Biochemical and Biophysical Research Communications 374 (2008) 17–21

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Ectopic expression of *tmie* transgene induces various recovery levels of behavior and hearing ability in the circling mouse

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ARTICLE INFO

Article history:
Received 17 June 2008
Available online 27 June 2008

Keywords:
tmie
Circling mouse
Organ of Corti
Hearing loss
Transgenic animal

ABSTRACT

The circling (*cir*/*cir*) mouse is one of the murine models for human non-syndromic deafness DFNB6. The mice have abnormal circling behavior, suggesting a balanced disorder and profound deafness. The causative gene was *transmembrane inner ear* (*tmie*) gene of which the mutation is a 40-kb genomic deletion including *tmie* gene itself. In this study, *tmie*-overexpression trasngenic mice were established. Individuals with germline transmission have been mated with circling homozygous mutant mice (*cir*/*cir*) in order to produce the transgenic mutant mice (*cir*/*cir*-tg) as a gene therapy. After the genotyping, phenotypic analyses were performed so that the insertion of the new gene might compensate for the diseases such as hearing loss, circling behavior, or swimming inability. Some individuals exhibited complete recovery in their behavior and hearing but the others did not show any amelioration in behavior or hearing. Individual mice had very different levels of *tmie* transgene expression in the cochlea. These results clearly indicate that *tmie* protein plays an important role when the appropriate expression level of *tmie* was expressed in the inner ear. The protein levels were variable in each individual and these are thought to induce the differences in disease amelioration levels.

Sensorineural hearing loss (SNHL) comprises hearing disorders with diverse pathologies of the inner ear and auditory nerve. Commonly the congenital hearing loss is caused by mutations in one or more genes which are related to the normal development of the inner ear tissues. At present, at least 30 syndromic and over 120 non-syndromic loci have been discovered that are implicated in causing hereditary hearing loss [1]. The primary cause of hearing loss is damage or death of the sensory receptor hair cells in the inner ear. Current research efforts are focused on gene manipulation, gene therapy for repairing or replacing damaged mammalian cochlear hair cells, which could lead to therapies for treating deafness in humans. Gene therapy may provide a way to restore cochlear function to deaf patients. Gene therapy to grow new auditory hair cells was used in adult guinea pigs [2,3]. This was achieved by inserting Math1 gene into cells lining the inner ear. Non-sensory epithelial cells in adult guinea pig cochlea could generate new sensory hair cells following the expression of Math1. When Math1 was overexpressesd in the non-sensory cells of the mature cochlea, it caused them to transdifferentiate into hair cells.

The circling (*cir*) mouse is a spontaneous mutant in the inner ear from ICR out-bred strain [4,5]. The circling mouse becomes hyperactive at about 7 postnatal days, and then shows a circling behavior. The most notable pathological phenotypes are the almost completely degenerated cochlea, and the remarkably reduced cellularity in the spiral limbus. An auditory test demonstrated hearing loss of the *cir*/*cir* mouse [5]. The genetic mapping demonstrated that the *cir* gene was mapped to 60.1 cM on mouse Chr 9. The distal portion of mouse Chr 9 encompassing the *cir* region is homologous with human chromosome 3p21, which contains DFNB6 locus [6]. And through the deletion analysis by genomic PCR of the candidate causative genes, the *cir*/*cir* mouse has a 40-kb genomic deletion that includes the transmembrane inner ear (*tmie*) gene and mRn49018 [7]. The spinner (*sr*) mice have similar phenotypes as the circling mice. The mutant mouse shows behavioral dysfunction including bidirectional circling and head-shaking [8]. The auditory function in *sr*/*sr* mice was found to be reduced, based upon the lack of a startle reflex to sound at any age [9]. The *sr*/*sr* mouse was identified to have two types of mutations: a 40-kb genomic deletion including 4 genes (*K007173, Tsp50, tmie, and mRn49018*) and a nonsense mutation expected to truncate the C-terminal end of its

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doi:10.1016/j.bbrc.2008.06.064

product. The *cir*/*cir* and *sr*/*sr* mice have a common deleted region, including *tmie* gene [7]. Therefore, *tmie* is identified as the causative gene of two mutants. However, the functional roles of *tmie* in the cochlea remain unclear. The amount, distribution, and time course of *tmie* protein have not been defined. Only the expression patterns in the adult mouse and rat were analyzed [10,11].

In this study, *tmie*-overexpression trasngenic mice were established. Individuals with germline transmission have been mated with circling homozygous mutant mice in order to produce the transgenic mutant mice as a gene therapy. After the genotyping, phenotypic analyses were performed so that the insertion of the new gene might compensate for the diseases such as hearing loss, circling behavior, or swimming inability. Some individuals exhibited complete recovery in their behavior and hearing but the others did not show any amelioration in behavior or hearing. Therefore the behavioral differences and transgenic *tmie* expression patterns in *cir*/*cir* mice with transgene have been assessed.

Materials and methods

Animals. The circling (*cir*/*cir*) mice are the spontaneous mutants in ICR out-bred strain [4] and have been maintained in the *cir*/*cir*-C57BL/6. In order to induce the systemic expression of mouse *tmie*, human cytomegalovirus (CMV) immediate-early promoter was used in the transgene construction and the transgenic mice were produced by microinjection (Fig. 1A). Hemagglutinin (HA) epitope tag was added to the end of *tmie* open reading frame (ORF) removing *tmie* stop codon. The transgenic mice were confirmed by PCR of the region from 3′ end of hCMV to 3′ end of *tmie* ORF. After screening of transgenic founder mice, we mated one founder mouse to *cir*/*cir* mice. Then the F1 +/*cir* progeny carrying transgene were mated to *cir*/*cir* mice for the production of N2 transgenic *cir*/*cir* mice (Fig. 1B). +/+ or +/*cir* heterozygous mice were used as wild type mice.

