Pre-Clinical Research Report

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
2019, Vol. 47(4) 1717–1730

DOI: 10.1177/0300060519829990
journals.sagepub.com/home/imr
SAGE

Efficient introduction of an isogenic homozygous mutation to induced pluripotent stem cells from a hereditary hearing loss family using CRISPR/Cas9 and single-stranded donor oligonucleotides

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Abstract

Background: Heterozygous purinergic receptor p2x gene (*P2RX2*) c.178G>T (p.V60L) mutations can lead to progressive hearing loss (HL) and increased susceptibility to noise. However, the underlying mechanisms remain unclear. A combination of human induced pluripotent stem cell (hiPSC) technology with clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein (Cas)9-mediated gene editing may provide a promising tool to study gene function and treat hereditary deafness in humans.

Methods: hiPSC technology and CRISPR/Cas9-mediated gene editing were used to generate heterozygous and homozygous *P2RX2* c.178G>T (p.V60L) cell models.

Results: We generated non-integrative hiPSCs from urine samples derived from three members of a large Chinese family carrying heterozygous *P2RX2* c.178G>T mutations (designated P2RX2$^{+/-}$) as a model to study P2RX2-mediated hereditary HL. Furthermore, we used

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CRISPR/Cas9 and single-stranded donor oligonucleotides to genetically establish homozygous *P2RX2* c.178G>T hiPSCs (designated P2RX2$^{-/-}$) from heterozygous patient-specific hiPSCs as a control to further study the pathological gene function.

Conclusions: Heterozygous and homozygous *P2RX2*-mutated hiPSC lines are good models to investigate the pathological mechanisms of *P2RX2* mutations in HL pathogenesis. Our findings confirmed our hypothesis that it is feasible and convenient to introduce precise point mutations into genomic loci of interest to generate gene-mutated hiPSC models.

Keywords

P2RX2, CRISPR/Cas9, iPS cells, hearing loss, gene editing, point mutations

Date received: 28 September 2018; accepted: 17 January 2019

Introduction

Hearing loss (HL) is the most common sensory disorder of humans,[1,2] with an estimated 299 million men and 239 million women affected worldwide.[3] Genetic etiology plays an important role in the pathogenesis of deafness, accounting for around 50% of HL cases.[4] With the development of biological informatics and next-generation sequencing technology, increasing numbers of genes involved in hereditary HL are being rapidly mapped and cloned. The identification of mutations that contribute to deafness and an understanding of the molecular mechanisms underlying HL will undoubtedly give new insights into genetic therapy, genetic counseling, and molecular diagnosis for the disease.

However, for many deafness genes, the detailed pathogenesis of HL remains unknown. Furthermore, various limitations preclude deciphering of the pathological mechanism, for example an ability to acquire a patient's cochlea and typical limitations of rodent modeling of human disorders. Therefore, we propose that the use of genetically modified human induced pluripotent stem cells (hiPSCs) differentiated into auditory neuron-like cells could provide a promising complementary tool for the study of hereditary HL. This would extend the possibilities of how we investigate the mechanisms of deafness-related genes in HL, as well as how we can pursue new cellular therapies for treating hereditary HL.

A large Chinese family with autosomal dominant deafness-41, a progressive sensorineural HL, was described in 2002.[5] After locus refinement in 2005,[6] the causative purinergic receptor p2x gene (*P2RX2*) was finally discovered in two Chinese families.[7] Mutations in *P2RX2* are inherited in an autosomal dominant manner, and have been implicated in age-related and noise-induced HL.[7] However, the detailed mechanism underlying pathophysiological changes in relation to HL is unknown. Previous studies showed that *P2RX2* p.V60L abolished the response of P2RX2 to ATP by patch clamp recoding of HEK293 cells transfected with a green fluorescent protein (GFP)-tagged P2RX2 p.V60L vector, and *P2RX2* was considered to be responsible for the development of a temporary threshold shift in *P2RX2* knockout mice.[7,8] *P2RX2* c.178G>T is a rare heterozygous allele that cosegregated with fully penetrant HL in a six-generation kindred living in Sichuan, China.[7] Three patients derived from this family were recruited for our present trial.

It is extremely difficult to study human temporal bone pathology in nonlethal diseases because biopsy is precluded by cochlear anatomy. Moreover, although transgenic mice are useful tools for hearing research, many studies have suggested that human deafness is not recapitulated in rodent models.[9] Additionally, the generation of mouse models carrying specific transgenes is costly and time-consuming. Furthermore, the differences between human and rodent P2RX2 gene and protein sequences mean that it is also necessary to develop novel complementary models for *P2RX2* pathophysiological studies.

In the present study, we first generated patient-specific hiPSC lines carrying the heterozygous *P2RX2* c.178G>T mutation. To better understand the genotype–phenotype relationship on the basis of HL pathogenesis, we introduced an isogenic mutation to the site of interest, thereby generating a unique homozygous *P2RX2* c.178G>T hiPSC line for pathological research by clustered regularly interspaced palindromic repeats (CRISPR)/ CRISPR-associated protein (Cas)9 and single stranded oligonucleotide (ssODN)-based gene editing.

