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The Lewis X-related α1,3-Fucosyltransferase, Fut10, Is Required for the Maintenance of Stem Cell Populations*

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Background: The Lewis X carbohydrate antigen is abundantly expressed in several stem cell populations.
Results: Fut10 is responsible for the synthesis of unique types of Lewis X on *N*-glycans and alters stem cell functions.
Conclusion: Fut10 are required for the maintenance of stem cells and neural development.
Significance: Learning Fut10 function is crucial for understanding how stemness is maintained.

Lewis X (Le^X, Galβ1–4(Fucα1–3)GlcNAc) is a carbohydrate epitope that is present at the nonreducing terminus of sugar chains of glycoproteins and glycolipids, and is abundantly expressed in several stem cell populations. Le^X antigen can be used in conjunction with fluorescence-activated cell sorting to isolate neurosphere-forming neural stem cells (NSCs) from embryonic mouse brains. However, its function in the maintenance and differentiation of stem cells remains largely unknown. In this study, we examined mice deficient for fucosyltransferase 9 (*Fut9*), which is thought to synthesize most, if not all, of the Le^X moieties in the brain. We found that the number of NSCs was increased in the brain of *Fut9*$^{-/-}$ embryos, suggesting that Fut9-synthesized Le^X is dispensable for the maintenance of NSCs. Another α1,3-fucosyltransferase gene, fucosyltransferase 10 (*Fut10*), is expressed in the ventricular zone of the embryonic brain. Overexpression of *Fut10* enhanced the self-renewal of NSCs. Conversely, suppression of *Fut10* expression induced the differentiation of NSCs and embryonic stem cells. In addition, knockdown of *Fut10* expression in the cortical ventricular zone of the embryonic brain by *in utero* electroporation of *Fut10*-miRNAs impaired the radial migration of neural precursor cells. Our data suggest that Fut10 is involved in a unique α1,3-fucosyltransferase activity with stringent substrate specificity, and that this activity is required to maintain stem cells in an undifferentiated state.

The Lewis X (Le^X)[2] (Galβ1–4(Fucα1–3)GlcNAc) carbohydrate epitope, which is present in antigens recognized by antibodies for stage-specific embryonic antigen-1 or leukocyte cluster of differentiation 15 (CD15), is a marker of the inner cell mass of the mouse blastocyst and its derivative, embryonic stem (ES) cells. Le^X expression is progressively down-regulated in the early mouse embryo as development proceeds, and in differentiating ES cells *in vitro* (1–4). Le^X is also expressed by neural precursor cells in the germinal zone of the embryonic mouse telencephalon (5–7). Neural stem cells (NSCs) and their progeny, neural progenitor cells, are collectively referred to be neural precursor cells and are present not only in embryonic brains but also in the subependyma of adult brains. When cultured in a colony-forming neurosphere assay, Le^X-positive cells from the adult mouse subependyma generated more neurospheres than Le^X-negative cells (8), suggesting that neurosphere-forming NSCs in the adult brain abundantly express Le^X. Although the expression of Le^X is considered to be tightly associated with the stemness of various types of stem cells, the functional roles of Le^X in neural development and the maintenance of stem cell pluripotency remain to be investigated.

Le^X is synthesized by adding fucose with an α1,3-linkage to the *N*-acetylglucosamine (GlcNAc) of the *N*-acetyllactosamine backbone at the nonreducing terminus of sugar chains (9). This reaction is catalyzed by α1,3-fucosyltransferases. Three active α1,3-fucosyltransferases, Fut4, Fut7, and Fut9, exist in rodents and are well characterized. *Fut4* and *Fut9* are expressed in the brain. Fut9 is thought to synthesize most, if not all, of the Le^X moieties on glycoproteins and glycolipids in the brain (10). Despite the expected roles of Fut9-synthesized Le^X in neural development, *Fut9*-deficient mice exhibit minor alterations in brain morphology and subtle behavioral abnormalities (11, 12). Recently, two novel putative α1,3-fucosyltransferases, Fut10 and Fut11, have been identified in mice and humans on the basis of structural homology to other α1,3-fucosyltransferases (13). Neither Fut10 nor Fut11 increased the amount of Le^X when overexpressed in cultured cells or exhibited enzymatic

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[2] The abbreviations used are: Le^X, Lewis X; ES, embryonic stem; NSC, neural stem cells; Fut, fucosyltransferase; VZ, ventricular zone; GE, ganglionic eminence; DIG, digoxygenin; PA, fluorophore 2-aminopyridine; SVZ, subventricular zone; LTA, *L. tetragonolobus* agglutinin; Pax6, paired box 6; Tbr2, eomesodermin homolog (*X. laevis*); E-PHA, erythroagglutinating phytohemagglutinin lectin from *Phaseolus vulgaris*; miRNA, microRNA; E, embryonic day.

activities with conventional oligosaccharide substrates (14, 15). However, Fut10 was reported to transfer fucose to GlcNAc at the innermost core position of *N*-glycans (16), in contrast to the conventional α1,3-fucosyltransferases that add fucose to GlcNAc at the nonreducing end. In this study, we present evidence that Fut10 increases the amount of Le^X structures on bisecting *N*-glycans of glycoproteins and plays an important role in the self-renewal of stem cells.

EXPERIMENTAL PROCEDURES

Mice and Genotyping—CD1 (ICR) mice (SLC, Japan) were used to isolate NSCs and for *in utero* electroporation except when specifically described. Noon of the plugged date was considered as embryonic day (E) 0.5. Mice heterozygous for *Fut9* were maintained in the C57/BL6 background. PCR primers used for genotyping *Fut9* mutants were: fut9 F300 (5′-ACAACAAATCCCATGCGGTC-3′), CB341R (5′-CATGTGATTCCCAAAACCG-3′), and fut9 KO R3 (5′-GCCATGATGGATACTTTCT-3′). DNA was obtained from the tip of the tail and DNA was amplified for 35 cycles in a thermal cycler (PerkinElmer Life Sciences) with denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 45 s. All experiments were carried out with the permission of the institutional Animal Research Committee.

Cell Culture and Fluorescence-activated Cell Sorting—The protocol used to generate neurospheres *in vitro* has been described (17, 18). Briefly, cells from the embryonic cortex or medial ganglionic eminence (GE) were mechanically triturated in serum-free media and cultured in the presence of 10 ng/ml of FGF-2 and 2 μg/ml of heparin (Sigma). After 7 days *in vitro*, the number of floating sphere colonies (neurospheres) possessing a diameter >0.08 mm was counted. Stem cell self-renewal was assessed by counting the number of new neurospheres that arose after primary single neurospheres were mechanically dissociated into single cells, and cultured for 6 days in 0.2 ml of serum-free media containing 20 ng/ml of EGF, FGF2, and heparin.

Neuro2a and COS1 cells were maintained in 10% FBS/DMEM culture medium. R1 ES cells (19) were grown on gelatin-coated plates and maintained in 15% FBS/DMEM medium containing 1000 units/ml of LIF (Millipore), 0.8 μM of the MEK inhibitor PD0325901, and 1.0 μM of the GSK3 inhibitor CT99021 (both from Axon Medchem BV). Cells were transfected with expression plasmids by using Lipofectamine 2000 transfection reagent (Invitrogen). A flow cytometer (FACS Aria Diva; BD Biosciences) equipped with an argon ion laser (488 nm, 200 milliwatt) was used to collect GFP^+ cells.

