Zinc finger genes Fezf1 and Fezf2 control neuronal differentiation by repressing Hes5 expression in the forebrain

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SUMMARY
Precise control of neuronal differentiation is necessary for generation of a variety of neurons in the forebrain. However, little is known about transcriptional cascades, which initiate forebrain neurogenesis. Here we show that zinc finger genes Fezf1 and Fezf2, which encode transcriptional repressors, are expressed in the early neural stem (progenitor) cells and control neurogenesis in mouse dorsal telencephalon. Fezf1- and Fezf2-deficient forebrains display upregulation of Hes5 and downregulation of neurogenin 2, which is known to be negatively regulated by Hes5. We show that Fezf1 and Fezf2 bind to and directly repress the promoter activity of Hes5. In Fezf1- and Fezf2-deficient telencephalon, the differentiation of neural stem cells into early-born cortical neurons and intermediate progenitors is impaired. Loss of Hes5 suppresses neurogenesis defects in Fezf1- and Fezf2-deficient telencephalon. Our findings reveal that Fezf1 and Fezf2 control differentiation of neural stem cells by repressing Hes5 and, in turn, by derepressing neurogenin 2 in the forebrain.

KEY WORDS: Corticogenesis, Neurogenesis, Zinc finger genes, Fezf1, Fezf2, Hes5, neurogenin 2, Mouse

INTRODUCTION
The mammalian forebrain is the most complex and organized structure that contains a variety of neurons and glia and that is required for higher neuronal functions, including memory, reasoning, emotion and planning. During development of the mammalian dorsal telencephalon (neocortex), neurons are generated sequentially from neural stem (progenitor) cells located in the ventricular zone (VZ) (Molyneaux et al., 2007). In mice, the Cajal-Retzius (CR) cells and subplate (SP) neurons are born around embryonic day (E) 11.5 or earlier (Allendoerfer and Shatz, 1994; McConnell et al., 1989). Layers VI (corticothalamic), V (subcerebral projection), IV, and II-III (intercortical projection) are then generated in the VZ around E12.5, E13.5, E14.5 and E15.5, respectively, and migrate to appropriate cortical layers (Leone et al., 2008; Molyneaux et al., 2007). Recent studies have identified transcription factors and gene networks that control specification of these cortical neurons (Alcamo et al., 2008; Arlotta et al., 2008; Britanova et al., 2008; Chen et al., 2005a; Chen et al., 2008; Hevner et al., 2001; Molyneaux et al., 2005; Zhou et al., 1999). It is thought that differentiation of the cortical neurons from the neural stem cells takes place in the VZ through asymmetric cell divisions (Chenn and McConnell, 1995; Gotz and Huttner, 2005). At least some cortical neurons are also differentiated from the TBR2 intermediate progenitors (also called basal progenitors; EOMES – Mouse Genome Informatics), which are derived from the neural stem cells in the VZ (Arnold et al., 2008; Kowalczyk et al., 2009; Sessa et al., 2008). However, little is known about the molecular mechanisms by which the neural stem cells are differentiated into neurons or intermediate progenitors during early cortical development.

Hes genes are vertebrate homologs of Drosophila hairy and enhancer of split, which encode basic helix-loop-helix (bHLH) transcriptional repressors, and Hes proteins function to repress the expression of the bHLH proneural genes, which promote neurogenesis (Kageyama et al., 2007; Kageyama et al., 2008a; Ross et al., 2003). Hes1, Hes3 and Hes5 are expressed in the neural stem cells of the central nervous systems of mice and their loss results in acceleration of neuronal differentiation and depletion of the neural stem cells (Hatakeyama et al., 2004). Hes genes are known as downstream effectors of Notch signaling (Ohsumi et al., 1999), and deficiency of Notch signal components leads to precocious neuronal differentiation from the neural stem cells (Gaiano and Fishell, 2002; Louvi and Artavanis-Tsakonas, 2006; Yoon and Gaiano, 2005). Thus, the Notch-Hes pathway plays an essential role in the maintenance of the neural stem cells. However, it remains elusive how the Notch-Hes pathway is controlled during the cortical development.

Fezf1 (Fez) and Fezf2 (Fez-like, Zfp312) are closely related genes that encode transcriptional repressors containing six C2H2-type zinc fingers and an EH1 (Engrailed homology 1) repressor motif, which is known to interact with Groucho or Tle (Transducin-like enhancer of split)-type transcriptional co-repressors (Shimizu and Hibi, 2009). Both Fezf1 and Fezf2 are expressed in the prospective forebrain region during early embryogenesis and they subsequently exhibit both overlapping and distinct expression domains in the mouse forebrain (Chen et al., 2005b; Hirata et al., 2006a; Hirata et al., 2006b; Hirata et al., 2004). Loss-of-function studies in mice reveal that Fezf1 is involved in development of the olfactory sensory system (Hirata et al., 2006b) and that Fezf2 is not only involved in...
the differentiation of SP neurons (Hirata et al., 2004) but also essential for specification of the subcerebral projection neurons in layer V of the cortex (Chen et al., 2005a; Chen et al., 2005b; Molyneaux et al., 2005). Fesf1 and Fesf2 redundantly function to prevent the rostral forebrain from being the caudal diencephalon as the caudal diencephalon is expanded rostrally during early neural patterning in Fesf1- and Fesf2-deficient mice (Hirata et al., 2006a). In zebrafish, fesf2 is required for the formation of dopaminergic (DA) neurons in the basal forebrain (Guo et al., 1999; Levkovitz et al., 2003; Rink and Guo, 2004).

Here, we have found that Fesf1 and Fesf2 directly repress the expression of Hes5 and thereby derepress the expression of neurogenin 2 in the mouse neocortex. We show that the gene cascade Fesf1/Fesf2 → Hes5 → neurogenin 2 plays an important role in early differentiation of the neural stem cells into TUJ1+ neurons or TBR2+ intermediate progenitors, which are required for proper cortical development.

