybridization. Sections were obtained from an anteroposterior level at which the subcortical ganglionic eminences are located. Asterisks in E–H show *Er81* expression in the lateral ganglionic eminence. *Erm* expression began to be detected in the CP cells located in the deep layers of the medial–dorsal sector of cerebral cortex by E16.5 (D). In addition, *Er81* expression began to be detected in a subpopulation of layer V CP neurons by E16.5 (H). Scale bars: A, E, I, 0.37 mm; B, F, J, 0.41 mm; C, G, K, 0.48 mm; D, H, L, 0.5 mm.

*Pea3-Ets* transcriptional factor full-length (*Ets-FL*) and DN forms. After 24 hr of transfection, luciferase activity was measured by extracting COS7 cells transfected with an appropriate amount of each DNA construct. The relative amounts of each DNA construct, *Pea3-Ets* responsive reporter, *Pea3-Ets* transcription factor full-length, and DN form that were transfected together were 45–50, 4–5, and 40–50%, respectively.

## Results

### Expression of *Pea3-Ets* transcription factors in the ventricular zone at the mid to late stages of neocortical development

To begin to address the issue of how *Pea3-Ets* transcription factors may play roles in cortical development, we first defined the spatial and temporal expression patterns of *Erm*, *Er81*, and *Pea3* in the mouse cerebral cortex during corticogenesis (Fig. 1A–L).

At E12.5, by which time the generation of deep-layer CP neurons is initiated, the expression of *Erm*, *Er81*, and *Pea3* was not yet detected at the neocortex (Fig. 1A, E, I). The expression of *Erm* and *Er81*, but not *Pea3*, was first detected at E13.5 in the VZ of the lateral neocortex (Fig. 1B, F, J). At E14.5, expression of all three transcripts was detected in the neocortical VZ in a lateral-high to dorsal-low gradient (Fig. 1C, G, K). Among the members of the *Pea3-Ets* transcription factors, *Erm* exhibited the most prominent expression (Fig. 1C) and *Er81* the next (Fig. 1G), whereas *Pea3* gave only a faint expression in the neocortical VZ (Fig. 1K). As a continuum of their prominent expressions at the lateral neocortex, the expressions of all the members of the *Pea3-Ets* transcription factors were detected in the VZ adjacent to the pallial–subpallial border (Fig. 1B–D, F–H, K, L). From E16.5 onward, faint expression of all three transcripts persisted uniformly in the neocortical VZ (Fig. 1D, H, L) (data not shown).

These results showed that, in the neocortex, the *Pea3-Ets* transcription factors *Erm*, *Er81*, and *Pea3* were expressed by cortical progenitors in the VZ in an overlapping pattern from E13.5 to 14.5 onward. Given that neocortical neurogenesis initiates at around E10.5–11.5 and declines at around postnatal day 0 (P0) in the mouse neocortex (Polleux et al., 1997; Caviness et al., 2000),

these results indicated that expression of *Pea3-Ets* transcription factors in the neocortical VZ was confined mostly to the mid to late stages of neocortical development when cortical progenitors are dedicated to the generation of intermediate- to upper-layer CP neurons, but not most of the deep-layer CP neurons (Caviness et al., 2000).

### Dominant-negative forms of *Pea3-Ets* transcription factors suppress proper cell body positioning of cortical neurons

To evaluate functionally the roles played by *Pea3-Ets* transcription factors expressed by cortical progenitors in the VZ, we prepared DN forms of *Pea3-Ets* transcription factors in which only the DNA-binding *Ets* domain was attained. By using an *in vitro* luciferase reporter assay, we first tested to determine whether each of the DN forms is sufficient to suppress the transcriptional activation mediated by the full-length forms of the *Pea3-Ets* transcription factors and whether it cross-reacts with other *Pea3-Ets* transcription factors to suppress their transcriptional

activities (supplemental Fig. 1, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). Each DN form was shown to decrease the transcriptional activities achieved by the full-length *Pea3-Ets* transcription factors to levels achieved by the reporter construct alone. Furthermore, each of the DN forms cross-reacted with all of the other *Pea3-Ets* transcription factors and suppressed their transcriptional activities to a similar extent, as expected from their sequence similarity among the DNA-binding domains of *Pea3-Ets* transcription factors (amino acids with >95% sequence identity). These results validated the use of these DN forms to suppress transcriptional activities that are mediated by *Pea3-Ets* transcription factors.

