In vivo genetic manipulation of cortical progenitors in gyrencephalic carnivores using in utero electroporation

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Introduction
Expansion and folding of the cerebral cortex are the most notable features of higher mammals such as carnivores and primates. A major underlying cause of these features seems to be the increase in population size of neural progenitors in the outer subventricular zone (OSVZ), a specialized germinal zone characteristic of the developing cerebral cortex in higher mammals. To better understand the OSVZ, it is therefore important to uncover the mechanisms regulating the differentiation and proliferation of neural progenitors in the cerebral cortex of developing higher mammals (Dehay and Kennedy, 2007; Rakic, 2009; Fietz and Huttner, 2011; Lui et al., 2011; Borrell and Reillo, 2012; Hevner and Haydar, 2012; Molnár and Clowry, 2012).

Cortical neurons arise from radial glial cells (RG cells, also known as apical progenitors/apical RG/ventricular RG), the epithelial stem cells that line the cerebral ventricles and extend apical fibers and basal fibers (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001). RG cells in the ventricular zone (VZ) undergo multiple rounds of asymmetric divisions and generate intermediate progenitor cells (IP cells/basal progenitors) that migrate into the subventricular zone (SVZ) and further proliferate to increase neuronal number (Haubensak et al., 2004; Noctor et al., 2004). Corticogenesis in higher mammals is distinguished by the appearance of the large SVZ that has an inner (ISVZ) and outer region (OSVZ), often split by a thin layer of fibers, the inner fiber layer (IFL) (Smart et al., 2002; Zecevic et al., 2005).

Recent studies identified a novel class of progenitor cells found in the OSVZ, termed OSVZ radial glial cells (oRG cells, also known as outer RG/basal RG/intermediate RG/translocating RG). Unlike RG cells in the VZ, oRG cells are unipolar, with a basal fiber that ascends toward the pia without an apical fiber that descends toward the ventricle (Fietz et al., 2010; Hansen et al., 2010).

Although there have been extensive anatomical and histochemical investigations of the OSVZ, and though previous pioneering studies uncovered the involvement of the Notch signaling and β3-integrin in the development of the OSVZ using cultured slices in vitro (Fietz et al., 2010; Hansen et al., 2010), a molecular understanding of the OSVZ in vivo is still limited. This is mainly because rapid and efficient genetic manipulations that can be applied to germinal zones of the cerebral cortex in higher mammals had been poorly available. In utero electroporation is a powerful tool for investigating the fundamental mechanisms underlying the formation and abnormalities of the cerebral cortex in higher mammals.

Summary
Brain structures such as the outer subventricular zone (OSVZ) and the inner fiber layer (IFL) in the developing cerebral cortex are especially prominent in higher mammals. However, the molecular mechanisms underlying the formation of the OSVZ are still largely unknown, mainly because genetic manipulations that can be applied to the OSVZ in higher mammals had been poorly available. Here we developed and validated a rapid and efficient genetic manipulation technique for germinal zones including the OSVZ using in utero electroporation in developing gyrencephalic carnivores. We also determined the optimal conditions for using in utero electroporation to express transgenes in germinal zones. Using our electroporation procedure, the morphology of GFP-positive cells in the OSVZ was clearly visible even without immunostaining, and multiple genes were efficiently co-expressed in the same cells. Furthermore, we uncovered that fibers, which seemed to correspond to those in the IFL of monkeys, also existed in ferrets, and were derived from newly generated cortical neurons. Our technique promises to be a powerful tool for investigating the fundamental mechanisms underlying the formation and abnormalities of the cerebral cortex in higher mammals.