MATERIALS AND METHODS

Mouse mutants

Previous research has described the generation of mice that are Fesf1-deficient, mice that are Fesf2-deficient and mice that are both Fesf1-deficient and Fesf2-deficient (Hirata et al., 2006a; Hirata et al., 2006b; Hirata et al., 2004) [the respective accession numbers of Fesf1-deficient mice and Fesf2-deficient mice in the RIKEN Center for Developmental Biology (CDB) are CDB0497K and CDB0498K; http://www.cdb.riken.jp/arg/mutant%20mice%20list.html]. Both Fesf1−/− and Fesf2−/− were originally established in a 129SV genetic background and backcrossed to the C57BL/6 background for several generations. Hes5-deficient mice were described previously (Cau et al., 2000; Hatakeyama et al., 2004). For the current study, we housed mice in an environmentally controlled room at the Animal Facility of the RIKEN CDB under the institutional guidelines for animal and recombinant DNA experiments. The genotypes of newborn mice and embryos were determined by PCR analysis (Hirata et al., 2006b; Hirata et al., 2004). Noon of the day on which the vaginal plug was detected was designated as E0.5.

Microarray analyses

The forebrain rostral to the caudal limit of the lateral ventricles was isolated manually from E9.5, E10.5 and E12.5 wild-type mice and from Fesf1−/−/Fesf2−/− mice. RNAs were isolated by Sepasol-RNA I (Nacalai Tesque) and were subjected to the One-Cycle Target Labeling procedure for biotin labeling by in vitro transcription (IVT; Affymetrix, Santa Clara, CA). The cRNA was subsequently fragmented and hybridized to the GeneChip Mouse Genome 430 2.0 Array (Affymetrix) according to the manufacturer’s instructions. The microarray image data were processed with the GeneChip Scanner 3000 (Affymetrix) to generate CEL data. Data obtained from wild-type and Fesf1−/−/Fesf2−/− mice were normalized according to the program’s default setting. Two criteria were set for exploring the candidates for FEZF1 and FEZF2 downstream genes. First, the candidates were to have two-fold or more changes in signal value between wild-type and Fesf1−/−/Fesf2−/− rostral forebrains. Second, the signal intensities of the higher value should be higher than 100. The microarray data have been deposited in Gene Expression Omnibus (GEO) under the accession number GSE21156.

RNA probes and in situ hybridization

Mouse embryos were fixed with 4% pafomaaldehyde (PFA) overnight at 4°C. Cryosections of the embryonic forebrain were prepared as described previously (Hirata et al., 2004). The samples were treated with 50 μg/ml Proteinase K for 8 minutes and then were post-fixed in 4% PFA for 15 minutes at room temperature. After the samples were washed with PBS, they were treated with 0.1 M triethanolamine-HCl (pH 8.0) followed by the addition of acetic anhydride. Hybridization and post-hybridization washing were performed as described previously (Shimizu et al., 2005). The samples were pre-incubated in the blocking solution (20% heat-inactivated goat serum in PBS, 0.1% Triton X-100) for 1 hour and incubated with 1/2000 diluted alkaline phosphatase (AP)- or peroxidase (HRP)-conjugated anti-digoxigenin antibodies (Roche Diagnostics Corp.) in the blocking solution at 4°C overnight. After undergoing three 30-minute washings with MABT (0.1 M maleic acid, 0.15 M NaCl and 0.1% Tween-20; pH 7.5), the samples underwent two 10-minute treatments with NTMT (0.1 M NaCl, 0.1 M Tris-HCl, 0.05 M MgCl2; and 0.1% Tween-20; pH 9.5). NBT and BCIP (Roche) were used as the substrate for AP. Tyramid signal amplification (TSA) kits with Alexa Fluor 555 tyramide (Molecular Probes) were used to visualize the fluorescent signals. The probes were as follows: FEZF1 (Hirata et al., 2006b), FEZF2 (Hirata et al., 2004), Hes5 (Ohtsuka et al., 1999), neurogenin 2 (Fode et al., 1998), p73 (Meyer et al., 2004), reelin (D’Arcangelo et al., 1995) and Rorb (Nakagawa and O’Leary, 2003). The NBT and BCIP and fluorescent signals were obtained with AxioPlan2 imaging and an LSM5 Pascal laser-scanning inverted microscope (Zeiss), respectively. The fluorescent images were constructed from Z-stack sections by a 3D projection program associated with the microscope. Alexa Fluor 488 and 555 signals were colored green and magenta, respectively, for the figures.

Immunohistochemistry

Cryosections of forebrains were blocked with 5% normal goat serum in PBS and then incubated with primary antibodies overnight at 4°C. After being rinsed with PBS, the sections were incubated with fluorescent secondary antibodies. The primary antibodies used in this study were anti-calretinin (1/400, Swant) (Schwaller et al., 1993), anti-NURR1 (NR4A2 – Mouse Genome Informatics; 1/100, R&D Systems) (Hoerder-Suabedissen et al., 2009), anti-PAX6 (1/200, Covance) (Marquardt et al., 2001), anti-TBR2 (1/500, Chemicon), anti-TBR1 (1/500, Abcam) (Englund et al., 2005), anti-CUX1 (1/100, Santa Cruz Biotech.), anti-TUJ1 (1/500, Sigma) (Lee et al., 1990), anti-chondroitin sulfate (1/200, Sigma) (Bicknese et al., 1994), anti-Ki67 (1/500, BD, Pharmingen), (Kubbutat et al., 1994), anti-neurogenin 2 (1/100, R&D Systems) (Lo et al., 2002), anti-BrduU (1/500, BD, Pharmingen) (Dolbeare et al., 1983) and anti-CTIP2 (1/500, Abcam) (Lai et al., 2008). The secondary antibodies were Alexa 488- or 555-conjugated goat anti-mouse, anti-rabbit or anti-rat IgG (Molecular Probes). For the anti-BrduU antibody, the Vectastain Elite ABC Kit (Vector) was also used for immunostaining with the HRP substrate diamino-benzidine (DAB). The DAB images were obtained with AxioPlan2 imaging.