Plasmids and Retrovirus Preparation—Full-length *Fut10* gene was amplified by RT-PCR from E14.5 mouse brain cDNA, sequenced, and subcloned into the pCX expression vector or pMXIE retroviral vector to generate retrovirus, which expresses *Fut10* and GFP as described (20). The BLOCK-iT™ PolII miR RNAi Expression Vector Kit (Invitrogen) was used for constructing miRNA expression vector. Three 21-nucleotide sequences, two (#1, 5′-GAGAGCTGGCGAGCTTCATTA-3′ and #2, 5′-CGGCTTCTGACAGCTCTCAAT-3′) from the open reading frame and one (#3, 5′-TACTGGCTGTCTTCTGTGGAA-3′) from the 3′ untranslated region of *Fut10*, were selected. DNA fragments containing each pair of sense and antisense together with loop sequences were subcloned into the pcDNA6.2-GW/EmGFP or pMXIE vectors to generate *Fut10* miRNA expression or retrovirus plasmids. A packaging cell line, ΦNX-Eco (ATCC 3443), was transiently transfected with the retrovirus plasmids using FuGENE 6 (Roche Applied Science) and cultured for 3 days at 30 °C. The supernatant was filtered, centrifuged to yield a titer more than 2×10^7 cfu/ml, and aliquoted at −80 °C until use. Cells were spin-infected at 1,000 rpm with 2×10^5 of retrovirus in the presence of 5 μg/ml of Polybrene (Sigma) at room temperature for 1.5 h.

Histochemistry and in Situ Hybridization—Brains were sectioned along the coronal plane into 14-μm thick sections, and then incubated with biotinylated lotus lectin (1:200; Vector Laboratories) at 4 °C overnight. Alternatively, cryosections were incubated with anti-Le^X mouse monoclonal antibody (Clone MMA, Lab Vision Corporation), followed by a biotinylated goat anti-mouse IgM antibody (Vector Laboratories) at 4 °C overnight. The sections were subjected to a Tyramide Signal Amplification (TSA™) biotin system (PerkinElmer Life Sciences). Le^X antigens were detected and visualized by incubating the sections with an avidin and biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) and a peroxidase substrate (3,3′-diaminobenzidine; Dojindo).

Digoxygenin (DIG)-labeled single-stranded riboprobes directed against the entire coding region of the *Fut4*, *Fut7*, *Fut9*, or *Fut10* genes were synthesized using the DIG RNA labeling mixture (Roche Applied Science). *In situ* hybridization was performed as described previously (21). Briefly, cryosections were treated with proteinase K (1 μg/ml, 60–90 min, room temperature), and then hybridized at 65 °C overnight in hybridization buffer with sense or antisense DIG-labeled RNA probes. DIG-labeled RNA probes were detected and visualized by incubating the sections with alkaline phosphatase-conjugated anti-DIG antibody (1:2000; Roche) and an alkaline phosphatase substrate (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate).

RT-PCR—Total RNA isolation, cDNA synthesis, and RT-PCR analysis were carried out as described (20). The sense and antisense primers used were summarized in Table 1.

N-Glycan Analysis—*N*-Glycans of glycoproteins from cells or tissues were analyzed as described previously (22, 23). Briefly, samples were precipitated with acetone, lyophilized, and hydrazinolized to release *N*-glycans. For purification and in-column *N*-acetylation, the hydrazinolized sample solution was loaded onto a graphite carbon column (GL-Pak Carbograph, GL Science), washed with 50 mM ammonium acetate buffer (pH 7.0), and then eluted with 50 mM triethylamine acetate buffer (pH 7.0) in 60% acetonitrile. The reducing ends of liberated *N*-glycans were tagged with the fluorophore 2-aminopyridine (PA) and the PA-tagged *N*-glycans were purified using a cellulose column (Takara Bio).

PA-sugar chains were separated by high-performance liquid chromatography (HPLC) as reported previously (22, 23). Briefly, PA-tagged *N*-glycans were applied onto a Microgranular DE52-packed column (GE Healthcare) to obtain neutral *N*-glycans. The neutral *N*-glycans were size fractionated by normal phase HPLC with a Shodex Asahipak NH2P-50 4E column

TABLE 1
PCR primers

Gene	Primer	Sequence
Fut4	Sense	5′-GACCTGGTGAAGGAACTCCA-3′
	Antisense	5′-AGGCACGAAGCGCTCATAGT-3′
Fut7	Sense	5′-TGTGTTCGGTCGCGCCAGCG-3′
	Antisense	5′-TCAAGCCTGGAACCAGCTTT-3′
Fut9	Sense	5′-ACAACAAATCCCATGCGGTC-3′
	Antisense	5′-GTGGGAATCAGATTTTTATC-3′
Fut10	Sense	5′-CAGGGTGTGGGCAAACAGTAG-3′
	Antisense	5′-GAATTAAGCTTACTCTGTCAGT-3′
Oct3/4	Sense	5′-CGTTCTCTTTGGAAAGGTGTTC-3′
	Antisense	5′-TACTCGAACCACATCCTTCTCT-3′
Nanog	Sense	5′-CACTGACATGAGTGTGGGTCTTC-3′
	Antisense	5′-GGAGGAGAGTTCTTGCATCTGCT-3′
Cdx2	Sense	5′-CAAGGAGAGGAAAATCAAGAAGAAG-3′
	Antisense	5′-CAAGGAGGTCACAGGACTCAAG-3′
Fgf5	Sense	5′-AAAGTCAATGGCTCCCACGAA-3′
	Antisense	5′-GAACAGTGACGGTGAAGGAAA-3′
Sox2	Sense	5′-TCTGTGGTCAAGTCCGAGGCCA-3′
	Antisense	5′-TGCGAAGCGCCTAACGTACCAC-3′
Brachyury	Sense	5′-AGTATGAACCTCGGATTCAC-3′
	Antisense	5′-CCGGTTGTTACAAGTCTCAG-3′
Gata4	Sense	5′-AGCCTACATGGCCGACGTGG-3′
	Antisense	5′-TCAGCCAGGACCAGGCTGTT-3′
Hnf4	Sense	5′-CCATGGTGTTAAAGGACGTGC-3′
	Antisense	5′-TAGGATTCAGATCCCGAGCC-3′
β-Actin	Sense	5′-AGGCCAACCGTGAAAAGATGAC-3′
	Antisense	5′-GTACATGGTGGTACCACCAGAC-3′

(Showa Denko), and each fraction was further separated by reverse-phase HPLC with a Develosil C30-UG-5 column (Nomura Chemical). PA-sugar chains were detected at excitation and emission wavelengths of 320 and 400 nm, respectively. *N*-Glycans were identified by calculating the mannose unit value from normal phase HPLC and the glucose unit value from reverse-phase HPLC.