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Key words: Ferret, Cerebral cortex, In utero electroporation, Outer subventricular zone, Inner fiber layer, Outer radial glia
uterine electroporation in gyrencephalic carnivore ferrets and demonstrated that genes of interest can be efficiently expressed in post-mitotic neurons (Kawasaki et al., 2012). Here we show that transgenes can be also efficiently expressed in neural progenitors in germinal zones including the OSVZ using in utero electroporation in the developing ferret. We also determined the optimal conditions for using in utero electroporation to express transgenes in germinal zones. Furthermore, using our electroporation procedure, we uncovered that fibers, which seemed to correspond to those in the IFL of monkeys, also existed in ferrets, and were derived from newly generated cortical neurons. Our method promises to be a powerful tool for investigating the molecular mechanisms underlying the development of the OSVZ in higher mammals. Uncovering the developmental processes of the OSVZ using higher mammals would help lead to the ultimate goal of understanding the human brain and its diseases.

Materials and Methods

Animals

Normally pigmented, sable ferrets (Mustela putorius furo) were purchased from Marshall Farms (North Rose, NY). Ferrets were maintained as described previously (Kawasaki et al., 2004; Iwai and Kawasaki, 2009; Iwai et al., 2012; Kawasaki et al., 2012). The day of birth was counted as postnatal day 0 (P0). All procedures were performed in accordance with a protocol approved by the University of Tokyo Animal Care Committee.

In utero electroporation procedure for ferrets

By modifying the procedure for in utero electroporation of rodents, we recently created a procedure for in utero electroporation to express transgenes in post-mitotic neurons of the ferret cerebral cortex (Kawasaki et al., 2012). Briefly, pregnant ferrets were anesthetized with sodium pentobarbital, and their body temperature was monitored and maintained using a heating pad. The uterine horns were exposed and kept wet with adding drops of PBS intermittently. The location of embryos were exposed and kept wet by adding drops of PBS intermittently. The location of embryos was visualized with transmitted light delivered through an optical fiber cable. The pigmented iris was visible, and this enabled us to assume the location of embryos. The aim was to avoid injury to the embryo.

Electroporation procedure

Electroporation was performed at E35, and GFP fluorescence was examined at P0. A dorsal view of the ferret brain is shown. GFP fluorescence was clearly visible in the cerebral cortex (arrowhead). A, anterior; P, posterior. (A) In utero electroporation was performed at E35, and GFP fluorescence was examined at P0. A dorsal view of the ferret brain is shown. GFP fluorescence was clearly visible in the cerebral cortex (arrowhead). a, anterior; p, posterior. (B) GFP-positive cells in the cerebral cortex. A coronal section is shown. GFP-positive cells were clearly visible even without immunostaining. *Lateral ventricle. (C) In utero electroporation was carried out at the indicated time points during development. Magnified images of the cerebral cortex are shown. When electroporation was performed at E35, and GFP fluorescence was examined at P0. GFP-positive cells were clearly visible even without immunostaining. *Lateral ventricle. Sections were made using a cryostat, permeabilized with 0.1-0.5% Triton X-100/ PBS, and incubated overnight with primary antibodies, which included an anti-Sox2 antibody (R&D Systems), anti-Pax6 antibody (Covance), anti-Thrb2 antibody (Abcam) and anti-neurofilament-M antibody (Chemicon). After incubation with secondary antibodies and Hoechst 33342, the sections were washed and mounted. Experiments were repeated at least three times in different animals and gave consistent results.

Results

Efficient expression of GFP in germinal zones of the ferret cerebral cortex using in utero electroporation

We recently established and reported in utero electroporation procedure for ferrets and successfully expressed GFP in post-mitotic neurons of the cerebral cortex (Kawasaki et al., 2012). We therefore assumed that our procedure is also applicable to manipulation of gene expression in the OSVZ. To identify the appropriate time point for performing in utero electroporation to express transgenes in germinal zones, we carried out in utero electroporation at various time points during development. Because the OSVZ is prominent soon after birth in ferrets (Fietz et al., 2010; Reillo and Borrell, 2012), we dissected ferret babies at postnatal day 0 (P0). Consistent with our previous report (Kawasaki et al., 2012), GFP fluorescence was clearly visible on the brain surface (Fig. 1A, arrowhead), and numerous GFP-positive cells were clearly visible even without immunostaining. *Lateral ventricle.
positive cells were observed in the cerebral cortex (Fig. 1B), indicating that cortical cells were efficiently transfected with GFP using our *in utero* electroporation procedure in ferrets.

