Inhibition of *Myo6* gene expression by co-expression of a mutant of transcription factor *POU4F3* (BRN-3C) in hair cells

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Abstract. An eight-base pair (bp) deletion in the *Pou4f3* gene in hair cells is associated with DFNA15, a hereditary form of hearing loss. To explore the pathological mechanisms underlying the development of DFNA15, the effect of the mutation in *Pou4f3* on the activity of the myosin VI (*Myo6*) promoter, was investigated. The upstream regulatory sequence of *Myo6* (2625 bp), consisting of an 1899 bp upstream sequence and a 727 bp intron 1 sequence, was amplified using polymerase chain reaction and subcloned into the pGL3-Basic vector expressing firefly luciferase. For verification of inserted fragments, plasmids were subjected to restriction analysis and then sequenced. HEK293T human embryonic kidney cells were transiently transfected with renilla luciferase-thymidine kinase vectors expressing *Renilla* luciferase and the *Myo6* promoter-driven firefly luciferase expressing vectors along with pIRES2-enhanced green fluorescent protein (EGFP)-*Pou4f3* (expressing wild-type *Pou4f3*) or pIRES2-EGFP-*Pou4f3* (expressing the truncation mutant of *Pou4f3*). The relative luciferase activities were measured to determine the activity of the *Myo6* promoter. The *Myo6* promoter activity was not affected by co-expression of wild-type *Pou4f3*, as indicated by the comparable relative luciferase activities in the presence of the pIRES2-EGFP-*Pou4f3* and the empty control vectors. However, co-expression of mutated *Pou4f3* significantly inhibited the activity of the *Myo6* promoter to almost half of that of the control (P<0.001). The data suggests that mutated *Pou4f3* has a negative role in the promoter activity of *Myo6*, and by extension, the expression of myosin VI, and this may be an underlying mechanism of DFNA15 hearing loss.

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Introduction

Hearing impairment is one of the most common types of genetic diseases, with an estimated prevalence of 1-3/1,000 newborns, at least half of which is attributed to genetic factors (1). ~One out of every 10 persons with a hearing impairment are carriers of mutant genes associated with deafness (1). Although not life-threatening, loss of hearing is a debilitating disorder which affects the life of patients in significant and occasionally devastating ways. Mutations in a large variety of genes have been identified as the cause of hereditary hearing loss. In 1998, Vahava *et al* (2) determined the molecular basis for an autosomal dominant progressive nonsyndromic hearing loss, DFNA15 (MIM 602459), in an Israeli Jewish family. A mutation in the *Pou4f3* gene (encoding for the POU domain transcription factor Brn3c/Brn3.1) was described, in which an eight-base pair (bp) deletion in exon 2 results in the formation of a premature stop codon in the first helix of this transcription factor. The truncation of the *Pou4f3* proteins affect their ability to bind and activate downstream targets (3). The mechanism by which the *Pou4f3* mutation leads to DFNA15 hearing loss remains to be elucidated. In *Pou4f3*$^{-/-}$ mice, the overall architecture of the cochlea is unaffected and hair cell markers are detected during early differentiation (4-6). However, hair cells in *Pou4f3*$^{-/-}$ mice undergo progressive loss compared with those of wild-type mice, with a disorderly arrangement, and are misplaced among supporting cells (6,7). Furthermore, the apical surface of hair cells in *Pou4f3*$^{-/-}$ mice is devoid of stereocilia, or with fused, disorganized, and occasionally giant stereocilia (4-8).

Stereocilia are a type of organ-pipe-arrayed giant stiff microvilli on the free surface of hair cells, containing a parallel bundle of actin filaments at the core. Deletion or mutation of the genes *Espn*, *Myo15*, *Myo6*, and *Myo7a* accounts for the malformation of stereocilia (9-14). Myosin VI, an anchoring protein encoded by *Myo6*, may aid to hold down the membrane against surface tension forces and to anchor the apical hair cell membrane to the actin cytoskeleton of the cuticular plate, thus permitting the stereocilia to remain as separate entities.

Disturbance of myosin VI may be implicated in the formation of fused and giant stereocilia, since inadequate anchoring of the apical membrane between stereocilia may result in the elevation of the membrane between adjacent stereocilia due to surface tension (11,15,16).