In Vitro Fucosyltransferase Assay—COS1 cells transfected with plasmids that express *Fut9* (pCX-Fut9) or *Fut10* (pCX-Fut10) were washed in 25 mM MES buffer (pH 7.4) containing 20 mM $MnCl_2$ for 10 min, and then harvested by scraping. Cells were pelleted by centrifugation and resuspended in 100 μl of cold 1% Triton X-100. The samples were sonicated briefly, then centrifuged at 17,800 × *g* for 10 min at 4 °C. The supernatant, which was used as a source of Fut9 and Fut10, was incubated with substrate (PA-sugar chains or Neuro2a cell extracts) in 50 mM cacodylate buffer (pH 6.8), 5 mM ATP, 75 μM GDP-L-fucose (Calbiochem), and 25 mM $MnCl_2$ at 37 °C for 2 h or overnight. The enzyme reaction was terminated by incubating the reaction mixtures at 98 °C for 3 min. The reaction mixtures were centrifuged at 17,800 × *g* for 5 min at 4 °C. Each supernatant was subjected to normal phase HPLC analysis. When Neuro2a cell extracts, prepared as described above, were used as a substrate, the *N*-glycans of glycoproteins in the reaction mixture were released by hydrazinolysis, and then tagged with PA. The PA-tagged *N*-glycans were analyzed by normal and reverse-phase HPLC.

In Utero Electroporation—In utero electroporation was performed as described previously (21). Three μg of plasmids in 1 μl of a solution containing 0.05% fast green was microinjected through the uterus into the lateral ventricle of E14.5 fetal brains. After injecting the plasmids, the embryos were placed between 2 electrodes (CUY650-P5, NEPA GENE). An electroporator (CUY21, NEPA GENE) was used to deliver six 50-ms pulses of 35 V at 75-ms intervals.

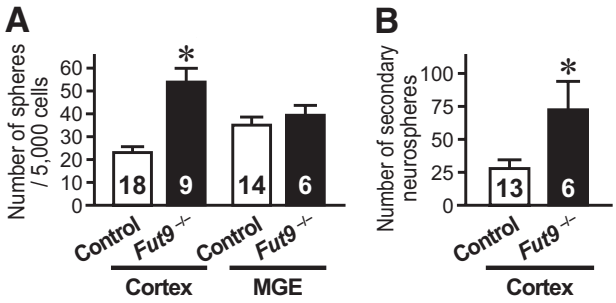

FIGURE 1. **Cortical neural stem cell numbers are increased in the E15.5 *Fut9*$^{-/-}$ brain.** *A*, NSCs isolated from the cortex or medial GE of E15.5 *Fut9*$^{-/-}$ embryos or littermate control embryos are shown. *B*, to assess the self-renewal capacity of primary sphere-forming NSCs, single primary neurosphere colonies of similar size were manually dissociated and recultured. More secondary spheres were formed from each primary *Fut9*$^{-/-}$ sphere than from each primary control sphere. *Error bars* indicate S.E. and *n* values are shown within the columns. *, $p < 0.05$ by Student's *t* test.

RESULTS

Increased Numbers of Neural Stem Cells in the Fut9$^{-/-}$ *Embryonic Brain*—To investigate the effects of *Fut9* gene disruption on the NSC pool size in embryonic mouse brains, we utilized a clonal colony-forming neurosphere assay (17, 18). Contrary to the expectation that Fut9-synthesized Le^X plays a significant role in the maintenance of NSCs, the number of NSCs in the brain of *Fut9*$^{-/-}$ embryos was greater than that in brains of their littermate controls (Fig. 1*A*). The difference between the number of NSCs in the dorsal forebrain of *Fut9*$^{-/-}$ embryos and in brains of control embryos was statistically significant ($t_{(25)} = 5.559$, $p < 0.001$), but the difference in the number of NSCs in the ventral forebrain of brains of *Fut9*$^{-/-}$ embryos and brains of control embryos was not statistically significant ($t_{(18)} = 0.705$, $p = 0.490$).

We assessed the self-renewal capacity of NSCs isolated from the embryonic brain by passaging single primary neurospheres and counting the resultant secondary neurospheres, because the number of secondary neurospheres reflects how many times an NSC undergoes symmetric expansive divisions during primary neurosphere formation. A greater number of secondary neurospheres was generated from *Fut9*$^{-/-}$ primary neurospheres than from control primary neurospheres ($t_{(17)} = 2.556$, $p = 0.021$; Fig. 1*B*). These results suggest that Fut9-synthesized Le^X is dispensable for the NSC maintenance in the dorsal forebrain of mouse embryos. If Le^X is required to maintain NSCs in an undifferentiated state, a specific Le^X could be synthesized by one of the other α1,3-fucosyltransferases.

Fut10 Is Expressed by Le^x*-positive Neural Precursor Cells in the Embryonic Brain*—The fucose-binding lectin *Lotus tetragonolobus* agglutinin (LTA) binds specifically to the Le^X sugar chain Galβ1–4(Fucα1–3)GlcNAcβ1–3-R. LTA does not bind glycans containing either sialyl Le^X or VIM-2 determinants, nor does it bind the isomeric Le^a, Galβ1–3-(Fucα1–4)GlcNAc-R (24). Thus, biotinylated lotus lectin was used to examine the expression pattern of Le^X in the embryonic brain. Lectin staining of E15.5 brains indicates that the Le^X epitope is present in the ventricular zone (VZ) and the subventricular zone (SVZ) around the lateral ventricles, the upper cortical layers, and the mantle zone of the GE (Fig. 2*A*). To examine

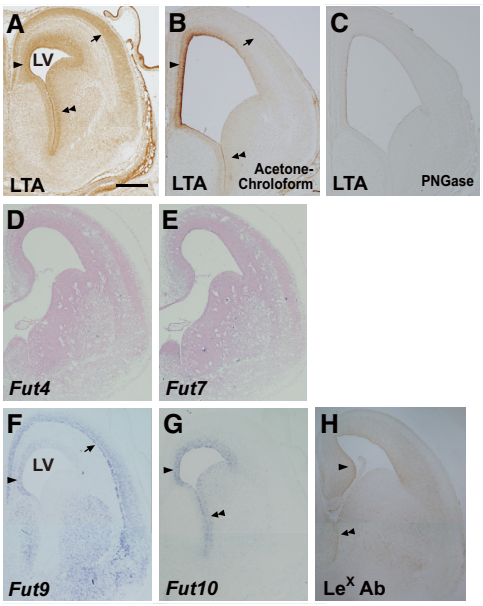

FIGURE 2. **Expression of Le^X and α1,3-fucosyltransferase genes.** *A–C*, coronal cryosections of E15.5 brains were stained with LTA lectin, which were non-treated (*A*) or pretreated with acetone/chloroform to release glycolipids (*B*) or peptide:*N*-glycosidase F (*PNGase*) to release *N*-glycans (*C*). *D–G*, the cryosections were subjected to *in situ* hybridization with probes directed against *Fut4* (D), *Fut7* (*E*), *Fut9* (*F*), or *Fut10* (*G*). LTA staining and an *in situ* hybridization signal for *Fut9* are visible in the upper cortical layers (*arrows* in *A* and *F*) and the dorsomedial portion of the VZ/SVZ (*single arrowheads* in *A* and *F*), whereas LTA staining and the *in situ* hybridization signal for *Fut10* overlap in the VZ/SVZ of the cortex and GE (*single* and *double arrowheads* in *A* and *G*). *H*, coronal cryosections of E15.5 $Fut9^{-/-}$ brains were immunostained with anti-Le^X antibody. Positive signal was detected in the VZ/SVZ (*single* and *double arrowheads*). *Scale bar* = 0.5 mm.

which glycoconjugates, glycoproteins or glycolipids, convey the lectin-binding Le^X moieties, we pretreated the sections with acetone/chloroform or peptide:*N*-glycosidase F before the LTA staining. We observed positive signals in the sections pretreated with acetone/chloroform, which were comparable with those in the non-treated one (Fig. 2*B*), but no signal in the peptide:*N*-glycosidase F-pretreated sections (Fig. 2*C*). These results suggest that Le^X epitopes attach to *N*-glycans of glycoproteins.