When ferrets were electroporated with pCAG-GFP at E31, we found that most GFP-positive cells had already moved into the cortical plate at birth (Fig. 1C, E31, arrowhead). Electroporation at E35 resulted in GFP-positive cells distributed throughout the developing cortex including the OSVZ (Fig. 1C, E35). When electroporation was performed at E40, germinal zones were prominently labeled with GFP (Fig. 1C, E40, arrow). These results suggest that our electroporation procedure is applicable for expressing transgenes in neural progenitors and that *in utero* electroporation between E35 and E40 is appropriate for obtaining transfected neural progenitors at birth.

Germinal zones in the cerebral cortex of developing ferrets can be distinguished by the expression patterns of progenitor marker molecules such as Sox2, Pax6 and Tbr2. We therefore compared the distribution pattern of GFP-positive cells with those of progenitor markers using immunohistochemistry. We performed *in utero* electroporation at E37, and sections prepared at E40 were stained with anti-Pax6, Sox2 and Tbr2 antibodies (Fig. 2). Pax6 and Sox2 are strongly expressed in the VZ, whereas Tbr2 is preferentially expressed in the ISVZ (Fietz et al., 2010; Reillo and Borrell, 2012; Martinez-Cerdeño et al., 2012). Our immunohistochemical studies showed that many GFP-positive cells were located not only in the ISVZ but also in the VZ and the OSVZ (Fig. 2). These results clearly indicate that our electroporation procedure is applicable to manipulation of gene expression in germinal zones of the developing ferret cerebral cortex.

Visualization of the morphology of GFP-positive cells in the OSVZ

Because the morphology of neural progenitors changes during development, it would be useful if our electroporation procedure were applicable for visualizing the morphology of single cells in the cerebral cortex of developing ferrets. We therefore examined whether GFP expression was strong enough for examining the morphology of cells without immunostaining. High magnification images clearly demonstrated that the morphology of individual GFP-positive cells in the OSVZ was clearly visible (Fig. 3C). When sections were immunostained with anti-Sox2 antibody, we found GFP-positive cells that were also positive for Sox2 in the OSVZ (Fig. 3D, arrowhead), suggesting that these GFP-positive cells are oRG cells. Consistently, these GFP/Sox2 double-positive cells extended their processes toward the pial surface without having apical fibers (Fig. 3D, arrowhead). Anti-Pax6 antibody gave results similar to those obtained with anti-Sox2 antibody (Fig. 3E, arrowhead). Combining our electroporation procedure and time-lapse imaging would contribute to uncovering the dynamics of the morphological changes and developmental lineages of progenitor cells in higher mammals during development.

![Fig. 2. Distribution of GFP-positive cells and progenitor markers.](image-url)

*In utero* electroporation was performed at E37, and sections were prepared at E40. The sections were immunostained with anti-Sox2, anti-Pax6 and anti-Tbr2 antibodies. The cerebral cortex is shown in A. The areas within the white boxes are magnified and are shown in B. CP, cortical plate; OSVZ, outer subventricular zone; ISVZ, inner subventricular zone; VZ, ventricular zone. Scale bars: 200 µm (A) and 100 µm (B).

