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Generation of *BAF53b-Cre* Transgenic Mice with Pan-Neuronal Cre Activities

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Abstract

Molecular and functional studies of genes in neurons in mouse models require neuron-specific Cre lines. The current available neuronal Cre transgenic or knock-in lines either result in expression in a subset of neurons or expression in both neuronal and non-neuronal tissues. Previously we identified BAF53b as a neuron-specific subunit of the chromatin remodeling BAF complexes. Using a bacteria artificial chromosome (BAC) construct containing the *BAF53b* gene, we generated a Cre transgenic mouse under the control of *BAF53b* regulatory elements. Like the endogenous *BAF53b* gene, we showed that *BAF53b-Cre* is largely neuron-specific. In both central and peripheral nervous systems, it was expressed in all developing neurons examined and was not observed in neural progenitors or glial cells. In addition, BAF53b-Cre functioned in primary cultures in a pan-neuron-specific manner. Thus, *BAF53b-Cre* mice will be a useful genetic tool to manipulate gene expression in developing neurons for molecular, biochemical, and functional studies.

Keywords

Mammal; Organism; Genetics

Introduction

During neural development, multipotent neural progenitors have the ability to differentiate into neurons, astrocytes, and oligodendrocytes. Despite the diversity of neuron subtypes, all neurons share common neuron-specific features; these include the permanent exit from the

cell cycle and adoption of neuronal specific morphology and physiological activities (Gage and Temple, 2013; Morrison and Kimble, 2006). Genetic studies are essential for the understanding of neuronal morphogenesis and functions. Therefore, neuron-specific Cre transgenic lines are desired to enable analysis of the functions of genes in neuronal development and function.

Several neuronal Cre transgenic mouse lines have been generated and are widely used for deletions of genes in neurons. The mis-expression of Cre in other neural or non-neural cell types or the restricted expression of Cre in subsets of neurons have limited the use of existing neuronal Cre lines in studying the general function of neurons. For example, *Nestin-Cre* transgenic lines have been widely used to delete genes in neural progenitors and consequently all neural progenies including neurons and glial cells (Tronche *et al.*, 1999). The *CamK2-Cre* is active in mature projection neurons in the forebrain but not in the developing neurons and other neuron subtypes (Chen *et al.*, 2001; Minichiello *et al.*, 1999; Tsien *et al.*, 1996). *Syn1-Cre* and *Nex-Cre* are expressed in developing neurons; however, *Syn1-Cre* is expressed in a mosaic pattern (Zhu *et al.*, 2001), and *Nex-Cre* is expressed only in principle neurons (Goebbels *et al.*, 2006). Several other Cre lines such as *Thy1-Cre*, *NSE-Cre*, and *CRE3* result in Cre activities in either specific brain regions or specific neuronal subtypes during development and in adults (Banares *et al.*, 2005; Campsall *et al.*, 2002; Cinato *et al.*, 2001; Heimer-McGinn and Young, 2011; Kwon *et al.*, 2006). Although these Cre lines are suitable for study of the functions of specific types of neurons, it remains difficult to perform certain molecular or biochemistry experiments that require the deletion of genes in all neurons as there have been no Cre mouse lines that display Cre activities in all neurons.

Primary neuronal cultures are an important tool for study of neuronal morphology and function and for molecular, biochemistry, and pharmacological experiments. Since primary cultures usually consist of both glial and neuron cells, a Cre system that enables neurons to be distinguished from other co-cultured cells would be valuable. Since most of the Cre transgenes mentioned above are only expressed in a subset of neurons *in vivo*, it is likely that they will only function in a subset of neuronal cells in culture.

The generation of neuronal Cre requires neuron-specific regulatory elements. The lack of pan-neuronal Cre activities is mostly due to the intrinsic properties of the driver gene or insufficient regulatory elements used in the transgenic construct. During our study of the neuron-specific chromatin remodeling complexes, we discovered that the *BAF53b* gene (also known as *Actl6b*), which encodes a subunit of the SWI/SNF-like BAF complexes, is expressed specifically in neurons and in all neurons examined (Lessard *et al.*, 2007; Olave *et al.*, 2002; Wu *et al.*, 2007). Using the regulatory elements of *BAF53b* in a BAC construct, we generated a Cre transgene that is specifically expressed in developing and mature neurons. The pan-neuronal expression pattern of the *BAF53b-Cre* transgene *in vivo* and in culture will make this transgenic mouse line a useful tool to study gene functions in neurons.

