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Stem cell factor induces heterotopic accumulation of cells (heterotopia) in the mouse cerebral cortex

Hitomi SOUMIYA, Hidefumi FUKUMITSU and Shoei FURUKAWA
Laboratory of Molecular Biology, Department of Biofunctional Analysis, Gifu Pharmaceutical University, Mitahora-higashi 5-6-1, Gifu 502-8585, Japan

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ABSTRACT

The stem cell factor (SCF)-c-kit signal transduction pathway plays an important role in the proliferation and migration of neural progenitor cells, but little is known about its function during the development of the cerebral cortex. We investigated the effects of SCF by directly administering it into the telencephalic ventricular space of 13.5-day-old mouse embryos. SCF produced the heterotopic accumulation of cortical cells in several distinct area of the cerebral cortex at the postnatal stage, including the subcortical periventricular area, marginal zone, and lateral ventricular space. Additional analysis revealed that the heterotopia included both neurons and astrocytes and that SCF initially increased the number of neural stem cells without affecting that of intermediate progenitors and also disturbed their organization. These results suggest that SCF alters the timing of the genesis and migration of neural stem/progenitor cells, which may lead to formation of the observed heterotopia.

Diverse neurons composing the cerebral cortex are sequentially generated from proliferating common cortical progenitors, and the postmitotic neurons then migrate to their destinations within the cortical plate (CP) (1). Impairment of either process causes ectopic accumulation of neurons (heterotopia), which is observed in the cerebral cortex of many patients with intractable epilepsy (4, 13). Although mutations of several genes encoding cytoskeletal molecules or their associated proteins are well-characterized and have been shown to alter the migration of cortical neurons, these mutate genes are currently thought to be responsible for only a small portion of the genetic disorders leading to the cortical heterotopia in humans (3). Therefore, for further understanding of the pathogenesis of cortical heterotopia, a larger number of animal models produced by different mechanisms are needed.

Currently, evidence exists indicating that other pathogenetic mechanisms could cause ectopic accumulation of neurons, such as modified proliferation and programmed cell death. Although intrinsic factors such as regulators of the cell cycle and cytoskeleton-related proteins are important in the development and behavior of cortical progenitor cells, growth factors and cytokines environmentally supplied by the cerebral cortex are thought to determine the timing of the genesis, survival, and directed migration of cortical neurons (19). Therefore, disturbance of the regulated availability of responsible growth factors or cytokines would alter these biological properties of the cortical neurons and be capable of causing heterotopia in the cerebral cortex.

Stem cell factor (SCF) is a ligand growth factor for c-kit, a member of the type III receptor tyrosine kinase (RTK) family that includes the platelet-derived growth factor receptor, the colony stimulating factor 1 receptor, and fms-like tyrosine kinase ligand 3 receptor (2). SCF is a probable candidate for such environmental cues that control the generation, survival, and behavior of the cortical neurons

Address correspondence to: Dr. Hidefumi Fukumitsu, Laboratory of Molecular Biology, Department of Biofunctional Analysis, Gifu Pharmaceutical University, Mitahora-higashi 5-6-1,Gifu 502-8585, Japan
Tel: +81-58-237-3931, Fax: +81-58-237-5979
E-mail: hfukumi@gifu-pu.ac.jp

for the following reasons: 1) SCF and c-kit are expressed in cells of the developing mouse cerebral cortex, including migrating neuronal progenitors and CP neurons (15, 20); 2) by regulating the proliferation, differentiation, and migration of a variety stem/progenitor cell types, the SCF/c-kit pathway plays key roles not only in hematopoiesis and embryonic development (31, 32) but also in the generation and progression of cancers (16); and 3) exogenous SCF promotes the survival and migration of cultured cortical progenitor cells (9). However, the precise roles of SCF in the developing cerebral cortex are poorly understood.