These data suggest that *Pou4f3* and *Myo6* are essential for the development and maintenance of stereocilia. Mutation of either gene may lead to abnormal stereocilial structures and hearing impairment in humans and mice. To elucidate the pathological mechanisms that contribute to the development of DFNA15, a form of hereditary deafness in humans, the *Myo6* promoter-driven expression in wild-type and mutant cells, carrying an eight-bp deletion in exon 2 of *Pou4f3*, were investigated in the present study using an *in vitro* mouse cell-based model.

Materials and methods

Plasmids, strains and cell lines. The pGL3-Basic, renilla luciferase-thymidine kinase (pRL-TK) control reporter, pIRES2-enhanced green fluorescent protein (EGFP)-*Pou4f3* (wild-type), and pIRES2-EGFP-*Pou4f3* (8-bp deletion in exon 2) vectors, C57/B6 mouse DNA, *E. Coli* DH5α competent cells, and the HEK293T human embryonic kidney (293T) cells used in the present study were kindly provided by Min-Sheng Zhu, Model Animal Research Center of Nanjing University (Nanjing, China).

Primer design and polymerase chain reaction (PCR) amplification. The promoter sequence of mouse *Myo6* was inferred from that of humans and consisted of a total of 2625 bp. The 1899 bp upstream and 727 bp intron 1 sequences of the mouse *Myo6* gene were analyzed using Vector NTI 10 software (Invitrogen Life Technologies, Carlsbad, CA, USA), which was also used to design primers for amplification of the *Myo6* promoter. The sequences of the forward and reverse primers were 5'-(ACGCGT)TTTAAAAACTAAAGTTCC CTTTCAG-3' and 5'-(AAGCTT)CAGTATCTCCACATT GGGAT-3', respectively, where ACGCGT and AAGCTT are restriction sites for *Mlu*I and *Hin*dIII, respectively. Primers were synthesized by GenScript Corporation (Nanjing, China). The *Myo6* promoter region was amplified from the C57/B6 mouse tail DNA with high-fidelity polymerase ExTaq using the above mentioned primers. The PCR conditions were as follows: An initial denaturation step of 98˚C for 5 min; followed by 30 cycles at 98˚C for 10 sec, 60˚C for 15 sec and 72˚C for 3.5 min, followed by a final elongation step at 72˚C for 10 min and maintenance at 16˚C until the reactions were completed. Amplified products were mixed with 0.5 μl Taq enzyme and incubated at 72˚C for 15 min followed by elongation at 4˚C to add an A-tail directly to the 3'-ends of the blunt-ended DNA fragment. Products were analyzed by electrophoresis on 0.8% agarose gels. Target bands were excised from the gel and the DNA was recovered using a DNA Gel Extraction Kit (Corning Life Sciences-Axygen Inc., Union City, CA, USA).

Plasmid constructs. The PCR products and pGL3-Basic vector were excised using *Mlu*I and *Hin*dIII (Takara Bio, Inc., Shiga, Japan) restriction enzymes. PCR products were ligated into the pGL3-Basic vectors containing the luciferase reporter gene using T4 DNA ligase (Takara Bio, Inc.). Competent *E. coli* DH5α cells were transformed with pGL3-Basic-*Myo6* promoter vectors, plated on Luria-Bertani (LB) medium containing ampicillin, and the plates were incubated at 37˚C for 12-20 h. Individual colonies were selected and grown in the liquid culture medium containing ampiccilin overnight. Plasmids were extracted using the AxyPrep Plasmid Miniprep Kit (Corning Life Sciences-Axygen, Inc.) and digested with *Mlu*I and *Hin*dIII, followed by purification of the inserted sequences for further sequencing by GenScript Corporation.

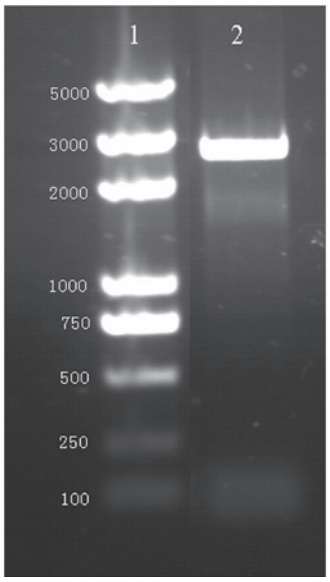

Figure 1. Polymerase chain reaction amplification of *Myo6* from mouse tail DNA. Lanes 1, Trans2K Plus DNA marker, with sizes indicated in bp on the left; and 2, amplified *Myo6*, 2625 bp. *Myo6*, myosin IV promoter.