Chromatin immunoprecipitation assay

A chromatin immunoprecipitation (ChIP) assay was performed according to the protocol previously reported (Molyneaux et al., 2005). Forebrains of E11.5 mouse embryos were mechanically dissociated and the cells were seeded on a poly-L-lysine-coated 24-well dish and cultured in DMEM containing 10% fetal calf serum overnight. The cells were fixed and used for ChIP assay. The PCR template was amplified with the following primers: 5′-GGATGCTAATGATGCAGGC-3′ and 5′-TGAGGATCTGGAGGC-GATTAGC-3′. To raise monoclonal antibodies against FEZF1 and FEZF2, we generated glutathione S-transferase (GST) fusion protein containing amino acids (aa) 39-205 of FEZF1 or 134-266 of FEZF2 (Shimizu et al., 2005). The GST fusion protein was purified by Glutathione Sepharose 4B (GE Healthcare) and used for immunization. Polyclonal antibodies against FEZF1 were generated by means of rabbit immunization with the synthetic peptide CTATPSAKDLARTVQS (the addition of the underlined C served to link the peptide covalently with keyhole limpet hemocyanin) as reported previously (Inoue et al., 2004). The control antibodies used for ChIP assays derived from pre-immune rabbit serum and control IgG (Santa Cruz) for the polyclonal and monoclonal antibodies, respectively.

BrdU incorporation assay

Pregnant females received a single intraperitoneal injection of BrdU (5′-bromo-2′-deoxyuridine, 100 mg/kg). The pups were sacrificed 5 minutes after the injection or allowed to develop to the indicated period and then were fixed with 4% PFA.

Luciferase reporter assay

Human embryonic kidney (HEK) 293 cells in a well of a 24-well plate were transfected with 0.1 μg of reporter plasmids pHEs5-luc (Takebayashi et al., 1995), pHEs1-luc (Nishimura et al., 1998) or 8xwtCBF1BS-luc (Molyneaux et al., 2005).
(Zhou et al., 2000), and an internal control plasmid, pBRL (Promega), together with the expression plasmids pCS2+Fezf1, pCS2+Fezf2 and pME-FNIC, using a HilyMax transfection reagent (DOJINDO). On the following day, luciferase activity was measured with a Dual Luciferase Reporter Assay System (Promega). The full coding cDNA fragment of Fezf1 and Fezf2 was inserted into pCS2+ (Turner and Weintraub, 1994) in pCS2+Fezf1 and pCS2+Fezf2, respectively. The expression plasmid for the intracellular domain of mouse NOTCH1 (pME-FNIC) had been previously published (Nishimura et al., 1998).

**Immunoprecipitation and immunoblotting**

HEK293 cells in a 6 cm dish were transfected with 5 μg of pCS2+Fezf1 or pCS2MT2−Fezf2 (contains six Myc tags). After 24 hours, the cells were lysed with 0.5 ml of a lysis buffer: 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40 and protease inhibitor cocktail (Nacalai). The lysates were immunoprecipitated with 10 μl of monoclonal antibodies (ascites) or 10 μl of polyclonal antibodies and blotted with anti-Myc (9E10, SantaCruz) or anti-FEZF1 antibodies. For direct detection (without immunoprecipitation), 10 μl of the lysates were used. The proteins were detected with HRP-conjugated goat anti-rabbit or anti-mouse IgG antibodies (TrueBlot, eBioscience) using a chemiluminescence system (Western Lightning; PerkinElmer Life Sciences).

**Statistics**

Student’s t-tests and ANOVA tests for comparisons involving two and more than two groups, respectively, were performed on the basis of GraphPad Prism 5.01 software.

**RESULTS**

**Upregulation of Hes5 and downregulation of neurogenin 2 in Fezf1- and Fezf2-deficient telencephalon**

To investigate molecular mechanisms by which Fezf1 and Fezf2 control forebrain development, we searched downstream genes of Fezf1 and Fezf2 by microarray analyses. We isolated rostral forebrains, which contain the telencephalon and the rostral part of the diencephalon, from embryonic day (E) 9.5, E10.5 and E12.5 wild-type control and Fezf1−/−Fezf2−/− embryos and compared their expression profiles. We picked up genes whose expression is up- or downregulated more than two-fold in the Fezf1−/−Fezf2−/− rostral forebrain (see Table S1 in the supplementary material). Many genes that are expressed in the caudal diencephalon (thalamus and pretectum) were upregulated in the Fezf1−/−Fezf2−/− rostral forebrain at E12.5. They include Tcf7l2, Dbx1, Ebf3, Brn3a (Pou4f1 – Mouse Genome Informatics) and Irx1/2/3/5 (see Table S1 in the supplementary material). The data are consistent with our previous report that the caudal diencephalon is expanded in Fezf1−/−Fezf2−/− mice (Hirata et al., 2006a) and validate our strategy toward gene-expression profiling of the rostral forebrain.

The microarray data suggest that Hes5 was upregulated at E9.5 and E10.5, and that the bHLH-type proneural gene neurogenin 2 was downregulated at E10.5 in the Fezf1−/−Fezf2−/− rostral forebrain in comparison with the wild type. To confirm this, we carried out in situ hybridization. Hes5 expression was indeed upregulated at both E9.5 and E10.5 in the telencephalon of Fezf1−/−Fezf2−/− mice (Fig. 1A,D,H), but not in Fezf1−/−Fezf2+/− and Fezf1+/−Fezf2−/− mice, in comparison with the control Fezf1−/−Fezf2+/− mice (Fig. 1A-C,E-G,I-K), suggesting that Fezf1 and Fezf2 function redundantly to repress Hes5 expression in the rostral forebrain. We also confirmed downregulation of neurogenin 2 expression at E10.5 in the telencephalon of Fezf1−/−Fezf2−/− mice in contrast to the control-mouse telencephalon (Fig. 1M,N). Simultaneous downregulation...
of neurogenin 2 and upregulation of Hes5 is consistent with the notion that neurogenin 2 is negatively regulated by Hes-family genes (Kageyama et al., 2005; Ross et al., 2003).