Results and Discussion

Construction of BAF53b-Cre BAC and generation of the transgenic line

By searching NCBI Clone database, we identified a BAC clone (RP23-127E17) that contains the entire *BAF53b* gene and approximately 100 kb upstream and downstream (Figure 1A). Using the recombineering method (Warming *et al.*, 2005), the BAC was first inserted with a *galK* selection marker replacing the first two coding exons of *BAF53b* gene downstream of the start codon. Then using negative selection, the *galK* cassette was replaced with the *Cre* gene coding region ligated with the *BAF53b* 3' UTR (Figure 1A). The correctly recombined BACs were identified by PCR using primers surrounding the recombined junctions (Figure 1A, 1B) and further confirmed by sequencing. The *BAF53b-Cre* containing BAC DNA was prepared and injected into the pronucleus of fertilized FVB/N mouse eggs. The positive transgenic mice were identified using PCR (Figure 1B). One founder line was generated with germline transmission of the transgene. *BAF53b-Cre* hemizygotes and homozygotes are normal and fertile.

BAF53b-Cre is active in developing nervous system

To characterize the *BAF53b-Cre* activities, the *BAF53b-Cre* mice were crossed to two reporter mice, *R26R-YFP* (Srinivas *et al.*, 2001) and *R26R-tdTomato* (*Ai9*) (Madisen *et al.*, 2010), both containing a LoxP sites-flanked stop signal upstream of the reporter genes at the *Rosa26* locus. Cre was expected to delete the stop signal and enable the expression of *YFP* or the *tdTomato*. The two reporter mice had identical expression patterns, and we used them interchangeably. Western blotting of young adult tissues indicated that *BAF53b-Cre* activity resulted in expression of YFP, which was detected in neural tissues such as brain and eyes (Figure 1C). Except a low expression of YFP in the testis, all the non-neural tissues examined are absent of the YFP signals (Figure 1C), indicating that *BAF53b-Cre* is largely neural specific.

During development, *BAF53b* gene expression in neurons is first detectable in E12.5 brain and spinal cord (Olave *et al.*, 2002). We observed *BAF53b-Cre* activities that were slightly delayed relative to this in both the developing central and peripheral nervous system. At E13.5, the reporter tdTomato expression was detected in the outermost marginal zone neurons in the cortex (Figure 2A). tdTomato was also expressed in the outer layer in mid-hindbrain where more mature neurons reside (Figure 2B). In the ventral brain regions, as well as in the spinal cord, high levels of tdTomato were detected in the differentiated areas (Figure 2C). In addition, strong tdTomato signals were detected in dorsal root ganglion, sympathetic ganglia, and nerve tracts (Figure 2C). Reporter signals were not detected in non-neural tissues (Figure 2C). At E15.5, reporter YFP signals were observed in the differentiated areas in cortex and mid-hindbrain regions (Figure 2D, 2E). In the cortex, the YFP signal was stronger in the more mature marginal zone and subplate neurons (Figure 2D). At E18.5, reporter tdTomato signals were observed in all differentiated brain areas, but were absent from undifferentiated areas such as the ventricular zone in the cortex (Figure 2F, 2G). Thus, we observed *BAF53b-Cre* activities in the nervous systems but not in most non-neuronal tissues.

BAF53b-Cre is specifically active in neurons

BAF53b is a neuron-specific gene that is expressed in most if not all neurons. To determine whether *BAF53b-Cre* is also expressed specifically in neurons, we co-stained the reporters with neural markers. In E13.5 spinal cord and peripheral ganglia/nerve tracts (Figure 3A), in E18.5 cortex, and in E18.5 cerebellum (Figure 3B, 3C), tdTomato reporter signals overlap completely with the neuronal marker beta-III tubulin detected using the Tuj1 antibody. In contrast, the tdTomato signal was mutually exclusive with the proliferation marker Ki67 detected in the external granule layer (EGL) in cerebellum (Figure 3D). In postnatal brain, we also observed differential localization of the reporter tdTomato with the oligodendrocyte marker MBP (Figure 3E—3E'') and the astrocyte marker GFAP (Figure 3F—3F''). In cerebellum, tdTomato signals were observed in both granule neurons in the internal granule layer (IGL) and in Purkinje neurons; little staining was present in the white matter where most glial cells reside (Figure 3E'', 3F''). These results indicate that *BAF53b-Cre* is expressed and active in all neurons examined but not in proliferating neural progenitors and glial cells.