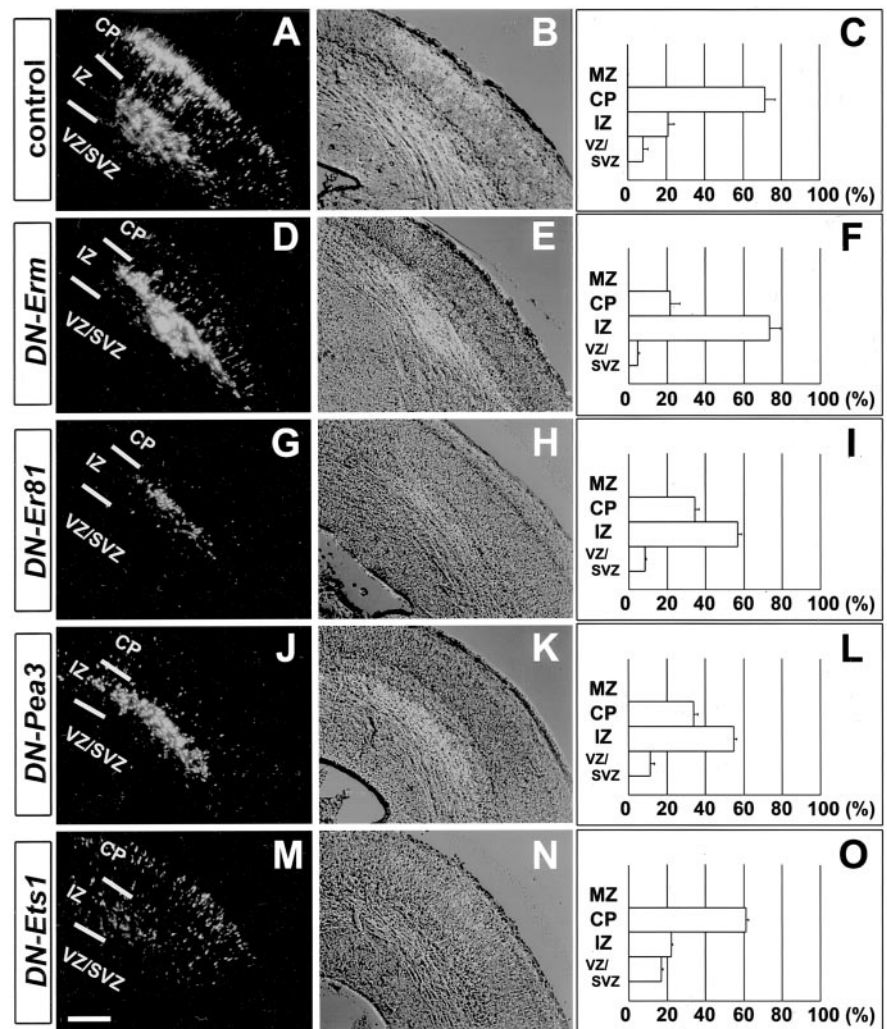
To suppress activities mediated by *Pea3-Ets* transcription factors in cortical progenitors in the VZ, we used *in vivo* electroporation-mediated gene transfer to introduce genes into cells located in the VZ. Because cortical neurons located at each layer in the CP are generated from common progenitors in a temporally precise manner, the *in vivo* electroporation-mediated gene transfer enabled us to specifically label and follow distinct cortical neurons that are generated from cortical progenitors and located at each layer by controlling the timing of electroporation (H.H. and Y.T., unpublished observations). We electroporated DN forms of *Erm* (Fig. 2D–F), *Er81* (Fig. 2G–I), or *Pea3* (Fig. 2J–L) in conjunction with an EYFP expression construct into VZ cells at the lateral neocortex where the expression of *Pea3-Ets* transcription factors is detected from E13.5 onward. We permitted the embryos to develop *in vivo* and followed the gene-incorporated descendants at the time when cortical neurons are expected to have left the ventricular zone and have registered tangentially in the CP.

When we electroporated EYFP alone at E13.5 into cells located in the VZ of the lateral neocortex and permitted embryos to develop *in vivo* until E16.5, most of the EYFP-positive descendants migrated radially from the VZ of the focal region where the electroporation was conducted (Fig. 2A, B). Most of their cell

bodies resided in the CP ( $71.4 \pm 5.5\%$ ) (Fig. 2A–C). These EYFP-positive descendants harbored extended neurites, with their cell bodies registered tangentially in the CP (Fig. 2A,B) (data not shown). When we electroporated *EYFP* together with DN forms of *Pea3-Ets* transcription factors, most of the descendants that incorporated genes left the VZ (Fig. 2D–L); however, they did not pass beyond the subplate and accumulated underneath it, and accordingly, they localized mainly in the intermediate zone but not in the CP (percentages of gene-incorporated descendants in the CP were as follows: DN-Erm:  $21.8 \pm 4.9\%$ ; DN-Er81:  $34.7 \pm 1.9\%$ ; DN-Pea3:  $33.8 \pm 2.3\%$ ) at E16.5 (Fig. 2D–L). These cells nevertheless expressed neuronal markers,  $\beta$ -III tubulin, and MAP2 (supplemental Fig. 2A, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material) (data not shown), and harbored neurite extensions, suggesting that the generation of cortical neurons was not perturbed even by the expression of DN forms of *Pea3-Ets* transcription factors. When DN forms of the distantly related members of *Ets* transcription factors *Ets1* (*Ets* domain amino acids with 64% sequence similarity with that of *Erm*) and *Elf1* (*Ets* domain amino acids with 45.3% sequence similarity with that of *Erm*), in which only the DNA binding domain was again attained, were electroporated with *EYFP*, most of the descendants migrated in the CP to an extent similar to that of the control (DN-Ets1:  $61.1 \pm 1.4\%$ ; DN-Elf1:  $60.2 \pm 6.5\%$ ) (Fig. 2M–O) (data not shown).