To determine which fucosyltransferases could synthesize Le^X in the embryonic brain, we performed *in situ* hybridization with probes directed against *Fut4, Fut7*, *Fut9*, and *Fut10*. *Fut4* and *Fut7* were barely expressed in the VZ/SVZ (Fig. 2, *D* and *E*). In contrast, *Fut9* was expressed in the dorsomedial aspect, but not the ventrolateral aspect, of the VZ/SVZ (Fig. 2*F*). This aspect of *Fut9* expression explains the above findings that the brains of $Fut9^{-/-}$ embryos have an increased number of NSCs in the cortical VZ/SVZ but not in the subcortical VZ/SVZ. The expression patterns of Le^X and *Fut9* overlapped completely in the upper cortical layers and the GE mantle zone, suggesting that migrating neuroblasts from the VZ/SVZ up-regulate *Fut9* expression and increase Le^X synthesis. We next examined the expression of *Fut10*, and discovered that mRNA for this gene was abundant throughout the entire VZ/SVZ of the embryonic brain (Fig. 2*G*). Finally, we investigated the expression of Le^X antigens in E15.5 $Fut9^{-/-}$ brains by immunostaining using an anti-Le^X antibody and detected positive signals in the VZ/SVZ (Fig. 2*H*). These results suggest that Fut10 could be an active α1,3-fucosyltransferase, although its enzymatic activities to synthesize Le^X antigens have not been provided.

To verify the presence of Le^X antigens in the embryonic brain, we used HPLC to analyze the *N*-glycans of the dorsomedial cortex and the VZ/SVZ of the GE of E15.5 brains. Le^X antigens were detected on the *N*-glycans of both tissues. The Le^X-containing bisecting *N*-glycan, A2G2Fo2FB, was more abundant in the VZ/SVZ of the GE than in the cortex (Fig. 3*A*), despite the fact that its substrate, A2G0FB, was expressed more strongly in the cortex than in the VZ/SVZ of the GE and that A2G2FB was comparable in these tissues (Fig. 3, *B* and *C*). The Le^X-containing biantennary *N*-glycan, A2G2FoF, was also expressed in the cortex (Fig. 3*C*). These findings suggest that Fut10 increases the amount of Le^X by preferentially attaching fucose to bisecting *N*-glycans. We cannot exclude the possibilities that a scarce amount of Fut9 is expressed and synthesizes A2G2Fo2FB in the VZ/SVZ of the GE or that the sample was contaminated with cells from the underlying mantle zone, which express Fut9 and could synthesize A2G2Fo2FB. However, these possibilities are unlikely because the amount of A2G2Fo2FB was much less in the dorsomedial cortex, where Fut9 is strongly expressed, than in the VZ/SVZ of the GE. Thus, these results suggest that Fut10 could be or be related to a unique α1,3-fucosyltransferase with stringent substrate specificity.

Fut10 Is Responsible for the Synthesis of Unique Types of Le^x on N-Glycans—To investigate whether Fut10 exhibits α1,3-fucosyltransferase activity and synthesizes Le^X antigens on *N*-glycans, we overexpressed *Fut10* in neurospheres derived from the GE of the E15.5 brain, which expresses both *Fut9* and *Fut10* (Fig. 4*A*). HPLC analysis of neurospheres infected with *Fut10*-expressing retroviruses revealed that a peak in fraction 8.2 (mannose unit 8.50 – 8.83) increased. We used mass spectrometry to determine that the peak consists of 5 hexoses, 5 *N*-acetylhexosamines, and 3 fucoses (Fig. 4*B*). Although we could not identify its structure, this *N*-glycan likely contains 2 or 3 outer fucoses (*e.g.* stereoisomers of A2G2Fo2FB).

We performed *in vitro* α1,3-fucosyltransferase assays to confirm the enzymatic activity of Fut10 and determine its substrate specificity (25). Cell extracts of COS1 cells that overexpress *Fut9* or *Fut10* were used as a source of enzymes because naive untransfected cells exhibit no α1,3-fucosyltransferase activities. The first sugar substrate we tested in the assay was PA-tagged lacto-*N*-neotetraose. Although Fut9 produced Le^X by attaching fucose to this substrate, Fut10 showed no activity (Fig. 4*C*). We then tested the bisecting *N*-glycan A2G2FB. Although Fut9 synthesized Le^X at the nonreducing terminal to generate A2G2Fo(3 or 6)FB and A2G2Fo2FB, Fut10 did not (Fig. 4*D*). Finally, we tested if Fut10 exhibited α1,3-fucosyltransferase activity with glycoprotein substrates present in Neuro2a neuroblastoma cell extracts, because Neuro2a cells express very little *Fut9* (Fig. 4*A*). After incubation, a reaction mixture of Neuro2a cell extracts and control COS1 cell extracts showed no apparent increase of Le^X-containing *N*-glycans. However, levels of both A2G1Fo(6)FB and A2G1Fo(6)B increased after incubating a mixture of Neuro2a cell extracts and *Fut10*-overexpressing COS1 cell extracts (Fig. 4, *E* and *F*). We did not detect increased levels of A2G1Fo(6)FB or

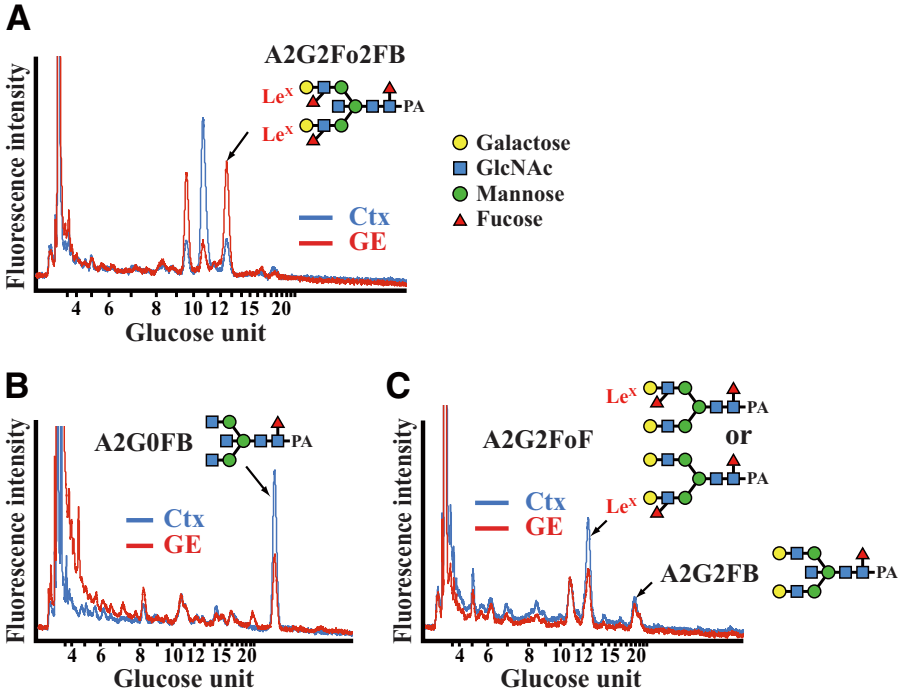

FIGURE 3. **Identification of Le^X-containing *N*-glycans.** *A–C*, *N*-glycans were isolated from the dorsomedial cortex (*Ctx*) and the VZ/SVZ of the GE of E15.5 brains, and then analyzed by HPLC. Reverse-phase HPLC charts of fraction 8.2 (mannose unit 8.50–8.83, *A*), fraction 5.2 (mannose unit 5.50–5.83, *B*), and fraction 7.2 (mannose unit 7.50–7.83, *C*) are shown.