The redundant function of Fezf1 and Fezf2 suggests that they are co-expressed in the early dorsal telencephalon. Fezf2 was reported to be expressed in the VZ of the dorsal telencephalon as early as E10.5 (Chen et al., 2005b). We found that Fezf1 was also expressed in the VZ of the dorsal telencephalon at E10.5 and E11.5 (Fig. 1O-S). All of these data suggest that, during early neurogenesis in the dorsal telencephalon, both Fezf1 and Fezf2 are expressed in the VZ and might function to repress Hes5 expression and thereby derepress neurogenin 2 expression.

**FEZF1 and FEZF2 directly repress Hes5 promoter activity**

Both FEZF1 and FEZF2 contain an EH1 repressor motif (Hashimoto et al., 2000; Hirata et al., 2006b) and zebrafish Fezf2 is shown to function as a transcriptional repressor in some context (Levkowitz et al., 2003). These findings suggest that FEZF1 and FEZF2 directly repress the promoter activity of Hes5. To address this issue, we examined the effect of Fezf1 or Fezf2 expression on Hes5 promoter activity by a luciferase reporter assay in non-neural human embryonic kidney (HEK) 293 cells (Fig. 2A). Basal Hes5 promoter activity in HEK293 cells was reduced by expression of either Fezf1 or Fezf2 (Fig. 2B,C). When an expression vector of the basal promoter region responsible for Notch-mediated repression with luciferase reporters containing a 5′ truncated promoter. The 95 bp promoter reporter [pHes5(95)-luc; Fig. 2A], which contains a CBF1(RBPjκ)-binding site, responded to expression of N1IC, and the basal and NOTCH1-mediated promoter activity was repressed by Fezf1 or Fezf2 (Fig. 2D,E). Expression of Fezf1 or Fezf2 suppressed the NOTCH1-dependent Hes5 promoter activity (Fig. 2D,E).

We further investigated a Hes5 promoter region responsible for Fezf1- or Fezf2-mediated repression with luciferase reporters containing a 5′ truncated promoter. The 95 bp promoter reporter [pHes5(95)-luc; Fig. 2A], which contains a CBF1(RBPjκ)-binding site (CBS), responded to expression of N1IC, and the basal and NOTCH1-mediated promoter activity was repressed by Fezf1 or Fezf2 (Fig. 2F; data not shown for Fezf2; see also Fig. S1 in the supplementary material). By contrast, the 66 bp promoter reporter [pHes5(66)], which lacks the CBS site, neither responded to N1IC expression nor was repressed by Fezf1 or Fezf2 (Fig. 2G; data not shown for Fezf2). The data suggest that a region responsible for Fezf1- or Fezf2-mediated repression is located between –99 and 65 bp in the Hes5 promoter. This region contains a CBS, suggesting that FEZF1 and FEZF2 repress the Hes5 promoter by inhibiting a Notch signaling pathway. However, Fezf1 or Fezf2 could suppress
the Hes5 promoter in the absence of Notch signaling (Fig. 2B,C) but did not suppress basal or NOTCH1-mediated promoter activity of Hes1, which is another downstream target of Notch signaling (Nishimura et al., 1998; Ohtsuka et al., 1999), or Notch-mediated activation of an artificial promoter containing multiple CBSs (see Fig. S2 in the supplementary material). These findings indicate that FEZF1 and FEZF2, rather than inhibit Notch cytoplasmic signaling, specifically repress the Hes-family and neurogenin-family bHLH genes are negative and Hes5 promoter from wild-type forebrain cells (Fig. 2H,I). These data reveal that FEZF1 and FEZF2 bind to and directly repress the Hes5 promoter in vivo.

**Fezf1 and Fezf2 control neurogenesis during early corticogenesis**

Hes-family and neurogenin-family bHLH genes are negative and positive regulators of neurogenesis (Kageyama et al., 2005; Kageyama et al., 2007; Kageyama et al., 2008a; Ross et al., 2003). Thus, we assumed that upregulation of Hes5 and downregulation of neurogenin 2 in the telencephalon of Fezf1+/−Fezf2−/− mice might lead to abnormal neurogenesis and might explain cortical developmental defects observed in Fezf1+/−Fezf2−/− mice (Hirata et al., 2006a). To address this issue, we performed immunostaining with anti-TUJ1 and anti-Ki67 antibodies, which are markers for differentiated neurons and proliferating progenitor cells (Fig. 3).

**Fezf1 and Fezf2 control generation of early-born telencephalic neurons**

The earliest born neurons in the dorsal telencephalon appear around E10.5-E11.5 in mice and form the pre-plate, which is later split into two regions: the more superficial marginal zone (MZ) and the deeply located subplate (SP) (Aboitiz et al., 2005; Molyneaux et al., 2007). The marginal zone (layer I) contains Cajal-Retzius (CR) cells, which are derived from three regions: the caudomedial cortical hem (Meyer et al., 2002; Takiguchi-Hayashi et al., 2004; Yoshida et al., 2006), the pallial-subpallial boundary and the septum (Bielle et al., 2006a). To address this issue, we performed immunostaining with anti-TUJ1 antibody of E10.5 control (Fig. 3A,E), Fezf1−/−Fezf2−/− (Fig. 3B,F), Fezf1−/−Fezf2−/− (Fig. 3C,G), and Fezf1+/−Fezf2−/− (Fig. 3D,H) forebrains. Sagittal sections with rostral to the left. E-H are higher-magnification views of the boxes in A-D. Note that TUJ1+ neurons were reduced in caudal telencephalon (arrows in H, n=5).

**Fig. 3. Reduced neurogenesis and increased proliferation in Fezf1−/−Fezf2−/− telencephalon.**

(A-H) Immunostaining with the anti-neuron-specific βIII tubulin antibody (TUJ1) of E10.5 control (Fezf1+/−Fezf2+/−, A,E), Fezf1−/−Fezf2−/− (B,F), Fezf1−/−Fezf2−/− (C,G) and Fezf1+/−Fezf2−/− (D,H) forebrains. Sagittal sections with rostral to the left. E-H are higher-magnification views of the boxes in A-D. Note that TUJ1+ neurons were reduced in caudal telencephalon (arrows in H, n=5).