Our present results suggest that exogenous SCF induced the formation of heterotopia in various areas of the cerebral cortex by modifying the development and/or migration of cortical progenitors.

MATERIALS AND METHODs

Animals. Pregnant ddY mice were purchased from Japan SLC (Shizuoka, Japan). The mice were handled in accordance with the Guidelines of Experimental Animal Care issued by the Office of the Prime Minister of Japan. Surgery and manipulation of animals were performed as described previously (11, 12, 22, 23).

Briefly, pregnant mice carrying embryonic day 13.5 (E13.5) embryos were deeply anesthetized with sodium pentobarbital (20 mg/g, i.p.), and the uterine horns were then exposed. After SCF or vehicle (phosphate-buffered saline, PBS) had been delivered via intrauterine injection, the uteri were placed back into the abdominal cavity to allow the embryos to continue normal development.

Semi-quantitative RT-PCR. Semi-quantitative RT-PCR was performed as described previously (12). The numbers of PCR cycles were optimized to be in the linear range of amplification by using specific primers. The following primer sets were used: β-actin, 5'-GATGGTGGGAATGGGTCAGAAG-3' and 5'-GAGTCCATCACAATGCCTGTGG-3'; SCF, 5'-ATGAAAAGCGGTCGTGCATTTT-3' and 5'-TTGGAGATGGCAGTTGTGCATT-3'; and c-kit, 5'-GTCTCAGCCATCTGCAAGTCCA-3' and 5'-GGTTTGGGACAAACGTCAGGTC-3'. An aliquot of the PCR products was resolved by agarose gel electrophoresis and visualized by ethidium bromide staining.

Western blotting. Cerebral cortices from E13.5 mouse embryos were dissected out in PBS. After having been pre-incubated in DMEM for 3 h, the cortices were incubated for 0.5, 1, 2 or 6 h in DMEM (Nissui, Tokyo, Japan) containing 100 ng/mL of SCF (R&D systems, Minneapolis, MN). These cortices were then lysed with 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2 mM EDTA, 1% NP-40, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethyl sulfonyl fluoride, 0.1% sodium dodecyl sulfate (SDS), and 1% Na deoxycholate. The lysates were centrifuged, and the protein concentration of the supernatant was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL). Each sample, containing 10 μg of protein, was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 10% acrylamide gels. The proteins were then transferred to a polyvinylidene fluoride membrane that was blocked for 1 h at room temperature with 5% skim milk in TTBS [20 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl and 0.05% Tween-20]. Next the membranes were incubated with primary antibody against serine/threonine kinase Akt (Cell Signaling, Beverly, MA) or phosopho-Akt (Cell Signaling) in Solution 1 (Canget Signaling, TOYOBO, Osaka, Japan), and then with alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI) in Solution 2. Finally, the specific protein bands were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indoryl-phosphate *p*-toluidine salt.

Tissue preparation. The animals were processed and analyzed as described previously (11, 12, 22, 23). Briefly, embryos were fixed in 4% paraformaldehyde solution (PFA), and postnatal mice were perfused via the heart with 4% PFA. Their brain tissues were removed and post-fixed overnight at 4°C. The brain tissues were soaked in PBS containing 20% (w/v) sucrose and frozen in embedding compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan). Coronal serial sections of 20-μm thickness were prepared with a cryostat (model CM 1800; Leica, Wetxlar, Germany), attached to adhesive-coated slides (Matsunami, Osaka, Japan), and dried before being used for immunofluorescence studies.

