IgSF Molecule MDGA1 Is Involved in Radial Migration and Positioning of a Subset of Cortical Upper-Layer Neurons

Takao Ishikawa, Naoya Gotoh, Chiaki Murayama, Takaya Abe, Misato Iwashita, Fumio Matsuzaki, Toshiharu Suzuki, and Tohru Yamamoto

Mdga1, encoding a GPI-anchored immunoglobulin superfamily molecule containing an MAM domain, is expressed by a specific subset of neurons, including layer II/III projection neurons, in the mouse neocortex. To investigate the function of Mdga1 in corticogenesis, we generated Mdga1-deficient mice and back-crossed them to obtain a congenic background. Gross anatomy of the Mdga1-deficient brain at postnatal day (P) 14 showed no obvious phenotype. However, the migration of Mdga1-mutant neurons to the superficial cortical plate was clearly delayed. Most Mdga1-mutant neurons reached the lower portion of the upper cortical layer by embryonic day 18.5 and stayed there through P0. By P7, the location of the mutant cells was the same as wild-type. The location of Cux2-expressing upper-layer neurons in the cortical plate was largely unaffected. These observations indicated that Mdga1 is involved in the migration and positioning of a subset of cortical neurons and suggested that the radial migration of upper-layer neurons might be differentially regulated. Developmental Dynamics 240:96–107, 2011. © 2010 Wiley-Liss, Inc.

Key words: cerebral cortex; radial migration; upper layer; Mdga1

Accepted 21 October 2010

INTRODUCTION

The cerebral cortex consists of neurons and glial cells born at different times and places that migrate to their ultimate destinations in an exquisitely orchestrated manner (Wonders and Anderson, 2006; Miller and Gauthier, 2007; Molyneaux et al., 2007). One prominent anatomical feature of the mammalian neocortex is its organization into six layers, which develop in an outside-in pattern, as later-born projection neurons migrate through the deep cortical layers, which are inhabited by earlier-born neurons (Rakic, 1974). This radial migration of projection neurons is precisely regulated by the concerted actions of multiple factors, including extracellular guidance molecules, such as Reelin, their receptors, and the intracellular molecules that coordinate the required cytoskeletal rearrangement (Marin, 2003; Ayala et al., 2007; Marin et al., 2010). In reelin mutant mice, the laminar organization of the neocortex is entirely disrupted (Tissir and Goffinet, 2003; Katsuyama and Terashima, 2009), indicating that the Reelin signal is prerequisite for virtually all projection neurons to migrate properly. However, Reelin’s receptors, Vldlr (very low density lipoprotein receptor) and ApoER2 (apolipoprotein E receptor 2), are differentially used to regulate the migration of projection neurons; Vldlr seems to mediate a stop signal for Reelin, whereas ApoER2 is largely required for the migration of later-born projection neurons to the upper layers (Hack et al., 2007).
Thus, neurons may have different requirements for these radial migration signals, or may interpret them differently, depending on their developmental fates and the circumstances at the time of signaling. Considering that a rich variety of neuronal subtypes is generated during corticogenesis, and that their developmental fates seem to be largely determined at their birth, it is plausible that the radial migration of projection neurons is regulated differentially, with some requiring additional signals on top of a common prerequisite machinery to ensure their arrival at the correct destination. Factors expressed by subsets of projection neurons during corticogenesis might be involved in activating cell-type-specific migration machinery.

Mdga1 (MAM [meprin, A5 protein, PTPα] domain-containing glycosylphosphatidylinositol anchor 1) is a GPI-anchored extracellular protein consisting of six tandem immunoglobulin-like repeats, a fibronectin-like region, and a MAM domain. Several groups including us have identified Mdga1 as a gene that is frequently up-regulated in human cancers (De Juan et al., 2002) and selectively expressed by a specific subset of neurons in rodent, chick, and fish nervous systems (Litwack et al., 2004; Fujimura et al., 2006; Takeuchi et al., 2007; Sano et al., 2009). Mdga1 proteins associate heterophilically with unidentified binding partners that are enriched in axon-rich regions, mainly through their MAM domain (Fujimura et al., 2006), suggesting that Mdga1 may function with coexisting molecules. In the mouse cerebral cortex, Mdga1 is expressed by neurons in layer II/III, and parts of layers IV and VI (Takeuchi et al., 2007). The introduction of a Mdga1-targeted siRNA expression vector into the progenitors of layer II/III neurons by in utero electroporation at embryonic day (E) 15.5 results in essentially all the transfected cells residing inappropriately in the deep layers of the cortical plate or below it at P0 (Takeuchi and O'Leary, 2006), strongly suggesting that Mdga1 is prerequisite for the radial migration of every superficial cortical layer projection neuron. However, detailed analyses of the radial migration of Mdga1-mutant cells in other genetic contexts have been lacking.

