Original Article

Estimating the concentration of therapeutic range using disease-specific iPS cells: Low-dose rapamycin therapy for Pendred syndrome

Makoto Hosoya a,1, Tsubasa Saeki b,1, Chika Saegusa a, Tatsuo Matsunaga c, d, Hideyuki Okano b, Masato Fujioka a,⁎, Kaoru Ogawa a

a Department of Otorhinolaryngology, Head and Neck Surgery, Keio University School of Medicine, 35 Shinanomachi Shinjuku-ku, Tokyo 160-8582, Japan
b Department of Physiology, Keio University School of Medicine, 35 Shinanomachi Shinjuku-ku, Tokyo 160-8582, Japan
c The Laboratory of Auditory Disorders and Division of Hearing and Balance Research, National Institute of Sensory Organs, National Tokyo Medical Center, 2-5-1 Higashigaoka, Meguro-ku, Tokyo 152-8902, Japan
d Medical Genetics Center, National Tokyo Medical Center, 2-5-1 Higashigaoka, Meguro-ku, Tokyo 152-8902, Japan

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A B S T R A C T

Introduction: Pendred syndrome is an autosomal-recessive disease characterized by congenital hearing loss and thyroid goiter. Previously, cell stress susceptibilities were shown to increase in patient-derived cells with intracellular aggregation using an in vitro acute cochlear cell model derived from patient-specific pluripotent stem (iPS) cells. Moreover, we showed that rapamycin can relieve cell death. However, studies regarding long-term cell survival without cell stressors that mimic the natural course of disease or the rational minimum concentration of rapamycin that prevents cell death are missing.

Methods: In this report, we first investigated the rational minimum concentration of rapamycin using patient-specific iPS cells derived-cochlear cells with three different conditions of acute stress. We next confirmed the effects of rapamycin in long-term cell survival and phenotypes by using cochlear cells derived from three different patient-derived iPS cells.

Results: We found that inner ear cells derived from Pendred syndrome patients are more vulnerable than those from healthy individuals during long-term culturing; however, this susceptibility was relieved via treatment with low-dose rapamycin. The slow progression of hearing loss in patients may be explained, in part, by the vulnerability observed in patient cells during long-term culturing. We successfully evaluated the rational minimum concentration of rapamycin for treatment of Pendred syndrome.

Conclusion: Our results suggest that low-dose rapamycin not only decreases acute symptoms but may prevent progression of hearing loss in Pendred syndrome patients.

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1. Introduction

Human-induced pluripotent stem (iPS) cell technology was first reported in 2007 by Shinya Yamanaka’s group [1]. iPS cells show very similar properties to embryonic stem cells and can be established from human peripheral blood cells [2]. Recent studies have reported the successful generation of iPS cells from patients having various diseases (i.e., patient-specific iPS cells), and this technology has been broadly applied in medical sciences to model diseases in many different organs cells [3]. Particularly, this technology has come to be a powerful tool for modeling diseases with no appropriate animal model, attributable to the ease by which cells expressing human disease can be obtained by inducing target cells from patient-specific iPS cells in vitro. Inspection of cellular phenotypes in these disease models has led to the elucidation of novel pathological mechanisms of various targeted diseases, including neurodegenerative and orthopedic diseases [3–7].

Recently, this technology has been applied to the discovery of a new modality of candidate therapeutics using diseased cells.

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Further, various drugs have been tested using this technology, and effective compounds have been easily screened using human cells, even when the target is a rare disease. For example, Yamashita et al. revealed that statin treatment rescues fibroblast growth factor receptor 3 skeletal dysplasia phenotypes [6], and Hino et al. reported that rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, prevents aberrant chondrogenesis in fibrodyplasia ossificans progressiva [7].

To date, we have applied this technology toward studying Pendred syndrome [8], an autosomal-recessive disease characterized by congenital hearing loss, vertigo, and thyroid goiter. The solute carrier family 26 member 4 (SLC26A4) gene encoding PENDRIN, an anion exchanger [9], was identified as a causative gene of this disease in 1997 [10]. The incidence of the disease is estimated at 7.5–10 in 100,000. Pendred syndrome may account for as many as 10% of the cases of hereditary hearing loss [11], making it one of the most common forms of syndromic hearing loss.

