INTRODUCTION

The Cre-loxP recombination system has been widely used for studying gene functions in animals by allowing region-specific knockout of target genes through site-specific expression of Cre. Inducible Cre such as CreER provides more specific control of spatiotemporal deletion or lineage labeling through timed administration of synthetic estrogen receptor (ER) ligands such as tamoxifen (TAM) or 4-hydroxytamoxifen (4-OHT) [1, 2]. Embryonic administration of tamoxifen rapidly induces abortion in pregnant mouse mothers and severely perturbs embryonic development, rendering the inducible Cre system inapplicable to the study of developmentally regulated genes in embryos [3]. In contrast, tamoxifen injection after birth is less harmful and relatively tolerable in neonates, suggesting that inducible Cre-loxP may provide genetic tools for the study of postnatal development [4]. While most major structures in the central nervous system develop before birth, cerebellar architecture develops actively during the first three weeks after birth [5]. During this period, neuronal progenitor cells proliferate, migrate, and terminally differentiate into the cerebellar cortex. Postnatal injection of tamoxifen to neonates may enable studies on developmentally regulated gene functions in mitotic and/or postmitotic cells in the cerebellum.

Cell Type-specific Knockout with Gli1-mediated Cre Recombination in the Developing Cerebellum

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The inducible Cre-loxP system provides a useful tool for inducing the selective deletion of genes that are essential for proper development and enables the study of gene functions in properly developed animals. Here, we show that inducible Cre-loxP driven by the Gli1-promoter can induce cell-type-specific deletion of target genes in cerebellar cortical neurons. We used reporter mice containing the YFP (yellow fluorescence protein) gene at the Git(ROSA)26Sor locus with a loxP-flanked transcriptional stop sequence, in which successful Cre-mediated excision of the stop sequence is indicated by YFP expression in Cre-expressing cells. Administration of tamoxifen during early postnatal days (P4–7) induces Cre-dependent excision of stop sequences and allows YFP expression in proliferating neuronal progenitor cells in the external granule layer and Bergmann glia in the Purkinje cell layer. A substantial number of YFP-positive progenitor cells in the external granule layer migrated to the internal granule cell layer and became granule cell neurons. By comparison, injection of tamoxifen during late postnatal days (P19–22) induces YFP expression only in Bergmann glia, and most granule cell neurons were devoid of YFP expression. The results indicate that the Gli1 promoter is temporarily active in progenitor cells in the external granule layer during the early postnatal period but constitutively active in Bergmann glia. We propose that the Gli1-mediated CreER system can be applied for the conditional deletion of genes of interest from cerebellar granule cell neurons and/or Bergmann glia.

Key words: Cerebellum, Cre recombinase, Tamoxifen, Gli1, Bergmann glia, Granule cell neuron

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lucal layer (ML), Purkinje cell layer (PCL), and granule cell layer (GCL). The somata of Purkinje cells (PCs) and Bergmann glia (BGs) are arranged in a single PCL layer. PCs are the only output neurons of the cerebellar cortex, and each sends a single, long axon to the deep cerebellar nuclei (DCN). ML contains inhibitory inter-neurons, parallel fibers of GCNs, PC dendrites, and BG radial fibers. The complex dendrites of PCs in the ML receive presynaptic inputs from parallel fibers (PFs) originating in GCNs in the GCL and climbing fibers (CFs) projecting from the inferior olivary nucleus [6]. This trilaminar architecture of the cerebellar cortex developed perinatally. During the late embryonic period (E17.5) and postnatal development, cerebellar granule cell progenitors (GCPs) rapidly proliferate in the external granule layer (EGL), radially migrate to the internal GCL, and eventually differentiate into GCNs. Sonic hedgehog (Shh) plays a key role in the proliferation of GCPs during cerebellar morphogenesis and histogenesis, and deletion of Shh induces hypoplasia of the cerebellar cortex [7-9]. Shh secreted by PC [9, 10] activates the Gli1 promoter in GCPs and BGs [7, 11-13]. Thus, Gli1CreERT2 mice with CreERT2 knocked into the Gli1 locus are widely used as a readout of Shh-positive signaling to study the contribution of Shh transcriptional activator function during postnatal development [14-16]. Recently, several proteins from the synaptic compartment have been shown to have a longer half-life than those from the cytoplasmic compartment [17]. The long half-life of proteins makes it difficult to sufficiently clear these gene products from post-mitotic neurons even after the mRNA and protein are no longer synthesized after genetic recombination. Gli1CreERT2 mediated recombination in proliferating GCPs may aid in the study of synaptic proteins with an extremely long half-life.

