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Generation and analysis of an improved *Foxg1-IRES-Cre* driver mouse line

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ABSTRACT

Foxg1 expression is highly restricted to the telencephalon and other head structures in the early embryo. This expression pattern has been exploited to generate conditional knockout mice, based on a widely used *Foxg1-Cre* knock-in line (*Foxg1*$^{tm1(cre)Skm}$), in which the *Foxg1* coding region was replaced by the *Cre* gene. The utility of this line, however, is severely hampered for two reasons: (1) *Foxg1-Cre* mice display ectopic and unpredictable Cre activity, and (2) *Foxg1* haploinsufficiency can produce neurodevelopmental phenotypes. To overcome these issues, we have generated a new *Foxg1-IRES-Cre* knock-in mouse line, in which an IRES-Cre cassette was inserted in the 3′UTR of *Foxg1* locus, thus preserving the endogenous *Foxg1* coding region and un-translated gene regulatory sequences in the 3′UTR, including recently discovered microRNA target sites. We further demonstrate that the new *Foxg1-IRES-Cre* line displays consistent Cre activity patterns that recapitulated the endogenous *Foxg1* expression at embryonic and postnatal stages without causing defects in cortical development. We conclude that the new *Foxg1-IRES-Cre* mouse line is a unique and advanced tool for studying genes involved in the development of the telencephalon and other *Foxg1*-expressing regions starting from early embryonic stages.

1. Introduction

The 'Cre/loxP' system is a powerful and widely utilized technology for the conditional gene manipulation and cell lineage tracing (Branda and Dymecki, 2004; Nagy, 2000). A key element of this approach is the creation of a suitable driver line that is phenotypically normal, but expresses Cre robustly, and reproducibly in a specific pattern. Even commonly used Cre driver lines, however, can have unexpected Cre activity patterns or show complicating phenotypes (Harno et al., 2013; Heffner et al., 2012; Schmidt-supprian and Rajewsky, 2007). For example, a *Nestin-Cre* mouse line (*Tg(Nes-cre)1Kln*), is one of the most commonly used Cre driver lines in the neuroscience field, but reports have revealed unexpected Cre expression in many tissues outside the central nervous system and a significant metabolic and behavioral phenotype in *Nestin-Cre* mice (Declercq et al., 2015; Giusti et al., 2014; Harno et al., 2013). These factors greatly complicate the interpretation of the derived phenotypes, and reduce the utility of this approach.

The transcription factor *Foxg1* (formerly called *BF-1*), a member of the forkhead box family proteins, is one of the earliest genes expressed in the emerging telencephalic region starting around E8.5–E9.0 (Hébert and McConnell, 2000; Shimamura et al., 1995; Xuan et al., 1995). Because of its very early and robust expression in the telencephalic progenitors and other embryonic regions, a knock-in mouse line in which the Foxg1 coding sequences was replaced with Cre gene (*Foxg1*$^{tm1(cre)Skm}$) has been widely used to manipulate function of genes expressed in the telencephalon, inner ear, olfactory epithelium, anterior retina, pharyngeal endoderm, Rathke's pouch, and foregut at early embryonic stages (Hébert and McConnell, 2000). However in the *Foxg1-Cre* mice, ectopic and unpredictable Cre activities have been observed. Analysis of the *Foxg1-Cre* line further revealed that Cre activity pattern varies significantly in each mouse, and the extent of variation is different depending on genetic background. The least variability has been found in the 129SvJ strain but even still, about 36% of *Foxg1-Cre* mice in the 129SvJ background show unintended and ectopic Cre recombination patterns that differed from the endogenous *Foxg1* expression (Hébert and McConnell, 2000). Due to its abnormal behavioral responses and congenital hypoplasia in the corpus callosum (Crawley et al., 1997; Wahlsten, 1982), the 129SvJ strain is not a preferred background strain in the neuroscience field. In a more widely used genetic background like C57BL/6, the *Foxg1-Cre* driver has been reported to generate widespread ectopic recombination, in some cases in the entire central nervous system and in others, in almost all tissues both in head and body regions (Hébert and McConnell, 2000). The dysregulation of expression in the *Foxg1-Cre* line precludes its use as a