Hearing loss in Pendred syndrome patients appears during childhood and is progressive. Using in vitro cochlear cell models derived from patient-specific iPSCs, we clarified the novel pathophysiology associated with Pendred syndrome and proposed a “degenerative cochlear disease model” [12]. In this model, cell stress susceptibilities leading to cell death are proposed to increase in patient-derived cells with intracellular aggregation. Moreover, we showed that rapamycin can relieve this cell death by activating autophagy. We concluded that this type of cell death explains the progression of hearing loss or fluctuations in hearing levels observed in patients with Pendred syndrome. Further, rapamycin could be a potential therapeutic drug for treating Pendred syndrome. However, studies regarding long-term cell survival in the absence of cell stressors that mimic the natural course of disease or the rational minimum concentration of rapamycin that prevents cell death are missing. Here, we evaluated the effective concentration of rapamycin using a fast drug-screening model with a cell stressor. In addition, we established an in vitro chronic disorder model of Pendred syndrome. Our results suggest that low concentrations of rapamycin can delay the progression of cell death, demonstrating the possibility of using low-dose rapamycin therapy as a therapeutic for Pendred syndrome.

2. Methods

2.1. Cell lines

Three Pendred syndrome-specific human iPSC cell lines (hiPSCs) (H723R #16, M147V #18, and T410M #12) generated from peripheral blood samples with episomal plasmids [12] were used in this study. H723R#16 was derived from a 7-year-old woman with a c.2168 A > G (p. His723Arg) homozygous missense mutation within the SLC26A4 gene. M147V#18 was derived from a 34-year-old female with c.439 A > G (p. Met147Val)/c.2168 A > G (p. His723Arg) compound heterozygous missense mutations within the SLC26A4 gene. T410M#12 was derived from a 4-year-old female with c.1229 C > T (p. Thr410Met) homozygous missense mutation within the SLC26A4 gene. Informed consent had been obtained from all patients. All experimental procedures for hiPSC production were approved by the ethics committee of the Keio University School of Medicine (#20140172) and the NHO Tokyo Medical Center (R13-097) and were in accordance with the guidelines of the National Institutes of Health, and the Ministry of Education, Culture, Sports, Science and Technology of Japan and declaration of Helsinki. For control experiments, two hiPSC lines were used, including one from a healthy 16-year-old girl (WD39) [13] and a SLC26A4 gene-specific site-corrected line (GE #21) derived from H723R #16.

2.2. Culture of hiPSCs

The hiPSCs were grown on mitomycin-C-treated SNL murine fibroblast feeder cells in gelatin-coated (0.1%) tissue culture dishes. The hiPSCs were maintained in standard hESC medium (Dulbecco’s modified Eagle medium [DMEM]/F12 Sigma, D6421) containing 20% knock-out serum replacement [KSR; Life Technologies], nonessential amino acids [NEAA, Sigma], 0.1 mM 2-mercaptoethanol [Sigma], and 4 ng/mL fibroblast growth factor 2 [FGF-2, PeproTech] at 37 °C in a humidified atmosphere of 5% CO2. For feeder-free culture conditions, the hiPSC/hESC lines were cultured in mTeSR1 medium (Stemcell Technologies) on matrigel-coated culture dishes (Corning, #354277).

2.3. Induction of cochlear outer sulcus cells (OSC)

We induced OSC-like cells expressing PENDRIN from undifferentiated iPSCs using previously reported methods [12]. In brief, after inducing otic progenitor cells, the medium was exchanged for LW medium containing 4 ng/mL FGF2, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 6% fetal bovine serum (FBS), and 100 mg/mL ampicillin in DMEM (Sigma, D5796). After 2 weeks to 1 month of culturing, OSC differentiation and selection were initiated by exchanging the medium for DMEM (Nacalai Tesque, 08459-64) containing 10% FBS, 0.75% NaHCO3, and 50 µM penicillin/streptomycin for 7 days. Subsequently, the cells were maintained in DMEM containing 10% FBS, 0.375% NaHCO3, and 50 µL penicillin/streptomycin, which yielded mature, induced cochlear OSCs after 1 week.