**MATERIALS AND METHODS**

**Mouse genetics**

Gli1™(CreERT2Alj)/J (referred to as Gli1CreERT2, #007913) and B6.129X1-Gt(ROSA)26Sor tm1(EYFP)Cos (referred to as R26R-YFP, #006148) have been previously described [14, 18]. Gli1CreERT2 mice were crossed with R26R-YFP mice to generate heterozygotes for each allele and used to determine Cre-mediated recombination. Gli1+/+::R26RYFP/+ was used as a negative control to demonstrate the specificity of tamoxifen (TAM) administration. Offspring were genotyped by polymerase chain reaction (PCR) with genomic DNA (gDNA) as previously described [19] using the primers shown in Table 1.

TAM (Sigma-Aldrich) was dissolved in corn oil to a final concentration of 10 mg/ml. To activate Cre recombinase, mice were force-fed TAM (50 μg/g weight/day) by oral pipet-feeding at the indicated time points and housed until euthanized.

All experimental procedures were approved by Ajou University Medical Center-Institutional Animal Care and Use Committee (AUMC-IACUC, Suwon, South Korea).

**Immunofluorescence analysis**

Immunohistochemical analyses were performed as described previously [20]. Briefly, mice were deeply anesthetized with 2.2.2

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**Table 1. The primer sequences for PCR reaction**

| Target                      | Primer sequence         | PCR product |
|-----------------------------|-------------------------|-------------|
| **R26R**                    |                         |             |
| Forward                     | 5'-AAA GTC GCT CTC ATG GAA ATG CAT GAT TAT-3' | WT: 600 bp (F+R1) |
| Reverse 1                   | 5'-GGA GCG GGA GAA ATG GAT ATG-3'       | Knock-in: 325 bp (F+R2) |
| Reverse 2                   | 5'-GCG AAG AGT TTG TCC TCA ACC-3'       |            |
| **ΔR26R-YFP (recombined)**  |                         |             |
| Forward                     | 5'-GGC AAG AGT TTG TCC TCA ACC-3'       | 750 bp      |
| Reverse                     | 5'-ATG GCG GAC TTG AAG ACG TG CG-3'     |             |
| **Gli1-CreERT2**            |                         |             |
| Forward                     | 5'-GCA TTA CCG GTC GAT GCA ACG AGT GAT GAG-3' | 404 bp      |
| Reverse                     | 5'-GAG TAG ACG AAC CTG GTC GAA ATC AGT GCG-3' |             |
tribromoethanol (200 mg/kg, i.p., Sigma-Aldrich), and then perfused transcardially with 10% neutral buffered formalin (BBC Biochemical). The brain was extracted, post-fixed in 10% neutral buffered formalin overnight at 4°C, and cryoprotected in 30% sucrose. The brain was sagitally divided into two halves, embedded in OCT compound (Tissue-Tek, Sakura Finetek), and sectioned into 30 μm-thick frozen sections using a Leica cryostat (Leica). The cryosections were air-dried, and the residual OCT compound was washed in PBS with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) (PBS-T). After incubation in blocking solution [10% (vol/vol) normal goat serum (Gibco), 1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS-T] for 1 h at room temperature, the sections were incubated with primary antibodies overnight at 4°C. The antibodies used in this study were as follows: anti-GFP (1:500, Abcam, #ab13970), anti-Pcp2 (1:500, Santa Cruz, #sc-137064), anti-GFAP (1:200, Dako, #Z0334), anti-S100β (1:500, Abcam, #ab41548), anti-NeuN (1:500, EMD Millipore, #MAB377), and anti-parvalbumin (PV; 1:500; Swant, #PV25). After unbound antibodies were washed with PBS-T, the sections were incubated with secondary antibodies conjugated with Alexa Fluor 405, 488, or 568 (1:500, Invitrogen). If necessary, nuclear counterstaining was performed using bisbenzamide (1:50,000, Hoechst 33258; Invitrogen). All fluorescence images were acquired using a Zeiss LSM710 confocal laser scanning microscope (Carl Zeiss) or Zeiss Axio Scan Z1 slide scanner (Carl Zeiss) at the Three-Dimensional Immune System Imaging Core Facility of Ajou University.