BAF53b-Cre is active in the peripheral nervous system and sensory organs

The expression of reporters in the dorsal root ganglion and sympathetic ganglia (Figure 3A) suggested that *BAF53b-Cre* is active in peripheral nervous system. We further examined the *BAF53b-Cre* activities in sensory organs, peripheral nervous system and other neuronal marker expressing cells. A co-staining of tissue sections from P8 *BAF53b-Cre tdTomato* mice with tdTomato and the neuronal marker β -III tubulin showed an extensive overlap between *BAF53b-Cre* reporters and neuronal markers. In the eye, tdTomato was observed in neurons in all layers including the ganglion cell layer, inner nuclear layer and outer nuclear layer (Figure 4A). In olfactory epithelium, tdTomato was observed in olfactory sensory neurons that are Tuj1 positive (Figure 4B). In the tongue, tdTomato overlapped with Tuj1 signals in taste bud sensory neurons (Figure 4C). In the inner ear, hair cells are positive for both tdTomato and Tuj1 (Figure 4D). In the skin, Merkel cells, which were identified around a whisker follicle, were also positive for tdTomato signals (Figure 4E). In the intestine, the enteric neurons were marked by both tdTomato and Tuj1 (Figure 4F). In pancreas, beta cells express a significant number of neuronal markers (Atouf *et al.*, 1997). Indeed, beta cells also express tdTomato reporters (Figure 4G). Thus, *BAF53b-Cre* is active in neurons in both the central and peripheral nervous systems.

BAF53b-Cre is active in all cultured cortical and hippocampal neurons

To evaluate expression of *BAF53b-Cre* at higher resolution, we generated primary hippocampal neural cultures from E18.5 *BAF53b-Cre R26R-YFP* or *R26R-tdTomato* embryos. In 14 day *in vitro* cultures, the reporter YFP signals were detected in all cultured neurons as indicated by the complete overlapping of YFP staining with staining for neuronal markers HuC/D and MAP2 in more than 500 neurons analyzed (Figure 5A—5A'', 5B—5B''). These results indicate that *BAF53b-Cre* is expressed specifically in neurons and in all neurons examined. Analysis of E16.5 cortical cultures also indicated exclusive expression in neurons (data not shown). Since a fraction of neurons are produced from neural progenitors in culture but all of the neurons were positive for the reporters, we concluded that *BAF53b-*

Cre is active in neuron cultures. The neuron-specific expression of *BAF53b-Cre* was further confirmed by the differential staining of reporter tdTomato with astrocyte marker GFAP and oligodendrocyte marker MBP (Figure 5C, 5D).

In summary, using the regulatory elements of a neuronal gene *BAF53b* in a BAC construct, we generated a pan-neuronal *Cre* transgenic mouse line. Using two reporter systems, we demonstrated that *BAF53b-Cre* is active in all developing neurons examined. Similar to the endogenous *BAF53b* gene, *BAF53b-Cre* was expressed specifically in neurons *in vivo* and in cultures, but not in progenitors, glial cells, or most non-neural cells. Thus *BAF53b-Cre* is a useful new tool that will enable manipulation of genes in developing neurons in mice and in cultures. The pan-neuronal expression pattern and the expression in developing neurons will make *BAF53b-Cre* especially useful in studies of the function of genes in common neuronal morphogenesis, development and physiologies.

Methods

BAC recombineering

BAC (RP23-127E17) containing the *BAF53b* gene was recombined at the first two coding exons downstream of the start codon with the *Cre* gene using the *galK*-positive and *galK*-negative selection method as previously described (Warming *et al.*, 2005; Yoo *et al.*, 2009). SW106 bacterial cells were transformed with RP23-127E17 BAC, selected and further transformed with *galK* PCR product amplified by primers with 50-bp arms homologous to the sites to be recombined (galK-53b-FWD: 5'-TGCTGATCCCGCCGCCCGCCACGGGCCGCGAGCAGCGTAGAGAGGGCACT CCTGTTGACAATTAATCATCGGCA-3'; galK-53b-REV: 5'-TCTGCGTCCAGAGTGATGGGATTTAAAGGAACGCACTACCCTGCCCAGCT TCAGCACTGTCTGCTCCTT-3'). Recombination was performed by heat-shocking the transformed SW106 cells, and colonies containing the recombined products were selected on minimal agarose plates plus galactose.