### Spatial and temporal correlation between the expressions of *FGFR3* and *Pea3-Ets* transcription factors in the neocortical VZ

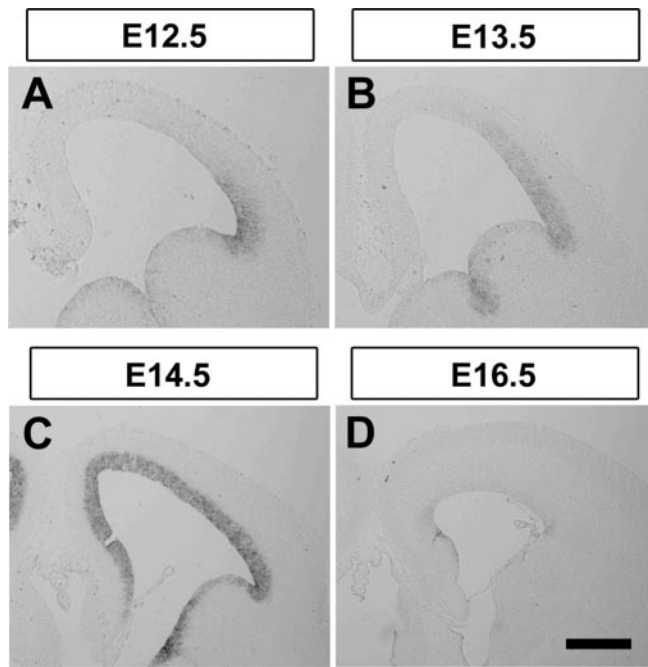
Previous studies have provided evidence that the expression of *Pea3-Ets* transcription factors in the CNS is controlled by the activities of several classes of cell-extrinsic signaling molecules, including neurotrophin-3, glial cell line-derived neurotrophic factors, Met-activating signals, and FGFs (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001; Haase et al., 2002; Helmbacher et al., 2003; Patel et al., 2003). At the caudal levels of the CNS, *Pea3-Ets* transcription factor expression in the developing motor neurons as well as proprioceptive sensory neurons appears to be induced by cell-extrinsic signals encountered by their axons at the peripheral target area. *Pea3-Ets* transcription factors appear to be critical for the acquisition of matured phenotypes, including proper cell body positioning and the precise axonal branching at the targets (Lin et al., 1998; Arber et al., 2000; Haase et al., 2002; Livet et al., 2002; Helmbacher et al., 2003; Patel et al., 2003). Our results presented above provided supportive evidence that *Pea3-Ets* transcription factors were critical for proper cell body positioning of newly generated cortical neurons, which prompted us to examine the identity of plausible environmental signals that might regulate the expression of *Pea3-Ets*



**Figure 2.** Disruption of proper radial migration of cortical neurons by suppression of activities mediated by *Pea3-Ets* transcription factors. Representative coronal sections of E16.5 cerebral cortex showing positions of cortical descendants after electroporation of *EYFP* (A–C), *DN-Erm* (D–F), *DN-Er81* (G–I), *DN-Pea3* (J–L), and *DN-Ets1* (M–O) at E13.5. C, F, I, L, O, Quantitative analyses of percentages of numbers of cells distributed at each zone. Each data point represents mean  $\pm$  SEM counted in  $>30$  sections. Images are obtained from  $>20$  electroporated embryos. Scale bar, 0.2 mm. MZ, Marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

transcription factors and control the proper cell body allocation of cortical neurons.

In a search for candidate receptors of the plausible environmental signals, we determined that *FGFR3*, which is one of the *FGFR* subtypes activated dominantly by the *FGF8* ligand subfamily (Ford-Perriss et al., 2001; Ornitz and Itoh, 2001), was expressed in a pattern tightly correlated with *Pea3-Ets* gene expression in the neocortical VZ (Fig. 3). *FGFR3* expression was first detected at E12.5, just before the onset of *Pea3-Ets* transcription factor expression, in the most lateral region of the neocortical VZ adjacent to the pallial–subpallial border where the expression of *Pea3-Ets* transcription factors was first detected (Figs. 1A–L, 3A). At E13.5, the expression of *FGFR3* in the VZ extended into a more dorsal region of the neocortex (Fig. 3B) in a lateral-high to dorsal-low gradient. At E14.5, prominent expression of *FGFR3c* covered nearly the entire region of the neocortical VZ (Fig. 3C). *FGFR3* expression was also detected in continuity in the VZ extending medially into the medial wall of the cerebral hemispheres and laterally into the lateral ganglionic eminence. From E16.5 onward, only faint *FGFR3* expression was detected in the neocorti-



**Figure 3.** Restricted expression of *FGFR3* in cortical progenitors in the ventricular zone of the developing neocortex. Coronal sections of E12.5 (*A*), E13.5 (*B*), E14.5 (*C*), and E16.5 (*D*) cerebral cortex, showing the expression patterns of *FGFR3*, determined by *in situ* hybridization. *In situ* hybridization analyses revealed the exclusive expression of *FGFR3* in the ventricular zone with the spatial and temporal expression patterns that are tightly linked with the expression of *Pea3-Ets* transcription factors (Fig. 1). Scale bars: *A*, 0.37; *B*, 0.41 mm; *C*, 0.48 mm; *D*, 0.5 mm.