Quantitative analysis

Confocal images of cerebellar sagittal sections were analyzed using ZEN software (Blue Edition, Zeiss). The specificity of Gli1-CreER2 were expressed as the ratio of NeuN+GCNs among YFP+ cells in GCL or the ratio of S100β+ cells in PCL/ML. The coverage was shown as the ratio of YFP+ cells in NeuN+ GCNs or in S100β+ cells as previously defined [21].

RESULTS

Cerebellum specific Cre-mediated recombination driven by Gli1 promoter

To assess the Gli1 promoter-mediated expression of the Cre enzyme, Gli1CreER1/2:: R26RYFP/+ mice were obtained by breeding Gli1CreER1/2 mice carrying the CreERT2 gene under the Gli1 promoter with R26R-YFP reporter mice that harbored a floxed stop cassette upstream of the enhanced yellow fluorescent protein gene (YFP) at the ubiquitously expressed ROSA locus [18]. Genotyping was performed by PCR using gDNA obtained from the tail biopsy or cerebellum. The wildtype and floxed alleles were detected as 600 bp and 325 bp fragments, respectively (Table 1).

The TAM-activated Cre enzyme excised the stop cassette flanked by two loxP sites and permitted YFP expression in Gli1-expressing cells. Cre-mediated recombination was validated using a 750 bp PCR product with gDNA isolated from the cerebellum. Such PCR products were not detected in the cerebral cortex, suggesting that the Gli1 promoter is active only in the cerebellum (Fig. 1C). Consistently, in the sagittal sections of Gli1CreERT2:: R26RYFP/+ brain, YFP expression was detected only in the cerebellar cortex, including the ML, PCL, and GCL, but not in the white matter or other brain regions (Fig. 1D). The results indicated that administration of TAM during early postnatal periods induces recombination in the cerebellar cortex.

Gli1 active cells in early postnatal cerebellum

The proliferation of GCPS reaches its peak at P4-8 in response to Shh produced by PCs [22]. To determine the cell types responsive to Shh in early postnatal period, we administered TAM at the peak time of the GCP proliferating period (P4–7) and sacrificed the mice at P9 (Fig. 2A). YFP expression was detected in proliferating GCPs in EGL. YFP+ cells migrated to the GCL and became fully differentiated NeuN+ GCNs. YFP expression was also detected in radially extending fibers in the ML and soma of GFAP+ BGs in the PCL (Fig. 2C and 2D). YFP was not expressed in Pcp2+ PCs in the PCL (asterisks in Fig. 2D) or parvalbumin (PV+) including GAB-Aergic inerneurons and PCs (Fig. 3). These results suggest that the administration of tamoxifen during early postnatal days induces expression of Cre recombinase in Gli1-expression GCPS and BGs, but not in other types of cells in the developing cerebellum.

Cerebellar granule cells- and Bergmann glial cells- specific expression of Cre recombinase activity

To determine the duration of Shhresponsiveness, we administered TAM at two distinct time points: during P4–7, when GCPs in the EGL migrated to the internal GCL and during P19–22 when EGL no longer existed [23]. YFP expression that was induced by TAM at P4–7 in GCPs and BGs was maintained in GCNs and BGs in the mature brain at P25 (Fig. 4B and 4D). In contrast, TAM administration at P19–22 induced YFP expression only in BGs, but not in GCPs (Fig. 4C and 4E). For quantification, we performed additional staining with anti-S100β. GFAP and S100β revealed the same cell population with distinctive immunoreactivity: S100β in the somata and proximal processes of BGs and GFAP in the arborized glial fibers. TAM injection at P4–7 induced YFP expression in 15.1±1.4% of NeuN+ GCNs and 18.0±1.8% of BGs (Fig. 4F and 4G). The specificity of YFP expression was high thus...
most YFP+ cells in GCL or PCL were NeuN+ GCN (98.6±0.46%) or BGs (100%), respectively. When TAM was injected at P19~22, the YFP+ expression was found in 45.8±2.9% to BGs in PCL with 99.5±0.47% specificity. None of GCNs were co-localized with YFP+ expression. The results indicated that Shh signaling is temporarily active in the proliferation of GCP and BG during the early postnatal period and constitutively active only in BGs.