![Fig. 3. The morphology of GFP-positive cells in the OSVZ.](image-url)

*In utero* electroporation was performed at E35, and coronal sections were prepared at P0 and were stained with Hoechst 33342. Many GFP-positive cells were distributed throughout the cortex (A). The areas within the white boxes in A are magnified and are shown in B. The areas within the white boxes in B are shown in C. Note that the morphology of GFP-positive cells was clearly visible even without immunostaining. (D,E) The sections were immunostained with anti-Sox2 antibody (D) and anti-Pax6 antibody (E), and high magnification images of the OSVZ are shown. The GFP-positive cells (arrowheads) expressed Sox2 (D) and Pax6 (E), and had basal fibers but not apical fibers, suggesting that these cells are oRG cells. GFP-positive fibers running tangentially in the inner OSVZ were also visible (arrow). *Lateral ventricle. CP, cortical plate; OSVZ, outer subventricular zone; ISVZ, inner subventricular zone; VZ, ventricular zone. Scale bars: 1 mm (A), 500 µm (B), 100 µm (C) and 20 µm (D,E).
Co-expression of GFP and mCherry in germinal zones of the ferret cerebral cortex
To investigate the molecular mechanisms underlying the formation of the OSVZ, it would be useful if multiple genes, such as GFP plus genes of interest, could be co-transfected in progenitor cells of ferrets. We therefore examined co-transfection efficiency by transfecting a mixture of pCAG-GFP and pCAG-mCherry at E35 and dissected the brain at birth. As we have shown, numerous GFP-positive cells were distributed throughout the cortex (Fig. 4A). We then examined co-localization of GFP and mCherry at the cellular level using high magnification images. We found that most of GFP-positive cells were also positive for mCherry (91.8±5.7%, 6 sections from 3 animals) (Fig. 4B). These results indicate that the co-transfection efficiency in the OSVZ is reasonably high.

Distribution of GFP-positive cells during development
Using in utero electroporation, we next examined changes in the distribution of GFP-positive cells during development in ferrets. We carried out in utero electroporation at E35–37 and dissected ferret babies at E40, P2 and P10. When ferret babies were dissected at E40, GFP-positive cells were distributed in the VZ, ISVZ and OSVZ (Fig. 5A,B). Basal fibers that ascend toward the pia were clearly visible (Fig. 5A,B, arrowheads). GFP-positive cells were located throughout the cortex at P2 and found mostly in the cortical plate at P10 (Fig. 5A), suggesting that most GFP-positive cells are migrating at P2, and the migration has been mostly completed by P10.

Identification and characterization of IFL-like fibers in ferrets
Although the IFL, which separates the ISVZ and the OSVZ, is one of the prominent features of the cerebral cortex of monkeys and humans (Smart et al., 2002; Zecevic et al., 2005), a previous study reported that an obvious fiber layer between the ISVZ and the OSVZ was difficult to distinguish in ferrets (Martínez-Cerdeno et al., 2012). It seemed possible that the IFL is specific to primates, and ferrets do not have fibers corresponding to those found in the IFL of monkeys. Conversely, it also seemed possible that ferrets do have fibers similar to those in the IFL of monkeys, but the fibers do not form a specific cytoarchitectonic layer and run within the neighboring OSVZ and/or ISVZ. Interestingly, we found GFP-positive fibers running tangentially in the OSVZ (Fig. 5A, P2 and P10, arrows; Fig. 5B, P2, square bracket. See also Fig. 3D, arrow). The GFP-positive fibers were predominantly located in the lower OSVZ (Fig. 5, P2 and P10).

This result is consistent with the idea that fibers, which correspond to those in the IFL of monkeys, also exist in ferrets and are running mainly in the lower OSVZ, although it is important to investigate whether the GFP-positive fibers found in this study are indeed homologous to fibers in the IFL of monkeys and humans. A previous study pointed out that the ferret OSVZ could be further divided into inner and outer subdivisions by Nissl staining, and the inner subdivision of the OSVZ had some cytoarchitectonic features similar to the IFL in monkeys (Reillo and Borrell, 2012). This inner subdivision of the OSVZ seems to correspond to the lower OSVZ, where we found the GFP-positive fibers.