Histological analysis. For analysis of the cellular architecture of the cerebral cortex, nuclei of the cells were visualized with Hoechst 33342 (Invitrogen, Carlsbad, CA). Immunohistochemical analysis was performed as described earlier (11, 12, 22, 23). Primary antibodies against MAP2 (microtubule-associated protein 2; 1 : 2000; Sigma, St. Louis, MO), GFAP (glial fibrillary acidic protein; DAKO,

Glostrup, Denmark), Sox2 (SRY-box containing gene 2; 1 : 1000; Millipore, Temecula, CA), Cux1 (orthologue of the Drosophila cut gene; 1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA), CTIP2 (COUP-TF [chicken ovalbumin upstream promoter transcription factor-interacting protein 2]; 1 : 500; Abcam, Cambridge, UK), and Tbr2 (T-brain gene-2; 1 : 1000; Millipore, Temecula, CA) were used. These primary antibodies were visualized with goat anti-mouse IgG conjugated to Alexa 488 or 546 (1 : 1000; Molecular Probes, Oregon), and the fluorescent signals were observed with a confocal laser microscope (Model LSM 510; Carl Zeiss, Jena, Germany). Fluorescent images were converted digitally into reversed monochrome by using Photoshop (Adobe System, SanJose, CA), if the sections had been immunostained with a single primary antibody.

RESULTS AND DISCUSSION

Expression of SCF and its receptor, c-kit, during development of cerebral cortex

We examined the expression of SCF along with that of its receptor, c-kit, in the developing cerebral cortex by using RT-PCR (Fig. 1). Tissues from the E15.5 cortex contained a level of SCF mRNA nearly 2-fold higher than that found in the E13.5 cortex (Fig. 1A, B). On the other hand, the mRNA expression of c-kit at E15.5 was slightly but significantly lower than that at E13.5 (Fig. 1A, C).

Next, we tested to see if SCF could activate c-kit expressing in the developing cortex. Cortical tissue was isolated at E13.5, and cultured from 30 min to 6 h in medium containing 100 ng/mL of SCF. Then, we analyzed the phosphorylation of Akt (pAkt), a representative SCF/c-kit signal transducer known to be activated in cultured spermatogonial stem cells (7), hematopoietic cells (17), and cortical neurons (6). The phosphorylation of Akt was rapidly enhanced by SCF within 30 min to 1 h, but pAkt decreased to its original level by 2 h of incubation (Fig. 2).

These results indicate that SCF and functional c-kit were expressed in the E13.5 cortex, *i.e.*, c-kit could transduce the SCF signaling into the cortical cells via phosphorylation of Akt.

SCF induced heterotopia in the cerebral cortex

To determine the effect of SCF on the development of the cerebral cortex, we injected 10 ng of SCF into the lateral ventricle of E13.5 mice and performed microscopic analysis on Hoechst-stained sections prepared from the P6 cerebral cortex. SCF induced the accumulation of cells in several distinct areas of the cerebral cortex, including the subcortical periventricular area (Fig. 3 A, b), marginal zone (mostly in the somatosensory area of the cortex; Fig. 3 A, c), and lateral ventricular space (data not shown, Table 1). Administration of a higher dose of SCF (20 ng/embryo) induced more severe phenotypes; *i.e.*, 2 mm-diameter heterotopia developed beyond the cranial bone over the cerebral cortex at birth. In this case, the pups had a balloon-like swollen belly and could not survive beyond 2 days after birth (data not shown). Because of our interest, we focused on the heterotopia in the subcortical periventricular area and in the marginal zone induced by the injection of 10 ng SCF. Immunohistochemical study revealed that the heterotopia included both neurons and astrocytes, independent of their location. In most cases, many neurons and fewer astrocytes were located in the center of the heterotopia (Fig. 3 B, C).