The *galK*-containing RP23-127E17 BAC was then recombined to replace *galK* with the *Cre* gene by transforming SW106 cells with the PCR product of Cre-53bUTR and homology arms. PCR products of the *Cre* coding region using PMC-CreN as the template (Cre-F: 5'-ATCGATAAGCTTACCATGCCCAAGAAGAAGAG-3'; Cre-R: 5'-GGTAAAGAATTCTGGCTAATCGCCATCTTCCAG-3') and the *BAF53b* 3' UTR from the stop codon to 100 bp downstream of the polyA signal (53bUTR-F: 5'-GGTAAAGAATTCAGTTGCCCCCTCCCCAAATAC-3'; 53bUTR-R: 5'-GGTAAAGGATCCTGAGGCCACACCCACTAGAGCAC-3') were digested with EcoRI and ligated. The ligation was used as the template for PCR of the Cre-53bUTR product with 50-bp homologous arms on both sides (53b-Cre-F: 5'-TGCTGATCCCGCCGCCCGCCACGGGCCGCGAGCAGCGTAGAGAGGGCACTAC CATGCCCAAGAAGAAGAGGAAGG-3'; 53b-Cre-UTR-R: 5'-TCTGCGTCCAGAGTGATGGGATTTAAAGGAACGCACTACCCTGCCCAGCTGACCGATCCGAACAAACGACCCAAC-3'). SW106 cells transformed with the Cre PCR product were grown on 2-deoxy-D-galactose-containing minimal plates for negative *galK* selection. Colonies with correctly recombined BACs were identified by PCR using primers

surrounding the recombined junctions as illustrated in Figure 1A (P1: 5'-AGAAGCGGCTGTACCTTTAAAGC-3'; P2: 5'-GATCCCTGAACATGTCCATCAG-3', P3: 53bUTR-F; P4: 5'-CATGAGCAGGGAGCTAGATTATTC-3'). BAC DNA was prepared from the isolated colonies, rechecked by PCR and further confirmed by sequencing.

Generating transgenic mice

BAC-transgenic mice were generated by pronuclear injections. The *BAF53b-Cre* containing BAC DNA was prepared and injected into the pronucleus of fertilized FVB/N mouse eggs. The positive transgenic mice were identified by PCR. One founder line was generated that has a germline transmission of the transgene. *R26R-YFP* (Srinivas *et al.*, 2001) and *R26R-tdTomato (Ai9)* (Madisen *et al.*, 2010) mice were purchased from Jax Laboratory and crossed to *BAF53b-Cre* mice. These mice are maintained on a mixed genetic background at UT Southwestern Medical Center Animal Facility. The *BAF53b-Cre* mouse will be available to the research community upon acceptance of the manuscript.

Immunoblotting

For immunoblotting, mouse tissues were lysed in RIPA buffer (50 mM Tris, pH 8, 250 mM NaCl, 0.1% SDS, 0.5% DOC, 1% NP-40), and cell lysates were separated on SDS-PAGE gels. The antibody used was the mouse monoclonal against GFP (JL-8, Clontech). HRP-conjugated secondary antibodies were purchased from Jackson Immunology.

Primary hippocampal neuron culture

E18.5 hippocampal cells were cultured as previously described (Wu *et al.*, 2007). Briefly, embryonic hippocampi were dissected, trypsinized, dissociated to single cells, and cultured on poly-L-ornithine and fibronectin-coated coverslips. Culture media contained DMEM/F12 with putrescine, 2-mercaptoethanol, transferrin, insulin, selenium, progesterone, MEM vitamin additive, and 5% FBS. Half of the medium in each well was replaced every other day. Typically 14 day cultures were used for immunostaining.

Immunofluorescent staining

Timed mouse pregnancies were determined by plugging date as day 0.5. Paraffin sections or PFA-fixed cultured neurons were stained with antibodies against RFP (rabbit, Rockland), GFP (rabbit, Molecular Probes), Tuj1 (Covance), Ki67 (BD Pharmingen), MBP (Millipore), GFAP (BD Pharmingen), MAP2 (Sigma), and HuC/D (Molecular Probes) and visualized using an Olympus BX50 microscope.