(LJ) Immunostaining with the anti-Ki67 antibody of the E10.5 control (I) and Fezf1−/−Fezf2−/− (J) forebrain. Note that Ki67+ proliferating cells were increased in Fezf1−/−Fezf2−/− telencephalon (n=4). (K,L) BrdU incorporation at E10.5. Control (Fezf1+/−Fezf2+/−, K) and Fezf1−/−Fezf2−/− (L) embryos were labeled with bromodeoxyuridine (BrdU) for 5 minutes and proliferating cells were analyzed by immunostaining with anti-BrdU antibody. (M-O) Number of TUJ1+ (M, n=5), Ki67+ (N, n=4) or BrdU+ (O, n=3) telencephalic cells in a comparable sagittal section for each genotype was counted. Data are represented as mean ± standard deviation. TUJ1+ cells were reduced (*, P<0.01, Student’s t-test), Ki67+ cells were increased (**, P<0.01) and BrdU+ cells were increased (***, P<0.01) in Fezf1−/−Fezf2−/− telencephalon in contrast to the control. Scale bars: 200 μm in A, 100 μm in E-I, K,. Magnifications of A and B-D, E and F-H, I and J, and K and L are the same.
2005). As neurogenesis was impaired in Fezf1+/Fezf2+/ telencephalon at E10.5, the formation of early-born telencephalic neurons might be affected in these mice. With this in mind, we examined expression of markers of the SP neurons and CR cells. Chondroitin sulfate proteoglycan (CSPG) normally accumulated in the pre-plate and was later concentrated in the SP region (Sheppard et al., 1991; Sheppard and Pearlman, 1997) (E16.5; Fig. 4A), and NURR1 was also a specific marker for the SP neurons (Arimatsu et al., 2003; Hoerder-Suabedissen et al., 2009) (Fig. 4C). In Fezf1+/Fezf2+/ telencephalon, the CSPG and NURR1 signals were strongly reduced (Fig. 4B,D). Furthermore, cells that had incorporated BrdU at E11.5 were located in the SP region in the control at E16.5 (Sheppard and Pearlman, 1997) (Fig. 4K) but were strongly reduced in Fezf1+/Fezf2−/− mice (Fig. 4L). These data suggest that differentiation of the SP neurons was affected in Fezf2-deficient mice (Hirata et al., 2004; Molyneaux et al., 2005). By contrast, from our data, the present study indicate that Fezf1+/Fezf2+/ mice had defects in generation of the SP neuron.

CR cells were stained with the anti-calretinin antibody at E11.5 (del Rio et al., 1995) or a reelin probe at post-natal day 0 (P0) in the control mice (Alcantara et al., 1998) (Fig. 4E,G), but calretinin+ or reelin+ CR cells were strongly reduced in Fezf1+/Fezf2−/− mice (Fig. 4F,H). An early marker for CR neurons derived from the cortical hem is p73 (Meyer et al., 2004; Meyer et al., 2002), which was detected in the MZ of the control mice at E13.5 (Fig. 4I). p73-positive CR cells were strongly reduced in the telencephalon of Fezf1+/Fezf2−/− mice (Fig. 4J). These findings indicate that Fezf1 and Fezf2 are involved in the generation of the early-born cortical neurons.

We further examined cortical-layer markers (Fig. 5). There were no significant differences in numbers of TBR1+ layer-VI neurons between Fezf1+/Fezf2−/− and Fezf1−/Fezf2−/− mice (Fig. 5D; Fezf1+/Fezf2−/−, 98±6.98 and Fezf1−/Fezf2−/−, 99±9.68 cells/field). CTIP2+ layer-V neurons were absent in both Fezf1+/Fezf2−/− and Fezf1−/Fezf2−/− mice (Fig. 5C). This phenotype was reported for Fezf2-deficient mice (Chen et al., 2005a; Molyneaux et al., 2005). In addition, Rorb+ positive-layer-IV neurons were reduced in the rostral part of the cortex of Fezf1+/Fezf2−/−, but not of Fezf1−/Fezf2−/−, mice (Fig. 5B). CUX1+ layer-II-IV neurons and SATB2+ corticocortical neurons (Alcamo et al., 2008; Britanova et al., 2008) were not significantly affected in Fezf1+/Fezf2−/− mice (Fig. 5A; data not shown for SATB2). Therefore, Fezf1 and Fezf2 redundantly function to generate the early-born neurons and the layer-IV neurons, but not the late-born neurons (see Fig. S4 in the supplementary material).

**Defects in the formation of intermediate progenitors in Fezf1- and Fezf2-deficient telencephalon**

Cortical neurons are differentiated from the neural stem cells in the VZ, and are also generated from TBR2+ intermediate progenitors that are derived from the neural stem cells in the VZ (Arnold et al., 2008; Kowalczyk et al., 2009; Sessa et al., 2008). Notch signaling has been shown to be involved in the generation of the intermediate progenitors (Mizutani et al., 2007; Yoon et al., 2008). In an attempt to reveal the role of Fezf1 and Fezf2 in the formation of the intermediate progenitors, we analyzed the neural stem cells and the intermediate progenitors in the mutant telencephalon by immunostaining with anti-PAX6 and anti-TBR2 antibodies (Englund et al., 2005; Gotz et al., 1998). PAX6+ neuronal stem cells were not strongly reduced in Fezf1+/Fezf2−/− mice from E11.5 through E13.5 (Fig. 5E-J). By contrast, the TBR2+ intermediate progenitors were decreased in the telencephalon of Fezf1+/Fezf2−/− mice in comparison with the control mice at these stages (Fig. 5K-R). These data suggest that differentiation of the neural stem cells into the intermediate progenitors was impaired in the Fezf1+/Fezf2−/− dorsal telencephalon.

