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Specific expression of FOXP2 in cerebellum improves ultrasonic vocalization in heterozygous but not in homozygous *Foxp2* (R552H) knock-in pups

Eriko Fujita-Jimbo[a,b], Takashi Momoi[a,*]

[a] Center for Medical Science, International University, Kitakanemaru, Ohtawara 3248501, Tochigi, Japan
[b] Department of Pediatrics, Jichi Medical University, Yakushiji, Shimotsukeshi 3290498, Tochigi, Japan

HIGHLIGHTS

- We prepared Tg mice expressing FOXP2 in the cerebellum by using *Pcp2* promoter.
- FOXP2 expression in the cerebellum partially improved USV in *Foxp2*(R552H)-KI mice.
- Foxp2 in the cerebellum pertains to USVs modification but not their production.

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ABSTRACT

The R553H mutation has been found in the FOXP2 gene of patients with speech-language disorder. *Foxp2*(R552H) knock-in (KI) mice exhibit poor dendritic development of Purkinje cells in the cerebellum and impaired ultrasonic vocalization (USV), which is related to human speech and language; compared with wild-type mice, heterozygous *Foxp2*(R552H)-KI pups exhibit the reduced number of whistle-type USVs and the increased short-type ones, while homozygous pups exhibit only click-type USVs but no whistle-type or short-type ones. To make clear the relationship between the role of Foxp2 in the cerebellum and whistle-type USVs activity, we prepared transgenic (Tg) mice specifically expressing human FOXP2-myc in cerebellum (*Pcp2-FOXP2-myc*-Tg mice) by using *purkinje cell protein-2* (*Pcp2*) promoter. FOXP2-myc expression in the cerebellum increased the relative numbers of whistle-type USVs in the heterozygous *Foxp2*(R552H)-KI pups and recovered their USVs but did not in the homozygous ones. Foxp2 in the cerebellum may pertain to the brain network engaged in whistle-type USVs activities including modification, but not their production. There may be common molecular contribution of Purkinje cells to human FOXP2-mediated speech-language and mouse Foxp2-mediated USVs.

1. Introduction

The phenotype of speech-language disorder segregates as an autosomal dominant trait [6]. One-half the members of the KE family with speech-language disorder have severe articulation difficulties accompanied by verbal and orofacial impairment. FOXP2 belongs to a forkhead box (FOX) subfamily with transcription activity [15], and a missense mutation (R553H) in the forkhead domain of FOXP2 co-segregates with the affected members of the KE family [12].

FOXP2 is extremely highly conserved. Compared with its mouse homologue, the human FOXP2 protein differs at three amino acids (D80E, N302T, and S325N); in addition, a single glutamine that is present in the human protein is absent from the polyQ tract in the mouse protein [4]. We have studied the communication ability of knock-in mice with the *Foxp2*(R552H) mutation, designated as *Foxp2*(R552H)-KI mice, which is related to the FOXP2(R553H) mutation [7].

When isolated from the mother and littermates, infant rodents emit ultrasonic vocalizations (USVs), whistle-like sounds with frequencies of 40–100 kHz [2]. These signals play an important communicative role in mother–offspring interactions because they elicit a prompt response from the dam in the form of

Abbreviations: BAC, bacterial artificial chromosome; FOX, forkhead box; KI, knock-in; KO, knock-out; Pcp2, purkinje cell protein-2; USV, ultrasonic vocalization.

* Corresponding author at: Center for Medical Science, International University, Kitakanemaru, Ohtawara, Tochigi, Japan. Tel.: +81 287 24 3162.
E-mail address: momoi@iuhw.ac.jp (T. Momoi).

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care-giving behaviors. *Foxp2*(R552H)-KI mice [7] and Foxp2-deficient knock-out (KO) mice [16] exhibit impaired USV communication, suggesting that human speech-language and mouse USV communication have a Foxp2-mediated common molecular basis. Therefore, the FOXP2-mediated molecular pathogenesis of the human speech-language disorder might be clarified by studying the impaired USV of *Foxp2*(R552H)-KI mice.

The poor dendritic development in Purkinje cells of *Foxp2*(R552H)-KI mice suggests that Foxp2-mediated development of Purkinje cells is associated with mouse USV activity and perhaps with human speech-language. In addition to Purkinje cells, however, Foxp2 is expressed in the cerebrum including the cortical-basal ganglia [5]. Their respective roles in USV communication are not clear. Therefore, we have not excluded the possibility that these two Foxp2-mediated events, USV activity and development of dendrites of Purkinje cells, occur independently and that USV activity may not be regulated by Purkinje cells alone.

To understand the role of Foxp2 in Purkinje cells in human speech-language and mouse USV activity, we generated the transgenic mouse line *Pcp2-FOXP2-myc*-Tg that specifically expresses human FOXP2-myc in the cerebellum, by using bacterial artificial chromosome (BAC) transgenesis of the *purkinje cell protein* (*Pcp2*/*L7*) gene, which specifically expresses in the Purkinje cells. In the present study, we examined the recovery of the impaired USV activity in the *Foxp2*(R552H)-KI pups by the expression of FOXP2 in the cerebellum.

2. Materials and methods

2.1. Ethics statement

We followed the fundamental guidelines for proper conduct of animal experiments and related activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, and all of the protocols for animal handling and treatment were reviewed and approved by the Animal Care and Use Committee of Jichi University (approval numbers, H22-179, 10–179) and International University of Health and Welfare (approval numbers, D1008; 10118).