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telencephalon specific Cre driver line, as ectopic Cre activity is invariably observed in regions around the mid- and hindbrain (Achim et al., 2012; Fuccillo et al., 2004; Hébert and McConnell, 2000; Kasberg et al., 2013; Li et al., 2008, 2012; Ma et al., 2002; Zembrzycki et al., 2007). The reasons behind these inconsistent Cre activities are not completely known but could be due in part by deletion of functional endogenous microRNA (miRNA) target sites in the *Foxg1* 3′UTR from the *Cre* coding messenger RNA (mRNA) (Miyoshi and Fishell, 2012) that would normally repress *Foxg1* expression (Choi et al., 2008; Garaffo et al., 2015; Shibata et al., 2011, 2008). In addition, the *Foxg1-Cre* allele contains a PGK-neo cassette, which in some case has been shown to result in unpredictable Cre activity (Iwasato et al., 2004; Pham et al., 1996).

The other notable caveat of using the *Foxg1-Cre* line is that intrinsic phenotypes due to the haploinsufficiency of *Foxg1* gene have been observed. Although earlier reports have claimed that heterozygous *Foxg1* mice appear normal (Dou et al., 1999; Hanashima et al., 2004, 2002; Hébert and McConnell, 2000; Xuan et al., 1995), recent papers have reported that the heterozygous *Foxg1-Cre* mice show a variety of significant neurodevelopmental defects including microcephaly, aberrant cortical area patterning, and impaired neurogenesis in the telencephalon (Eagleson et al., 2007; Frullanti et al., 2015; Shen et al., 2006; Siegenthaler et al., 2008). Therefore, phenotypes observed in the *Foxg1-Cre*-mediated conditional knockout mice could at least in part be caused by *Foxg1*-haploinsufficiency.

To overcome these limitations, we have generated a new *Foxg1-IRES-Cre* mouse line. We show here that this new driver produces consistent Cre recombination patterns that faithfully recapitulate the endogenous *Foxg1* expression at embryonic and postnatal stages without causing *Foxg1*-haploinsufficiency. We conclude that the *Foxg1-IRES-Cre* line is a new tool for manipulating gene expression involved in the development and function of telencephalon and other *Foxg1*-expressing tissues from early embryonic stages.

2. Materials and methods

2.1. Mice

All animal experiments were approved and conducted following the guidelines of the Institutional Animal Care and Use Committee at the Salk Institute and were in full compliance with the guidelines of the National Institutes of Health for the care and use of laboratory animals. The day of insemination and the day of birth are designated as embryonic day 0.5 (E0.5) and postnatal day 0 (P0), respectively. *Foxg1-IRES-Cre* mice were backcrossed to C57BL/6 background mice (Harlan Laboratories) for at least six times except for Fig. 6B in which we used mice backcrossed for four times. To assess specificity of Cre recombination, *Rosa26-LacZ* mice (Soriano, 1999) were crossed to *Foxg1-IRES-Cre* mice.