cal VZ (Fig. 3*D*) (data not shown). Other FGFRs, *FGFR1c* and *FGFR2c*, that preferentially react with FGF8 ligand subfamily members as does *FGFR3c* also exhibited expressions in the neocortical VZ in a pattern partially overlapping those of *Pea3-Ets* transcription factors (supplemental Fig. 3, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). *FGFR4* expression was not detected in the neocortical VZ throughout the developmental period (data not shown). Together, these results indicated that there was a tight spatial and temporal correlation between the expression of *FGFR3* and that of *Pea3-Ets* transcription factors in the developing neocortex. The onset of *FGFR3* expression appeared to precede those of *Pea3-Ets* transcription factors in the neocortical VZ, and accordingly, the spatially broader area of *FGFR3* expression in the neocortical VZ appeared to contain the areas of *Pea3-Ets* transcription factor expression. Thus, these observations raised the possibility that FGFR-mediated signals in cortical progenitors might regulate the expression of *Pea3-Ets* transcription factors and control the proper cell body positioning of their neuronal progeny.

#### Activation of FGFR is required for proper cell body allocation of cortical neurons and for expression of *Pea3-Ets* transcription factors

We first examined whether FGFR-mediated signals in cortical progenitors were critical for newly generated cortical neurons to acquire proper radial migratory behaviors. FGFR dimer formation is known to be a critical step for FGFR activation and signaling, and thus an FGFR form that does not have cytosolic kinase domain inactivates FGFR-mediated signal transduction by forming nonfunctional homodimers as well as heterodimers with other subtypes of FGFRs (Ueno et al., 1992; Plotnikov et al.,

1999). Among the FGFRs that we examined for expressions, *FGFR3c* gave the most prominent expression at the mid to late phase of corticogenesis (Fig. 3) (supplemental Fig. 3, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). We thus prepared an *FGFR3c-ΔC* terminus form in which the cytosolic domain was deleted. We electroporated *FGFR3c-ΔC* in lateral neocortical VZ at E12.5 when and where the expression of *FGFR3c* began to be detected and analyzed the cell body positioning of gene-incorporated descendants at E15.5.

When *EYFP* alone was electroporated at E12.5, most of the *EYFP*-positive descendants again appeared to migrate properly to cross the subplate and locate their cell bodies in the CP at E15.5 ( $67.2 \pm 2.8\%$ ) (Fig. 4*A–C*). In contrast, when *FGFR3c-ΔC* was electroporated in conjunction with *EYFP*, most of the *FGFR3c-ΔC*-incorporated descendants appeared to be located in the intermediate zone and did not pass beyond the subplate ( $31.0 \pm 3.3\%$  in the CP) (Fig. 4*D–F*), an essentially similar phenotype obtained by electroporating DN forms of *Pea3-Ets* transcription factors. These results indicate that the proper migration and cell body positioning of newly generated cortical neurons are perturbed by the loss of FGFR-mediated activities in the VZ. *FGFR3c-ΔC*-incorporated descendants exhibited several neuronal characters including neurite extensions and the expressions of  $\beta$ III-tubulin and MAP2 (supplemental Fig. 2*B*, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material), suggesting that the generation of cortical neurons was not perturbed by the loss of FGFR-mediated signals. Together, these results indicated that activities mediated by FGFRs in cortical progenitors were critical for nascent neuronal progeny to acquire proper migratory behavior and cell body allocation at the mid to late stages of neocortical development.

Next, we attempted to determine whether FGFR-mediated signals might control the expression of *Pea3-Ets* transcription factors in the neocortical VZ (Fig. 4*G–L*). To suppress the activities mediated by FGFRs, we prepared an *FGFR3c-ΔTM* form, in which only the extracellular domain of *FGFR3c* was retained (Ornitz and Leder, 1992) to scavenge FGFR-activating environmental signals in the neocortical VZ. Because *Pea3* expression was detected only faintly in the neocortical VZ, we analyzed the expressions of *Ern* and *Er81* to evaluate the downregulation of expressions, because these two transcripts can be detected reliably in the neocortical VZ. When we electroporated *FGFR3c-ΔTM* into the neocortical VZ at E12.5 and analyzed the patterns of *Ern* and *Er81* expressions at E14.5 when the level of their expressions is at its peak during neocortical development, their expressions were downregulated in the neocortical VZ at the region where the electroporation was conducted (Fig. 4*J–L*). Prominent expression of *Ern* and *Er81* was detected in the neocortical VZ on the contralateral side of telencephalic vesicles (Fig. 4*G–I*).