Next, to examine the phenotype of the cortical

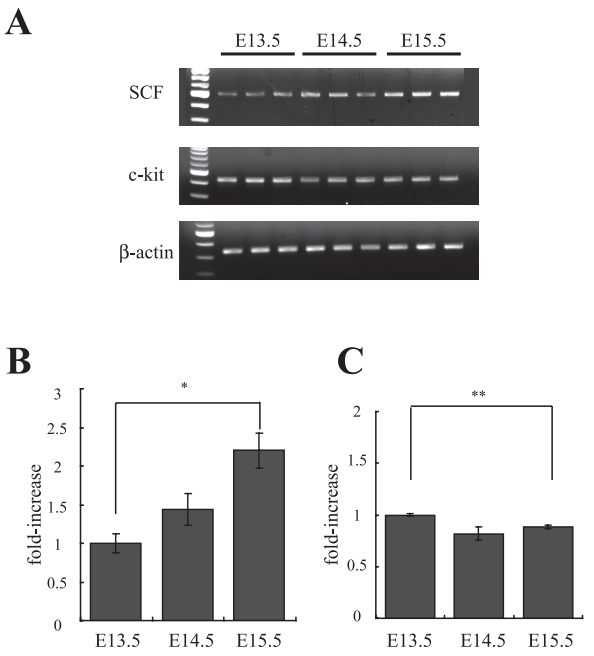

Fig. 1 Expression of the genes for SCF and its receptor, c-kit, in the embryonic mouse cortex during the neurogenesis period. (**A**) Total RNAs from E13.5, 14.5, and 15.5 cortices were subjected to RT-PCR using primers specific for SCF, c-kit, and β-actin. The PCR products were electrophoresed on 2% agarose gels, and the gels were stained with ethidium bromide. The intensity of the target bands for SCF (**B**) and for c-kit (**C**) was densitometrically quantified, and the ratio of the intensity of each stage band to that of the β-actin band was calculated. Values represent the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t* test; n = 3.

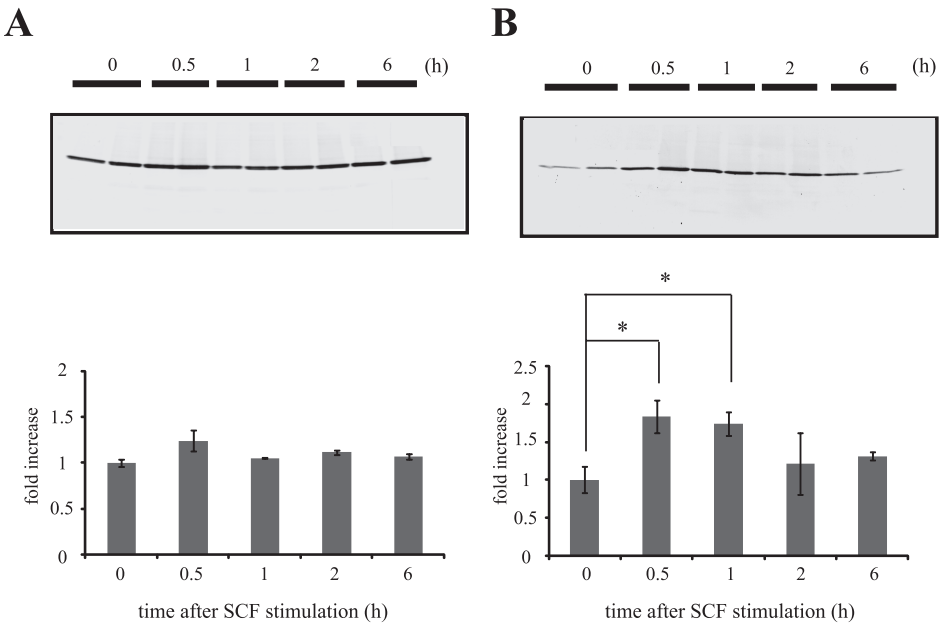

Fig. 2 SCF stimulated the phosphorylation of Akt in the E13.5 mouse cerebral cortex. Western blots for Akt (**A**, upper panel) and phosphorylated Akt (pAkt; **B**, upper panel) from freshly isolated E13.5 cortical tissue stimulated for 30 min to 6 h with SCF. The intensity of the target band was densitometrically quantified (lower panel of **A**: Akt or **B**: pAkt). Values represent the mean ± SE. $^{*}P < 0.05$, Student's *t* test; n = 3.