Materials and methods

Subjects, clinical evaluations, and DNA sequencing

The patient pedigree is shown in Figure 1. Pure-tone audiometry, distortion product otoacoustic emission, auditory steady state responses, auditory brainstem responses, and vestibular tests were carried out to assess patient HL and vestibular function. Peripheral blood samples were collected and genomic (g)DNA was extracted using the RelaxGene Blood DNA System (TIAGEN Biotech, Beijing, China). The DNA fragment flanking *P2RX2* c.178G>T was amplified by PCR using specific forward (5′-TGGGACTCGGGG TGCTGG-3′) and reverse (5′-GGCTT CACGTACTCCTCCACG-3′) primers. gDNA samples were subjected to a deafness genetic screen to exclude the most common four deafness genes using a universal array (CapitalBio, Beijing, China) for nine mutations causing hereditary HL (GJB2: c.35delG, c.176del16, c.235delC, c.299-300delAT; GJB3: c.538C>T; SLC26A4: c.IVS7-2A>G, c.2168A>G; mtDNA: m.1555A>G, and m.1494C>T). Then, a custom capture panel (MiamiOtoGenes) was used to exclude 180 known and candidate genes associated with sensorineural HL. This study was approved by the Medical Ethics Committee of the Second Xiangya Hospital, Central South University, and informed consent was obtained from all individual participants.

hiPSC generation and feeder-free culture

Renal epithelial cells derived from II-1, II-2, and III-1 (Figure 1a) were collected and cultured as described previously by Zhou et al.[10] These are an ideal resource for reprogramming, being readily available, simple, noninvasive, and cost-effective. The CytoTune®-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) containing the 'Yamanaka' factors (Oct, Sox2, Klf4, and c-Myc) was used to reprogram renal epithelial cells into iPS cells according to the manufacturer's instructions. Briefly, 1×10^5 urine epithelial cells were plated into one well of a gelatin-coated 6-well plate 48 hours before viral transduction at the appropriate density to achieve 2.5×10^5–3.5×10^5 cells per well on the day of transduction. Cells were transfected according to the manufacturer's recommended multiplicity of infection (MOI) value (KOS MOI=5, hL-Myc MOI=5, hKlf4 MOI=3). After 7–8 days, cells were