Here, we report the initial characterization of Mdga1-mutant neurons during corticogenesis, in the context of their radial migration and ultimate destination. We generated Mdga1 knockin-knockout mice, and backcrossed them for more than 10 generations to the C57BL/6 strain, which meets the standard for establishing a congenic strain (Silver, 1995). Unexpectedly, the Mdga1-deficient neocortex showed a normal laminar structure at P14. However, during the formation of the cerebral cortex, the migration of Mdga1-mutant neurons was delayed, and their position in the upper layer was altered. However, these changes were no longer discernible by postnatal day (P) 7. Together with the observation that the localization of Cux2-expressing upper-layer neurons was largely unaffected, these results collectively indicated that Mdga1 is involved in the proper migration and positioning of a subset of upper-layer projection neurons and suggest that the radial migration of upper-

---

**Fig. 1.** Generation of Mdga1-mutant mice. **A:** Schematic diagram illustrating the organization of the wild-type and disrupted allele near the first exon of Mdga1. The disruption of Mdga1 was carried out by replacing the coding sequence of the first exon with a LacZ-pA-PGKNeo-pA cassette. **B:** Southern blot analysis of the tail DNAs prepared from Mdga1 wild-type or Mdga1+/lacZ mice. The probe detected the 9-kbp and 14-kbp Neo inserts, respectively. **C:** The disrupted Mdga1 is functionally null. Presence of MDGA1 proteins in the brains of Mdga1 wild-type (+/+), Mdga1+/lacZ (+/−), and Mdga1lacZ/lacZ (−/−) embryonic day (E) 15.5 embryos and postnatal day (P) 14 mice were detected by an anti-mouse MDGA1 antibody. **D:** Expression of the inserted LacZ reflects the expression of endogenous Mdga1. The expression of Mdga1 and LacZ in adjacent coronal sections of a P14 Mdga1+/lacZ mouse brain was detected by in situ hybridization (ISH). Scale bar = 1 mm.
layer neurons may be differentially regulated.

RESULTS

Generation of Mdga1-Mutant Mice

To analyze the function of Mdga1 in mouse neural development, we disrupted the endogenous gene in transgenic knock-in mice, in which the first exon, which largely encodes the N-terminal signal sequence, was replaced by a LacZ-encoding selection cassette (PGK-Neo; Fig. 1A,B). The cassette was used for embryonic stem (ES) cell screening, and contained a Neo selection gene flanked by flippase recognition target (FRT) sequences that was removed by crossing the resultant mutant mice with transgenic mice ubiquitously expressing FLPe recombinase (Tian et al., 2002). We verified that the expression patterns of the β-galactosidase activity and the inserted LacZ mRNA in the heterozygous P14 mice matched the expression of Mdga1 determined by in situ hybridization (ISH; Fig. 1D). The resultant heterozygous mice were backcrossed to C57BL/6 mice for more than 10 generations to obtain a functionally congenic strain. The following investigations were carried out using the progeny of the transgenic strain.

The ratio of the genotypes of the progeny obtained by crossing heterozygous mice was Mendelian (+/+ 141, +/+ 254, /−/− 142), and all the progeny reached adulthood without displaying any obvious abnormalities, and were fertile. To confirm that these mice were functionally null for Mdga1 expression, we performed ISH. The homozygous mice showed no detectable Mdga1 expression (see the panels of ISH detection of Mdga1 in Mdga1−/− mice shown in Figs. 2–8). To further verify the lack of Mdga1 protein in the mutant mice, we generated an anti-mouse Mdga1 antibody, because our previously prepared anti-chick Mdga1 antibody (Fujimura et al., 2006) recognized mouse Mdga1 poorly (data not shown). Our antibody specifically recognized endogenous Mdga1, which was undetectable in the homozygous mutant mice (Fig. 1C). These results indicated that the expression of Mdga1 was abolished in the homozygous mice and that Mdga1 is not essential for the viability or reproduction of mice.