Clinical observations. The circling behavior was observed using an open-field apparatus (75 × 75 × 30 cm), where vertical and horizontal lines were drawn every 15 cm. The circling counts were defined as the number of times the mouse ran in circles in the 5 min after being placed in an open field. Swimming test was done by placing mice into the water to observe their swimming behavior.

Auditory brainstem response tests. Mice were anesthetized with xylazine (4 mg/kg) and ketamine (40 mg/kg) by i.m. prior to measurement. The animals were placed in a sound-isolated, electrically shielded booth. Needle electrodes (Grass E2 platinum) were subcutaneously placed below the tested ear (reference electrode), in the vertex (active electrode), and below the contralateral ear (ground electrode). The sound stimulus consisted of 15/ms tone bursts (rise-fall time 1 ms) at 4, 8, 16, and 32 kHz and were generated by Tucker-Davis hardware. The sound stimuli were delivered into the ear canal from an encased, shielded Beyer earphone through a 13 mm tube. Response waveforms (1,000,000 gain, filtered from 0.3–3.0 kHz) were averaged (1024 epochs) using a Tucker-Davis data acquisition system. The response threshold was defined as the interpolated value between the last level at which no response was observed. The sound delivery system was calibrated with a 1/4 in. ACO Pacific condenser microphone (Belmont, USA) in a volume approximating the mouse external ear canal and expressed as dB SPL.

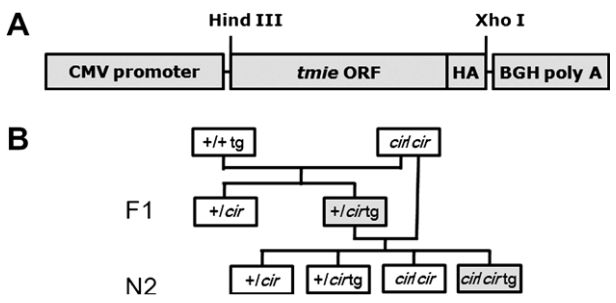

Fig. 1. Construction for the production of *tmie*-overexpressing mice (A) and breeding strategy (B). *cir*/*cir*-tg represents the *cir*/*cir* mice carrying *tmie* transgene.

tmie antibody production and Western blot analysis. *tmie* antibodies were generated by immunizing a rabbit (Peptron, Daejeon, Korea) with a synthetic peptide (aa118-133, GenBank Accession No. NP666372) of mouse *tmie*. Western blot was performed as follows. An equal volume of 1× SDS sample buffer was added and the samples were then boiled for 5 min. Sample (50 μg) was subjected to electrophoresis on 13% SDS–polyacrylamide gels for 2 h at 200 mA and then transferred onto nitrocellulose. The membrane was incubated for 1 h in 5% (w/v) skim milk in PBS containing 0.05% (v/v) Tween 20 (PBS-T), washed in PBS-T and then incubated for 2 h in the presence of primary antibody (1:1500). The membrane was washed extensively with PBS-T and then incubated with anti-rabbit IgG antibody (1:1500, Amersham) for 1 h. After extensive washing, immunoreactive bands on the membrane were visualized using chemiluminescent reagents according to the manufacturer's protocol (Supersignal Substrate; Pierce, Rockford, IL).

Immunohistochemical analysis of the organ of Corti. The removed temporal bone was fixed in 4% paraformaldehyde for 16 h at 4 °C, decalcified with 10% EDTA in PBS for 1 week, dehydrated, and embedded in paraffin wax. Sections of 4 μm were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. For immunohistochemical study, the LSAB-kit Universal K680 (DAKO, Carpinteria, CA, USA) was used and all the procedures were carried out according to the manufacturer's instructions. The endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min at room temperature. Afterward, specimens were washed in PBS and non-specific binding was blocked with 1% goat serum for 1 h. Then, primary antibody (anti-*tmie*, 1:50) was added to the slides, and incubation proceeded for 2 h. After repeated washes with PBS, the section was incubated with Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Invitrogen) at 1:150 dilution in 1% goat serum in PBS for 2 h at room temperature, followed by three washes with PBS. In the final step, the nuclei of immunostained cells were counterstained with DAPI.

Surface preparation of the cochlea. The organ of Corti and vestibular systems were prepared for histological analysis. The temporal bone was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C. Following fixation, the otic capsule was removed and the cochlea was microdissected into individual turns. The specimens were rinsed in 0.1 M PBS, then incubated in 0.25% Triton X-100 for 2 min and immersed in TRITC-labeled phalloidin (Sigma P1951, 1:4000) in PBS for 20 min. After three washes with PBS, the specimen was examined under fluorescence microscope with appropriate filters for TRITC (excitation: 510–550 nm, emission: 590 nm).

Statistics. Data were analyzed by chi-square analysis to determine the differences between groups. A value of $P < 0.05$ was considered to be statistically significant.

Results

Clinical observation and hearing tests

According to the previous studies about the expression pattern of mouse *tmie*, *tmie* gene is expressed in many kinds of organs as well as the inner ear [9,10]. We produced *tmie*-overexpressing transgenic mouse model using CMV promoter for the phenotypic rescue of the circling (*cir*/*cir*) mouse. Through the continuous