Although the IFL is a prominent feature of the cerebral cortex in higher mammals (Smart et al., 2002; Zecevic et al., 2005), the origin of the IFL was unclear (Molnár and Clowry, 2012). One possibility was that the IFL consists of the fibers derived from newly generated cortical neurons, while it was also possible that early thalamocortical projections constitute the IFL (Molnár and Clowry, 2012). Because we expressed GFP in cortical cells but not in the thalamus, our results indicate that IFL-like fibers in ferrets consists of, at least partially, fibers derived from newly generated cortical neurons and/or neural progenitors. Interestingly, at E40, when GFP expression was restricted to neural progenitors, IFL-like fibers were invisible (Fig. 5, E40). This result suggests cortical neurons rather than neural progenitors are responsible for IFL-like fibers in the developing ferret cortex. Consistently, neurofilament-M was found...
in IFL-like fibers (Fig. 6). Because both the IFL and the increased number of cortical neurons are features of higher mammals, it seems plausible that the fibers in the IFL are derived from evolutionarily new cortical neurons that were added through cortical expansion during evolution. In addition, because the IFL is prominent in higher mammals, it would be intriguing to uncover its functional role.

**Discussion**
Here we have shown that transgenes can be efficiently expressed in neural progenitors in developing ferrets. We also determined the optimal experimental conditions to express transgenes in neural progenitors. Our procedure should provide a rapid means to investigate the molecular mechanisms underlying the development of the cerebral cortex in higher mammals. In addition, our results suggest that IFL-like fibers also exist in ferrets and are derived from newly generated cortical neurons.

**Development of the cerebral cortex in higher mammals**
Corticogenesis in higher mammals is characterized by the appearance of the OSVZ and the IFL. Earlier studies suggested that the proliferation of cells within the OSVZ contribute to the expansion of the cerebral cortex in higher mammals. Although anatomical and histochemical investigations have been performed extensively, the molecular mechanisms underlying the formation of the OSVZ are still unclear. Because previous pioneering studies reported the involvement of the Notch signaling and β3-integrin in the development of oRG cells using cultured slices in vitro (Fietz et al., 2010; Hansen et al., 2010), it would be important to examine the roles of the Notch signaling and β3-integrin in vivo using our method. Our method promises to be a powerful tool for investigating the molecular mechanisms underlying the proliferation and differentiation of neural progenitors in higher mammals.

Importantly, our results showed that the expression level of GFP was high enough to examine the morphology of transfected cells without using immunostaining. Therefore, it seems plausible that morphological changes of progenitors can be examined in living ferret neonates. Because RG cells, IP cells and oRG cells have distinct characteristic morphologies, it would be intriguing to examine the morphological transitions of these cells in vivo. It would also be interesting to investigate other features in vivo such as the cleavage plane during mitosis and cellular locomotion such as interkinetic nuclear migration and mitotic somal translocation.

**The advantages of ferrets**
 Usually more than 6 ferret babies are born from one pregnant mother. This large number of babies per pregnant mother relative to other higher mammals such as the marmoset is an important advantage of ferrets. This enables us to examine various experimental conditions and to obtain a sufficient number of experimental samples.

Brain structures such as the OSVZ, the IFL, ocular dominance columns (ODCs) and the parallel visual pathways (i.e. the magnocellular, parvocellular and koniocellular pathways) are especially prominent in higher mammals including ferrets. Although these structures have been believed to be important, the molecular mechanisms underlying the formation of these structures and their functional significance are still elusive. This is mainly because rapid and efficient genetic methods for manipulating genes in the brains of higher mammals had been poorly available. Therefore, our in utero electroporation procedure for ferrets should open the door to the next generation of neuroscientific experiments using higher mammals.