2.2. Tg mice expressing FOXP2 in the cerebellum

Using BAC transgenesis of the *purkinje cell protein-2* (*Pcp2/L7*) gene, we generated Tg mice specifically expressing human FOXP2-myc in cerebellum (*Pcp2-FOXP2-myc*-Tg); *human FOXP2* was under the control of the *Pcp2*/*L7* gene within Purkinje cells *in vivo* because Pcp-2/L7 is expressed exclusively in cerebellar Purkinje cells [8,14], and BAC transgenesis can integrate the transgene independent of position [9]. We transferred the *FOXP2-myc* gene by BAC recombination and injected the linearized BAC transgene construct into pronucleus-stage mouse embryos to establish independent transgenic lines.

2.3. PCR for genotype assay

In *Foxp2*(R552H)-KI mice, one LoxP sequence (ATAACTTCGTATAGCATACATTATACGAAGTTAT) still remained in the intron between exon 17 and 18 in the targeted alleles after Cre-mediated removal of the PGK-neo Marker cassette. To identify the wild-type and targeted alleles, we performed PCR using primer pairs: Foxp2In17-18(692F) 5′-GATGGTCAAGACCCACTAGT-3′ for forward primer and Foxp2In17-18(1018R) 5′-AGGAGGAGACAGCATGCATT-3′ for reverse primer [7]. For *Pcp2-FOXP2-myc*-Tg mice, we performed using primer pairs: FOXP2F 5′-ATGCATCCAGTGGCCTACTG-3′ for forward primer and Myctag-R 5′-CAGATCCTCTTCTGAGATGAG-3′ for reverse primer. The amplification protocol were as follows: 1 cycle at 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 64 °C 60 °C for 30 s, 72 °C for 30 s, and 1 cycle at 72 °C for 7 min.

To detect the expression of FOXP2-myc by RT-PCR analysis, the cDNA were prepared from the cerebellum of the wild and *Pcp2-FOXP2-myc*-Tg mice as described previously [7] and fragments were amplified at the same condition.

2.4. Detection of FOXP2-myc expression

2.4.1. Immunobloting

Mouse brains were lysed with 5 times volume of tissue weights of the butter (10 mM HEPES pH 7.6, 60 mM KCl, 1 mM EDTA, 0.1% IGEPAL CA630, and protease inhibitors), incubated on ice for 30 min and centrifuged at (16,000 × *g*, 4 °C) for 30 min. The extracts were subjected to SDS-PAGE (12%) electrophoresis and immunoblot analysis using the rabbit anti-Foxp2 (Abcam, CA) and monoclonal mouse anti-myc (Nacarai tesque, Japan) antibodies. Immune reactivities were visualized by alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG antibodies, respectively, using Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate (Roche Diagnostics, Switzerland).

2.4.2. Immunostaining

Cerebellums of 9-day-old pups were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4 °C overnight, and then soaked in 30% sucrose/PBS at 4 °C overnight, embedded in optimal cutting temperature (O.C.T.) compound (Sakura Finetec, CA), and frozen. Frozen sections (10 μm thick) were cut on a cryostat and attached to MAS-coated slides (Matsunami Glass, Japan). Sections were immunostained with monoclonal mouse anti-myc (Nacarai tesque), polyclonal rabbit anti-calbindin (Sigma, MO) in PBS containing 0.1% skim milk and 0.1% Triton X-100 at 4 °C for 1 day as described previously [7]. Alexa Fluor 488 and Alexa Fluor 568 conjugated secondary antibodies against rabbit and mouse IgG were purchased from Molecular Probes. Nuclei were detected by Hoechst 33342 at 37 °C for 15 min. The reactivity was viewed using a Leica TCS SP5 confocal microscope (Leica Microsystems, Germany).

2.5. Vocalization

Animals were assessed for USV monitoring before behavioral testing on postnatal day 9 (P9). Each pup was separated from the litter one at a time and placed in a shallow beaker in a soundproof chamber, in which the pup was then positioned below a microphone connected with the UltraSoundGate 116 detector set (Avisoft Bioacoustic, Germany) to detect USVs at 40–100 kHz. Analysis started after the pup habituated to the chamber for 60 s, and sounds were recorded for 3 min, and then saved for later analysis. Here, in addition to whistle-type and click-type USVs whose duration was shorter than 4 ms, we defined short-type USVs as USVs whose duration was shorter than 8 ms [7].

3. Results and discussion

We generated *Pcp2-FOXP2-myc*-Tg mice expressing *human FOXP2-myc* under the control of the *purkinje cell protein-2* (*Pcp2/L7*) gene using BAC transgenesis of the *Pcp2/L7* gene (Fig. 1A), and one to thirty copies of the FOXP2-myc BAC transgenic construct were integrated into each transgenic line (Fig. 1B). The FOXP2 line (30 copies) was used in the following experiments. Expression of FOXP2-myc was detected in the cerebellum of *Pcp2-FOXP2*-myc-Tg mice by RT-PCR but not in the wild-type animals (Fig. 1C). Endogenous Foxp2 (110 kDa) was expressed in both the cerebrum and cerebellum, while FOXP2-myc (110 kDa) was detected in the cerebellum alone, using anti-myc antibody (Fig. 1D). FOXP2-myc expression