**Loss of the Hes5 gene suppresses defects in neurogenesis in Fezf1- and Fezf2-deficient telencephalon**

In light of the above observations, the neurogenesis defects observed in the Fezf1+/Fezf2−/− cortex might stem from upregulation of Hes5 expression. To genetically prove this hypothesis, we carried out an epistatic analysis by crossing Fezf1- and Fezf2-deficient mice and Hes5-deficient mice. TUJ1+ neurons and neurogenin 2+ intermediate progenitors and neurons were not affected in the Fezf1+/Fezf2−/−/Hes5−/− telencephalon at E10.5 (Fig. 3A; Fig. 6A,D,G). This is consistent with the previous report that Hes5 deficiency induces upregulation of Hes1, which compensates for loss of Hes5 (Hatakeyama et al., 2004). TUJ1+ or neurogenin 2+ cells were reduced in the Fezf1+/Fezf2−/−/Hes5−/− telencephalon (Fig. 6B,E,H), whereas they were recovered in the
Regulation of forebrain neurogenesis by FEZF1 and FEZF2

DISCUSSION

Role of Fezf1 and Fezf2 in differentiation of neural stem cells

An important question about neural development is how the differentiation of neural stem cells is precisely controlled in the forebrain. Asymmetric cell division of neural stem cells is thought to contribute to the differentiation of neural stem cells (radial glial cells) into either neurons or intermediate progenitors (Gotz and Huttner, 2005). Recent reports suggest that the orientation of stem cell division in the VZ might not directly control which of the two asymmetrically divided cells becomes a stem cell and which of the two becomes a differentiated cell (Konno et al., 2008; Morin et al., 2007). Although asymmetric centrosome inheritance during the asymmetric cell divisions was reported to play a role in the maintenance of the neural stem cells (Wang et al., 2009), it is not clear what factors determine cell fate. It is known that oscillation of Hes1 and neurogenin 2 expression in the telencephalic VZ plays an important role in maintenance of the neural stem cells and that stabilization of neurogenin 2 expression supports differentiation of the neural stem cells (Kageyama et al., 2008b; Shimojo et al., 2008). However, it is still not understood what factor(s) control differentiation. These reports imply that, besides asymmetric distribution of cell-fate determinants, extrinsic and intrinsic factors might bias the neural stem cells toward differentiation. Notch signaling plays an essential role in maintenance of the neural stem cells (Gaiano and Fishell, 2002; Louvi and Artavanis-Tsakonas, 2006; Yoon and Gaiano, 2005). Thus, regulators of Notch signaling and its downstream effectors might be involved in the decision as to whether to be a stem cell or a differentiated cell. In this report, we demonstrate that Fezf1 and Fezf2, which are expressed in the neural stem cells at the beginning of mouse cortical development (Chen et al., 2005b; Hirata et al., 2006b; Hirata et al., 2004) (Fig. 1O-5), inhibit the expression of the Notch effector Hes5 and

Fezf1+/Fezf2+/Hes5+/ telencephalon (Fig. 6C,F,I,J). These data indicate that the Hes5 deficiency suppressed neurogenesis defects in the Fezf1–/–Fezf2–/– telencephalon.

We then sought to analyze the phenotypes of the Fezf1–/–Fezf2+/–/Hes5+ telencephalon in more detail. As reported previously (Hirata et al., 2006a), Fezf1–/–Fezf2–/– mice showed defects in rostro-caudal polarity of the forebrain: loss of olfactory bulbs and prethalamus, and reduction in thalamus (Fig. 7A,B). These defects were not recovered in the Fezf1–/–Fezf2–/–Hes5–/– mice (Fig. 7C). The generation of TBR2+ layer-V neurons was also not recovered in the Fezf1–/–Fezf2–/–Hes5–/– forebrains (Fig. 7J-L). However, the generation of calretinin+ CR cells, CSPG-expressing subplate neurons, Rorb-positive layer-IV neurons and TBR2+ intermediate progenitors were recovered in the Fezf1–/–Fezf2–/–Hes5–/– telencephalons in comparison with the telencephalic VZ (Fig. 6C,F,I,J). These data indicate that the Hes5 deficiency suppressed neurogenesis defects in the Fezf1–/–Fezf2–/– telencephalon.

**DISCUSSION**

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FEZF1 and FEZF2 directly repress the Hes5 promoter

Expression of Fezf1 or Fezf2 repressed both NOTCH1-dependent and NOTCH1-independent Hes5 promoter activity, but did not repress the Hes1 promoter or the artificial CBS-dependent promoter (Fig. 2; see Fig. S1 and S2 in the supplementary material). Hes1 expression was not upregulated in the telencephalon of Fezf1+/Fezf2+/– mice (data not shown). Furthermore, FEZF1 and FEZF2 bound to the Hes5 promoter in vivo in the mouse forebrain (Fig. 2). All of these data indicate that FEZF1 and FEZF2, rather than inhibit Notch cytoplasmic signaling, specifically bind to and directly repress the Hes5 promoter. FEZF1 and FEZF2 have an EH1 repressor motif (Hashimoto et al., 2000; Hirata et al., 2006b; Shimizu and Hibi, 2009). Our data support the assertion that FEZF1 and FEZF2 function as transcriptional repressors and repress the Hes5 promoter at least during early cortical development. Hes5 deficiency suppressed neurogenesis defects in Fezf1–/–Fezf2–/– telencephalon (Figs 6, 7), supporting the hypothesis that Fezf1 and Fezf2 suppress the expression of Hes5 and thereby control differentiation of the neural stem cells.

FEZF1 and FEZF2 repress only Hes5. Hes1 and Hes5 function redundantly in the maintenance of neural stem cells in the mouse central nervous system (Hatakeyama et al., 2004), whereas only Hes1 is reported to exhibit oscillatory expression in the neural stem cells (Shimojo et al., 2008), suggesting that Hes1 and Hes5 might have distinct roles in neurogenesis. Previous research has revealed that oscillation of Hes1 is involved in the maintenance of neural stem cells (Kageyama et al., 2008b; Shimojo et al., 2008) and, in the current study, we speculate that Hes5 plays a different role in neurogenesis; specifically, we propose that Hes5, in contrast to Hes1, sets up the overall expression levels of Hes genes and neurogenin 2 in the forebrain. Once Fezf1 and Fezf2 expression exceeds a threshold, FEZF1 and FEZF2 might repress Hes5 expression, stabilize neurogenin 2 expression and thereby bias the neural stem cells toward differentiation.