A2G1Fo(6)B in a reaction consisting only of *Fut10*-overexpressing COS1 cell extracts, indicating that Fut10 increases the amount of Le^X on the bisecting *N*-glycans of glycoproteins that are specifically expressed on Neuro2a neuroblastoma cells. Although it remains to be investigated whether or not Fut10 itself is an α1,3-fucosyltransferase, these results suggest that Fut10 is involved in an α1,3-fucosyltransferase activity with strict substrate specificity and that this activity synthesizes Le^X only on restricted glycoprotein substrates.

Fut10 Is Required for the Maintenance of ES Cells and Neural Stem Cells—To investigate the function of *Fut10* and the Le^X that it synthesizes, we used miRNAs to knockdown gene expression. Three miRNA-expressing vectors were constructed: two target sequences within the coding region and one targets a sequence in the 3′ UTR (Fig. 5*A*). All miRNAs also expressed enhanced green fluorescent protein. Individually or in combination, the miRNAs efficiently down-regulated endogenous *Fut10* expression in Neuro2a cells (Fig. 5, *B* and *C*). Next, we transfected ES cells with the *Fut10*-miRNA vectors, and collected the GFP$^+$ cells 48 h later by using fluorescence-activated cell sorting. We confirmed that *Fut10* expression was knocked down in the transfected ES cells ($t_{(8)} = 2.852$, $p = 0.021$; Fig. 6*A*). The expression of pluripotent stem cell-specific genes was significantly down-regulated in the *Fut10*-miRNA transfectants ($t_{(8)} = 4.758$, $p = 0.001$ for *Oct3/4* and $t_{(8)} = 4.072$, $p = 0.004$ for *Nanog*; Fig. 6*B*), suggesting that *Fut10* expression is required to maintain ES cells in an undifferentiated state. We also examined the expression of *Sox2*, an early neural marker, and *Gata4*, a primitive endodermal marker. Both genes were selectively up-regulated in *Fut10*-miRNA overexpressing ES cells ($t_{(6)} = 3.366$, $p = 0.015$ for *Sox2* and $t_{(4)} = 3.500$, $p = 0.025$ for *Gata4*; Fig. 6*C*). It is possible that knockdown of *Fut10* caused the ES cells to differentiate into cells that are equivalent to those of the cylinder stage epiblast, which expresses both *Sox2* and *Gata4* but not *Brachyury*, a mesodermal marker.

To investigate the function of *Fut10* in NSCs, we infected primary neurospheres with retrovirus expressing *Fut10*, *Fut10*-miRNA, or *Fut9*. First, we examined expression levels of active α1,3-fucosyltransferase genes and found that overexpression or knockdown of *Fut10* did not induce the expression of *Fut4*, *Fut7*, or *Fut9* (Fig. 7*A*). We then assessed the function of *Fut10* expression in the maintenance of NSCs by passaging single retrovirus-infected neurospheres derived from the embryonic medial GE, and then counting the resultant secondary neurospheres. Although overexpressing *Fut9* in primary neurospheres showed little effects on the formation of secondary neurospheres, overexpressing *Fut10* increased the number of secondary neurospheres compared with that of neurospheres infected with control retroviruses ($F_{(3,11)} = 11.40$, $p = 0.001$; Fig. 7*B*). These findings suggest that *Fut10* enhances the self-renewal of NSCs. To exclude the possibility that *Fut10* overexpression increases Fut9 activity, we utilized neurospheres derived from the GE of E15.5 *Fut9*$^{-/-}$ embryos. The difference between the number of secondary neurospheres obtained from *Fut9*$^{-/-}$ primary neurospheres infected with either control retroviruses or *Fut10*-expressing retroviruses was similar to the difference between the number obtained from *Fut9*$^{+/-}$ primary neurospheres infected with either control retroviruses or Fut10-expressing retroviruses ($F_{(2,6)} = 18.06$, $p = 0.003$ for *Fut9*$^{+/-}$ and $F_{(2,12)} = 17.33$, $p < 0.001$ for *Fut9*$^{-/-}$; Fig. 7*C*). These results indicate that the effects of *Fut10* overexpression are independent of Fut9. Conversely, when we used *Fut10*-miRNA-expressing retroviruses to knockdown *Fut10* expression in *Fut9*$^{+/-}$ and *Fut9*$^{-/-}$ primary neurospheres, the

