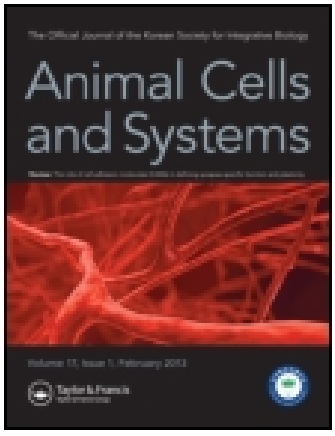

Animal Cells and Systems
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tacs20

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Published online: 19 Jun 2014.

To cite this article: C. Justin Lee, Joo Ok Min & Bo-Eun Yoon (2014) Generation of Best1 knockdown mice using lentiviral vector expressing small hairpin RNA, Animal Cells and Systems, 18:3, 178-182, DOI: 10.1080/19768354.2014.924994

To link to this article: http://dx.doi.org/10.1080/19768354.2014.924994

Animal Cells and Systems, 2014
Vol. 18, No. 3, 178–182, http://dx.doi.org/10.1080/19768354.2014.924994

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Generation of *Best1* knockdown mice using lentiviral vector expressing small hairpin RNA

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(*Received 23 March 2014; received in revised form 2 May 2014; accepted 13 May 2014*)

We demonstrate that pronuclear injection of lentiviral vectors expressing small hairpin RNAs (shRNAs) that silence the expression of specific genes can be used to generate knockdown mice. A lentiviral vector capable of generating shRNA that is specific for the target gene bestrophin 1 (*Best1*) encodes for an anion channel that is permeable to glutamate and gamma amino butyric acid (GABA) and that also regulates intracellular calcium signaling. We confirmed that cultured cerebellar glia from these *Best1* knockdown mice showed attenuation of GABA release induced by an increase in intracellular calcium. Therefore, we propose that a combined approach, the use of transgenesis together with lentiviral vectors expressing shRNAs, can successfully generate a large number of mice in which the expression of a specific gene can be downregulated gradually. We also suggest that the *Best1* knockdown mouse can be a useful tool for studying *Best1* gene function.

Keywords: astrocyte; cerebellum; tonic inhibition

Introduction

Bestrophin 1 (*Best1*) is believed to function as a chloride channel that may also serve as a regulator of intracellular calcium signaling (Hartzell et al. 2008). *Best1* has been shown to be permeable to chloride, thiocyanate, bicarbonate, glutamate, and gamma amino butyric acid (GABA), and *Best1*-mediated GABA release has recently been demonstrated to be responsible for tonic inhibition in cerebellar granule cells (Lee et al. 2010). Therefore, a molecular tool for *Best1* knockdown in cerebellum would be useful for the study of tonic inhibition. Indeed, several previous reports have shown that tonic inhibition has great implications. Blocking tonic $GABA_A$ receptor-mediated conductance increases the firing frequency of granule cells and Purkinje cells in cerebellum (Hamann et al. 2002). Enhanced tonic inhibition by alcohol ingestion shows impairment of motor behavior (Hanchar et al. 2005). Tonic inhibition was increased in pharmacological models of genetic absence epilepsy (Cope et al. 2009) and reducing excessive GABA-mediated tonic inhibition in motor cortex promotes functional recovery after stroke (Clarkson et al. 2010). Tonic inhibition is clearly important as a physiological and pathological function and we can expect there to be further, as yet undiscovered, roles for tonic inhibition in the brain. Although we have established an acute gene silencing method by lentivirus injection into mouse brain (Lee et al. 2010), it is hard to infect the whole cerebellar cortex region by local virus injection because the cerebellar cortex is composed of 10 lobules. The development of tools for whole cerebellar knockout or knockdown of target genes is essential, as opposed to local virus infection methods for functional studies. We use pronuclear injection (Gordon & Ruddle 1983) of our construct, which induces gene silencing of *Best1* acutely for generating *Best1* knockdown mice. In these mice, *Best1* expression in cerebellum disappears and the green fluorescence protein (GFP) signal, which reflects small hairpin RNA (shRNA) expression, is clearly intense. In addition, we observed attenuation of Ca^{2+}-induced GABA release from cerebellar glia in these mice. We thus confirmed *Best1* gene silencing of both the expression and the function of *Best1* in our mice.

Materials and methods

Animals and housing

Adult (aged 8–10 week), male or female, wild-type (WT) and *Best1* shTG (transgenic) mice genotypes were used. All experimental procedures described below were performed in accordance with Korea Institute of Science and Technology (KIST) animal welfare regulations (Seoul, Korea).