It has recently been reported that the Drosophila homolog of Fezf1/2 (dFezf or Earmuff) restricts the developmental potential of intermediate progenitors by negatively regulating Notch signaling (Weng et al., 2010). Although the mechanism by which dFezf represses Notch signaling is unknown, Fezf family genes function to negatively regulate Notch signaling in both vertebrates and invertebrates.

**Fezf1- and Fezf2-mediated cortical development**

Fezf1 and Fezf2 function to repress the caudal diencephalon fate and their function is involved in proper rostro-caudal patterning of the forebrain (Hirata et al., 2006a; Jeong et al., 2007). The prospective telencephalon domain is already smaller in Fezf1/2–/– embryos than in the wild type at E9.5, before neurogenesis is initiated in the telencephalon (Hirata et al., 2006a) (Fig. 1A,D). Therefore, the defect in rostro-caudal patterning is attributable to reduction of the telencephalon domain. In addition, Fezf2–/– or Fezf1–/– Fezf2–/– telencephalon lacks layer-V subcerebral projection neurons (Chen et al., 2005a; Molyneaux et al., 2005) (Fig. 5). Hes5 deficiency did not suppress the defects in rostro-caudal patterning of the forebrain or specification of layer-V neurons in Fezf1+/–Fezf2–/– forebrains (Fig. 7). Therefore, Fezf1/2-mediated downregulation of Hes5 is not involved in the rostro-caudal patterning of the forebrain and the specification of layer-V neurons (Fig. 7S). Fezf1 and/or Fezf2 probably control genes other than Hes5 to elicit these functions.

**Fig. 6. Loss of Hes5 suppresses neurogenesis defects in Fezf1+/–Fezf2+/– telencephalons.** (A–I) Immunostaining with anti-TUJ1 (A–C) and anti-neurogenin 2 antibodies (D–I) of E10.5 Fezf1+/–Fezf2+/–Hes5–/– (A,D,G), Fezf1+/–Fezf2+/–Hes5+/+ (B,E,H) and Fezf1–/–Fezf2–/–Hes5+/– (C,F,I) telencephalons. Sagittal sections with rostral to the left. G–I are higher-magnification views of the boxes in D–F. TUJ1+ neurons (arrows) and neurogenin 2+ cells (arrowheads) in the telencephalon were increased in Fezf1+/–Fezf2+/–Hes5+/– embryos (C,F) in comparison with Fezf1+/–Fezf2+/–Hes5+/+ embryos (B,E). (J) Number of TUJ1+ telencephalic cells. TUJ1+ cells in a comparable sagittal section were counted for each genotype. Data are represented as mean ± standard deviation. *, P<0.001 (n=3, Student’s t-test). Scale bars: 100 μm.

Fezf1–/–Fezf2–/–Hes5–/– telencephalon exhibited reduced formation of the telencephalon. This finding is consistent with a reduction of differentiated (TUJ1+) neurons in the Fezf1+/–Fezf2–/– telencephalon at E10.5 (Fig. 3), when SP neurons and CR cells are born in the VZ. Hes5 deficiency rescued neurogenin 2
expression at E10.5 and the generation of SP neurons and CR cells (Figs 6, 7) in Fezf1−/−Fezf2−/− telencephalon, indicating that Fezf1- and/or Fezf2-mediated repression of Hes5 plays an important role in the generation of these early-born cortical neurons (Fig. 7S). It is reported that formation of CR cells in the choroid plexus region, near the cortical hem, is controlled by a Hes-neurogenin cascade but that the Notch-signaling-mediated lateral inhibition is not involved in regulation of the Hes-neurogenin cascade in the CR cell development (Imayoshi et al., 2008). Fezf1 and Fezf2 are expressed in the dorsomedial telencephalon (Hirata et al., 2006b; Hirata et al., 2004). Our data suggest that Fezf1 and Fezf2 might control the development of CR cells by regulating Hes5 and neurogenin 2 expression in the choroid plexus domain.

Fezf1−/−Fezf2−/− telencephalon had normal upper-layer (layer II, III) neurons but displayed a reduction of layer-IV neurons (Fig. 5). There are two plausible explanations for this finding: Fezf1 and Fezf2 regulate the specification of layer-IV neurons or Fezf1 and Fezf2 control the generation of layer-IV neurons (Chen et al., 2005a; Chen et al., 2005b; Molyneaux et al., 2005). Neither Fezf1 nor Fezf2 is expressed in differentiated layer-IV neurons (Chen et al., 2005a; Chen et al., 2005b; Hirata et al., 2006b; Inoue et al., 2004; Molyneaux et al., 2005), but both are expressed in their progenitors (neural stem cells or intermediate progenitors). Layer-IV neurons are normally born (differentiated) from E13.5 through E15.5 (Molyneaux et al., 2007). Birthdate analysis indicated that Fezf1−/−Fezf2−/− telencephalon contained a reduced number of Rorb-positive neurons that were born at E13.5 (data not shown), suggesting that Fezf1 and Fezf2 control the generation of layer-IV neurons either from the neural stem cells or the intermediate progenitors. In Fezf1−/−Fezf2−/− telencephalon, differentiation of the neural stem cells into the TBR2+ intermediate progenitors was impaired (Fig. 5). Thr2 is an essential regulator of the intermediate progenitors (Arnold et al., 2008; Sessa et al., 2008) and is directly regulated by neurogenin 2 (Ochiai et al., 2009). These data suggest that the gene cascade Fezf1/Fezf2 → Hes5 → neurogenin 2 regulates the expression of Thr2 and controls differentiation of the neural stem cells into the intermediate progenitors. The reduction of the TBR2+ intermediate progenitors in the Fezf1−/−Fezf2−/− telencephalon might contribute to a reduction of layer-IV neurons. Consistent with this idea, Hes5 deficiency rescued the development of TBR2+ intermediate progenitors as well as layer-IV neurons in Fezf1−/−Fezf2−/− telencephalon (Fig. 7). It is reported that TBR1+ layer-VI neurons are increased in Fezf2−/− telencephalon (Molyneaux et al., 2005), suggesting the trans fate of layer-V to layer-VI neurons. However, they were not increased in Fezf1−/−Fezf2−/− telencephalon (Fig. 5), implying that the gene cascade Fezf1/Fezf2 → Hes5 → neurogenin 2 controls the generation of layer-VI neurons. Future studies will clarify these issues.