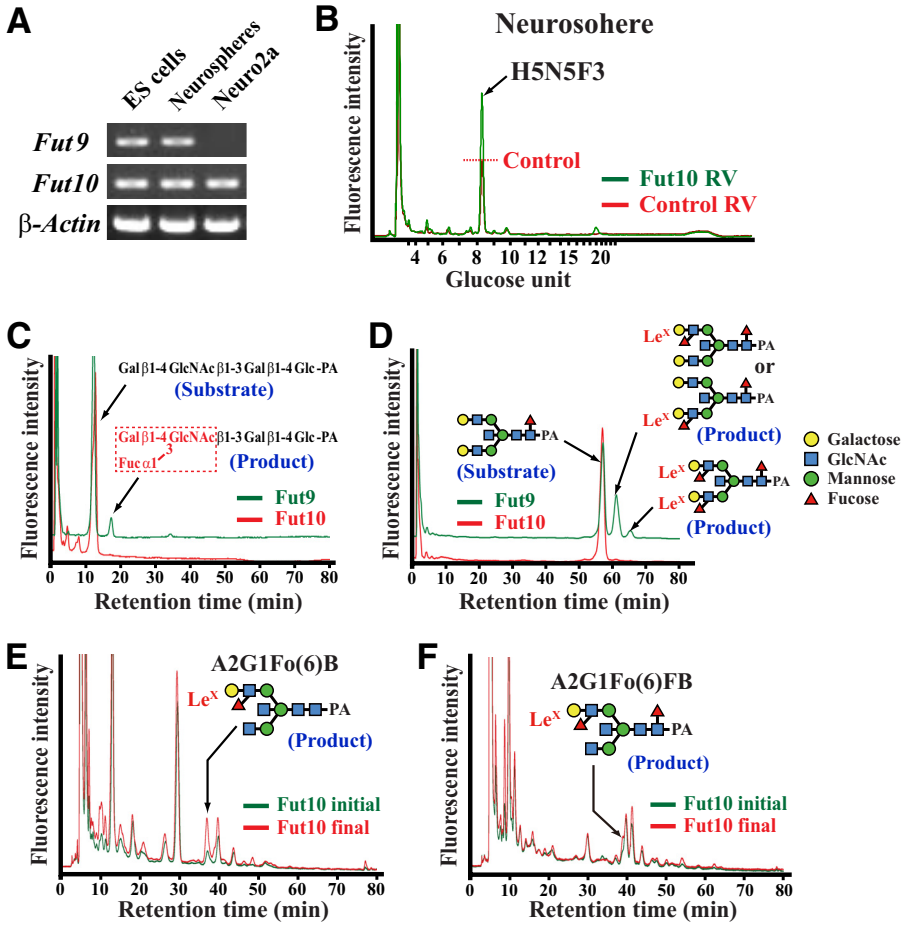

FIGURE 4. **Fut10-related α1,3-fucosyltransferase activities.** *A*, RT-PCR for *Fut9*, *Fut10*, and *β-actin* expression in ES cells, Neuro2a cells, and neurospheres derived from the E15.5 ganglionic eminence. *B*, reverse-phase HPLC elution patterns of PA-tagged *N*-glycans from neurospheres infected with *Fut10* (*green*) or control (*red*) retroviruses (*RV*) are shown. Reverse-phase HPLC was conducted on normal phase HPLC fraction 8.2 (mannose unit 8.50–8.83). *C* and *D*, sugar chain substrates were mixed with COS1 cell extracts, which served as a source of Fut9 or Fut10 enzyme activity, and then the reaction mixture was analyzed by normal phase HPLC. Whereas Fut9-mediated Le^x synthesis generated lacto-*N*-fuconeopentaose from lacto-*N*-neotetraose (*C*) and A2G2Fo(3 or 6)FB and A2G2Fo2FB from the bisecting *N*-glycan substrate A2G2FB (*D*), no Fut10 activity was detected on these substrates. *E* and *F*, Neuro2a cell extracts were used as a source of substrates and mixed with the Fut10 enzyme source. *N*-Glycans were extracted from the reaction mixture and analyzed by HPLC. Normal phase HPLC fractions that contain *N*-glycans with mannose unit 7–8 (*E*) and 8–9 (*F*) were further separated by reverse-phase HPLC. Incubation with Fut10 increased the peak of Le^x-containing A2G1Fo(6)B (*E*) and A2G1Fo(6)FB (*F*).

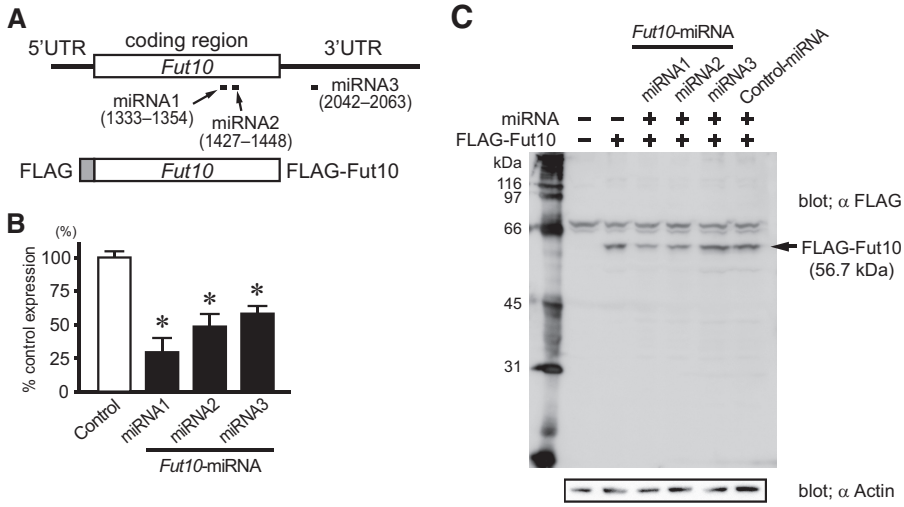

FIGURE 5. ***Fut10*-miRNAs knockdown the expression of *Fut10*.** *A*, full-length *Fut10*, FLAG-tagged *Fut10*, and the target sequences of the miRNAs are shown. *B*, *Fut10*-miRNAs were transfected into Neuro2a cells and *Fut10* mRNA was quantified by quantitative RT-PCR 3 days later. The expression level of *Fut10* relative to that of *β-actin* is shown. Values are mean ± S.E. ($n = 3$). *, $p < 0.05$. *C*, *Fut10*-miRNAs were transfected into Neuro2a cells and the cells were subjected to Western blotting. *Fut10*-miRNA1 and miRNA2, but not miRNA3, knocked down the expression of FLAG-tagged *Fut10*.

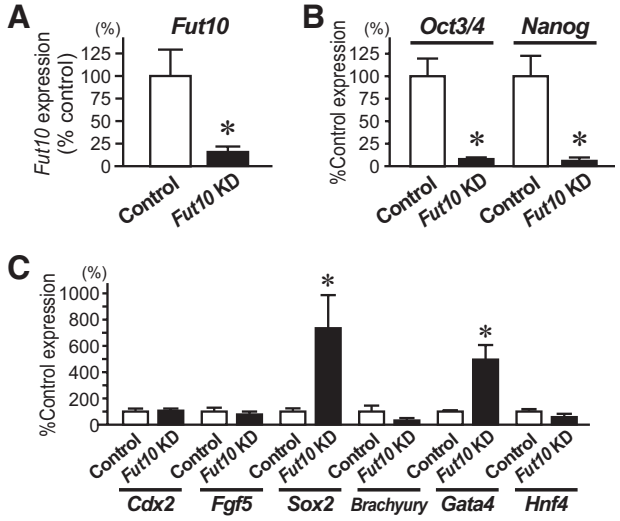

FIGURE 6. **Knockdown of *Fut10* enhances ES cell differentiation.** *A–C*, ES cells were transfected with *Fut10*-miRNA expression plasmids and transfectants were collected 48 h later by cell sorting for GFP fluorescence. Quantitative RT-PCR was performed for *Fut10* (*A*), undifferentiated cell markers (*B*), and differentiated cell markers (*C*) and the expression levels of those genes relative to that of *β-actin* are shown. *Error bars* indicate S.E. from three or more independent experiments. *, $p < 0.05$ by Student's *t* test.

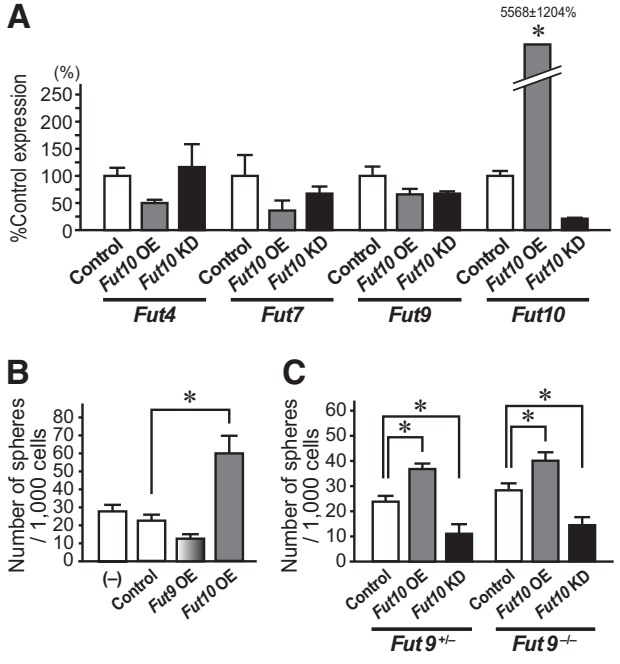

FIGURE 7. **Expression of *Fut10* regulates neural precursor cell differentiation.** *A–C*, neurospheres derived from the E15.5 medial GE were infected with retroviruses expressing *Fut10*, *Fut10*-miRNA, or *Fut9*. *A*, retrovirus-infected GFP$^+$ cells were collected by cell sorting from secondary neurospheres 7 days after the infection. Quantitative RT-PCR was performed for *Fut4*, *Fut7*, *Fut9*, and *Fut10* and the expression levels of those genes relative to that of *β-actin* are shown. *B*, retrovirus-infected neurospheres were passaged and the number of secondary neurospheres that were obtained from 1000 plated cells was counted. Overexpression of *Fut10*, but not *Fut9*, in neurospheres derived from the medial GE of E15.5 CD1 wild-type embryos enhanced secondary neurosphere formation. *E*, overexpression of *Fut10* increased, and knockdown of *Fut10* decreased, the number of secondary neurospheres obtained from *Fut9*$^{+/-}$ and *Fut9*$^{-/-}$ primary neurospheres. *Error bars* indicate S.E. from three or more independent experiments. *, $p < 0.05$ by one-way analysis of variance followed by post hoc Tukey's comparison.

number of secondary neurospheres obtained after passage decreased when compared with *Fut9*$^{+/-}$ and *Fut9*$^{-/-}$ primary neurospheres that had been infected with control retroviruses (Fig. 7*C*). These results suggest that *Fut10*, but not *Fut9*, is required for the maintenance of ES cells and NSCs.