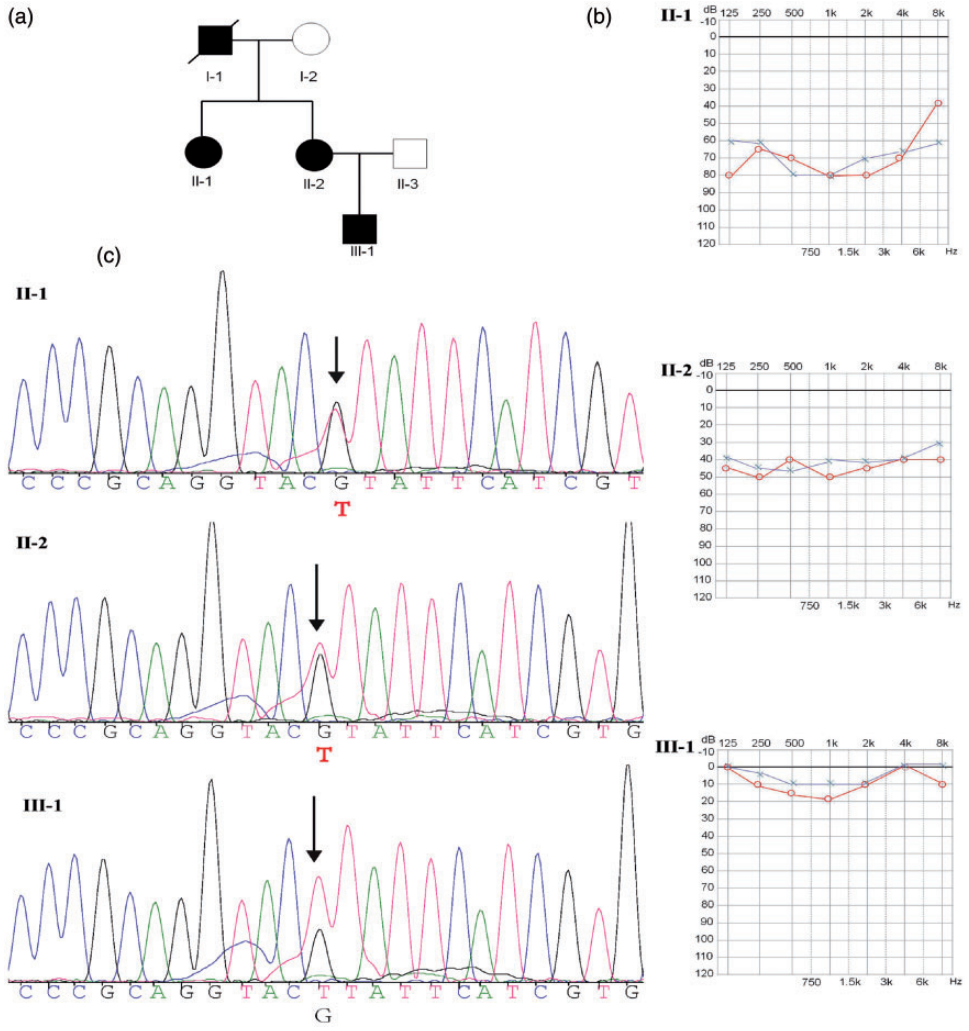

Figure 1. Three members from a large Chinese family suffering from deafness. (a) Pedigree of family members recruited in the study with hereditary HL. (b) Audiograms; red indicates the right ear and blue indicates the left ear. (c) Identification by Sanger sequencing of the *P2RX2* c.178G>T mutation.

plated onto culture dishes coated with vitronectin (Gibco Cell Culture, Carlsbad, CA, USA) containing complete E8 medium (Thermo Fisher Scientific). Spent medium was replaced daily. Colonies had typically grown to an appropriate size for transfer 3–4 weeks after transduction. Undifferentiated colonies were manually picked up and transferred onto prepared vitronectin-coated 6-well culture plates for further expansion.

CRISPR/Cas9-mediated gene knock in to generate homozygous P2RX2 c.178G>T hiPSC lines

The GeneArt® CRISPR Nuclease Vector Kit (Invitrogen Corp., Carlsbad, CA,

USA) was used for genetic editing according to the manufacturer's instructions. Briefly, three pairs of oligos were synthesized by Integrated DNA Technologies (Coralville, IA, USA) as follows: gRNA-1 forward 5′-ATGAATACGTACCTGCGG GCGTTTT-3′ and reverse 5′-GCCCGCA GGTACGTATTCATCGG TG-3′; gRNA-2 forward 5′-GCACGATGAATACGT ACCTGGTTTT-3′ and reverse 5′-CAGG TACGTATTCATCGTGCCGGTG-3′; gRNA-3 forward 5′-CACGATGAATAC GTACCTGCGTTTT-3′ and reverse 5′-GC AGGTACGTATTCATCGTGCGGTG-3′.

After annealing the above single-stranded oligos to generate double-stranded oligos, the gRNAs-Cas9-OFP-expressing vector was generated by cloning double-stranded oligos into the CRISPR Nuclease Vector bearing both a single guide RNA scaffold backbone and Cas9. Vectors were then transformed into One Shot® Chemically Competent TOP10 *Escherichia coli* cells (Invitrogen) and positive clones were selected. Transformants were analyzed for the presence of inserts by Sanger sequencing using the U6 primer (5′-GGACTATCATATGCTTACCG-3′). The gRNAs-Cas9-OFP vector was functionally validated in 293T cells using the GeneArt® Genomic Cleavage Detection Kit (Invitrogen), and the most efficient plasmid was selected for further transfection of hiPSC lines. A 130 bp ssODN carrying the mutation site was synthesized by IDT as follows: 5′-GCGGGCGGGACT CAGCCTTCCCAGGGTCGCCTCCGG AGCCGGCGCCGCCCCTGCCCGCAG GTACTTATTCATCGTGCAGAAAAGC TACCAGGAGAGCGAGACGGGCCCC GAGAGCTCCAT CATCACCAAG-3′.

Heterozygous *P2RX2* c.178G>T hiPSCs were transfected with the gRNA2-Cas9-OFP plasmid and ssODN. For targeting, hiPSCs were cultured in one well of 6-well plates until the cells were 60%–70% confluent. ROCK inhibitor Y-27632 (Sigma-Aldrich, St Louis, MO, USA) was added to a final concentration of 10 μM 1 hour before transfection. The Lipofectamine™ 3000 Kit (Invitrogen) was used for lipid-based transfection. Briefly, dissociated hiPSCs were incubated for 12 minutes with transfection mix (50 μL Opti-MEM medium with 1.5 μg gRNA-Cas9 plasmid, 1.5 μg ssODN, and 5 μL P3000 mixed with 50 μL Opti-MEM medium and 3.75 μL Lipofectamine 3000). Cells together with the transfection mix were seeded onto 6-well plates coated with Matrigel (Gibco) in the presence of Y-27632. After 24 hours transfection, the medium was replaced with complete E8 medium without Y-27632. Then, the cells were cultured for an additional 48 hours and transferred into 96-well plates in the presence of Y-27632 for single-cell selection with limited dilution. Typically around 10–15 days after single-cell seeding, colonies had grown to an appropriate size for harvest. Half of the cells were transferred to 12-well plates for further expansion and the remainder was used for gDNA extraction. Sanger sequencing was used for mutation identification. Primers flanking the *P2RX2* locus for PCR amplification and sequencing were: forward (5′-TGGGACTCGGGGTGCTG G-3′) and reverse (5′-GGCTTCACGTAC TCCTCCACG-3′).