In summary, FEZF1 and FEZF2 are transcriptional repressors that repress Hes5 expression and subsequently activate neurogenin expression. The Fezf1/Fezf2 → Hes5 → neurogenin 2 gene cascade controls differentiation of the neural stem cells into neurons or intermediate progenitors and contributes to the generation of a variety of neurons in the forebrain.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References


Table S1. Microarray data

<table>
<thead>
<tr>
<th>Expression level (ratio to control)</th>
<th>Abbreviation of gene name</th>
<th>Gene name</th>
<th>Accession number</th>
</tr>
</thead>
</table>
| (A) Genes upregulated in Fezf1
  –/–Fezf2
  –/– rostral forebrain at E9.5 | Lhx8 | LIM homeobox protein 8 | D49658.1 |
| 0.258314 | Hes5 | hairy and enhancer of split 5 | BBS61515 |
| 0.278952 | 3830406C13Rik | RIKEN cDNA 3830406C13 gene | BB763097 |
| 0.37488 | Crym | crystallin | NM_016669.1 |
| 0.380651 | LOC382156 | similar to F-box- and WD40-repeat-containing protein | BM229128 |
| 0.400377 | Dchs1 | dachsous 1 | AK014167.1 |
| 0.408742 | Igf1 | insulin-like growth factor 1 | BG075165 |
| 0.4137254 | Synpo | synaptopodin | BB426294 |
| 0.43194 | Insm1 | insulinoma-associated 1 | BB468410 |
| (B) Genes downregulated in Fezf1
  –/–Fezf2
  –/– rostral forebrain at E9.5 | Psmd6 | proteasome 26S subunit, non-ATPase, 6 | BC006869.1 |
| 7.842105 | Cnnm3 | Cyclin M3 | B236001 |
| 2.625724 | 3110069A13Rik | RIKEN cDNA 3110069A13 gene | AK014242.1 |
| 2.501182 | Strbp | Spermatid perinuclear RNA binding protein | W488249 |
| 2.126619 | Wbcsr1 | Williams-Beuren syndrome chromosome region 1 homolog | AU018978 |
| 2.104 | Lhx5 | LIM homeobox protein 5 | NM_008499.1 |
| 2.08 | Plekha2 | pleckstrin homology domain-containing, family A member 2 | BC010215.1 |
| 2.039887 | Tcf3 | transcription factor 3 | BE994269 |
| (C) Genes upregulated in Fezf1
  –/–Fezf2
  –/– rostral forebrain at E10.5 | 3830406C13Rik | RIKEN cDNA 3830406C13 gene | BB763097 |
| 0.305256065 | Cnnm3 | Cyclin M3 | B236001 |
| 0.307469181 | Arnt | aryl hydrocarbon receptor nuclear translocator | AV233793 |
| 0.307507508 | Smarcb1 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1 | AV310148 |
| 0.315825565 | Usp7 | ubiquitin specific peptidase 7 | ARS4816 |
| 0.326007326 | Aldhl1a3 | aldehyde dehydrogenase family 1, subfamily A3 | NM_053080.1 |
| 0.327475103 | 1810014B01Rik | RIKEN cDNA 1810014B01 gene | AK017634.1 |
| 0.332034929 | Rpl27a | ribosomal protein L27a | BG141806 |
| 0.33595711 | Hes5 | hairy and enhancer of split 5 | BBS61515 |
| 0.34977683 | Tmem69 | transmembrane protein 69 | BB24992 |
| (D) Genes downregulated in Fezf1
  –/–Fezf2
  –/– rostral forebrain at E10.5 | Psmd6 | proteasome 26S subunit, non-ATPase, 6 | BC006869.1 |
| 3.470720721 | Arf5 | ADP-ribosylation factor 5 | NM_007480.1 |
| 2.507236684 | Neurog2 | neurogenin 2 | NM_009718.1 |
| 2.348773349 | Ptprr1 | protein tyrosine phosphatase, receptor type Z, polypeptide 1 | BC002298.1 |
| 2.009538951 | 2610017I09Rik | RIKEN cDNA 2610017I09 gene | AW045679 |
| (E) Genes upregulated in Fezf1
  –/–Fezf2
  –/– rostral forebrain at E12.5 | Dlx1 | developing brain homeobox 1 | AI426026 |
| 0.25706371 | Irx5 | iriroid related homeobox 5 | NM_018826.1 |
| 0.28193535 | Tcftf12 | transcription factor 1-like 2, T-cell specific, HMG-box | AF112981.1 |
| 0.28193535 | Irx2 | iriroid related homeobox 2 | AF295691.1 |
| 0.30982019 | Shox2 | short stature homeobox 2 | AV32957 |
| 0.32046332 | Irx3 | iriroid related homeobox 3 | NM_008393.1 |
| 0.32046332 | Evl1 | ectropic viral integration site 1 | AI467591 |
| 0.35974026 | Ebf3 | early B-cell factor 3 | AK014058.1 |
| 0.38888889 | Irx1 | iriroid related homeobox 1 | AF165984.1 |
| 0.39520028 | Fgfn15 | fibroblast growth factor 15 | NM_008003.1 |

List of genes, the expression of which was downregulated or upregulated in Fezf1
  –/–Fezf2
  –/– rostral forebrains at E9.5, E10.5 or E12.5 in comparison with wild-type rostral forebrains. The genes were selected according to the following criteria: (1) they display two-fold or more change in signal value between wild-type and Fezf1
  –/–Fezf2
  –/– rostral forebrains, (2) the signal intensities of the higher value should be greater than 100, and (3) the top-ten genes are shown in tables and raw data have been deposited in Gene Expression Omnibus (GEO) under the accession number GSE21156.