Mdga1-mutant Mice Exhibit Essentially Normal Gross Brain Anatomy

We next examined the gross anatomy of the Mdga1-mutant mouse brain, because introducing an siRNA-expressing vector against Mdga1 into the developing mouse cerebral cortex by in utero electroporation severely retards the migration of the transfected cortical projection neurons (Takeuchi and O’Leary, 2006). Surprisingly, however, the neurons in the Mdga1-negative homozygotes were in their normal locations at P14 (Fig. 2). To analyze the
laminar construction of the cerebral cortex of these mice in more detail, the brains of wild-type and homozygous Mdga1-negative littermates of heterozygous parents were dissected, and the cerebral cortical structure was visualized by Nissl staining. The positions of specific layers were identified by the expression of Cux1, Cux2 (Layer II–IV; Nieto et al., 2004; Zimmer et al., 2004), Unc5d (Layer II–IV, weak in layer II–III at this age; Zhong et al., 2004), RORβ (Layer IV; Scharen-Wiemers et al., 1997), Er81 (Layer V; Hevner et al., 2003), FoxP2 (layer VI; Ferland et al., 2003), and Syt6 (Layer VI; Ulrich and Sudo, 1995), which were detected by ISH in adjacent sections. The Mdga1-mutant cells in the homozygous-null mice were detected by the expression of LacZ. No readily discernible difference was found in the overall laminar structures, including the positions of the Mdga1-negative mutant cells, between the homozygous and wild-type littermates (Figs. 2B, 3). These observations collectively indicated that Mdga1 is not prerequisite for the proper construction of the laminar structure of the cerebral cortex or for the correct localization of the Mdga1-expressing cells.

**Loss of Mdga1 Affects the Radial Migration and Positioning of Mutant Neurons During Corticogenesis**

Next, we examined the behavior of Mdga1-mutant cells during corticogenesis. We crossed Mdga1 heterozygous mutant mice, and collected embryos at E16.5, E17.5, and E18.5.
and pups at P0 and P7. The positions of the 
Mdga1-expressing (wild-type mice) and LacZ-expressing (homozygous mutant mice) cortical neurons of the littermate embryos and pups were detected by ISH. To clarify the positions of the deep- and upper-layer neurons, the locations of the Er81- and Cux2-expressing cells of adjacent sections were visualized by ISH. As reported previously (Takeuchi and O’Leary, 2006), obvious expression of Mdga1 was observed around E16.5, and Mdga1-expressing cells were found throughout the cortical plate in the wild-type embryos at this age (Fig. 4). However, in the homozygous mutant littermates, the majority of the LacZ-expressing Mdga1-negative cells resided in the deeper region of the cortical plate, beneath the earlier born, Er81-expressing future layer V neurons (Fig. 4). At E17.5, in the wild-type embryos, the great majority of 
Mdga1-expressing cells had migrated to the upper region of the cortical plate, where the Cux2-expressing cells reside (Fig. 5). In the mutant littermates, although some of the LacZ-expressing cells migrated to the upper cortical layers, most remained below the Er81-expressing cells (Fig. 5). By E18.5, however, the majority of the LacZ-expressing cells had migrated above the Er81-expressing cells (Fig. 5). These observations collectively indicated that the migration of the Mdga1-negative cells to the superficial layers was delayed, suggesting that Mdga1 is involved in the proper radial migration of these cells.