Cell culture. 293T cells were grown and maintained in Dulbecco's modified Eagle medium (Gibco-BRL, Carlsbad, CA, USA) containing 10% fetal bovine serum (PAA Laboratories, Cölbe, Germany) supplemented with 100 IU/ml penicillin and 100 mg/l streptomycin at 37˚C in an atmosphere of 5% CO_2.

Plasmid transfection and luciferase reporter assays. The day prior to transfection, 293T cells were suspended in fresh medium and plated in 96-well plates at a density of $2x10^4$ cells/well. When the cells reached 70% confluency, they were transfected with 50 ng pGL3-Basic-*Myo6* promoter and 0.5 ng pRL-TK (internal control), along with either pIRES2-EGFP, pIRES2-EGFP-*Pou4f3* (wild-type), or pIRES2-EGFP-*Pou4f3* (8-bp deletion in exon 2), using the Lipofectamine™ 2000 Transfection Reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. The total amount of plasmid DNA was maintained at 200 ng in each well. The cells were harvested and lyzed 24 h post-transfection. Firefly and *Renilla* luciferase activities were determined with the Dual-Luciferase Reporter Assay System in a GloMax96 luminescence reader (both from Promega, Madison, WI, USA) according to the manufacturer's instructions. Relative luciferase activity was expressed as the ratio