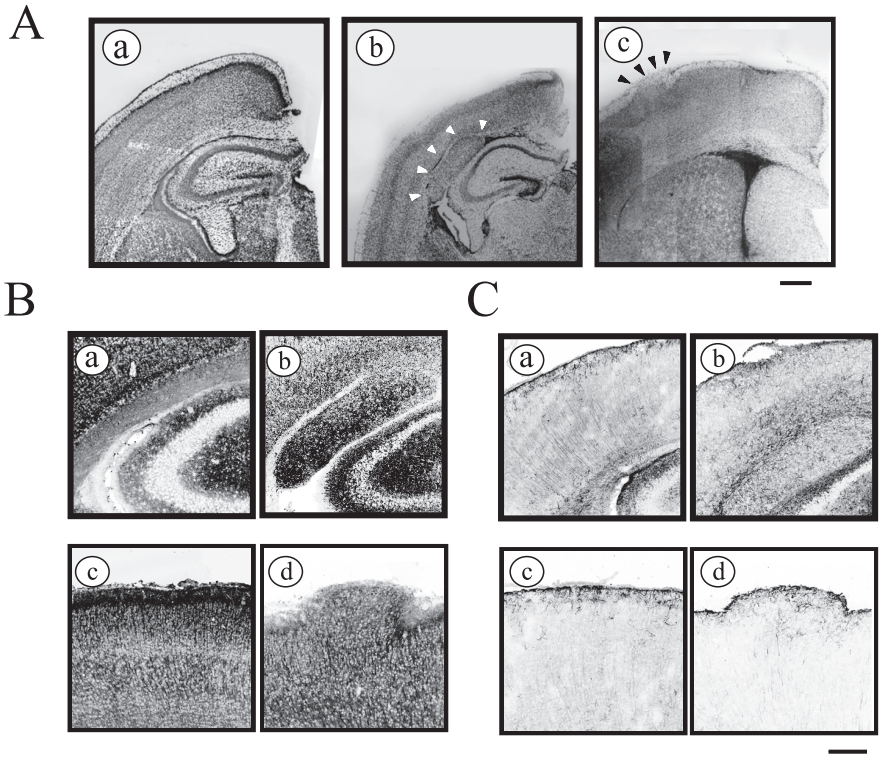

Fig. 3 SCF induced heterotopic accumulation of cortical cells. (**A**) Coronal sections of vehicle-treated (a) or SCF-treated (b, c) cortex were stained with Hoechst. The SCF-treated cortex shows periventricular (b) or marginal zone heterotopia (c). The arrowheads indicate the edge of each area of heterotopia. Scale bar is 500 μm. The periventricular area (a, b) and marginal zone area (c, d) were immunostained with anti-MAP2 antibody (**B**) or anti-GFAP (**C**). The photographs shown in (a, c) and (b, d) indicate the vehicle-treated and SCF-treated cortex, respectively. The scale bar is 200 μm.

Table 1 *Summary of phenotypes induced by SCF administration*

Phenotype	number of the animals
Heterotopia	8
Subcortical periventricular heterotopia	2
Marginal zone heterotopia	6
Accumulation of cells in the lateral ventricular space	4
Total	12 (total 15)

