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Characterization of the *Frizzled10*-CreERTM Transgenic Mouse: An Inducible Cre Line for the Study of Cajal-Retzius Cell Development

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Summary

Cajal-Retzius cells are an enigmatic class of neurons located in the most superficial layer of the cerebral cortex, and they play an important role in cortical development. Although many studies have indicated that CR cells are involved in regulating cell migration and cortical maturation, the function of these cells is still not fully understood. Here we describe an inducible Cre mouse line in which CreERTM is driven by the promoter for the Wnt receptor *Frizzled10*. Consistent with our previous studies on *Frizzled10* expression and transgenic mouse lines using the *Frizzled10* promoter, we found that in the developing telencephalon, Cre was mainly detected at the cortical hem, the largest source of CR cells. By crossing the Cre line to R26R reporter mice and injecting tamoxifen at different time points, we were able to detect via X-gal staining CR cells produced from the cortical hem at distinct stages during development. Thus, this transgenic Cre mouse line is a valuable tool for studying the molecular and cellular mechanisms of CR cell development.

Keywords

Cajal-Retzius cell; reelin; CreERTM; *Frizzled10*; Wnt; cortical hem; cortical development; neuron migration

Cajal-Retzius (CR) cells are among the earliest born cortical neurons and are located in the marginal zone of the cerebral cortex, the most superficial cortical layer adjacent to the pial meninges. These cells play a major role in regulating both migration of other neurons and cortical network maturation by regulating synapse and dendrite formation during murine and human cortical development (Soriano and Del Rio, 2005).

A number of studies have indicated that CR cells and their best characterized gene product, the secreted glycoprotein reelin, are involved in neuronal migration. Reelin plays a key role in the normal inside-out formation of neocortical layers (D'Arcangelo *et al.*, 1995; Ogawa *et al.*, 1995; Rice and Curran, 2001). Neocortical layers are inverted and cells are abnormally dispersed in reeler mice, which lack normal reelin function (Caviness, 1976, 1982; D'Arcangelo *et al.*, 1995; Goffinet, 1984). Similar defects are observed in mice lacking the

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two reelin receptors, the very-low-density lipoprotein receptor and the apolipoprotein E receptor 2 (Hiesberger *et al.*, 1999; Trommsdorff *et al.*, 1999). These developmental defects are also observed in mice deficient in the downstream intracellular adaptor protein Dab1, which is required for reelin signaling (Howell *et al.*, 1997; Sheldon *et al.*, 1997; Ware *et al.*, 1997). On the other hand, emerging data show that the CR cells are a critical part of an early and complex network of neural activity in layer I. Recent studies have shown that CR cells contribute to spontaneous neural activity, which modulates cortical maturation during the very early stages of development (Aguilo *et al.*, 1999; Radnikow *et al.*, 2002; Soda *et al.*, 2003).

Although considerable research effort has been devoted to CR cells since their discovery by S. Ramon y Cajal and G. Retzius more than a century ago, the function of these cells during cortical development is still not fully understood. Notably, the precise molecular and cellular mechanisms by which these intriguing neurons regulate the development of the cerebral cortex remain to be elucidated. Part of the difficulty is that CR cells do not serve a single function but instead exert several disparate effects at distinct stages of development, and tools have been lacking to dissect these events.

In recent years, the Cre recombinase from bacteriophage P1 has become an essential tool for conditional gene activation and inactivation in the mouse for the study of gene functions and the downstream molecular mechanisms (Nagy, 2000). Furthermore, to allow conditional modification of gene activity at distinct stages of development, a fusion protein composed of Cre and a mutated form of the ligand binding domain of the estrogen receptor (Cre-ERTM) have been generated (Hayashi and McMahon, 2002) in which the Cre activity can be induced efficiently by the addition of tamoxifen (TM).

In our previous study, we reported a transgenic mouse line using the promoter of one of the Wnt receptors (*Frizzled10*) to specifically drive a reporter gene (*Tau-LacZ*) in the cortical hem (the most caudal-medial edge of the telencephalic neuroepithelium). We found that CR cells originating from the cortical hem were specifically labeled by β -galactosidase (β -gal) (Zhao *et al.*, 2006). Here, we generated a transgenic Cre-ERTM mouse line using the same promoter, and Cre was detected at the cortical hem in the developing telencephalon. Cre was also detected in the dorsal thalamus, cerebellum, dorsal spinal cord and DRG during development. By crossing the Cre line to R26R reporter mice and injecting TM at different time points, we could detect X-gal staining from CR cells produced from the cortical hem at distinct developmental stages. Thus, this transgenic Cre mouse line may be a valuable tool for studying the molecular and cellular mechanisms of CR cell development.