Best1 shRNA production

The *Best1* nucleotide sequence from 774 to 793 (5-tttgccaacttgtcaatgaa-3) was selected for the target region of *mBest1* shRNA. For lentivirus-based shRNA expression,

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mBest1 shRNA was synthesized as follows: 5-tttgccaacttgt caatgaattcaagagatcattgacaagttggcaatttttc-3 and 5-tcgagaaa aaatcgcatagcgtatgccgtttctcttgaaaacggcatacgctatgcgaa-3. The annealed double-stranded oligonucleotide was inserted into HpaI–XhoI restriction enzyme sites of the pSicoR lentiviral vector (Addgene) and verified by sequencing.

Generation of transgenic mice

Six-week-old females were superovulated with 5 units of pregnant mare serum gonadotropin (Sigma), followed by an injection of 5 units of human gonadotropin (Sigma) 48 hours later, and were then mated with males. Morulae (8- to 16-cell embryos) were isolated by flushing the oviduct 2.5 days postcoitus with M2 medium (Sigma). Removal of the *zona pellucida* was achieved by Acidic Tyrode's treatment. Morulae were infected overnight with 20 ng of lentiviral, 24 ml in a 5-liter volume, covered with light paraffin oil (Fisher). Thirty hours after infection, blastocysts were transferred into the uteri of pseudopregnant mice. The presence of the lentiviral vector DNA was detected by polymerase chain reaction (PCR), which amplifies a 700-bp fragment of the GFP gene.

Immunohistochemistry

Rabbit polyclonal mouse *Best1* IgG was produced using a previously described (Barro Soria et al. 2009) antigen (AbFrontier). B6 adult mice were deeply anesthetized with 2% avertin and perfused with 0.1 M phosphate buffered saline (PBS) at room temperature, followed by a perfusion with ice-cold 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde at 4°C for 24 hours and 30% sucrose at 4°C for 48 hours. Brains were then cut into 30-µm-thick coronal cryosections. Sections were blocked in 0.1 M PBS containing 0.3% Triton X-100 (Sigma) and 2% serum from species of the secondary antibody for 1 hour. Primary antibody was then applied at the appropriate dilution: mouse *Best1* IgG at 1:200 and GFP at 1:1000 (Millipore Bioscience Research Reagents) and incubated overnight at 4°C. After overnight incubation, the sections were washed three times in PBS and then incubated in secondary antibody Alexa 555 goat anti-rabbit IgG at 1:400 (Invitrogen) and Alexa 488 goat anti-chicken IgG at 1:400 (Invitrogen) for 2 hours. After three rinses in PBS, the sections were mounted onto glass slides. Images were acquired on an Olympus FluoView FV1000 confocal microscope.

Cerebellar glia culture

Cultured cerebellar astrocytes were prepared from P0 to P3 postnatal mice. The cerebellar cortex was dissected free of adherent meninges, minced, and dissociated into a single cell suspension by trituration through a Pasteur pipette. Dissociated cells were plated onto either 12-mm glass coverslips or into six-well plates coated with 0.1 mg ml^{-1} of poly-D-lysine. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, cat. no. 11960-044) supplemented with 25 mM glucose, 10% heat-inactivated horse serum, 10% heat-inactivated fetal bovine serum, 2 mM glutamine and 1000 units ml^{-1} penicillin–streptomycin. Cultures were maintained at 37°C in a humidified 5% CO_2-containing atmosphere.

Sniffer patch

Sniffer-patch assays were performed with calcium-sensitive Fura-2 imaging techniques in cultured cerebellar astrocytes from *Best1* shTG or WT mice, and current recordings were made from HEK293T cells expressing $GABA_C$ receptor ($GABA_CR$). To induce GABA release from astrocytes, protease-activated receptor 1 (PAR1) was