At E18.5 in the wild-type embryos, the Mdga1-expressing cells were located in the same layer as the Cux2-expressing cells. However, in their homozygous littermates, the migrated Mdga1-negative mutant cells were mostly located in a deeper layer (Fig. 6). This altered localization of the Mdga1-mutant cells was still visible at P0 (Fig. 7), indicating that the migrated Mdga1-negative mutant cells never attained their intended destination, but became improperly positioned in the lower portion of the upper layers. Some LacZ-expressing cells were observed beneath the Er81-expressing cells at P0, but some layer VI neurons normally start expressing Mdga1 around P0 (Fig. 7). By P7, when the cortical layers are defined, the positions of the LacZ-expressing Mdga1-negative cells in the homozygous mutant mice were indistinguishable from the positions of the Mdga1-expressing cells in their wild-type littermates (Fig. 8). To examine whether or not later-born Mdga1 mutant cells exist in the deep layer, bromodeoxyuridine (BrdU) was injected into 15.5 days post coitum (dpc) pregnant mutant mice. BrdU–β-galactosidase double-positive cells were readily detected in the upper layer; however, they were very rarely observed in the deep layer at P7 (Fig. 9), suggesting that great majority of Mdga1-mutant cells migrated to the upper layer.

DISCUSSION

Here, we present evidence that Mdga1 is functionally involved in mouse corticogenesis. A comparison of the locations of the Mdga1-expressing and Mdga1-negative cells during mouse cerebral cortex formation is schematically summarized in Figure 10. Because the migration of the Cux2-expressing upper-layer neurons was unaffected in the Mdga1-deficient embryos, even when the majority of Mdga1-mutant cells resided beneath the Er81-expressing future layer V neurons, Mdga1 appears to be
expressed by and required for only a subset of upper-layer neurons. To our knowledge, our observation may be the first evidence suggesting that the migration of upper-layer neurons is differentially regulated among cell types, during the time that they pass through the earlier born neurons in the cortical plate.

We attempted to observe the Mdga1-expressing cells simultaneously with other upper-layer markers at single-cell resolution at E18.5, but were unable to do so, because of the limited sensitivity of our antibodies and of ISH for Mdga1 and LacZ. Therefore, we verified the population of LacZ-expressing Mdga1-positive cells among layer II/III neurons at a later stage of development, when the expression of β-galactosidase was readily detectable. At P14, the great majority of layer II/III neurons expressed Cux1 (91.2 ± 1.4% [mean ± standard error; n = 18]), as reported previously (Nieto et al., 2004), and approximately half (48.2 ± 3.2%; mean ± standard error; n = 18) of the Cux1-positive cells in layer II/III were β-galactosidase-negative (Supp. Fig. S1, which is available online), suggesting that Mdga1 was expressed by only some of the upper-layer neurons. This proportion might be an underestimate, because some layer II/III neurons may start expressing Mdga1 later in development, as observed in some layer VI neurons, and, therefore, after they reach the upper layers.

Because Mdga1 seems to be expressed by a subset of projection neurons, a remaining question is, do other projection neuron subtypes require an Mdga1-like activity for their migration? Because Mdga1 has a closely related family member, Mdga2, which encodes a protein with a similar primary structure (53% identity) and the same structural domain organization, Mdga2 is a possible candidate for this role in other projection neuron subsets. Examining the radial migration of Mdga1-Mdga2 doubly-expressing cells in single-cell resolution may provide insights into the mechanisms underlying radial migration of upper-layer neurons.

**Fig. 5.** Delayed radial migration of Mdga1-mutant cells to the upper cortical plate at embryonic day (E) 17.5. The positions of Mdga1-expressing cells in wild-type (+/+; top) and LacZ-expressing mutant cells in homozygous mutant (Mdga1LacZ/LacZ; bottom) littermates in adjacent sections. Vertical orange bars highlight the positions of the Mdga1-expressing or Mdga1-mutant cells. The Mdga1-expressing cells of the wild-type littermates migrated to the uppermost cortical plate, where the Cux2-expressing cells reside. The majority of the Mdga1-mutant cells remained beneath the Er81-expressing cells, although some Mdga1-mutant cells were found in the upper area (dotted line). No obvious differences in the distribution of Cux2-expressing cells in the homozygous-mutant and wild-type cortical plates were seen. Four litters were analyzed and showed essentially the same phenotype. Representative images obtained from one litter are shown. MZ, marginal zone; CP, cortical plate; IZ, intermediate zone. Scale bar = 100 μm.
deficient mice will help delineate the importance of the Mdga family molecules in corticogenesis.