Recently, several groups including us have reported molecules expressed in the structures mentioned above that are unique to higher mammals (Kawasaki et al., 2004; Murray et al., 2008; Johnson et al., 2009; Yamamori, 2011; Bernard et al., 2012; Iwai et al., 2012). For example, we uncovered that the Forkhead transcription factor FoxP2 was selectively expressed in the parvocellular pathway in the dLGN of ferrets and monkeys (Iwai et al., 2012). Furthermore, recent studies provided lists of genes expressed in the VZ and the SVZ of mice and various cortical regions in monkeys (Ayoub et al., 2011; Bernard et al., 2012). Combining these molecules and our electroporation procedure, gain-of-function and loss-of-function studies using higher mammals are now ready to be conducted.

**Advantages of in utero electroporation**
It seems plausible to make transgenic ferrets using virus vectors because the successful application of virus vectors to make transgenic monkeys and marmosets was reported (Chan et al., 2001; Lois et al., 2002; Sasaki et al., 2009). However, compared with virus vectors, in utero electroporation has several advantageous features. First, it does not take a long time to obtain transfected animals. Transfected ferrets should be available within a couple of days. Second, multiple genes can easily be introduced simultaneously, as shown in this study. Co-transfection of GFP plus genes of interest should be useful. Third, transgenes can be selectively expressed in appropriate brain regions, even without using specific promoters, by modifying the direction of electrodes and the age when electroporation is performed. Finally, if necessary, cell type-specific promoters can be utilized. Because germinal zones of the cerebral cortex contain a heterogeneous population of progenitor cells (Hansen et al., 2010; Reillo and Borrell, 2012), it might be important to express transgenes in a cell type-specific manner to investigate the mechanisms underlying development of each progenitor. We recently reported that in utero electroporation is useful for exploring promoter regions and found that the newly generated Thy1S promoter resulted in sparse neuronal labeling in the cerebral cortex and the hippocampus of mice (Ako et al., 2011). Thus, our in utero electroporation procedure for ferrets described here could contribute to identifying cell type-specific promoters for each progenitor.

Theoretically, using our in utero electroporation procedure, it should be possible to express transgenes in neural progenitors.
including shRNA constructs, optogenetic molecules (e.g., channelrhodopsin and halorhodopsin), neuronal activity reporters (e.g. GFP-based Ca²⁺ sensors) and activity-modifying channels (e.g. Kir2.1 and NaChBac) (Boyden et al., 2005; Deisseroth et al., 2006; Arentiel et al., 2007; Gradinaru et al., 2010). Combining these molecules with in utero electroporation in ferrets would contribute toward an understanding of the formation, evolution and function of the gyrencephalic brains of higher mammals. In addition, because our results indicate that in utero electroporation can be used not only in rodents but also in ferrets, it seems reasonable to speculate that in utero electroporation is applicable to other higher mammals such as primates. It would be intriguing to establish in utero electroporation protocols for primates.

Acknowledgements
We are grateful for Drs Shoji Tsuji, Haruhiko Bito, Takashi Kadokawi (The University of Tokyo), Eiisuke Nishida (Kyoto University), Yoshiki Sasai (RIKEN-CDB) and Shigetada Nakashima (Osaka Bioscience Institute) for their continuous encouragement. We thank Zachary Blalock and Kawasaki lab members for their helpful discussion and support. This work was supported by the 21st Century COE Program “Center for Integrated Brain Medical Sciences” from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), the Global COE Program “Comprehensive Center of Education and Research for Chemical Biology of the Diseases” from MEXT, Grant-in-Aid for Scientific Research from MEXT, PRESTO from Japan Science and Technology Agency, and Human Frontier Science Program. This work was also supported by Takeda Science Foundation, Takeda Medical Research Foundation, Astellas Foundation for Research on Metabolic Disorders, the Life Science Foundation of Japan, the Kurata Hiramichi Science and Technology Foundation, Mitsubishi Foundation, Fukuda Foundation for Medical Technology, Yamada Science Foundation, Hokuto Foundation, Daichi-Sankyo Foundation of Life Sciences, Research Foundation for Opto-Science and Technology, and Santan Pharmaceutical.

Competing Interests
The authors have no competing interests to declare.

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