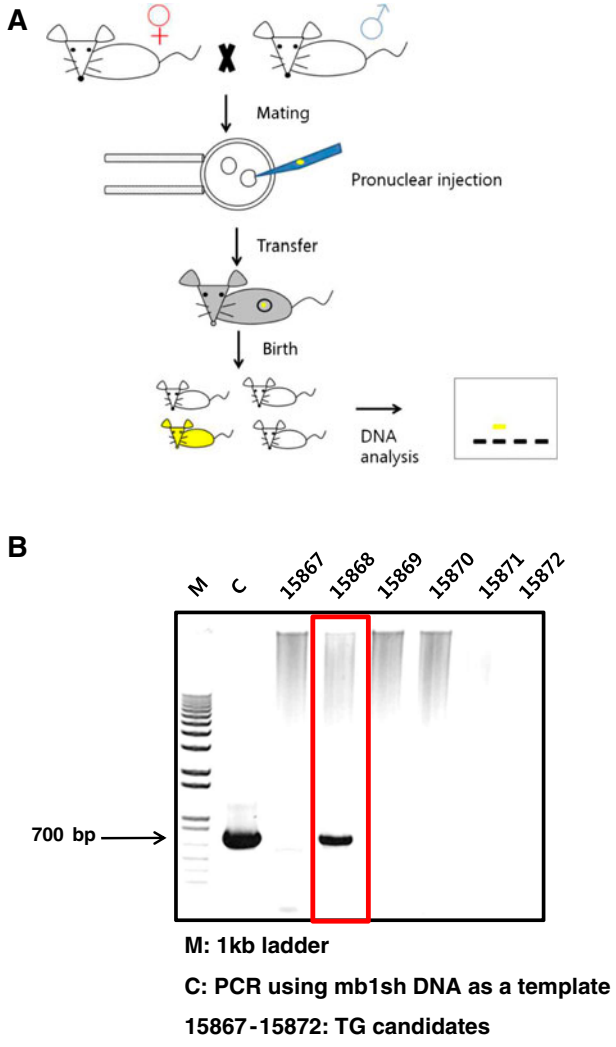

Figure 1. (A) Schematic diagram of embryonic pronuclear injection method for generation of knockdown mice. (B) Identification, with PCR, of transgenic founder mouse from several candidates.

activated by pressure application of the TFLLR (Thr-Phe-Leu-Leu-Arg, peptide). On the day of the experiment, cultured mouse astrocytes (HEK293T cells added) were incubated with 5-mM Fura-2 AM (mixed with 5 ml of 20% pluronic acid; Invitrogen) for 40 minutes, washed at room temperature, and subsequently transferred to the microscope stage for imaging. External solution contained (in millimolar): 150 NaCl, 10 HEPES, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 5.5 glucose (pH adjusted to pH 7.3 and osmolarity adjusted to 325 mOsmol/kg). Images detected at a wavelength of 510 nm (emission wavelength) were taken at excitation wavelengths 340 nm and 380 nm, using an iXon high speed EMCCD camera (Andor). Two of the resultant images were used for ratio calculations with Imaging Workbench 6.2 software (Indec Systems). $GABA_C$R-mediated currents were acquired from HEK293T cells under voltage clamp (Vh = −70 mV) using a MultiClamp 700B Amplifier and were digitized with pCLAMP 9.2 software (Molecular Devices). Recording electrodes (4–7 MU) were filled with (in millimolar): 110 Cs-gluconate, 30 CsCl, 0.5 $CaCl_2$, 10 HEPES, 4 Mg-ATP, 0.3 Na_3-GTP, and 10 BAPTA (pH adjusted to 7.3 with CsOH and osmolarity adjusted to 290–310 mOsmol/kg with sucrose). For simultaneous recordings, Imaging Workbench software was synchronized with that of pCLAMP 9.2.

Results and discussion

We have used the U6 promoter to drive expression of shRNA targeting *Best1*. We have confirmed silencing of the target gene and have designed lentiviral vectors for delivery of shRNA expression cassettes in previous reports (Park et al. 2009). We have also shown that specific silencing of target genes is induced in virus-infected cells in injected brain regions (Lee et al. 2010). To that end, we have generated a transgenic founder mouse using pronuclear injection of lentiviral vector expressing shRNA against *Best1* (Figure 1A). From the