Regardless of the functional significance of \textit{Mdga2}, the question of why some upper-layer neurons require \textit{Mdga1} for their proper migration but others do not remains an interesting question. Considering that the cortical projection neurons are largely generated by two different progenitors, the apical and basal progenitors, one explanation could be that \textit{Mdga1} is required by the subset of neurons derived from only one of these progenitors. A recent study indicates that the migratory behaviors of the progeny of the apical and basal progenitors differ. Postmitotic neurons derived from apical progenitors exit the ventricular zone rapidly, but stay within the subventricular/intermediate zone (Tabata et al., 2009). The factors regulating the migration of these different cell types may well be different, and \textit{Mdga1} might be one of them. Interestingly, \textit{Cux2} is expressed by a subset of basal progenitors (Nieto et al., 2004; Zimmer et al., 2004), and may contribute progeny to the \textit{Cux2}-expressing upper-layer neurons. Given that the localization of the \textit{Cux2}-expressing cells in the cortical plate was not altered in \textit{Mdga1}-deficient mice, it is tempting to speculate that \textit{Mdga1} might be selectively expressed by neurons derived from apical progenitors. Further investigations are required to test this possibility.

In addition to the timing of their migration, the position of the \textit{Mdga1}-deficient cells in the upper layer at E18.5 and P0 was also altered. They settled in a relatively deep region of the upper layer, while the wild-type \textit{Mdga1}-expressing cells were in the superficial part of the upper layer, with the \textit{Cux2}-positive neurons. It is unclear whether \textit{Mdga1} actively plays a role in determining the position of the neurons in the upper layer, or if the \textit{Mdga1}-mutant cells simply failed to inhabit the more superficial layer because of their delayed migration. Interestingly, the \textit{Mdga1}-mutant cells appeared to have attained their appropriate position by P7, suggesting that the upper-layer neurons may be re-distributed between P0 and P7, which might reflect the flexibility of neural network formation in the brain. However, it cannot be excluded that other upper-layer neurons, which did not express \textit{Mdga1}

---

\begin{figure}
\centering
\includegraphics[width=\textwidth]{mdga1-deficient_mice.png}
\caption{\textit{Mdga1}-mutant cells reach the lower portion of the upper cortical plate at embryonic day (E) 18.5. The positions of the \textit{Mdga1}-expressing cells in wild-type (+/+) and LacZ-expressing mutant cells in homozygous (\textit{Mdga1}\textsuperscript{LacZ/LacZ}) littermates in the developing forebrain along with those of the \textit{Er81-} and \textit{Cux2}-expressing cells at E18.5 are shown. Most \textit{Mdga1}-mutant cells migrated to the upper cortical plate, although a minor population remained in the deeper region. The \textit{Mdga1}-mutant cells in the upper cortical plate were still situated relatively deeper than the \textit{Mdga1}-expressing cells in the wild-type littermates. Three litters were analyzed and showed essentially the same phenotype. Representative images obtained from one litter are shown. MZ, marginal zone; CP, cortical plate; IZ, intermediate zone. Scale bar = 100 \textmu m.}
\end{figure}
until then, start expressing \textit{Mdga1} at P0, as observed in layer VI, and that this may have altered the localization pattern of the \textit{Mdga1}-mutant cells at P0. Further studies are required to address these issues.

A previous report showed that the introduction of siRNA vectors against \textit{Mdga1} by in utero electroporation, believed to be a random process, severely retarded the radial migration of virtually every siRNA-expressing cell (Takeuchi and O'Leary, 2006). Our studies on \textit{Mdga1}-deficient mice support their main conclusion, that \textit{Mdga1} is involved in the radial migration of cortical projection neurons. However, there are discrepancies in the results obtained from their siRNA experiments and our transgenic mice. In the \textit{Mdga1}-deficient mice, the migration of only some upper-layer neurons was retarded; the \textit{Mdga1}-mutant cells accumulated in the lower region of the cortical plate, whereas the migration of the \textit{Cux2}-expressing neurons to the superficial layer of the cortical plate was largely unaffected. Furthermore, the majority of \textit{Mdga1}-mutant cells reached the upper cortical layer by P0, whereas the siRNA-expressing neurons were largely located in deep layers at P0. These discrepancies do not seem to be due to the difference in the genetic background of the mice, because the siRNA experiments were performed using C57BL/6 mice, and our mice were backcrossed more than 10 generations to C57BL/6 mice and can be regarded as congenic. Future detailed characterizations of the \textit{Mdga1}-expressing cells may resolve the apparent discrepancies.