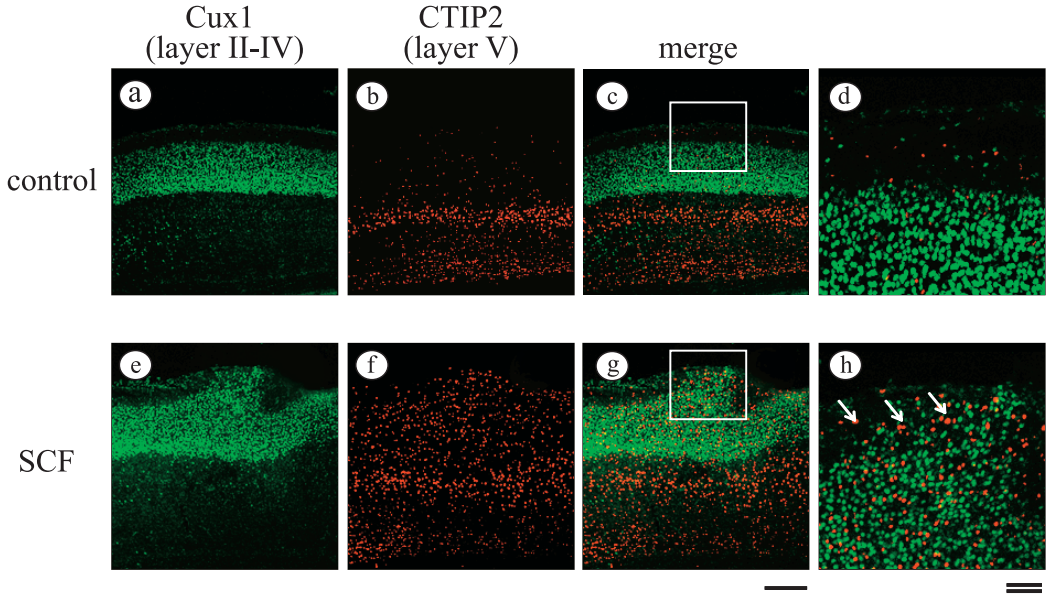

Fig. 4 Expression of lamina-specific marker proteins in the marginal heterotopias induced by SCF. P6 mouse cerebral cortex that had been treated with SCF (e–h) or vehicle (a–d) at E13.5 was double immunostained with anti-Cux1 (a marker of layer II–IV neurons) and anti-CTIP2 antibody (a marker of layer V neurons). The images in d and h show the enlarged views of the boxed area shown in c and g, respectively. Note that the heterotopia induced by SCF contained both deep (arrows) and upper layer neurons. Single scale bar is 200 μm. Double scale bar is 100 μm.

neurons comprising the marginal zone heterotopia, we performed double-immunolabeling with anti-Cux-1 antibody (a marker of layer II–IV neurons) and anti-CTIP2 antibody (a marker of layer V neurons). Consistent with previous knowledge, most of the Cux-1-positive cells were observed in layer II to IV but hardly detected in the other layers of the control cortex including the marginal zone (Fig. 4 a–d). Many CTIP2-positive cells with large nuclei were found in layer V and those with small nuclei were mainly found in the layer VI and sparsely observed in layers I–IV (Fig. 4 b). On the other hand, the SCF-induced heterotopia in the marginal zone contained a large number of Cux-1-positive neurons and many CTIP2-positive cells with large and small nuclei (Fig. 4 e–h). The results indicate that the SCF-induced heterotopia, regardless of its location, mainly consisted of neurons and that the marginal zone heterotopia contained both upper and deep layer neurons.

SCF altered the development and migration of neural stem/progenitor cells

In order to analyze how SCF induced the heterotopic accumulation of neurons, we examined its effect on the differentiation of cortical progenitors. In the mouse cerebral cortex, the generation of neurons extends from E11 to early E17 (30). During this period, intermediate neuronal progenitors are generated from neural stem cells, have limited mitotic activity of 1–3 cycles, and produce only neurons as daughter cells (8, 25). These 3 types of cortical cells, *i.e.*, neural stem cells, intermediate neuronal progenitors, and cortical neurons, specifically express Sox2, Tbr2, and βIII-tubulin, respectively (8, 25). Consistent with previous studies, many Sox2- and Tbr2-