Immunofluorescence staining of hiPSC lines

The Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit (Invitrogen) containing four antibodies, octamer-binding transcription factor (OCT)3/4, sex determining region Y-box 2 (SOX2), stage-specific embryonic antigen 4 (SSEA4), and TRA-1-60, was used to confirm pluripotency in all hiPSC lines according to the manufacturer's instructions.

Teratoma formation and embryoid body (EB) formation

Teratoma analysis was performed as previously described.[4]For EB formation, hiPSCs were cultured in E6 medium (Gibco) for 7 days of floating culture using low attachment 6-well plates (Corning, Corning, NY, USA). EBs were then cultured in gelatin-coated 6-well plates for another 7 days.

Real-time PCR and gene expression analysis

Total RNA was extracted from hiPSCs using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). cDNA synthesis was performed with the SuperScript™ III CellsDirect™ cDNA Synthesis Kit (Invitrogen). Primers are listed in Table 1. The PCR reaction was performed using 15 ng cDNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 U Taq DNA polymerase, 0.2 μM of each primer, and 1× reaction buffer under the following conditions: 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute.

Off-target analysis

Eight potential off-target sites predicted to cause site-specific cleavage by the CRISPR/Cas9 system were analyzed according to an online design tool (https://zlab.bio/guide-design-resources/). PCR products of the potential off-target sites were confirmed by sequencing. Primers for off-target amplification are listed in Table 1.

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of Second Xiangya Hospital, Central South University (Reference number: (2010) IRB NO. (191)). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from adult subjects and the parents of minor subjects. Informed consent was obtained from all individual participants included in the study.

Results

Clinical features and hearing evaluation

The four most common deafness genes were excluded from the family members using universal array (CapitalBio), and 180 known and candidate genes associated with sensorineural HL were excluded using a custom capture panel (MiamiOtoGenes). Sanger sequencing of *P2RX2* showed that the family members carried the heterozygous *P2RX2* c.178G>T mutation (Figure 1 and Table 2).

Generation and characterization of hiPSCs from patients with the heterozygous P2RX2 c.178G>T mutation

Renal epithelial cells were isolated and cultured from the urine of three patients with the *P2RX2* c.178G>T (p.V60L) mutation and a healthy donor. Morphologically, type I and type II cell types were observed as described by Zhou et al.[10] Type I colonies had a more regular appearance with smooth-edged contours and cobblestone-like morphologies, whereas type II colonies were more randomly arranged. After 10 days of culture, some renal epithelial cell clones showed a high level of proliferation. Although urine-derived cells from individuals with *P2RX2* c.178G>T (p.V60L) mutations expanded *in vitro* as efficiently as the healthy donor's cells, they went into senescence earlier than healthy cells.

Table 1. Oligonucleotide sequences of primers.

Gene	Primers (5′–3′)	Amplification product size (bp)
SOX2	GGGAAATGGGAGGGGGTGCAAAAGAGG TTGCGTGAGTGTGGATGGGATTGGTG	152
c-MYC	TGCACTGGAACTTACAACACCCGA TAAGCAGCTGCAAGGAGAGCCTTT	133
OCT-4	GACAGGGGGAGGGGAGGAGCTAGG CTTCCCTCCAACCAGTTGCCCCAAAC	144
KLF4	GAGGGAAGACCAGAATTCCCTTGA AGAACCAAGACCTCACCAAGCACCA	181
NANOG	ACCTATGCCTGTGATTTG AGAAGTGGGTTGTTTGC	169
PAX6	ACCCATTATCCAGATGTGTTTGCCCGAG ATGGTGAAGCTGGGCATAGGCGGCAG	317
AFP	GAATGCTGCAAACTGACCACGCTGGAAC TGGCATTCAAGAGGGTTTTCAGTCTGGA	281
SOX17	AAGATGCTGGGCAAGTCGT CTCTGGCAGTCGCGGTAG	326
SOX1	CAACCAGGACCGGGTCAAAC CCTCGGACATGACCTTCCAC	146
MSX-1	CGAGAGGACCCCGTGGATGCAGAG GGCGGCCATCTTCAGCTTCTCCAG	307
TBX1	GCCCTCTCCCTCCCCTCCACGCACAG CGGCGCCGTTGCTCACAGACCACAGG	274
GAPDH	GAAGGTCGGAGTCAACGG GGAAGATGGTGATGGGATT	221
OFF TARGET 1	CAGACTCCTCCATACCCCCA CATACCCCTCCCAGAGGGAA	682
OFF TARGET 2	GGCTACTGAGGACCCACCTA TGCCATCTAAGGAAGCTACACC	713
OFF TARGET 3	GGACAGATGCTTGAGGCCAT GCCTGAGGGTTCTCTGTCAC	496
OFF TARGET 4	AGGAGATCAGGCTGGAGGTT GTGACACAGCTCTCTGGTCC	661
OFF TARGET 5	CCTCTGTCCCCACCCATAGA TGGCCTTCCTGCCTACCTAT	680
OFF TARGET 6	ATGTGTACTGCAGGGCTTCC GCATCTCATGGGCCTTCTGT	645
OFF TARGET 7	TCCTCTCACCCAGGTAGCTC TGCTGACCCTGTGATTTCAAGT	560
OFF TARGET 8	CAGCTGAGACACCTCACAGT TAAACTCAGGAGGCTGGCAC	319

Renal epithelial cells were transfected with four "Yamanaka factors" encoded by the non-integrating Sendai virus system using Lipofectamine 3000 to reprogram them into hiPSCs. To improve the transfection efficiency, we used somatic cells at passage 3 from the healthy donor and at passage 2 from HL patients. Approximately 3 weeks later, hiPSC-like colonies with a high nuclear–cytoplasmic

Table 2. Clinical data of participants.