2.2. Generation of the Foxg1-IRES-Cre knock-in mouse line

For generation of *Foxg1-IRES-Cre* mice, *Foxg1* gene targeting was carried out using homologous recombination in embryonic stem cells (derived from 129 Sv/ter mouse strain) at the Salk Genome Manipulation Core Facility. The 9154 bp genomic region of the mouse *Foxg1* gene (flanked by EcoRI sites) was used for the targeting vector. The 5′ fragment (flanked by EcoRI and AclI sites) and the 3′ fragment (flanked by AclI and EcoRI sites) were isolated from BamHI flanked genomic region (13634 bp) of the mouse *Foxg1* gene. A DNA cassette containing an internal ribosormal entry site (IRES) preceding a *Cre recombinase* gene followed by an frt-flanked PGK-neo was inserted at the AclI site, 27 bp downstream of the stop codon in the 3′UTR of *Foxg1* gene. A PGK-Diphtheria toxin (DTA) was included to select against random insertion events. Targeted embryonic stem cell clones were screened by Southern blot with 5′ and 3′ probes to identify *Foxg1-IRES-Cre*/+ clones. These identified clones were injected into C57BL/6J blastocysts at the Salk Transgenic Core Facility and the resulting chimeras were mated to C57BL/6J females to obtain germ-line transmission. Heterozygous mice were mated with FLPe mice (Rodríguez et al., 2000) to remove the PGK-neo cassette. Genotyping PCR was performed using three primers for detecting wild type allele and *Foxg1-IRES-Cre* allele (*Foxg1* forward (WT-F): 5′-GGGGGACCA-GACTGTAAG-3′, *Foxg1* reverse (WT-R): 5′-CTCCCACATTGCACCTCG-3′, *Cre* forward (Cre-F): 5′-GTGTTGCCGCGCCATCTGC-3′).

2.3. Western blot analysis

Western blot analysis was performed as described previously (Lim et al., 2008). Brain lysates were prepared from P0 neocortex using RIPA buffer, and the concentration of protein in the lysates was measured using DC protein assay (Bio-Rad). The following antibodies were used: rabbit anti-Foxg1 (1:5000, Abcam) and mouse anti-Gapdh (1:5000, GeneTex).

2.4. X-gal staining, Nissl staining, immunohistochemistry, in situ hybridization

X-Gal staining was performed on whole mount embryo or 14 μm cryosections. β-galactosidase activity was developed in staining solution (PBS containing 1 mg/ml X-Gal, 2 mM MgCl2, 0.01% SDS, 0.02% NP40, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6) for several hours or overnight at 32 °C or 37 °C. Specimens were then washed in PBS and postfixed in 4% PFA. Sections were counterstained with nuclear fast red (Vector lab). Nissl staining and immunostaining were carried out as described (Zembrzycki et al., 2015). The following primary antibodies were used: rabbit anti-Cux1 (1:1000, Santa Cruz), rabbit anti-Foxp2 (1:3000, Abcam), and rabbit anti-serotonin (1:50,000; Immunostar). In situ hybridization was done using digoxigenin (DIG)-labeled riboprobe for *Cad8* on whole brains as described previously (Sahara et al., 2007).

2.5. Statistical analysis

Quantitative data in Figs. 1, 5 and 6 are means ± s.e.m for the numbers of brains (n) indicated in the corresponding figures. Data were compared between groups with one-way ANOVA (Fig. 1) or unpaired Student's *t* test (Figs. 5 and 6). A *P* value of < 0.05 was considered statistically significant.

3. Results

3.1. Generation of a Foxg1-IRES-Cre knock-in allele

To generate a mouse line that expresses the Cre recombinase in *Foxg1*-expressing cells without disturbing its endogenous *Foxg1* gene expression, we targeted an IRES-Cre cassette into the 3′UTR of the *Foxg1* gene (Fig. 1A). This targeting strategy was designed to generate a bicistronic messenger RNA (mRNAs), encoding both endogenous *Foxg1* and the transgenic *Cre*. Moreover, the targeting strategy was designed to insert the IRES-Cre and frt-flanked PGKneo cassettes 27 bp after the stop codon of the full length *Foxg1* gene, thus maintaining the previously described miRNA target sites in 3′UTR of *Foxg1* gene (e.g., *miR-9* and *miR-200*) (Choi et al., 2008; Garaffo et al., 2015; Shibata et al., 2011, 2008). Importantly, this strategy did not incorporate an exogenous polyadenylation (polyA) sequence, which causes abnormal miRNA regulation in the *Foxg1-Cre* line due to the deletion of *Foxg1* 3′UTR

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