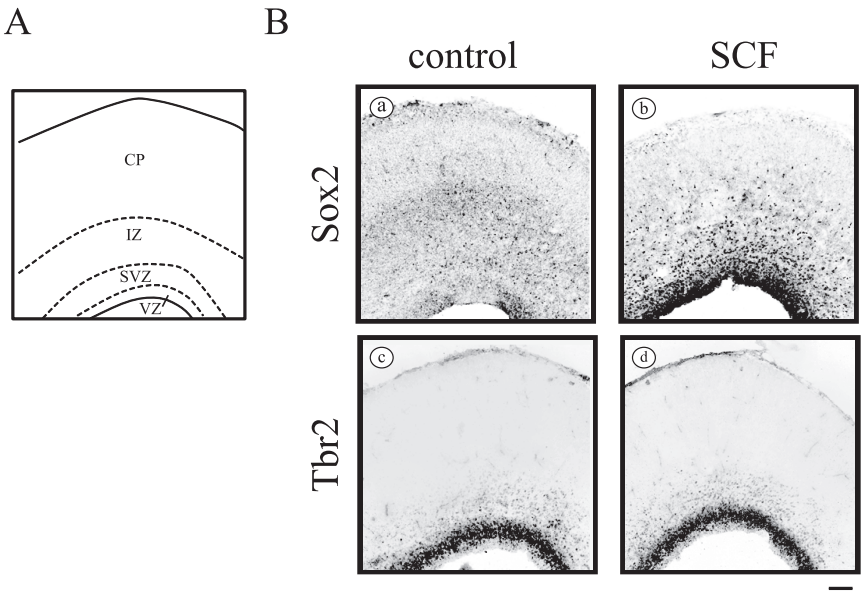

Fig. 5 SCF increased the number of Sox2-positive cells without affecting that of Tbr2-positive cells. (**A**) This illustration shows the locations of the ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ), and cortical plate (CP). (**B**) Coronal sections of the E17.5 cortices were immunostained with anti-Sox2 (a, b) or anti-Tbr2 (c, d) at 4 day after vehicle (a, c) or SCF (b, d) administration on E13.5. Scale bar is 200 μm.

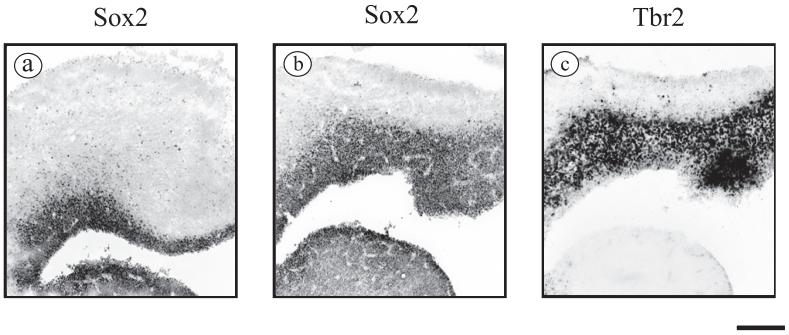

Fig. 6 SCF altered the distribution of ventricular progenitor cells. Coronal sections of E14.5 cortices prepared 1 day after SCF (20 ng) administration on E13.5 were immunostained with anti-Sox2 antibody (a, anterior cerebral cortex; b, mid-posterior cerebral cortex) or anti-Tbr2 antibody (c, mid-posterior cortex, section adjacent to that shown in b. Scale bar is 200 μm.

positive cells were observed in the ventricular zone (VZ) and subventricular zone (SVZ), respectively, of the control cortex at E13.5 (data not shown). As neurogenesis approaches completion, neural stem and neuronal progenitor cells decrease in the number. At that time (E17.5) only a few Sox2-positive cells were detected in the intermediate zone (IZ), and fewer Tbr2-positive cells in the SVZ, of the control cortex (Fig. 5). In the SCF-treated cortex, most of the cortical progenitor cells strongly expressed Sox2 in the VZ without affecting the number or distribution of Tbr2-positive cells (Fig. 5). Taken together with the fact that SCF promotes the self-renewal and the proliferation of hematopoietic stem cell (21), these results indicate that SCF may have a similar effect on the cortical progenitor cells, making possible an extension of the period of neurogenesis and an increase in the number of neurons (Fig. 5).