**DISCUSSION**

This study showed that timed postnatal administration of TAM differentially regulates cell-type-specific excision of floxed genes in the developing cerebellar cortex. TAM administration at early postnatal days (P4~7), when the GCP proliferation peaks in the EGL, can induce the expression of Gli1-mediated Cre recombinase in GCPs of EGL and BGs in PCL. Thus, tamoxifen administration during postnatal days (P19~22), when EGL is almost depleted, leads to the expression of YFP only in BGs. Our results are consistent with the previous finding that Gli1 expression is restricted to proliferating GCPs and BGs in the developing cerebellar cortex in response to Purkinje-derived Shh in postnatal stages through adulthood [10, 13, 24, 25]. Importantly, YFP+ GCPs in the EGL inwardly migrate to and differentiate NeuN+ GCNs in the GCL, where they remain as YFP+ GCNs to adulthood (Fig. 2).
As mentioned earlier, proteins with long half-lives in the synaptic compartment [17] may remain in postmitotic GCNs even after the mRNA and protein are no longer synthesized. Gli1-CreERT2 may be advantageous for inducing genuine deficiency of the gene products by targeting GCPs, and the protein products are diluted during cell division.

**Shh-Gli1 signaling in the developing cerebellum**

All cerebellar neurons are generated from progenitors in two distinct germinative centers in the hindbrain: the rhombic lip and the ventricular zone [5, 26]. The progenitor cells in the rhombic lip express Math-1 (mouse homolog-1 of Drosophila Atonal) and generate glutamatergic neurons, including projection neurons in deep cerebellar nuclei, unipolar brush cells, and GCNs in the GCL.

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**Fig. 2.** Gli1-mediated Cre recombination in proliferation EGL, ML, and GCL. (A) Schematic experimental design for the tamoxifen injection time points used in Gli1CreERT2+/::R26R<sup>YFP</sup> mice. Pups were orally injected TAM at P4-7 and sacrificed at P9 to analyze the YFP-expressing cells. (B) YFP-labeled cells following tamoxifen injection are dominantly distributed in EGL, ML, and GCL in cerebellar cortex of the P9 Gli1CreERT2+/::R26R<sup>YFP</sup> pups. The lobules of vermis are identified by Roman numerals (II-X). Boxed region denotes enlarged area in C and D. (C, D) Triple staining for YFP, NeuN (a marker for GCs), PCP2 (a marker of PCs) and/or GFAP (a marker of BGs) of sagittal cerebellum of Gli1CreERT2+/::R26R<sup>YFP</sup> showed that YFP signal was colocalized with NeuN<sup>+</sup> proliferating GCPs in EGL (C), differentiated GCNs in ML (C’–C”), GFAP<sup>+</sup> BGs in ML/PCL (D–D”), but not in PCP2<sup>+</sup> PCs (D and D”). Boxed region in C and D denotes enlarged area in C, C’, D, and D”. In D”, Asterisks indicated soma of PCs. P, postnatal day; TAM, tamoxifen; IHC, Immunohistochemistry; EGL, external granule cell layer; ML, Molecular layer; PCL, Purkinje cell layer; GCL, Granule cell layer. Scale bars=500 μm in B, 50 μm in C and D, 20 μm in C’–D”.

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The progenitor cells in the ventricular zone generate all GABAergic phenotypes, including PCs, nucleo-olivary projection neurons, and all inhibitory interneurons, astrocytes, and oligodendrocytes in the white matter [29, 30]. Shh produced by PCs acts as a mitogen on progenitor cells originating from the rhombic lip and ventricular zone [24, 31] affects Bergmann glial differentiation [12]. Based on Gli1 expression, a high level of positive Shh signaling is restricted to the proliferating GCPs and BGs in developing cerebellar cortex [13, 24]. Only BGs have been shown to be capable of responding to PC-derived Shh signals in postnatal stages through adulthood [25]. Consistently, TAM activates Gli1-CreERT2 in Shh-responsive GCPs in the EGL and BGs in the PCL (Fig. 1 and 2). Shh is also known to exert a proliferative function on neural stem cell-like progenitors in the white matter around P1–2 [9, 15, 32]. These progenitor cells (Tnc', CD133') give rise to GABAergic progenitor cells (Ptf1a') and astrocyte precursors (Tnc', CD15'), which eventually differentiate into interneurons, oligodendrocytes, astrocytes, and BGs [9, 30, 33]. Thus, TAM administration at P1–3 activates Gli1-CreERT2 in these progenitor cells, leading to the expression of reporter genes in interneurons and astrocytes [15]. However, we did not detect YFP expression in GABAergic neurons when TAM was administered at P4–7 (Fig. 3). Our results are consistent with the notion that the entire repertoire of GABAergic interneurons in the cerebellar cortex is generated before P7 with a peak around P5 [29, 30, 34]. Importantly, timed administration is critical to conditionally knockout the target genes in glutamatergic GCNs without affecting gene expression in GABAergic interneurons.