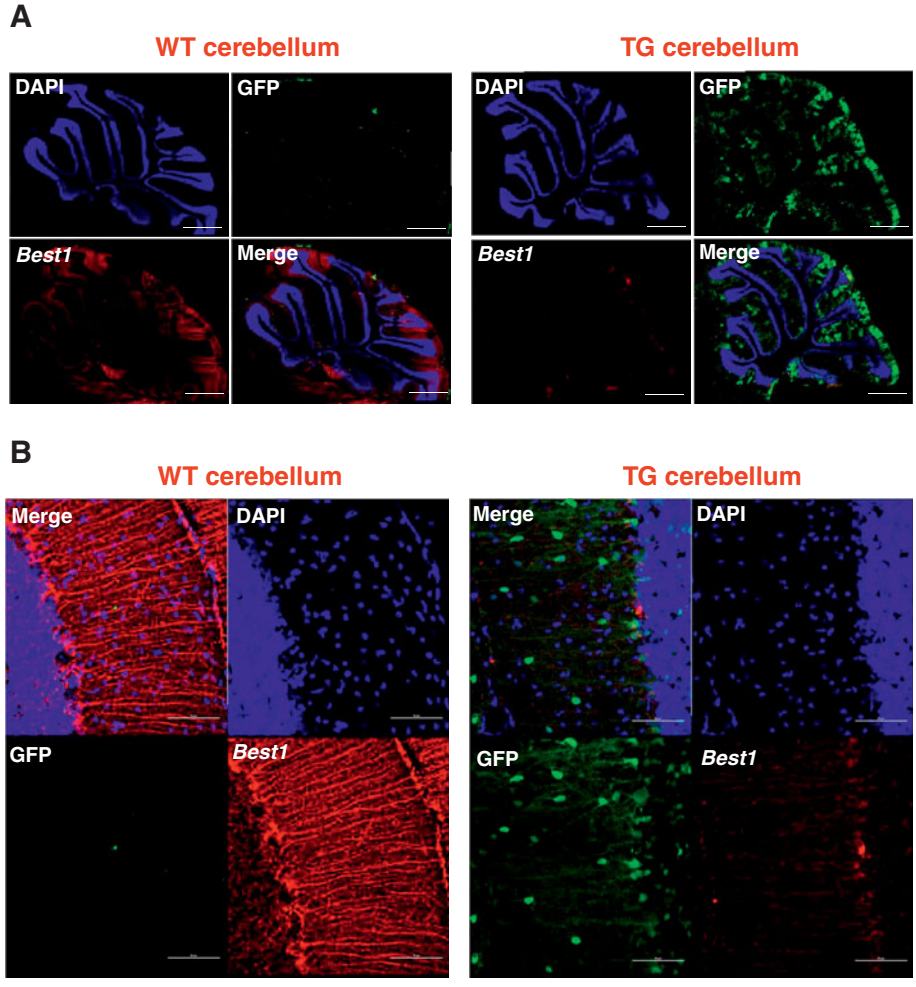

Figure 2. Immunohistochemistry of *Best1* using anti-mouse bestrophin 1 (*Best1* IgG) antibody (1:200, AbFrontier) and amplified GFP signal with anti-GFP antibody (1:1000, Millipore) in whole cerebellum (A: scale bar: 500 μm) and cerebellar cortex (B: scale bar: 50 μm).

first injection, we acquired one female founder mice, which we confirmed by PCR of GFP (Figure 1B). After mating and breeding, we can thus generate a number of transgenic mice. To show the gene silencing effect in these transgenic mice, we performed immunohistochemistry using anti-mouse bestrophin 1 antibody and anti-GFP antibody. As a result, in transgenic mouse cerebellum, a very intense amplified GFP signal was detected and expression of *Best1* was very diminished. On the other hand, in WT cerebellum, *Best1* intensity is normal and there was no native GFP fluorescence (data not shown) nor was there an amplified GFP signal from anti-GFP antibody (Figure 2). These observations were made both in whole cerebellum (Figure 2A) and in a more magnified version that differentiated individual cells in the molecular and granule cell layer (Figure 2B). Consequently, we confirmed widespread expression of *Best1* shRNA from the intense GFP signal and a simultaneous extensive gene silencing effect of that shRNA expression from a reduced *Best1* intensity in *Best1* knockdown transgenic mouse cerebellum.

Next, we tested functional gene silencing of *Best1* in this transgenic mouse cerebellum. Using the sniffer-patch technique, we measured intracellular calcium-induced robust GABA release from cultured cerebellar astrocytes in WT mice and we observed a decrease in GABA release