RESULTS AND DISCUSSION

As described previously, the construct used to generate the *Frizzled10*-CreERTM transgenic line contained a 6.7 kb fragment upstream of the transcriptional start site of the mouse *Frizzled10* gene followed by a CreERTM cassette carrying a polyA sequence (Fig. 1a). The DNA construct was injected into B6/FvB oocytes and five founders were obtained. To determine the recombination efficiency in the transgenic mice, each line was crossed with the R26R mouse line (Soriano, 1999). This reporter line contains a Cre-dependent, loxP-inactivated *lacZ* cassette within the ubiquitously expressed ROSA26 locus. Cre-mediated recombination results in β -galactosidase (β -gal) expression that is stably inherited by all daughter cells regardless of their differentiated fate. Pregnant bigenic mice were treated with a single intraperitoneal injection of TM at gestation point E10.5. Embryos were harvested at E12.5 and subjected to X-gal staining. Through a whole-mount analysis of all five lines, we observed that three lines exhibited the same *lacZ* expression pattern similar to that in our previous studies of the *Frizzled10*-*TauLacZ* transgenic mouse (Zhao *et al.*, 2006) (Fig. 1b,c). We therefore chose one of these lines for further characterization. We performed anti-Cre immunostaining and

Frizzled10 mRNA in situ hybridization at E12.5 and found that both Cre protein and *Frizzled10* mRNA could be detected in the cortical hem and dorsal thalamus (Fig. 1d,e). This indicated that Cre expression was very similar to the expression of endogenous *Frizzled10*.

To characterize the *Frizzled10*-CreERTM mouse line, we first performed immunohistochemical staining to examine Cre expression during development. In the developing forebrain, Cre expression was strictly detected at the cortical hem from E10.5 to E16.5 (Fig. 2a–f). The cortical hem, which expresses multiple Wnts and BMPs, is a transient neuroepithelial structure that forms the boundary between the hippocampus and the choroid plexus epithelium (CPE) during embryonic development (Grove *et al.*, 1998). Additionally, previous studies indicated that the cortical hem is a source of CR cell production (Meyer *et al.*, 2002; Takiguchi-Hayashi *et al.*, 2004). From E17.5 to P0, Cre was expressed in the outer marginal zone (OMZ) and fimbria (Fi) of the hippocampus (Fig. 2g,h), but rapidly declined to undetectable levels by P8 (data not shown). Interestingly, Cre expression was also observed at the thalamocortical axon (TCA) and internal capsule (IC) (Fig. 2g).

To examine the temporal pattern of Cre activity, *Frizzled10*-CreERTM mice were crossed to Cre reporter R26R, and the embryos were subjected to X-gal staining after TM injection at different time points. When TM was administered, Cre mediated a recombination event that activated the reporter allele. This event occurred within 6–12 h of TM administration and continued for up to 36 h (Hayashi and McMahon, 2002; Zervas *et al.*, 2004), which was consistent with the pharmacokinetics of TM in vivo (Robinson *et al.*, 1991). We injected TM at E4.5, E7.5, and E10.5 and performed X-gal staining at E12.5 and E13.5. Weak and diffuse LacZ activity was observed when Cre activity was induced at E4.5 (Fig. 2j), indicating that Cre worked in at least as early as E4.5. This result was consistent with that of a previous study that showed that *Frizzled10* gene expression began at E3.5 (Kemp *et al.*, 2007). Much stronger staining was observed when Cre activity was induced at E7.5 and E10.5 (Fig. 2k,l). Importantly, the distribution of LacZ activity after E7.5 TM injection demonstrated that Cre expression at this early stage was much more extensive than later stages, whereas the E10.5 injection yielded a pattern quite similar to the Cre expression pattern between E10.5 and E13.5.

The most important feature of the inducible Cre activity was that temporal aspects of lineage allocation could be addressed at different time points. After administration of TM, the population marked initially was identified 24–48 h later by detecting the LacZ protein. The fate of the initial population was then visualized at a later time point. In this way, the long-term fate and contribution of not only cells from a distinct genetic lineage, but also from cells marked during a defined 24-h period could be assessed (Joyner and Zervas, 2006). In this study, through marking by X-gal staining and through TM injection into the *Frizzled10*-CreERTM mice, we were able to mark cells originating from the cortical hem at different time points. Interestingly, many β -gal positive cells migrated laterally from the cortical hem to form an increasingly dense layer close to the pial surface of the cortex where CR cells are located (Fig. 3b,c,e,f). When TM was injected at E13.5 followed by examination of the whole brain by X-gal staining at P0, we observed a large population of β -gal positive cells covering the neocortex (Fig. 3g–i).