Fut10 Is Crucial for the Radial Migration of Neural Precursor Cells—We assessed the role of *Fut10* in neural development by using *in utero* electroporation to introduce *Fut10*-miRNAs into the embryonic mouse cortex. GFP$^+$ neural precursor cells that had incorporated the control miRNA expression plasmid migrated radially from the VZ/SVZ to the cortical plate during a 3-day period (E14.5-E17.5; Fig. 8*A*). In contrast, radial migration was impaired in GFP$^+$ neural precursor cells that had been electroporated with *Fut10*-miRNA1 (Fig. 8*B*). Similar results were obtained in brains that received *Fut10*-miRNA2 or *Fut10*-miRNA3 (Fig. 8*C*). Quantification of migration by bin counting revealed a statistically significant increase in the percentage of GFP$^+$ cells in the basal regions of the cortex of control brains compared with that of brains that were electroporated with either *Fut10*-miRNA1 or *Fut10*-miRNA3. Additionally, the percentage of GFP$^+$ cells in the apical regions of the cortex of control brains was significantly decreased compared with that of brains that were electroporated with either *Fut10*-miRNA1 or *Fut10*-miRNA3 ($F_{(2,6)} = 227.4$, $p < 0.001$ for bin 6 and $F_{(2,6)} = 322.3$, $p < 0.001$ for bin 1; Fig. 8*E*). We performed rescue experiments with a *Fut10* expression vector and *Fut10*-miRNA3, which targets a 3′ UTR sequence that is absent in the *Fut10* expression vector (Fig. 5*A*). Co-electroporation of the *Fut10* expression vector with *Fut10*-miRNA3 restored radial migration in cortical neural precursor cells (Fig. 8*D*): the percentage of GFP$^+$ cells in the basal regions of the cortex of brains that were electroporated with both vectors was increased compared with that of brains that were electroporated with *Fut10*-miRNA3 only. Conversely, the percentage of GFP$^+$ cells in the apical regions of the cortex of brains that were electroporated with both *Fut10*-miRNA3 and *Fut10* vectors was decreased compared with that of brains that were electroporated with *Fut10*-miRNA3 only ($t_{(4)} = 11.67$, $p < 0.001$ for bin 6 and $t_{(4)} = 7.658$, $p = 0.002$ for bin 1; Fig. 8*E*). The percentage of GFP$^+$ cells in the basal and apical cortical regions of brains that were electroporated with both the *Fut10* expression vector and *Fut10*-miRNA3 were similar to those of control brains (Fig. 8*E*).

As paired box 6 (Pax6)$^+$ neural precursor cells in the VZ differentiate, they exit the cell cycle, migrate into the SVZ, and start to express eomesodermin homolog (*Xenopus laevis*) (Tbr2). We first examined the incorporation of bromodeoxyuridine (BrdU), which had been administered 2 h prior to perfusion, in the cortex of embryos that had previously (24 h) been electroporated with *Fut10*-miRNA1 (Fig. 8, *F* and *G*). After *Fut10* knockdown, GFP$^+$ neural precursor cells incorporated less BrdU (Fig. 8*L*). We also determined the proportion of GFP$^+$/Tbr2$^+$ (Fig. 8, *H* and *I*) and GFP$^+$/Pax6$^+$ (Fig. 8, *J* and *K*) cells in the cortex of *Fut10*-miRNA1- or control miRNA-electroporated brains. We found more GFP$^+$, Tbr2$^+$ cells and less GFP$^+$, Pax6$^+$ cells in the *Fut10*-miRNA1-electroporated cortex than in controls (Fig. 8*L*). These results suggest that the expression of *Fut10* is crucial for preventing neural precursor