Subject	Gender	Age (years)	Noise exposure	PTA (left ear)	PTA (right ear)	Audiogram shape	Tinnitus	Vertigo	Severity
II-1	Female	54	Yes	70dB	75dB	U-shape	Yes	No	Profound
II-2	Female	31	No	45dB	45dB	Flat	Yes	No	Moderate
III-1	Male	7	No	10dB	15dB	Flat	No	No	Normal

ratio were observed. Single colonies were then selected and separately subcultured. Multiple assays were performed to fully characterize the generated hiPSC lines (Figure 2). hiPSCs formed colonies with a morphology similar to that of embryonic stem cells. There were no observable morphological differences between patient and control hiPSCs.

Generation of the homozygous P2RX2 c.178G>T mutation

We next generated a homozygous *P2RX2* c.178G>T hiPSC line to compare the pathological, morphological, and functional effects of the homozygous versus the heterozygous mutation. We used CRISPR/Cas9 and ssODN to introduce the isogenic *P2RX2* c.178G>T mutation to wild-type alleles in patient-derived hiPSCs carrying the heterozygous *P2RX2* c.178G>T mutation. All 23 bp genomic sites of the form 5′-N20NGG-3′ near *P2RX2* c.178G of the intended target site (ideally ±50 bp) were analyzed. Sanger sequencing revealed that out of 120 hiPS clones, three had been successfully generated with the homozygous *P2RX2* c.178G>T mutation (Figure 3).

Characterization of hiPSCs carrying the homozygous P2RX2 c.178G>T mutation using CRISPT/Cas9-mediated genetic editing and ssODN

Examination of the homozygous *P2RX2* c.178G>T hiPSC line revealed that it expressed hiPSC endogenous marker genes including *OCT4*, *SOX2*, Kruppel-like factor 4 (*KLF4*), *c-MYC*, and *NANOG* (Figure 4c), as well as marker proteins characteristic of hiPSC such as OCT4, SOX2, SSEA4, and TRA-1-60 (Figure 4a). The hiPSC line also formed EBs and expressed markers for the three germ layers (ectoderm: SOX1 and paired box [PAX]6; endoderm: alpha-fetoprotein [AFP] and SOX17; and mesoderm: T-box 1 [TBX1] and msh homeobox 1 [MSX1][4,11]) (Figure 4d). Off-target sequencing revealed that no double peaks adjacent to the top eight suspected off-target sites were found in the homozygous hiPSC clones, indicating that no off-targeting had occurred.

These results showed that the *P2RX2* c.178G>T point mutation was successfully introduced into the wild-type allele in the heterozygous *P2RX2* c.178G>T hiPSC line, generating a new hiPSC line with the homozygous *P2RX2* c.178G>t (p. V60L) genotype.

Discussion

CRISPR/Cas9-mediated gene editing has emerged as one of the most useful tools to study gene functions, and also has the potential to treat genetic disorders. Combining the cellular versatility of hiPSC differentiation with the ease of CRISPR/Cas9-mediated genome editing has proven to be a very powerful experimental approach, and has become a standard tool in stem cell research and human disease modeling.[12,13] However, although this technology has been broadly applied