The molecular mechanism of the \textit{Mdga1}-mediated regulation of radial migration remains to be elucidated. \textit{Mdga1} is sometimes referred to as a cell-adhesion molecule; however, no evidence showing that \textit{Mdga1} indeed possesses such activity has been formally reported. We previously presented evidence that \textit{Mdga1} associates heterophilically with unidentified binding partner(s): in \textit{trans} through its Ig domains, and probably in \textit{cis} (lateral) through its MAM domain (Fujimura et al., 2006). Deletion of the four N-terminal Ig domains enhanced the MAM-domain-mediated binding activity approximately 30-fold (T.Y., unpublished observations), collectively suggesting that the interaction with binding partner(s) through the MAM domain may be hindered by the N-terminal Ig domains, and that the interaction with ligand(s) through the Ig domains might facilitate MAM-domain interactions on the cell surface. \textit{Mdga1} may act as a co-receptor for external signals, and the identification of its binding partner(s) would help elucidate the regulation of radial migration by \textit{Mdga1}.

Our observations indicated that \textit{Mdga1} is involved in radial migration and the final location of a subset of cortical upper-layer neurons, and further suggest that the regulation of radial migration and positioning of projection neurons may differ even

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{\textit{Mdga1}-mutant cells reside in the lower portion of the upper cortical layer at P0. The positions of \textit{Mdga1}-expressing cells of wild-type (+/+) and \textit{LacZ}-expressing mutant cells of homozygous (\textit{Mdga1}\textsuperscript{LacZ/LacZ}) littermates in the motor area of the cerebral cortex along with the positions of \textit{Er81}- and \textit{Cux2}-expressing cells at P0 are shown. Most of the \textit{Mdga1}-mutant cells still resided in the deeper part of the upper cortical layer. Three litters were analyzed and showed essentially the same phenotype. Representative images obtained from one litter are shown. I, layer I; UL, upper layer; DL, deep layer; WM, white matter. Scale bar = 100 \textmu m.}
\end{figure}
among cell types belonging to the same cortical layer. Future investigation on the Mdga-mediated regulation of radial migration will help reveal the underlying mechanisms that ensure proper formation of the highly structured mammalian neocortex.

EXPERIMENTAL PROCEDURES

Generation of Mdga1-Mutant Mice

Mouse genomic DNA including the first exon of Mdga1 was obtained from a C57BL/6 BAC clone (Invitrogen, Carlsbad, CA). The coding sequence in the exon was replaced by a LacZ-pA-PGK-Neo-pA cassette (http://www.cdb.riken.jp/arg/cassette.html) to construct the targeting vector, which was electroporated into TT2 ES cells. The successful recombinants were identified by polymerase chain reaction (PCR; 5’-ACC GCTTCCTCGTCTTACGTTATC-3’ and 5’-AGACTGAGAGCTGAAAT TCCATCCC-3’), and further confirmed by Southern blot analysis. The recombinant ES cells were injected into ICR eight-cell stage embryos to generate chimeric founders, which were crossed to C57BL/6 females to obtain the mice carrying the disrupted allele (http://www.cdb.riken.jp/arg/Methods.html). The resultant mice were backcrossed to C57BL6 mice for more than 10 generations. The pGK-Neo region of the cassette was removed by crossing the Mdga1 transgenic mice to C57BL6 mice ubiquitously expressing Flippase (Tian et al., 2002). The presence of the wild-type allele, LacZ-pA-PGK-Neo-pA allele, and floxed LacZ-pA-pA allele was verified by PCR using the following primer sets: 5’-CATCGGTGGCCTGTTGG ATC-3’ and 5’-GCACTTTGAGCAAG