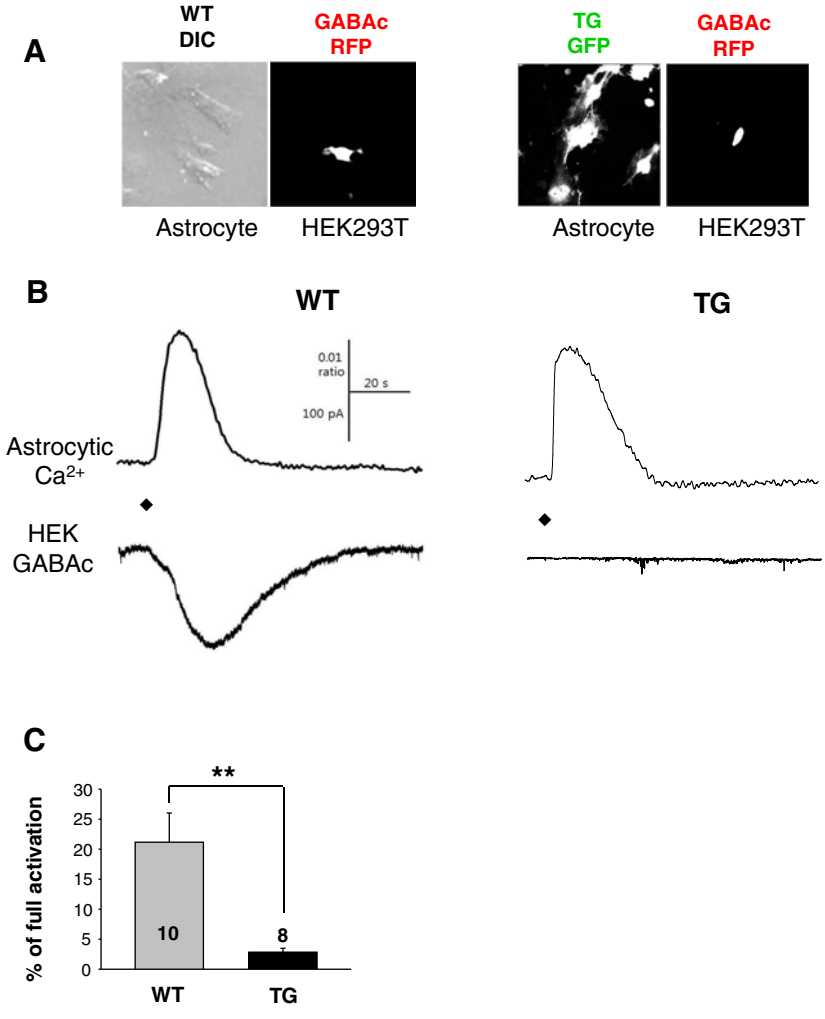

Figure 3. (A) Sniffer-patch assay. $GABA_C$Rs expressed in HEK293T cells (RFP) and cultured astrocytes (wild-type [WT]: DIC, *Best1* shTG: GFP). Left pipette, pressure application of TFLLR, a selective agonist of PAR1 receptor. Right pipette, recording pipette for HEK293T cell expressing $GABA_C$Rs. GABA is released from cultured astrocytes upon TFLLR application. Concluding these experiments, $GABA_C$Rs receptors of the sensor cell were fully activated by bath application of GABA (100 μm) so that the response to released GABA could be normalized according to the number of GABA receptors expressed in the sensor cell. Time-dependent reductions in sensor cell currents are due to desensitization of $GABA_C$Rs. (B) Representative traces recorded from sniffer-patch assay under each condition (Left: WT mice; Right: *Best1* shTG mice). Upper trace: Ca^{2+} transient recorded from astrocyte. Lower trace: whole-cell current recorded from sensor cell ($Vh = -70$ mV) upon TFLLR pressure application. Diamond: TFLLR application (10 psi, 100 ms, 500 mM). (C) Summary bar graph of GABA release measured under the indicated conditions, with values normalized as described above. Significance was determined by a paired student's *t*-test.

from cerebellar astrocytes in *Best1* knockdown mice (Figure 3). These results are consistent with the previous reports that show GABA to be released via the *Best1* channel in cerebellar glial cells, for the purpose of mediating tonic inhibition in cerebellum (Lee et al. 2010).

We have demonstrated that a lentivirus vector system expressing shRNA against bestrophin 1 can efficiently transduce preimplantation mouse embryos. Furthermore, the resulting progeny express shRNA and show a reduced expression of specific genes in transgenic mouse cerebellum. We also confirmed the functional knockdown of *Best1* by measuring GABA receptor-mediated currents induced by GABA release from cultured cerebellar glial cells in cerebellum. This *Best1* knockdown mouse could be used not only to investigate the alteration of tonic inhibition and regulation of glial GABA concentrations in cerebellum but also to probe the function of tonic inhibition in vivo.

Acknowledgments

We thank Dr Daesoo Kim at KAIST for the generation of transgenic mice. This work was supported within the framework of the International Cooperation Program managed by the National Research Foundation of Korea (2013K2A2A4003724), the World Class Institute (WCI 2009-003) programs of the National Research Foundation (NRF) funded by the Korean Ministry of Science, Education and Technology (MEST), by National Agenda Project (NAP) of the Korea Research Council of Fundamental Science and Technology (NAP-09-04), and by KIST's Internal Grant (2E24480).

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