#### Spatial and temporal correlation between the expressions of FGF18 in the CP and *Pea3-Ets* transcription factors in the VZ

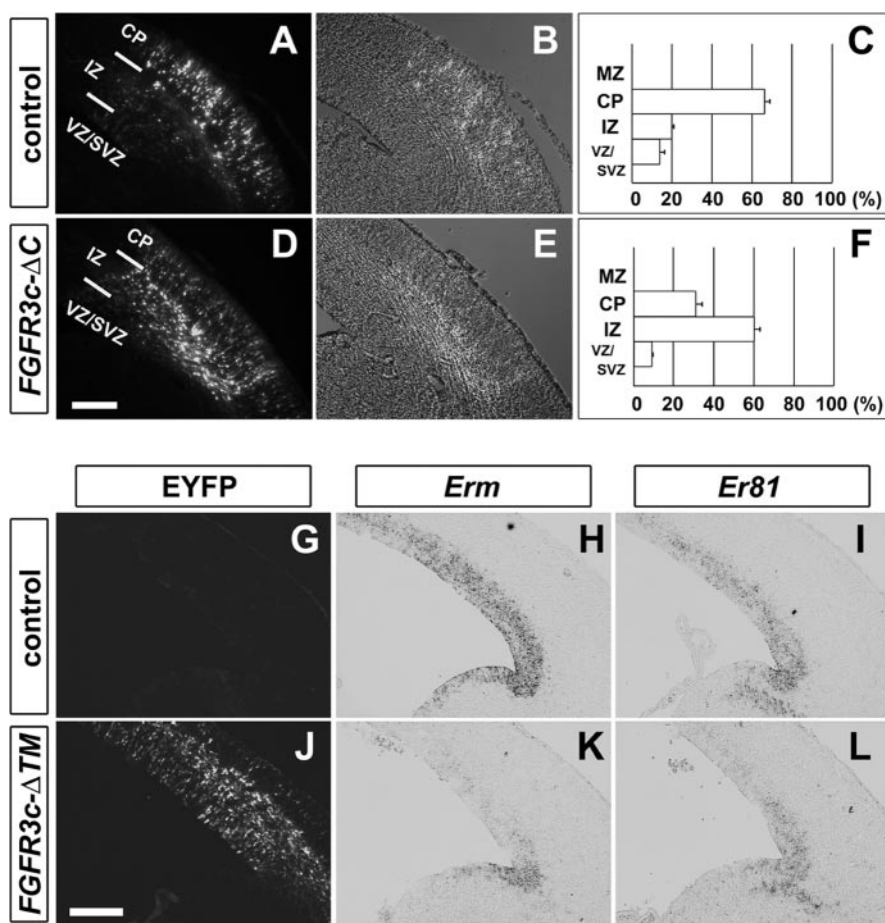
The results presented above showed that activation of FGFR in cortical progenitors was critical for conferring proper migratory behavior on nascent cortical progeny. FGFR-mediated signals were also responsible for the expression of *Pea3-Ets* transcription factors in the neocortical VZ. We next examined whether any endogenous FGF environmental signals might spatially and temporally accompany the expression of *Pea3-Ets* transcription factors and coordinate activation of FGFRs with expression of *Pea3-Ets* transcription factors at the mid to late phase of neocortical development.

We analyzed the temporal profiles of the expression patterns

of preferential ligands of FGFR3c, FGF8 ligand subfamily members (Ford-Perriss et al., 2001; Ornitz and Itoh, 2001), FGF8, FGF17, and FGF18, in the developing neocortex (Fig. 5). Among FGF8 ligand subfamily members, prominent expression of *FGF18*, but not of *FGF8* and *FGF17*, was detected in the CP exclusively in the developing neocortex (Fig. 5A–C) (data not shown). *FGF18* was expressed in the CP in a lateral-high to dorsal-low gradient in a pattern similar to *FGFR3c* expression in the VZ at E13.5 (Fig. 5A). At E14.5 when the expression of Pea3-Ets transcription factors is peaked in the VZ, prominent expression of *FGF18* was detected exclusively throughout the CP along nearly the entire dorsolateral axis of the neocortex (Fig. 5B) in a pattern tightly linked with the expression of Pea3-Ets transcription factors in adjacent VZ. Persistent expression of *FGF18* was detected weakly in the entire CP of neocortex from E16.5 onward (Fig. 5C) (data not shown). Together, these results demonstrated that FGF18 was expressed by early-generated cortical neurons after their cell body positioning in the CP, with a pattern that was cohesive with the expression of Pea3-Ets transcription factors by cortical progenitors in the VZ. Thus, these results raised the possibility that FGF18 signals by early-generated cortical neurons located in the CP might temporally coordinate the expression of Pea3-Ets transcription factors in the VZ at the mid to late phase of neocortical development.

#### FGF18 temporally coordinates the expressions of Pea3-Ets transcription factors

To test this possibility, we electroporated *FGF18* into the VZ cells at E11.5 and analyzed the expression of *Pea3-Ets* transcription factors at E13.5 when normally the expression of *Pea3-Ets* transcription factors is detected only faintly. Misexpression of *FGF18* resulted in robust expressions of all *Pea3-Ets* transcription factors in E13.5 cerebral cortices, suggesting that FGF18 signaling elicits the expression of Pea3-Ets transcription factors and sets up the timing of their expressions (Fig. 5D, E) (data not shown). To further examine whether FGF18 derived from the CP was the environmental signal that contributed to the expression of Pea3-Ets transcription factors in the VZ, we analyzed embryonic (E14.5–15.5) *FGF18* knock-out mouse cerebral cortices (Ohbayashi et al., 2002). Virtually no expression of all members of the *Pea3-Ets* transcription factors was detected in the *FGF18* (–/–) neocortical VZ, sparing the expressions in other regions of the telencephalon (Fig. 5F–I, insets) (data not shown). These results indicated that FGF18 derived from the early-generated CP neurons acted on the cortical progenitors in a feedback manner and temporally controlled the timing of Pea3-Ets transcription factor expression in the VZ. Together with evidence that activities mediated by FGFRs and Pea3-Ets transcription factors appeared to