The increase in the number of Sox2-positive cells is not likely the sole reason for the ectopic cell accumulation. Since SCF is also known to act as a chemoattractant toward cultured cortical neurons (9) and hematopoietic cells (31), we examined the distribution of Sox2- and Tbr2-positive cells 1 day after the injection of a high dose of SCF (20 ng/em-

bryo). Examination of serial sections revealed that Sox2- and Tbr2-positive cells were ectopically increased in number in the anterior cortex, although the number of these cells was decreased in the mid-posterior cortical sections prepared from the same SCF-treated cortex, suggesting that SCF would stimulate the accumulation of these cells ectopically (Fig. 6). The obvious disturbance of the distribution of cortical progenitor cells was not detected at 1 day after 10 ng of SCF injection, but a similar but weaker effect probably occurred close to the injection site. These results indicate that SCF affected not only the maturation but also the migration of the cortical progenitor cells, which may have led to the ectopic accumulation of neurons.

We found that the administration of SCF induced the heterotopic accumulation of neural cells in various areas of the cerebral cortex. This is the first report that examined the function of SCF in the developing cerebral cortex by *in utero* injection of SCF. The SCF-induced heterotopia in the marginal zone (Fig. 3) was structurally similar to that seen in dyslexia and in BXSB and NZB mice, which are animal models for autoimmune-disorder with developmental learning impairment (28); *i.e.*, 1) the heterotopia was mostly present in the somatosensory area of the SCF-treated cortex; 2) the heterotopias consisted of a mushroom-shaped extrusion of neuronal cell bodies into the molecular layer of the cortex (Fig. 3, ref. 29); and 3) these neurons included both upper and deep layer cortical neurons (Fig. 4, ref. 14). On the other hand, the periventricular heterotopia induced by SCF was similar to a severe form of diffuse subcortical heterotopia, also called double cortex, which is commonly associated with epilepsy and a delay in mental retardation, and also similar to the cortical heterotopia of spontaneous mutant mice reported as a model for refractory epilepsy (5).

Recent genetic linkage studies have provided evidence that several genes may be associated with dyslexia, and others with periventricular heterotopia. The former include Dcdc2, Dyx1c1, and Kiaa0319 (18, 24, 27); and the latter, DCX, ARFGEF2 and FLNA (26). These genes have been shown to regulate neuronal migration in the developing cortex in rodent models (18, 24, 27). Although none of these genes are known to interact directly with the downstream signaling cascade of SCF/c-kit, some sort of common mechanisms might be engaged to cause ectopic accumulation of neurons. Down-regulation of the Dyx1c1 or DCX gene in cortical progenitor cells, by use of the RNA interference technique *in utero*, impairs the neuronal migration and induces periventricular heterotopia (26). These heterotopias involve not only gene-transfected cells but also a large number of non-transfected neural cells. If the transfected cells may secrete a growth factor or a cytokine such as SCF, it is possible that they would attract the non-transfected cells or stimulate the proliferation and survival of these non-transfected cells. SCF is known to act as a chemoattractant for various types of the cells including cortical progenitor cells (9) and hematopoietic stem cells and mast cells (31) via the c-kit/PI3K/Akt pathway (10, 33). Moreover, we showed that SCF induced the phosphorylation of Akt in the developing cortex (Fig. 2), stimulated the self-renewal and/or proliferation of the cortical progenitor cells (Fig. 5), and disturbed the structure of the proliferating zone, probably via migration of cortical progenitor cells (Fig. 6). Further investigations are necessary to be clarity the molecular mechanism underlying the SCF-induced formation of heterotopia. This SCF-induced mouse model, however, would be a useful tool to better understand the pathogenesis of cortical heterotopia in patients with refractory epilepsy.

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