Our previous study has shown that CR cells originating from the cortical hem could be labeled in *Frizzled10*-TauLacZ transgenic mice (Zhao *et al.*, 2006). CR cells are one of the main cellular constituents of Layer 1 and are defined as being strongly reelin-positive cells in the marginal zone (Hevner *et al.*, 2003; Meyer *et al.*, 1999). Here we examined whether these β -gal positive cells were CR cells through X-gal staining and immunostaining with anti-reelin antibody. We found that all β -gal positive cells were reelin positive regardless of TM injection at E10.5 (Fig. 4a–c) or E13.5 (Fig. 4d–i). This was consistent with previous studies showing that a large percentage of CR cells originated from the cortical hem and migrated tangentially

to cover the cortex (Takiguchi-Hayashi *et al.*, 2004), and expanded upon previous findings that the hem was one of the CR cell sources (Meyer *et al.*, 2002; Takiguchi-Hayashi *et al.*, 2004).

Additionally, Cre activity in the cerebellum, dorsal root ganglia (DRG) and spinal cord was also observed. High levels of Cre expression and recombination activity were detected in Purkinje cells in the cerebellum, in the granule cell layer (GL) (Supporting Information Fig. a,b), in the DRG (Supporting Information Fig. c,d) and in the dorsal spinal cord (Supporting Information Fig. e,f).

In summary, we generated an inducible Cre transgenic mouse line in which Cre recombination activity could be induced by TM injection at different stages of development. Cre was detected mainly at the cortical hem, one of the sources of CR cells in the developing telencephalon. We also detected additional Cre activity in the dorsal thalamus, cerebellum, dorsal spinal cord and DRG. Thus this transgenic Cre mouse line is a valuable tool to study the molecular and cellular mechanisms of CR cell development and is useful for conditional inactivation of target genes in the cortical hem at different development stages.

MATERIALS AND METHODS

Mouse Manipulations

A 7.7 kb fragment containing promoter region of mouse *Frizzled10* gene was subcloned into Topo XL vector (Invitrogen). *CreERTM* gene and a polyadenylation site were excised from *CreERTM*-pBluescript SK(+) by *ApaI* and *EcoRI*, and inserted into the *EcoRV* site behind *Frizzled10* promoter. When the transgenic construct was linearized using *SalI* and *NotI* and purified by Qiagen kit (Cat No: 20021), a 6.7 kb fragment upstream of the *Frizzled10* transcriptional start site was used as the promoter. The fragment DNA was then microinjected into the pronuclei of B6/FVB oocytes using standard procedures. All offspring were genotyped by PCR using primers (5'-CATACCTGGAAAATGCTTCTGTCC-3', and 5'-ATTGCTGTCACTTGGTCGTGGC-3') to amplify a 300 bp *Cre* fragment.

X-Gal Staining

Embryos or embryonic brains were immersed in freshly prepared 2% paraformaldehyde (PFA) at 4°C for 2 h. Brains older than P0 were perfused by 2% PFA. Tissues were then cryoprotected in 30% sucrose, embedded in OCT and sectioned at 16–25 µm (embryonic brains) on a cryostat or at 40 µm (older than P8) on a sliding microtome. After washing and permeabilizing in PBS containing 0.02% NP-40, 2 mM MgCl₂ for 30 min, the tissue was stained with 1 mg/ml X-gal in PBS containing 0.02% NP-40, 5 mM of K₃Fe(CN)₆, 5 mM of K₄Fe(CN)₆, and 2 mM of MgCl₂ at 37°C for about 6–16 h. Sections were then rinsed with PBS, refixed in 4% formalin about 4 h, and coverslips were applied.

Immunohistochemistry

Tissues were fixed in 4% PFA and sections were prepared as described above. Sections were then permeabilized with 0.1% Triton in PBS, blocked in 10% calf serum, incubated with first antibody at 4° overnight, and detected by fluorescent secondary antibodies. For X-gal/immunohistochemistry double-staining, after X-gal staining the sections were refixed in 4% PFA and processed with a standard immunohistochemistry procedure. Instead of fluorescent secondary antibodies, staining was detected by biotinylated secondary antibodies and was viewed by DAB staining. The following antibodies and reagents were used for immunostaining: rabbit anti-Cre (Covance, PRB-106C), mouse anti-reelin (Chemicon, MAB536), FITC goat anti-Rabbit IgG (Jackson Immuno-Research, 111-095-144), FITC goat anti-mouse IgG (Jackson ImmunoResearch, 115-095-146), and biotinylated goat antirabbit IgG (Vector Laboratories, BA1000). VECTASTAIN ABC kit (Vector Laboratories, pk-6100).

For microscopy of X-gal/immunohistochemistry, the bright-field views of the images were inverted and then merged with the fluorescent images.