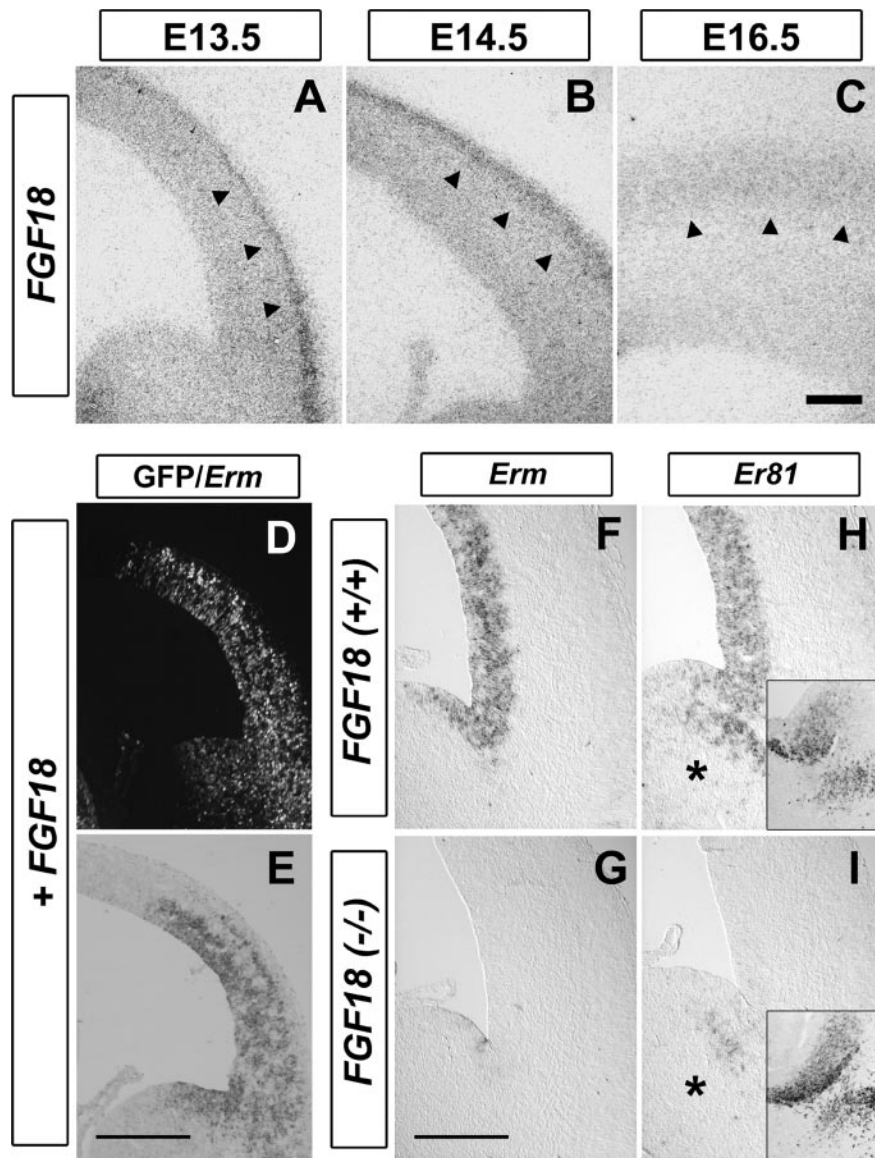


**Figure 4.** Disruption of proper radial migration of cortical neurons and inhibition of Pea3-Ets transcription factor expression by suppression of FGFR-mediated activities. *A–F*, Representative coronal sections of E15.5 cerebral cortex after the electroporation of either *EYFP* alone (*A–C*) or *FGFR3c-ΔC* (*D–F*) together with *EYFP* at E12.5. *C, F*, Quantitative analyses of percentages of number of cells distributed at each zone. Each data point represents mean  $\pm$  SEM counted in  $>30$  sections. Images are obtained from  $>20$  electroporated embryos. Scale bar, 0.2 mm. *G–L*, Hierarchical relationships between FGFR3 and Pea3-Ets transcription factors in the suppression of radial migration of cortical neurons were analyzed. Representative coronal sections of E14.5 cerebral cortex after the electroporation of *FGFR3c-Δ TM* construct at E12.5, showing the electroporated side (*J–L*) and the contralateral side (*G–I*) of telencephalic vesicles. Electroporation of *FGFR3c-Δ TM* construct in conjunction with *EYFP* construct at E12.5 into the ventricular zone of the lateral neocortex repressed *Erm* and *Er81* expression in the ventricular zone at E14.5 (*K, L*). Fluorescent images of adjacent coronal sections are shown in *G* and *J*. Images are representative of  $>10$  electroporated samples. Images in *G–I* were processed to show mirror images for the ease of comparison with the images of *J–L* using Photoshop. Scale bar, 0.2 mm.

be critical for conferring proper migratory behavior and cell body allocation on nascent neuronal progeny, these results suggested that a feedback mechanism mediated by FGF18 controls the proper cell body positioning of cortical neurons generated at the mid to late stages of neocortical development (supplemental Fig. 4, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material).