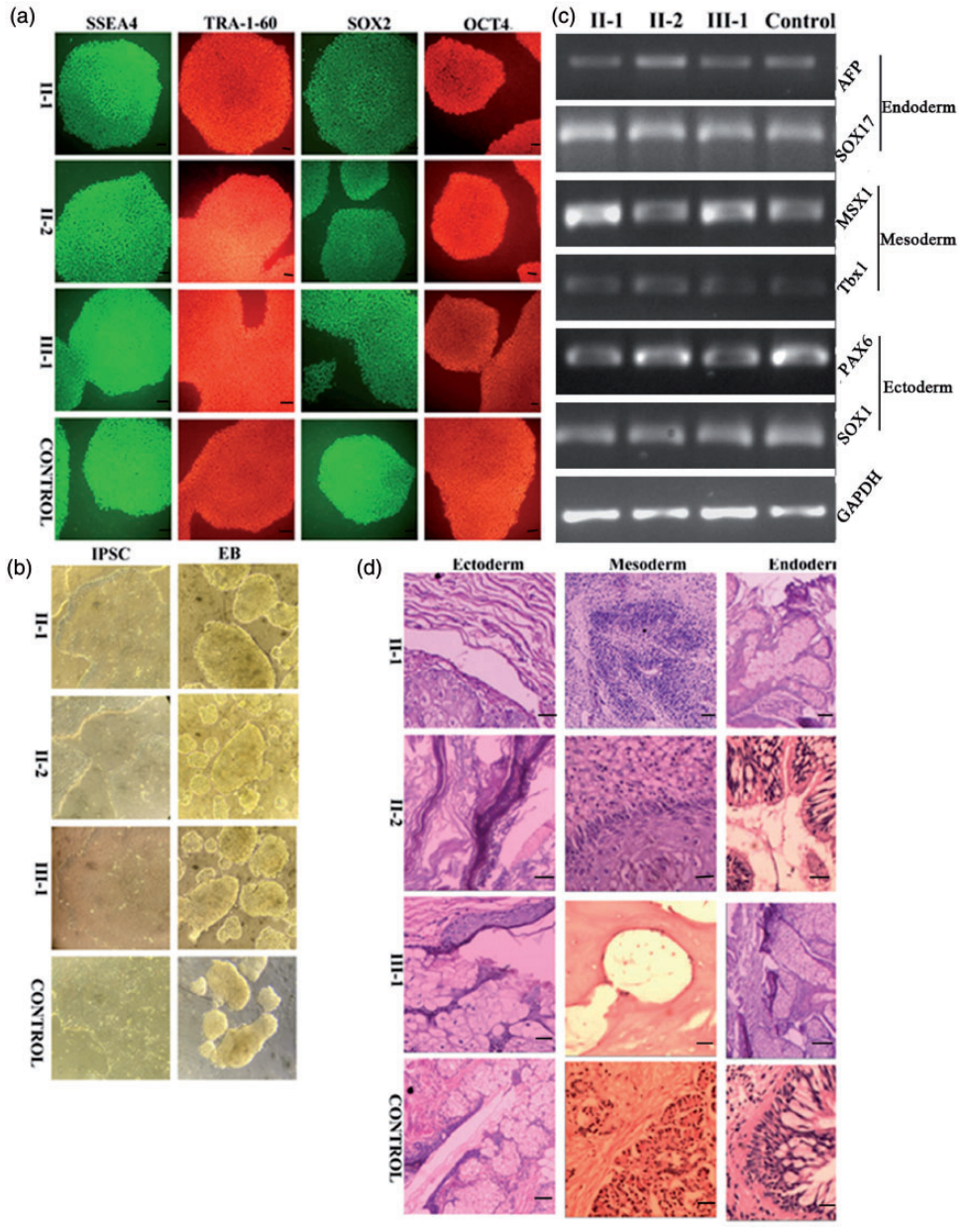

Figure 2. Characterization of hiPSCs generated from three family members carrying *P2RX2* c.178G>T. (a) Immunofluorescence for pluripotency markers SSEA4, TRA-1-60, SOX2, and OCT4. (b) hiPSCs and embryoid body formation. (c) RT-PCR analysis of markers for the three germ layers (ectoderm: SOX1 and PAX6; endoderm: AFP and SOX17; mesoderm: TBX1 and MSX1). (d) Teratoma formation.