In Situ Hybridization

Brains were fixed in 4% PFA overnight and cryoprotected in 30% sucrose/DEPC-PBS at 4°C, and then embed in OCT. Coronal sections were obtained with a Leica CM 3050S cryostat and stored at -70°C until use. In situ hybridization on sections was performed as described previously (Zhao *et al.*, 2006). The concentration of the probe used in this study was 1 µg/µL, and sense probe was used as a negative control.

TM Injection

TM (Sigma, T5648) was dissolved in corn oil (Sigma, C8267) at a concentration of 10 mg/ml. Two milligrams of TM/40 g body weight was injected into pregnant mothers at E10.5, 13.5, 16.5 days postcoitum (dpc), 1 mg of TM/40 g body weight at 7.5 days postcoitum (dpc), and 0.1 mg of TM/40 g of body weight at 4.5 dpc.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Schematic drawing of the *Frizzled10*-CreERTM construct and the generation of transgenic mouse. **(a)** The transgenic construct of *Frizzled10*-CreERTM mouse. **(b, c)** Screening of transgenic founders. **(b)** X-gal stained E12.5 transgenic embryo after TM injection at E10.5. **(c)** Wild-type control. **(d, e)** Cre expression mimicked expression of endogenous *Frizzled10* in transgenic mice. **(d)** Cre expression in E12.5 transgenic telencephalon. **(e)** In situ hybridization of endogenous *Frizzled10* mRNA at E12.5. Arrows indicate the cortical hem of cortex. Cx, Cortex; Th, thalamus. Scale bars: 400 μ m.

FIG. 2.

Analysis of Cre expression in developing transgenic cortex by immunostaining. (a-f) Cre was expressed at the cortical hem (CH) in the developing mouse cortex from E10.5 to E16.5. Arrows indicate the cortical hem. Cre expression was also observed at the dorsal thalamus (c, d, f, g). (g-i) From E17.5 to P0, Cre was detected at OMZ and Fimbria in transgenic brains. (h) High magnification view of (g). (j-l) X-gal staining after TM administration showed that the Cre initial working time is at least as early as E4.5. (j) TM injected at E4.5 and embryos harvested and X-gal stained at E13.5. (k, l) Transgenic embryos were X-gal stained at E12.5 after TM injection at E7.5 (k) and E10.5 (l). Cx, Cortex; Th, thalamus; CH, cortical hem; OMZ, outer

marginal zone; Fi, Fimbria; TCA, thalamocortical axon. IC, internal capsule. Scale bars: 400 μm .

FIG. 3.

X-gal staining shows a large population of β -gal positive cells originating from the cortical hem at different developmental stages. (a–c) TM was injected at E10.5 and X-gal staining was carried out at E14.5. High lacZ activity was detected at cortical hem and thalamus. (b) High magnification view of large boxed region in (a), showing strong X-gal stained cells originating from the hem (arrowheads). (c) High magnification view of small boxed region in (a), showing β -gal positive cells in the surface of the developing cortex (arrowheads). (d–f) X-gal stained E14.5 transgenic brain after TM administration at E12.5. (d) Whole-mount staining of a hemisphere showing strong LacZ activity at the cortical hem. (e) High magnification view of boxed region in (d), showing cells migrating out of the hem (arrowheads). (f) A coronal section of E14.5 brain showing the cortical hem and cells originating from the hem (arrowhead). (g–i) X-gal stained whole P0 brain after TM administration at E13.5. Large populations of β -gal positive cells covering the surface of cortex were observed. (h) High magnification view of boxed region in (g). (i) High magnification view of boxed region in (h) and arrows indicate the β -gal positive cells on the surface of cortex. Cx, Cortex; CH, cortical hem; MHB, medial habenular nucleus. LHB, lateral habenular nucleus. Scale bars: a, e, h: 1 mm; b, i: 500 μ m; c, f: 200 μ m.

FIG. 4.

All β -gal positive cells covering the cortex were reelin positive. (a–c) Double-stained bi-transgenic E14.5 brains by X-gal and anti-reelin immunostaining after TM injection at E10.5 (b, c) High magnification view of boxed regions in (a). Arrows indicate that β -gal positive cells are reelin positive. (d–i) Double-stained P0 brains after TM injection at E13.5. (e, f) High magnification view of boxed regions in (d). Arrows indicate that all β -gal positive cells are reelin positive. (g–i) Characterization of β -gal positive cells by immunofluorescence staining. (g) Immunofluorescence of anti-reelin. (h) Inverted dark-field view of X-gal staining by adding false color. (i) Merged view of (g) and (h). Arrows indicate the double labeled cells. Scale bars: a: 400 μ m; d: 1 mm; others: 200 μ m.