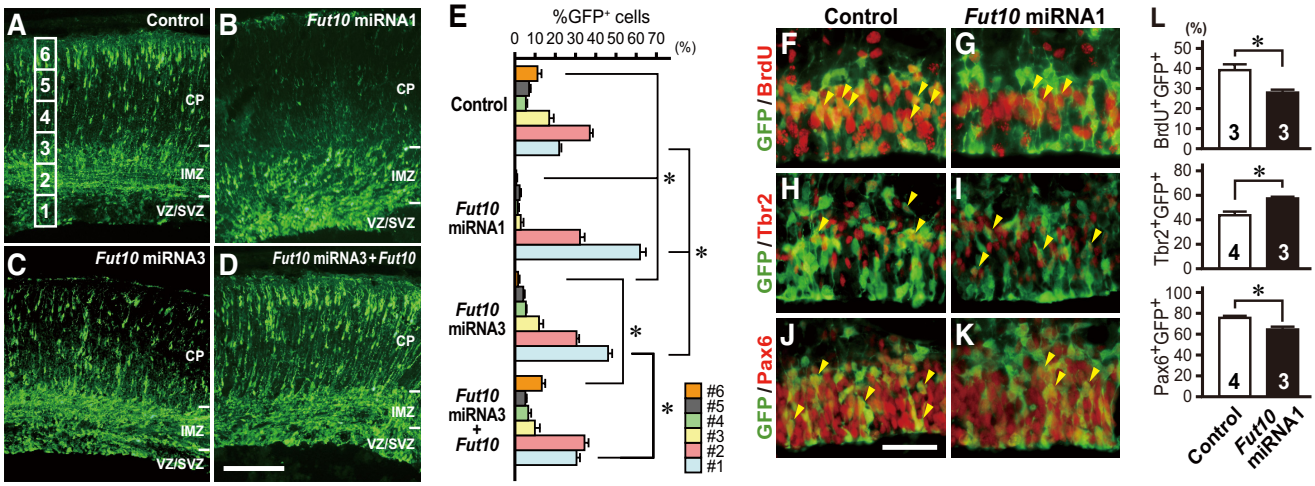

FIGURE 8. **Knockdown of *Fut10* in the cortex impairs radial migration.** *A–E, Fut10*-miRNAs (miRNA1, miRNA2, and miRNA3) or control miRNA vectors were electroporated into the dorsal cortex of E14.5 embryos *in utero* and the brains were analyzed at E17.5. *A,* GFP^+ neural precursor cells that received control miRNA migrated radially to the outer cortical plate (*CP*) during the 3-day period after electroporation. *B* and *C,* because the three *Fut10*-miRNAs showed similar phenotypes, only the results of electroporation with *Fut10*-miRNA1 (*B*) and *Fut10*-miRNA3 (*C*) are shown. *D,* co-electroporation of *Fut10*-miRNA3 and a *Fut10* expression plasmid restored radial migration. *Scale bar* = 250 μm. *IMZ*, intermediate zone. *E,* the entire cortex of E17.5 brains that had been electroporated at E14.5 was divided into 6 bins and the GFP^+ cells in each bin were counted. *Error bars* indicate S.D. from three independent experiments. *F–L*, *Fut10*-miRNA1 or control miRNA vectors were electroporated into the dorsal cortex of E14.5 embryos *in utero*, and then the embryos were fixed and the brains were analyzed 24 h later. Double label immunofluorescence of coronal cryosections was used to detect expression of GFP and BrdU (*F* and *G*), Tbr2 (*H* and *I*), or Pax6 (*J* and *K*). *Arrowheads* indicate double-positive cells. *L,* quantification of BrdU/GFP (*upper graph*), Tbr2/GFP (*middle graph*), or Pax6/GFP (*bottom graph*) double-positive cells. *Scale bar* = 50 μm. *Error bars* indicate S.E. and *n* values are shown within the columns. *, $p < 0.05$ by one-way analysis of variance followed by post hoc Dunnett's comparison (*E*) or by Student's *t* test (*L*).

cells from differentiating and migrating radially to the cortical plate.

DISCUSSION

In this study, we provide evidence that Fut10 is involved in an α1,3-fucosyltransferase activity with stringent substrate specificity, and that this activity synthesizes Le^X structures on bisecting *N*-glycans of glycoproteins. First, the Le^X-harboring bisecting *N*-glycan, A2G2Fo2FB, was detected in the VZ/SVZ of the GE of E15.5 mouse brains, where *Fut10*, but not *Fut9*, is expressed. In contrast, in the dorsal cortex, where *Fut9* mRNA is abundant, Le^X is found predominantly on biantennary *N*-glycans, not bisecting *N*-glycans. Second, overexpression of *Fut10* in neurospheres increased the amount of an *N*-glycan that harbors 2 or 3 outer fucoses. Third, an *in vitro* α1,3-fucosyltransferase assay revealed that although Fut10 could increase the amount of A2G1Fo(6)FB and A2G1Fo(6)B, which are both Le^X-containing bisecting *N*-glycans, by fucosylating the *N*-glycans of glycoproteins, it could not fucosylate the oligosaccharide lacto-*N*-tetraose or PA-tagged bisecting *N*-glycans such as A2G2FB. These findings suggest that the Fut10-related α1,3-fucosyltransferase activity requires the peptide portion of the glycoprotein in addition to the bisecting *N*-glycan.

In previous studies, an anti-CD15 Le^X antibody did not detect any Le^X antigens on *Fut10*-overexpressing COS cells (14). A possible explanation for this result is that COS cells express very low levels of bisecting *N*-glycans. Alternatively, immunohistochemical analysis might be less sensitive than our HPLC system for detecting Le^X-containing bisecting *N*-glycans. It is also possible that the CD15 antibody binds less preferentially to the Le^X residues of bisecting *N*-glycans, because the GlcNAc that is attached to the inner mannose with an α1,4-linkage may make it more difficult for the antibody to access the Le^X antigen. Recently, Mollicone *et al.* (16) reported that the enzymatic activity of Fut10 transfers a fucose onto the innermost GlcNAc of the core chitobiose. This type of activity is clearly distinct from the Le^X-synthesizing α1,3-fucosyltransferase activity detected in our study. Their enzymatic assay conditions may not have been optimized to detect α1,3-fucosyltransferase activities that synthesize Le^X at the peripheral GlcNAc residue, because they did not use bisecting *N*-glycans. It remains to be determined whether or not Fut10 itself is an α1,3-fucosyltransferase because neither Mollicone *et al.* (16) nor we used purified preparations of Fut10 to detect the enzymatic activity. Fut10 may function as a chaperone to another α1,3-fucosyltransferase as does core 1 synthase-specific molecular chaperone (Cosmc) to core 1 β3-galactosyltransferase, both of which show 26% homology (26, 27).

The expression of Le^X in various types of stem cells and its subsequent down-regulation in cells that are differentiating suggests that Le^X plays an important role in maintaining stem cell in the undifferentiated state. However, analyses of mice deficient for other α1,3-fucosyltransferase genes argue against this possibility: *Fut4* and *Fut7* double knock-out mice show only subtle immunological defects (28), and mice that lack *Fut9*, which is responsible for the synthesis of most of the Le^X antigens on glycoproteins and glycolipids, develop normally until adulthood (11) and the only neurological phenotypes these mice exhibit are a few behavioral changes (12). The expression of *Fut9* and the Le^X antigens it synthesizes in the outer cortical plate and the mantle zone of the ventral forebrain of E15.5 embryos suggests a role for *Fut9* in migrating neuroblasts rather than in the self-renewing NSCs in the VZ/SVZ. In addition, our findings that the number of NSCs and their self-renewal capability are increased in the cortical VZ/SVZ of

Fut9$^{-/-}$ brains suggests that the expression of Le^X synthesized by Fut9 facilitates the differentiation and migration of neural precursor cells. This notion appears inconsistent with the fact that neurosphere-forming NSCs are enriched in Le^X-positive cells from the adult mouse subependyma (8). However, this inconsistency disappears if one considers that neurosphere-forming NSCs express Le^X epitopes, which are synthesized by Fut10-related α1,3-fucosyltransferase activity. Indeed, we demonstrated that immuno-positivity by mouse monoclonal anti-Le^X antibody remains in the VZ/SVZ of *Fut9*$^{-/-}$ brains. We propose that different Le^X-containing epitopes synthesized by Fut9 and Fut10 could be required for distinct cellular functions such as promoting their differentiation and maintaining NSCs in an undifferentiated state.

miRNA-mediated knockdown of *Fut10* expression in ES cells as well as NSCs attenuated the self-renewal capacity of both stem cell populations. Although we could not exclude the possibility that *Fut10* enhances stemness through a nonenzymatic action, it is likely that the unique Le^X-containing bisecting *N*-glycans synthesized by Fut10 modify the function of the glycoproteins they are attached to, including those expressed on stem cells. Several glycoproteins that have been identified in neurospheres contain Le^X antigens (29, 30). Moreover, blocking the *N*-glycans on the EGF receptor was reported to modify EGF signaling and affect the proliferation and differentiation of EGF receptor-positive cells. The erythroagglutinating phytohemagglutinin lectin from *Phaseolus vulgaris* (E-PHA) binds specifically to the bisecting *N*-glycans on the EGF receptor in U373 MG cells. In doing so, E-PHA prevents EGF from binding to its receptor and suppresses EGF-induced autophosphorylation of the receptor (31). It would be intriguing to determine whether the glycoproteins present on the EGF receptor express Le^X and contain bisecting *N*-glycans. Knockdown of *Fut10* reduced the number of GFP$^+$ cells that migrated radially into basal regions of the cortex. This defect could occur if the differentiation of neural precursor cells was suppressed or, alternatively, if the migratory ability of the cells was impaired independent of their state of differentiation. We think the latter possibility is more likely for the following reasons. First, knockdown of *Fut10* in neurospheres attenuated their capacity to self-renew. Second, compared with electroporation of control miRNAs, electroporating *Fut10*-miRNAs into the embryonic cortex induced less neural precursor cells of the VZ/SVZ to express Pax6 as opposed to Tbr2, which is a marker for differentiating cells in the SVZ.

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