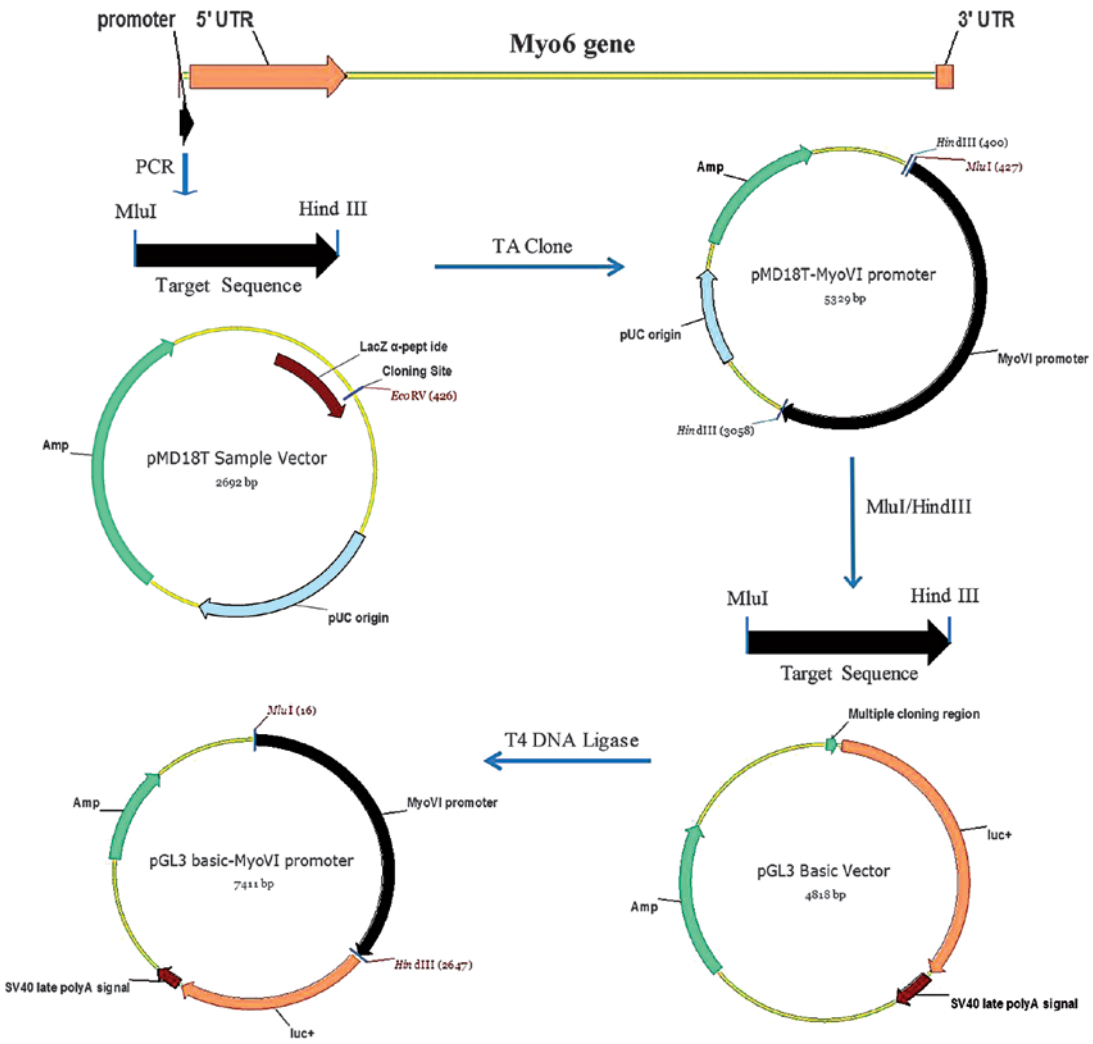

Figure 2. Schematic diagram of the strategy for constructing the *Myo6* reporter-driven luciferase expression vector. The promoter sequence of mouse *Myo6* was inferred and amplified from mouse tail genomic DNA using PCR. Purified target fragments were subcloned into pMD-18T. For verification of the length and orientation of the inserted fragments, the plasmids were digested with *Mlu*I and *Hin*dIII and then sequenced. Finally, fragments whose sequences were verified by sequencing analysis were ligated into the pGL3-Basic vectors using T4 DNA ligase to construct the pGL3-Basic-*Myo6* promoter vector. *Myo6*, myosin IV promoter; UTR, untranslated region; PCR, polymerase chain reaction; luc, luciferase; polyA, polyadenylation.

of firefly luciferase activity to the *Renilla* luciferase activity in each sample. All values were obtained from at least three independent repetitions of the transfection, with six wells for each transfection mixture/sample in every experiment.

Statistical analysis. Data analysis was performed using the SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). The Student's t-test was used for comparing means between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Myo6 promoter amplification. PCR was performed using primers with restriction sites. Gel electrophoresis analysis revealed the presence of a 2625 bp fragment in the PCR product, as expected (Fig. 1). Non-specific amplification was not detected (Fig. 1). Furthermore, the sequencing analysis confirmed that the sequence of the amplified *Myo6* promoter was consistent with that of the template.

Construction and confirmation of pGL3-Basic-Myo6 promoter vectors. The *Myo6* promoter-driven luciferase reporter construct, pGL3-Basic-*Myo6* promoter, was constructed as described in the materials and methods section (Fig. 2). To verify the sequences introduced into the pGL3-Basic-*Myo6* promoter vector, the plasmid DNAs were digested with *Mlu*I and *Hin*dIII and the DNA fragments were re-introduced into the same vectors, i.e., pGL3-Basic vector, followed by sequencing analysis. The sequences were confirmed to be correct and devoid of any mutations, demonstrating that the *Myo6* promoter was successfully inserted into pGL3-Basic vectors (Fig. 3).

Dual luciferase reporter assay. The activity of the *Myo6* promoter in 293T cells was assayed using the pGL3-Basic *Myo6* promoter reporter, in the presence of wild-type *Pou4f3* or a truncated mutant of *Pou4f3*, with the dual luciferase assay system. The relative luciferase activity was expressed as the ratio of firefly luciferase activity to *Renilla* luciferase activity to evaluate the effect of *Pou4f3* on the promoter