#### Discussion

Our results indicate FGFR-mediated activities in cortical progenitors are critical for nascent neuronal progeny to acquire proper migratory behaviors at the mid to late stages of neocortical development. Our results support the idea that Pea3-Ets transcription factors in cortical progenitors mediate FGFR activities and confer the proper migratory behaviors on neuronal progeny. Moreover, FGF18 expressed by early-generated cortical neurons in the CP controls the expression of Pea3-Ets transcription factors in mid to late phase cortical progenitors at the VZ. Thus, the results of our study lead to the suggestion that a temporally regulated feedback mechanism mediated by FGF signaling underlies the coord-



**Figure 5.** Feedback regulation of Pea3-Ets transcription factor expressions in the VZ by FGF18-mediated signals derived from the CP. *A–C*, Coronal sections of E13.5, E14.5, and E16.5 cerebral cortices, showing the restricted expression of *FGF18* in the CP (arrowheads). Scale bar, 0.3 mm. *D, E*, Induction of expression of Pea3-Ets transcription factors by FGF18. Misexpression of *FGF18*, detected by the coexpression of EYFP (*D*), resulted in an early and ectopic expression of *Erm*. Images are representatives obtained from more than six electroporated embryos. Scale bar, 0.4 mm. *F–I*, *FGF18* expression in the CP is required for *Pea3-Ets* transcript expression in the VZ. Coronal sections of E15.5 neocortex derived from *FGF18* knock-out mice [*FGF18*( $-/-$ )] and the wild-type littermates [*FGF18*( $+/+$ )] are shown. Asterisks in *H* and *I* show *Er81* expression in the lateral ganglionic eminence in both *FGF18* knock-out mice and the wild-type littermates. Insets in *H* and *I* show *Er81* expression at subcortical ganglionic eminences in both *FGF18* knock-out mice and the wild-type littermates. Scale bar, 0.1 mm.

ordinated spatial and temporal migratory behaviors of intermediate to upper layer cortical neurons at the mid to late stages of neocortical development and thereby contributes to the laminar patterning of developing neocortex.

#### FGF signaling as a feedback control of migratory behavior of cortical neurons

During development of the CNS, temporal patterns of distinct progeny generation from common progenitors are controlled by the intimate interplay between cell-extrinsic and -intrinsic mechanisms (Cepko, 1999; Edlund and Jessell, 1999; Qian et al., 2000). In the developing neocortex, where the major period of cortical neurogenesis precedes the onset of gliogenesis, the temporal con-

trol of timing and choice between neuronal and glial developmental pathways in common progenitors appears to be mediated by FGF2-like signals derived from cortical neurons (Qian et al., 1997). These signals are proposed to act on common progenitors in a feedback manner and trigger the switch for the generation of glial cells (Qian et al., 2000). Similar to the developmental programs of neocortical gliogenesis, the acquisition of laminar identities of cortical neurons appears to be controlled by the activities of environmental cues at around the time when progenitor cells leave the final cell cycle to generate cortical neuronal progeny (McConnell and Kaznowski, 1991; Bohner et al., 1997). These studies using heterochronic transplantation of cortical progenitors led to the proposal that a feedback mechanism may operate for the specification of cortical neurons with distinct laminar identities (McConnell, 1995).

Our results suggest that a feedback mechanism mediated by FGF signaling controls the laminar allocation of cortical neurons generated at the mid to late stages of neocortical development. These results imply that early-generated cortical neurons behave as a temporal switch to provide a feedback environmental cue, FGF18. FGF18 acts on progenitors expressing FGFRs and temporally coordinates the expression of Pea3-Ets transcription factors that in turn control the radial migratory behavior and cell body positioning of mid- to late-generated cortical neurons. Thus, FGF signaling operating at the mid to late stages of neocortical development, in addition to its roles in gliogenesis, appears to be involved in the laminar patterning of neocortex. Moreover, our results suggest that, as with the specification of laminar identities of cortical neurons (McConnell and Kaznowski, 1991; McConnell, 1995), the acquisition of distinct migratory behaviors and the laminar allocation of cortical neurons are controlled at least partly in cortical progenitors before the radial migration. Our results do not rule out the possibility that FGF signaling in cortical progenitors is involved in the specification of laminar identities of mid- to late-generated cortical neurons.