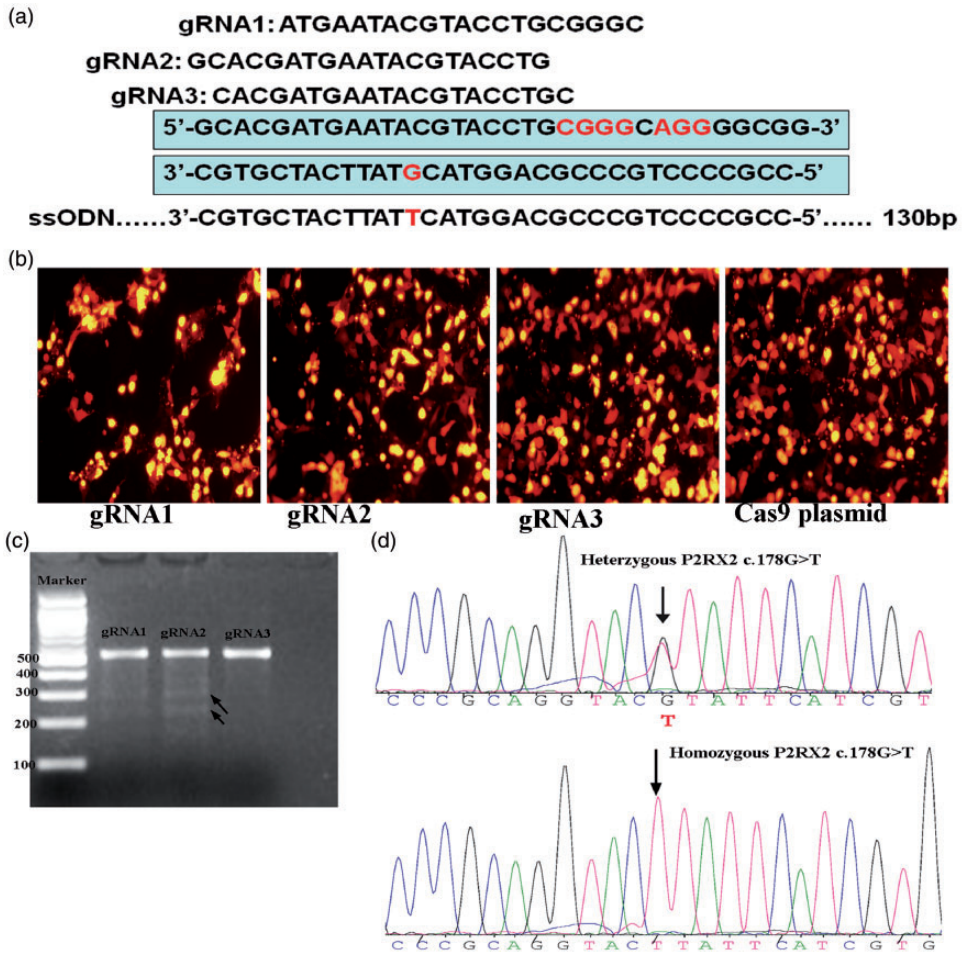

Figure 3. Establishment of homozygous *P2RX2* c.178G>T point mutation in heterozygous *P2RX2* c.178G>T hiPSCs with CRISPR/Cas9 and ssODN. (a) Schematic of gRNA targeting. Top: gRNAs targeting site. PAM is highlighted in red. Bottom: 130 bp ssODN sequence; mutation site T is highlighted in red. (b) Fluorescence images of 293T cells co-transfected with Cas9-gRNAs plasmid carrying the OFP gene. (c) T7E1 assay to assess Cas9-gRNA activity in 293T cells, indicating that gRNA2 has higher cleavage activity. (d) Sequencing analysis. Top: heterozygous *P2RX2* c.178G>T. Bottom: homozygous *P2RX2* c.178G>T (black arrow).

to neurodegenerative diseases, blood disorders, and retinal degenerative diseases, little is known about hereditary HL.[14,15] Most hereditary HL is a monogenetic disorder that is ideally suited to iPS-based disease modelling. hiPSCs reprogrammed from patient somatic cells could then be differentiated into disease-relevant cells such as spiral ganglions, hair cells, and as a cell model to further study the pathological mechanism of deafness genes, drug screening, and safety pharmacology.

P2RX2 encodes a receptor protein that assembles as a trimer to form an ATP-gated ion channel. The P2X2 receptor is broadly expressed in cochlear epithelial cells such as

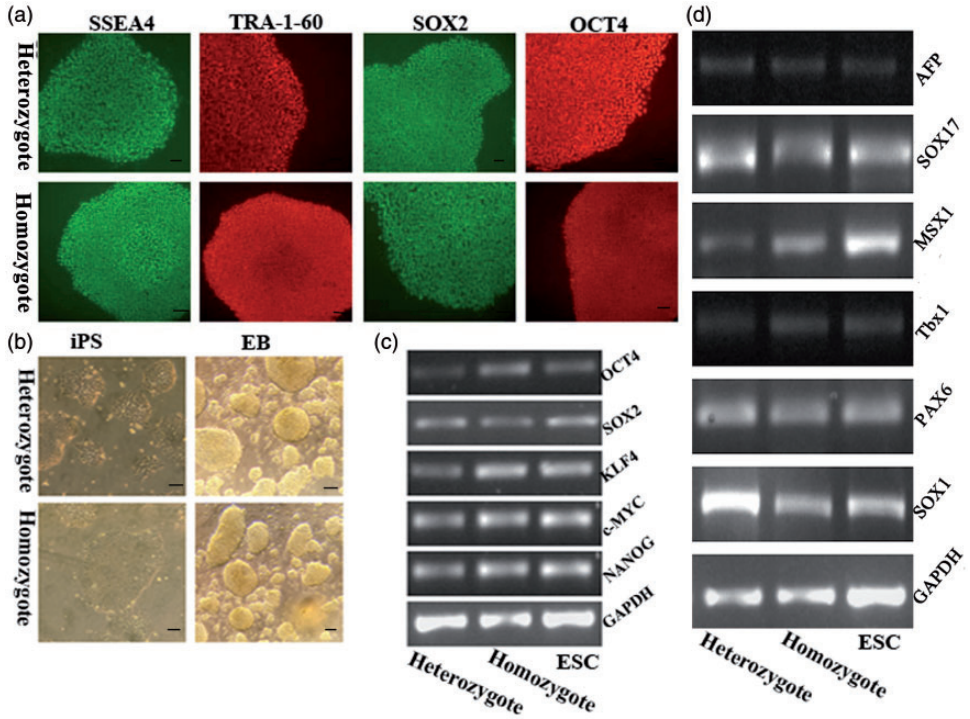

Figure 4. Characterization of hiPSCs carrying heterozygous and homozygous *P2RX2* c.178G>T. (a) Immunostaining for pluripotency markers SSEA4, TRA-1-60, SOX2, and OCT4. (b) hiPSCs and embryoid body (EB) formation. (c) RT-PCR analysis of pluripotency markers OCT4, SOX2, KLF4, c-MYC, and NANOG. (d) RT-PCR analysis of EB markers for the three germ layers (endoderm: AFP and SOX17; mesoderm: TBX1 and MSX1; ectoderm: SOX1 and PAX6).