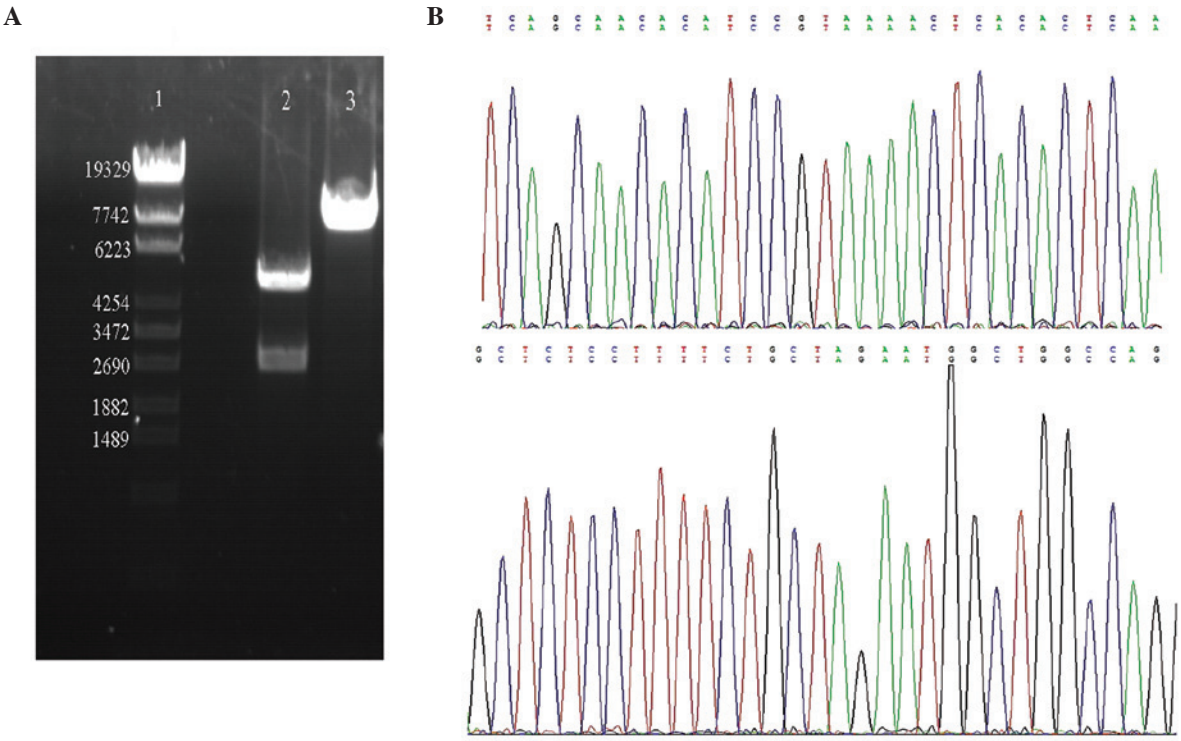

Figure 3. Identification of recombinant expression vector by restriction enzyme analysis and the inserted sequences by sequencing analysis. (A) Lanes 1, λ-EcoT14 I digest DNA Maker, with sizes indicated in bp on the left; 2, restriction analysis with *Mlu*I and *Hin*dIII, the upper 4818-bp band corresponds to the pGL3-Basic vector, the lower 2625 bp band corresponds to the *Myo6* promoter-containing target fragment; and 3, restriction enzyme analysis by *Hin*dIII, the linearized 7411-bp pGL3-Basic-*Myo6* promoter vector. (B) Bidirectional sequencing for the insert sequences, the sequences were confirmed to be correct and devoid of any mutations. *Myo6*, myosin IV promoter.

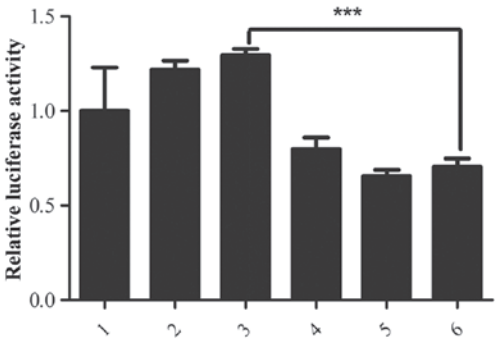

Figure 4. Regulation of the *Myo6* promoter by *Pouf4f3* as assessed using a luciferase reporter assay. 293T cells were transfected with the indicated combinations of vectors and the relative luciferase activity was measured to evaluate the *Myo6* promoter activity. 1, pIRES2-EGFP empty vector, the ratio of firefly/*Renilla* luciferase activities for this empty vector, used as the control, was normalized to 1.0 and the relative luciferase activity in all other samples is calculated following normalization to the control value; 2-3, 100 and 150 ng of pIRES2-EGFP-*Pouf4f3* (wild-type), respectively; 4-6, 50, 100 and 150 ng of pIRES2-EGFP-*Pouf4f3* (8-bp deletion in exon 2), respectively. No difference between in relative luciferase activity was observed in cells transfected with the wild-type *Pouf4f3*-expressing plasmid and the control. In contrast, co-transfection with the mutant *Pouf4f3*-expressing plasmid significantly inhibited the expression of luciferase from the *Myo6* promoter, reducing the relative luciferase activity to almost half of that of the control (P<0.001). ***P<0.001, vs. control. *Myo6*, myosin IV promoter; EGSP, enhanced green fluorescent protein.

activity of *Myo6*. The relative luciferase activity of cells expressing wild-type *Pou4f3* was comparable to that of the control, wherein the empty vector was co-transfected with the luciferase reporters. Co-transfection of the construct expressing mutated *Pou4f3* downregulated the expression of luciferase from the *Myo6* promoter to almost half of that of the control (P<0.001; Fig. 4), indicating that mutated *Pou4f3* has a negative role in the expression of *Myo6*. These data indicate that the inhibition of expression of *Myo6* in the presence of the mutated *Pou4f3* may be an underlying mechanism of DFNA15 hearing loss.