Our analyses of the brains of *FGF18* knock-out mice, however, we could not detect any migration defects in cortical neurons by the age of P0, at least, when *FGF18* knock-out mice die because of an unknown mechanism (H.H., N.O., S.T., and Y.T., unpublished observations). Nevertheless, our studies of FGFR function in cortical progenitors clearly show an essential role for FGF signaling in the acquisition of proper migratory behaviors and cell body allocation, consistent with a critical role for FGFRs and FGFRs in cell migration in many other developmental systems, including invertebrate organisms such as *Drosophila* and

*Caenorhabditis elegans* (Montell, 1999). The precise mechanism of how the lack of FGF18 is compensated remains unknown. Our results show that high levels of FGFR1–3, but not FGFR4, are expressed in the VZ during the period of corticogenesis (Ornitz and Itoh, 2001; Dono, 2003). In the telencephalon, many ligands of FGFRs, including FGF2, FGF7, FGF8, FGF15, and FGF17, are expressed mostly in the VZ, except FGF18, which is expressed in the CP. Thus, in the *FGF18* knock-out mice, genetic compensation potentially mediated by these FGFRs and FGFs, together with alternatively spliced isoforms of FGFRs and heparan sulfate proteoglycans, might have resulted in the changes in the requirement of FGFR signal transduction and rescued the radial migration defect. Future studies are needed to determine whether any other FGFs are expressed in the CP and/or VZ, and the potential roles of those FGFs expressed in the developing neocortex in the control of radial migration of cortical neurons generated at mid- to late-developmental periods. Given the restricted expression of most FGFs in the VZ, however, these studies may reveal rather a cell-autonomous control mechanism mediated by FGF signaling that is operated in the progenitors in the VZ in an autocrine manner, possibly accompanied by temporal changes in their competence to the FGF signals.

#### Pea3-Ets transcription factors as mediators of FGF signals in cortical progenitors

Our approaches using *in vivo* electroporation-mediated gene transfer to introduce DN forms of *Pea3-Ets* transcription factors, in which only the DNA-binding Ets domain was retained, appear to suppress the transcriptional activities mediated by Pea3-Ets transcription factors in cortical progenitors, given the suppressive activities of DN forms of *Pea3-Ets* transcription factors shown in the *in vitro* studies. Moreover, given that DN forms of distantly related Ets transcription factors do not perturb the proper migratory behaviors of cortical neurons *in vivo*, our study supports the idea that Pea3-Ets transcription factors, but not other Ets transcription factors, are critical for the acquisition of proper migratory behavior of nascent cortical progeny; however, approaches using these DN forms with >95% amino acid sequence similarity did not reveal any potentially distinct activities mediated by each of the members of the Pea3-Ets transcription factors. Moreover, we are left with the question of whether these DN forms of Pea3-Ets transcription factors might have perturbed activities other than those mediated by Pea3-Ets transcription factors.

Nevertheless, our results show that activities mediated by FGFRs are critical for the expression of Pea3-Ets transcription factors in cortical progenitors and for the proper migration of cortical neurons. Thus, these results are consistent with the idea that, in cortical progenitors at the mid to late stages of neocortical development, Pea3-Ets transcription factors act as key mediators that interpret FGF signals to confer proper migratory behavior on nascent neuronal progeny. Furthermore, these results are consistent with the idea that Pea3-Ets transcription factors define a temporal identity of cortical progenitors for the specification of proper migratory behaviors of cortical progeny generated at the mid to late stages of neocortical development. Combinatorial expressions of distinct cell-intrinsic cues, superimposed on the expression of Pea3-Ets transcription factors, in cortical progenitors may further specify their distinct temporal identities for the generation of spatially distinct progeny.

#### Indirect control of radial migration of cortical neurons by FGF signaling

Previous studies using molecular genetic approaches in invertebrate organisms such as *Drosophila* and *Caenorhabditis elegans* provided insights into the molecular mechanisms of cell migration (Montell, 1999). These studies revealed FGFs and FGFRs as key mediators of cell migration, playing direct roles, for example, in chemoattraction, and/or indirect roles, for example, in the control of cell differentiation. Our results presented above show that FGF signaling is critical for the proper radial migration of cortical neurons. Indeed, the way migratory behaviors of cortical neurons are perturbed by the inhibition of FGF signaling is reminiscent of the phenotypes observed in mouse mutants with cell migration defects (Gupta et al., 2002; Nadarajah and Parnavelas, 2002; Olson and Walsh, 2002), in which cortical neurons cannot pass beyond the subplate and accumulate underneath it. Importantly, however, the expressions of FGFRs and Pea3-Ets transcription factors are confined to cortical progenitors, not detected by radially migrating cortical neurons, suggesting that FGF signaling is involved only indirectly in the control of radial migration through the activities mediated by Pea3-Ets transcription factors that would regulate downstream genes that could play more direct roles in migration.

What are the molecules that are involved more directly in the control of migratory behavior under the control of the FGF signaling pathway? Previous studies in mouse mutants, together with studies in human congenital lissencephaly, have identified several molecules that are critical for the radial migration of cortical neurons (Gleeson and Walsh, 2000; Gupta et al., 2002; Nadarajah and Parnavelas, 2002; Marin and Rubenstein, 2003). At least two distinct modes of radial migration appear to be adopted by cortical neurons, which are controlled by at least two distinct but cooperatively interacting signaling pathways, the reelin- and p35-Cdk5-signaling pathways. Early-generated cortical neurons adopt the nuclear translocation mode, whereas mid- to late-generated cortical neurons adopt mostly the locomotion mode. Future studies are needed to determine how molecular descendants expressed in migrating neurons under the FGF signaling pathway work together with the reelin- and p35-Cdk5-signaling pathways for the control of microtubule rearrangement and actin cytoskeleton dynamics and eventual laminar formation of neocortex.

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