hair cells, supporting cells, and spiral ganglion neurons. P2X receptors mediate complicated cellular responses such as outer hair cell electromotility,[16] auditory neurotransmission,[17] gap junctions,[18] and K^+ recycling in the inner ear,[19] as well as excitatory postsynaptic responses in sensory neurons,[20] and cell proliferation, differentiation, and death during development and regeneration in the nervous system.[21] Although *P2RX2* was recently identified as a deafness gene, the heterozygous mutations V60L and G353R were shown to cause nonsyndromic HL so it is an essential but challenging problem to study the physiopathologic function of HL-related genes.

Here, we established a method to rapidly generate hiPSC lines from disease-specific patients with mutated deaf genes and to introduce CRISPR/Cas9-mediated mutations to heterozygous mutant cell lines to study the pathological mechanism of deaf genes in developing HL. We efficiently generated mutant hiPSC lines with the heterozygous *P2RX2* c.178 G>T mutation. However, because most hereditary HL is inherited in an autosomal dominant manner, the stem cells generated from affected patients would carry heterozygous *P2RX2* alleles. Therefore, the normal allele would partially compensate for the function as an iron channel, adding to the difficulty of studying the *P2RX2* function. This is why we focused on introducing the same mutation into heterozygous hiPS cells to achieve homozygous mutant cell lines,

offering an internal control for phenotypic analyses.

After gene editing, the manipulated hiPSCs retained the potential to differentiate into three germ layers. More importantly, no mutations were detected at predicted off-target sites, which is essential for future studies of gene function and clinical applications. Out of 120 single-cell clones, we obtained three with the homozygous c.178G>T mutation. Although the efficiency of lipid-based transfection is lower than that of electroporation and nucleofection, it has the advantages of ease of use and higher survival of targeted hiPSCs. Electroporation usually results in massive cell death and loss of stemness after transfection, while nucleofection requires expensive equipment and reagents. [4,22]

Multiple tissues have reportedly been used to reprogram somatic cells into iPS cells. The speed and convenience of acquiring donor cells is essential for clinical use, and a noninvasive procedure is a major consideration. Renal epithelial cells present in urine meet all these requirements. Besides the integrated retrovirus transfection of four "Yamanaka factors", non-integrated methods have been developed more recently such as Sendai viruses, episomal vectors, mRNAs, minicircle DNAs, microRNAs, proteins, and small molecules. The derivation of hiPSCs by methods that are integration-free and xeno-free is a basic requirement for clinical trials because random integrations could unexpectedly silence or activate vital genes, which is a risk to patient safety.

In our study, we considered ways of reducing unnecessary stress on hiPSCs. For example, although the efficacy of precise genetic modification was low and may have been improved using a puromycin-based expression plasmid or a GFP-expressing plasmid to allow the transient selection of cells, we chose to perform subsequent single-cell clone and sequencing without additional cell manipulation.

The heterozygous and homozygous *P2RX2* mutated hiPS cell lines generated via patient-specific iPS technology and CRISPR/Cas9 gene editing are good models for further investigating the pathological mechanisms of *P2RX2* mutations during HL pathogenesis. The current findings confirmed our hypothesis that it is feasible and convenient to introduce precise point mutations into genomic loci of interest to generate gene-mutated hiPSC models.

Conclusion

This is the first report of the establishment of an hiPSC line from patients carrying the heterozygous *P2RX2* c.178G>T mutation and the specific introduction of this c.178G>T point mutation into the heterozygous line, thereby establishing a homozygous hiPSC lines by CRISPR and ssODN-based gene editing. This resulted in a unique cell line for P2RX2 pathologic research that will be beneficial to help understand the genotype and phenotype relationship of *P2RX2* mutations and their contribution to HL. This method combines the cellular versatility of hiPSC differentiation with the ease of CRISPR/Cas9-mediated genome editing, greatly expanding the current resources of genetic tools.

Author contributions

X.Z.L., D.H.X., and Y.P.D. conceived and designed the experiments. Y.P.D. and X.Z.L. conducted the experiments. H.P.X., D.H.X., and Y.P.D. analyzed the results. Y.P.D. drafted the manuscript. All authors participated in the discussion of results and reviewed the manuscript.

Data availability statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This work was supported by the National Basic Research Program of China (973) (grant no. 2012CB967904) to Dinghua Xie. This study was also supported by R01 DC05575 and R01 DC012115 from the National Institutes of Health/National Institute on Deafness and Other Communication Disorders, the National Natural Science Foundation of China (grant no. 81771024) to Xuezhong Liu, and by a local government foundation to Donghui Tan (grant no. 2016JJ6144 and 201558).

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