Fig. 8. Distribution of Mdga1-mutant cells becomes apparently normal by postnatal day (P) 7. The positions of the Mdga1-expressing cells of wild-type (+/+ ) and LacZ-expressing mutant cells of homozygous (Mdga1LacZ/LacZ) littermates in the motor area of the cerebral cortex, along with the positions of Er81- and Cux2-expressing cells, at P7 are shown. No obvious differences were seen between the distribution of Mdga1-expressing and Mdga1-mutant cells. Four litters were analyzed and showed essentially the same phenotype. Representative images obtained from one litter are shown. Roman numerals indicate the positions of the corresponding layers. WM: white matter. Scale bar = 100 μm.
ATCTTG-3' for wild-type; 5'-GAT CGGCCATTTGAAACAGATG-3' and 5'-AGAGCGAGCGGATTGCTGTTG-3' for \( \text{LacZ-pA-PGK-Neo-pA} \); 5'-GCTGG CTGCCATGAACAAAGGTTGG-3' and 5'-GCACTTTGAGCAAGATCTTG-3' for \( \text{LacZ-pA-pA} \). Mice were housed in environmentally controlled rooms of an animal facility of Hokkaido University, and handled according to the institution's guidelines. Noon of the day when the vaginal plug was observed was defined as E0.5.

**Fig. 9.** Later born neurons rarely reside in the deep layer. Bromodeoxyuridine (BrdU) was injected into the 15.5 days post coitum (dpc) pregnant heterozygous (\( \text{Mdga1}^{+/\text{LacZ}} \)) mice that have been crossed with the heterozygous males. The brains of homozygous (\( \text{Mdga1LacZ/LacZ} \)) postnatal day (P) 7 pups were fixed and the anti-\( \beta \)-galactosidase- and BrdU-containing cells were visualized by fluorescein isothiocyanate (FITC) anti-rabbit (\( \beta \)-galactosidase) and Cy3 anti-mouse (BrdU) antibodies. Arrowheads indicate the cells colabeled with \( \beta \)-galactosidase and BrdU. Scale bar = 100 μm.

**Fig. 10.** Schematic diagram illustrating the positions of \( \text{Mdga1} \)-expressing and \( \text{Mdga1} \)-mutant cells during mouse corticogenesis. \( \text{Mdga1} \)-mutant cells accumulated transiently beneath the \( \text{Er81} \)-expressing cells, and the majority reached the lower portion of the upper cortical plate by embryonic day (E) 18.5. At postnatal day (P) 0, the \( \text{Mdga1} \)-mutant cells resided in the lower portion of the upper cortical layer, but by P7, the locations of the \( \text{Mdga1} \)-mutant cells in the cerebral cortex were indistinguishable from those of the \( \text{Mdga1} \)-expressing neurons in wild-type mice. The fate of the \( \text{Mdga1} \)-mutant cells that remained below the \( \text{Er81} \) cells is unclear, because some layer VI neurons started expressing \( \text{Mdga1} \) at P0. The distribution of the \( \text{Cux2} \)-expressing neurons was essentially unaltered by the \( \text{Mdga1} \) deficiency, suggesting that \( \text{Mdga1} \) is expressed in only a subset of future upper-layer neurons, and is involved in only these neurons’ radial migration and positioning. Roman numerals indicate the corresponding layers. MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; UL, upper layer; DL, deep layer; WM, white matter.
by PCR using the following primer set: 5’-GAATTCCCATGATTTACAG CATA-3’ and 5’-CTCGAGTACCGGG GACACTCTCCGTTTCT-3’. The resultant amplified fragment was inserted into pET21a (Merck, Germany), to produce the His-tagged recombinant proteins in E. coli. The purified recombinant proteins were used to immunize rabbits, and the resultant antisera were affinity purified.