Discussion

Stereocilia of hair cells perform the process of auditory transduction. They receive mechanical inputs derived from airborne sound waves and transduce these signals into electrical responses that are then relayed to the brain for interpretation. Only stereocilia, arranged in rows of increasing height, are capable of converting mechanical activity to electrical activity (17-19). Damage to, or deterioration of the staircase-like arrangement of stereocilia accounts for hearing loss or balance disorders (15,20,21). With the help of transgenic and knock-out mouse models of human hearing loss (22), a variety of genes responsible for inner ear development and deafness, including genes encoding transcription factors, actins, ion channels, membrane proteins, and structural proteins, have been identified (2,21,23,24). Specifically, transcription factors including Math1 (25-28) and *POU4f3* have been shown to be crucial for hair cell development, differentiation, maturation and maintenance. Structural proteins, including Myo6 and Myo7a are important for the stability of the structure and function of stereocilia.

The POU-domain family of transcription factors is widely expressed in the nervous system. For example, *Pou4f1*, *2* and *3* are essential for the development of sensory ganglion, retinal ganglion and hair cells, respectively (29-33). *Pou4f3* (also known as Brn3.1 and Brn3c), an important transcription factor of the POU-domain family, specifically binds to a 9-bp recognition element, ATAATTAAT to activate downstream gene expression and thus cell development (34,35). *Brn3c*$^{-/-}$ mice deficient in *Pou4f3* are hearing impaired and exhibit vestibular dysfunction. Furthermore, the hair cells fail to mature and stereociliary bundles are formed or only malformed and disorganized stereocilia exist (6-8,36).

Mutation of unconventional myosins, including myosins VIIa, VI, Ic, and XV, have been shown to be associated with deafness. Normally present on cilia and actin-rich microvilli, including the stereocilia on mammalian hair cells, unconventional myosins are required for the cohesion and structural integrity of the bundle (11,37-39). Myosin VI, encoded by *Myo6*, is expressed in the inner and outer hair cells of mouse cochlea, concentrating in the actin-rich cuticular plate associated with stereociliary rootlets, pericuticular necklaces, and cytoplasm (11,15). As an anchoring protein, myosin VI may help anchor the membrane against surface tension forces and to anchor the apical hair cell membrane to the actin cytoskeleton of the cuticular plate, thus permitting the stereocilia to remain as separate entities. Myosin VI is crucial for the motility and shape change of hair cells during the development of stereocilia, and mutation of myosin VI results in the formation of giant stereociliary bundles and degenerated hair cells (11).

The luciferase reporter assay system is widely used to investigate the interaction between *cis*-acting regulatory elements, including promoters, enhancers, silencers and trans-acting regulatory proteins. This reporter assay has become the choice of the majority of investigations due to the advantages of rapidity, reliability and sensitivity. In the present study, the *Myo6* promoter was successfully inserted into an expression vector and the *Myo6* promoter-driven luciferase reporter construct was transiently transfected into human embryonic kidney 293T cells, along with expression vectors carrying wild-type and mutated *Pou4f3* using liposomes. The present study showed no differences between the Myo6 promoter activity in cells expressing the wild-type *Pou4f3* and the control cells. However, co-transfection with the mutated *Pou4f3*-expressing construct significantly inhibited the *Myo6* promoter activity to almost half of that of the control, indicating that mutated *Pou4f3* has a negative role in the regulation of *Myo6* expression. The data suggest that the inhibition of the *Myo6* promoter and consequently the expression of myosin VI due to mutation of *Pou4f3* may be an underlying mechanism of DFNA15. These data provide insights into the molecular basis of hearing impairment in DFNA15 hearing loss and suggest that loss of expression from the *Myo6* promoter may explain, at least in part, the hearing loss phenotype in the presence of the *Pou4f3* truncation mutation.

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