**Cre-mediated knockout for the study of cerebellar cortex**

Several Cre lines have been used to knock out genes in specific cell types in the cerebellum (Table 2): GABAa6-Cre and Math1-Cre for deletion in GCNs [35–38], and Pcp2/L7-Cre and Shh-Cre for deletion in PCs [15, 39–41]. While GABAa6-Cre is useful
Fig. 4. Gli1-mediated Cre recombination in GCNs and BGs. (A) Schematic experimental design for the tamoxifen injection time points used in Gli1<sup>CreERT2/+</sup>:<sup>R26RYFP/+</sup> mice. Pups were orally injected with TAM at P4–7 (left, for B and D) or P19–22 (right, for C, E), and sacrificed at P25 to analyze the YFP-expressing cells. (B–E) Tamoxifen injection at different time point led to YFP labeled cells that were cerebellar cell-type and specifically regulated Cre-mediated recombination in P25 Gli1<sup>CreERT2/+</sup>:<sup>R26RYFP/+</sup> cerebellum. Similar to P9 cerebellum, injection of TAM at P4–7 allowed YFP expression which was induced in NeuN<sup>+</sup> GCNs (Fig. B–B”), GFAP<sup>+</sup> BGs (D–D”), but not in PCP2<sup>+</sup> PCs (D), whereas late administration of TAM at P19–22 led to detection of YFP signal in GFAP<sup>+</sup> BGs (E–E”), but not in any of GCNs (C–C”) and PCs (E) in P25 Gli1<sup>CreERT2/+</sup>:<sup>R26RYFP/+</sup> cerebellum. Boxed region in C–E denotes enlarged area in C’–E”. Scale bars=50 μm in B–E, 20 μm in B’–E”. (F–G) Colocalization of YFP<sup>+</sup> cells were assessed with confocal images from 4–6 from sagittal sections of 2–3 animals per group as mentioned in the Methods. The specificity and coverage of YFP expression in NeuN<sup>+</sup> GCNs and in S100β<sup>+</sup> are shown means±SEM.
for late-onset Cre expression in mature GCNs, Math1-CreER and NSE-CreERT2 are useful for targeting GCPs that give rise to GCNs in GCL [35, 38]. However, Cre enzymes are also expressed in the non-cerebellar area in these mouse lines, such as the hippocampus, midbrain, medulla, spinal cord, and inner ear [27, 38, 42]. Unlike GCPs or GCNs, the Gli1 promoter is constitutively active in BGs. Thus, TAM administration around the weaning phase (P19~22) induces YFP expression in BGs. Several inducible Cre lines have been utilized for the study of gene function in BGs and specific types of astrocytes. While GFAP-CreER [21, 43] and GLAST-CreER [44] induce recombination in BGs and astrocytes in a wide area of the brain, TNC-CreER can limit the reporter gene expression to the BGs and/or GABAergic interneurons in the cerebellum, depending on TAM administration [15].

**The perspective application of Gli1-CreERT2**

The cerebellum is the largest sensorimotor structure in the brain and has extensive connections with the brainstem and spinal cord. The cerebellum plays an important role in coordinating skilled voluntary movements by influencing muscle activity and controlling equilibrium and muscle tone through connections with the vestibular system and the spinal cord and its gamma motor neurons. Recently, there has been rapidly increasing evidence indicating the role of the cerebellum in emotion and cognition in addition to movement [49, 50]. Long-term depression (LTD) [53]. Intensive studies using Pcp2/L7-Cre have revealed that the molecular machinery, including Ca^{2+} influx, protein kinase C, and endocytosis of AMPA-type glutamate receptors play critical roles in postsynaptic PCs [41]. By comparison, the presynaptic roles of PF in LTD induction are relatively unknown.

We show that Gli1-CreERT2 system can be applicable to conditional deletion of genes from cerebellar GCNs and/or BGs without altering gene expression in the non-cerebellar area and TAM treatment window of P4~7 allows excitatory GCN-specific deletion without affecting gene expression in GABAergic interneurons or PCs. We also propose that Gli1-CreERT2 can provide a tool to identify the molecular and cellular events in presynaptic PFs by allowing selective deletion of synaptic proteins with long half-lives in GCNs.

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