In Situ Hybridization, Histochemistry, and Immunohistochemistry

In situ hybridization was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993; Tsuchida et al., 1994). Briefly, deeply anesthetized animals were perfused with ice-cold phosphate-buffered saline (PBS: 10 mM sodium phosphate pH 7.4, 137 mM NaCl) followed by ice-cold 4% paraformaldehyde (PFA; Nacala Tesque, Kyoto, Japan)/PBS. The brains were isolated and further fixed in 4% PFA/PBS overnight at 4°C. The brains were successively placed in 10%, 20%, and 30% sucrose solutions, overnight. The fixed and cryo-protected brains were embedded in OCT compound (Sakura Finetech, Tokyo, Japan) and sectioned into serial 20-μm coronal sections on a CM3000 cryostat (Leica Microsystems, Wetzlar, Germany). The resultant sections were post-fixed in 4% PFA/PBS at room temperature (RT) for 5 min, washed three times with PBS, and incubated in 1 μg/ml Proteinase K (Roche Applied Science) in 6.25 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0 (Dojindo Laboratories, Kumamoto, Japan) and 50 mM Tris pH 7.5 (Wako Pure Chemical Industries, Osaka, Japan) at RT for 5 min. The sections were re-fixed in 4% PFA/PBS at room temperature for 5 min, washed three times with PBS, and acetylated in 1.33% triethanol amine (Sigma-Aldrich) at 37°C for 10 min. The acetylated sections were washed three times with PBS and incubated in hybridization buffer (50% formamide (Sigma-Aldrich), 0.25 mg/ml Yeast RNA (Sigma-Aldrich), 0.5 mg/ml herring sperm DNA (Roche Applied Science), 5× Denhardt’s (Sigma-Aldrich), 5× SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0) at room temperature for 2 hr, then with digoxigenin-labeled cRNA probes in hybridization buffer at 72°C for 16 hr. The hybridized sections were washed in 5× standard saline citrate (SSC) at 72°C for 10 min and then in 0.2× SSC for 1 hr. The washed sections were incubated with 10% heat-inactivated goat serum (Roche Applied Science) in 100 mM Tris pH 7.5, 0.15 M NaCl solution at RT for 1 hr, followed by incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science) in the same solution at 4°C overnight. The sections were washed three times with 100 mM Tris pH 7.5, 0.15 M NaCl solution, and twice with 100 mM Tris pH 9.5, 0.1 M NaCl, 50 mM MgCl2 solution, followed by incubation with NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; Roche Applied Science) in the same solution containing 0.24 mg/ml levamisole (Sigma-Aldrich) at room temperature in the dark. The reaction was stopped by immersing the sections in PBS-5 mM EDTA.

The β-galactosidase activity staining and antibody staining were performed using fixed brain prepared as above except for the post-fixation time (2 hr). For β-galactosidase activity staining, the sections were incubated with 100 mM sodium phosphate pH 7.4, 2 mM MgCl2, 0.1% sodium deoxycholate (Wako Pure Chemical Industries), 0.02% NP40 (Nacala Tesque) at room temperature for 30 min × 2. The sections were incubated in 100 mM sodium phosphate pH 7.4, 2 mM MgCl2, 0.1% sodium deoxycholate, 0.02% NP40, 5 mM ferricyanide, 5 mM ferrocyanide, 1 mg/ml X-Gal (Wako Pure Chemical Industries) at 37°C. The reaction was stopped by washing the sections with PBS. To detect Cux1 and β-galactosidase by immunohistochemistry, anti-β-galactosidase (1:1,000, Promega; Madison, WI) and anti-Cux1 (1:1,000, Santa Cruz, Santa Cruz, CA) antibodies were used and visualized by donkey secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA) as described (Fujimura et al., 2006). For labeling the cells with BrdU, BrdU solution in PBS (Sigma, 10 mg/ml) was injected into abdominal cavities of the 15.5 dpc heterozygous pregnant mice at 50 μg/g body weight. The pups of the mice were killed, and their brains were fixed as described above. The cryosectioned slices were treated as described (Tabata et al., 2009) for immunostaining with anti-BrdU (1:100, Sigma) and anti-β-galactosidase (1:1,000, Biogenesis, Poole, UK) antibodies.

ACKNOWLEDGMENTS

We thank Dr. Thomas M. Jessell (Columbia University) for encouraging us to pursue this project. T.Y. was funded by a Grant-in-Aid for Scientific Research and a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Science, Culture, Sports, and Technology, Japan.

REFERENCES


MDGA1 IN MOUSE CORTICOGENSESIS 107

Developmental Dynamics