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A. The construction of the BAC containing *BAF53b*-*Cre*-53bUTR expression cassette.

B.

C.

Figure 1. Generation of *BAF53b*-*Cre* BAC transgenic mouse line

A. Construction of the BAC-containing *BAF53b*-*Cre*-53bUTR expression cassette. The genomic structure of the *BAF53b* gene is shown. Recombineering methods were used to replace the first two exons of the *BAF53b* gene with the *Cre* gene and the *BAF53b* 3'UTR; this places the *Cre* gene immediately downstream of the start codon. The diagram is not drawn to scale. The positions of the primers used for cloning confirmation are shown (P1–P4).

B. PCR confirmation of the correct BAC construct and positive transgenic mouse.

C. Western blot of protein extracted from indicated tissues in P21 *BAF53b*-*Cre* *R26R*-YFP mouse. YFP was detected at high levels in brain tissue and at low levels in eyes and testis. YFP was not detected in other tissues examined. The asterisk indicates a non-specific band.

Figure 2. *BAF53b-Cre* is active in developing nervous system

A–C. Sections of E13.5 *BAF53b-Cre R26R-tdTomato* brain and trunk regions were stained with anti-RFP antibodies to visualize tdTomato expression. Scale bars: 200 μ m.

D–E. Sections of E15.5 *BAF53b-Cre R26R-YFP* brain regions were stained with anti-GFP antibodies to visualize reporter YFP expression. Scale bars: 200 μ m.

F–H. Sections of E18.5 *BAF53b-Cre R26R-tdTomato* brain regions were stained with anti-RFP antibodies to visualize tdTomato expression. Scale bars: 100 μ m.

CTX: cortex, DRG: dorsal root ganglion, SG: sympathetic ganglion, MB: midbrain, CB: cerebellum, VZ: ventricular zone, EGL: external granule layer.

Figure 3. *BAF53b-Cre* activity is neuron-specific

Sections of *BAF53b-Cre R26R-tdTomato* brain and trunk regions at different developing stages were stained with anti-RFP antibodies to visualize tdTomato expression and antibodies against markers for (A–C) neurons (Tuj1), (D) neural progenitors (Ki67), (E) oligodendrocytes (MBP), and (F) astrocytes (GFAP).

A'' is the overlay of A and A'; E'' is the overlay of E and E'; F'' is the overlay of F and F'.

DRG: dorsal root ganglion, SG: sympathetic ganglion, VZ: ventricular zone, EGL: external granule layer; IGL: internal granule layer; WM: white matter, PL: Purkinje neuron layer.

Figure 4. *BAF53b-Cre* is active in the peripheral nervous system and sensory organs

Sections of various tissues and sensory organs of P8 *BAF53b-Cre R26R-tdTomato* mice were co-stained with anti-RFP antibodies and the Tuj1 antibody against the neuronal marker β -III tubulin. Scale bars: 50 μ m.

A. Eye. tdTomato was observed in neurons in all layers. GCL: ganglion cell layer, INL: inner nuclear layer, ONL: outer nuclear layer.

B. Nose olfactory epithelium. tdTomato was observed in Tuj1+ olfactory sensory neurons (OSN).

C. Tongue. tdTomato was observed in taste bud sensory neurons.

D. Inner ear. tdTomato was observed in hair cells (HC).

E. Skin in the whiskers area. tdTomato and Tuj1 staining overlap in the Merkel cells (MC) around a whisker follicle.

F. Intestine. tdTomato was observed in the enteric neurons.

G. Pancreas. tdTomato was observed in beta-cells that were marked by Tuj1 antibodies.

Figure 5. *BAF53b-Cre* is active in cultured neurons but not in glial cells
 A–D. Cultures of E18.5 *BAF53b-Cre R26R-YFP* or *BAF53b-Cre R26R-tdTomato* hippocampal neurons were co-stained with anti-XFP antibodies and antibodies against markers for neurons (HuC/D and MAP2), astrocytes (GFAP), and oligodendrocytes (MBP). Note the coexpression of YFP and HuC/D or MAP2, and the non-overlap between tdTomato and GFAP or MBP.