2.4. Cellular stress susceptibility assay: acute stress model

Induced OSCs from each cell line were incubated in DMEM containing 10% FBS, 0.375% NaHCO3, and 50 µL penicillin/streptomycin with or without a proteasome inhibitor, epoxomicin (Peptide Institute), which was broadly used as a cell stressor [12,14,15]. For the high-stress/long-term model, we used 0.5 µM epoxomicin, and cells were fixed after incubating for 24 h. For the high-stress/short-term model, we used 0.5 µM epoxomicin, and cells were fixed after incubating for 20 h. For the moderate-stress/long-term model, we used 0.3 µM epoxomicin, and cells were fixed after incubating for 24 h. Prior to 24 h of treatment with epoxomicin, induced OSCs were incubated in DMEM containing 10% FBS, 0.375% NaHCO3, and 50 µL penicillin/streptomycin containing various concentrations of rapamycin for 48 h. The cells were fixed and then subjected to immunocytochemical analyses using anti-PENDRIN and anti-cleaved caspase-3 antibodies. PENDRIN-positive cells or PENDRIN- and cleaved caspase-3 double-positive cells were counted using a confocal laser scanning microscope (LSM700; Carl Zeiss) as previously reported [12]. Viable cells were defined as PENDRIN-positive and cleaved caspase-3-negative, and cell viability was defined as the number of viable cells divided by the number of viable cells in the untreated (control) sample.

2.5. Cellular stress susceptibility assay: chronic stress model

OSC-like cells derived from iPSCs were maintained in DMEM containing 10% FBS, 0.375% NaHCO3, and 50 µL penicillin/streptomycin. The medium was changed every 2 or 3 days. We did not passage the cells during this assay. We compared long-term cell survival ratios and assessed the effects of low-dose rapamycin (0.002 µM). Cells were fixed after culturing for 14–42 days and then subjected to immunocytochemical analyses using anti-PENDRIN and anti-cleaved caspase-3 antibodies. PENDRIN-positive cells or PENDRIN- and cleaved caspase-3-
double-positive cells were counted using a confocal laser scanning microscope (LSM700; Carl Zeiss) as previously reported [12]. Viable cells were defined as PENDRIN-positive and cleaved caspase-3-negative, and cell viability was defined as the number of viable cells divided by the number of viable cells in the Day \(= 0\) sample.

2.6. Intracellular aggregation counting

The induced OSCs were fixed and subjected to immunocytochemical analysis with anti-PENDRIN antibody. Intracellular PENDRIN aggregations (>2 \(\mu\)m) were counted using a confocal laser scanning microscope (LSM700; Carl Zeiss). We counted the number of the cells dividing three groups according to the number of the aggregations per one cell; 0 or 1 aggregation, 2–5 aggregations, and 6 or more aggregations.

2.7. Immunocytochemical analysis

For immunocytochemical analyses, the cells were fixed with 4% paraformaldehyde. After boiling in 0.1 mM citrate buffer (pH 6.0) for 1 h and blocking in blocking buffer (phosphate-buffered saline [PBS] containing 10% normal donkey serum) for 1 h at room temperature, the cells were incubated with primary antibodies at 4 °C overnight. After three washes with PBS, the cells were incubated with Alexa 488- or Alexa 555-conjugated secondary antibodies (Life Technologies) for 1 h at room temperature. The nuclei were stained with 10 \(\mu\)g/mL Hoechst 33258 (Sigma). After washing with PBS, the cells were examined using a confocal laser scanning microscope (LSM700; Carl Zeiss).

2.8. Antibodies for immunostaining

The following primary antibodies were used for these analyses: anti-PENDRIN (goat, 1:100, Santa Cruz, SC23779) and anti-cleaved caspase 3 (rabbit, 1:300, Cell Signaling, D175).

2.9. Quantification and statistical analysis

Treatment effects on the cells were analyzed using a two-tailed paired student’s t-test. Tukey’s test was used to compare three or more groups. Treatment effects on the cell aggregations were analyzed using a two-tailed non-paired student’s t-test. Data are presented in the text and figures as the means ± S.E.M. All P-values less than 0.05 were considered significantly different.

3. Results

3.1. Acute stress model

First, we investigated the minimum effective concentration of rapamycin using three acute stress models with an existing fast drug screening assay using a disease specific iPS line, H723R#16, including a high-stress/long-term model (0.5 \(\mu\)M epoxomicin, 24 h), a high-stress/short-term model (0.5 \(\mu\)M epoxomicin, 20 h), and a moderate-stress/long-term model (0.3 \(\mu\)M epoxomicin, 24 h) (Fig. 1). Previously, we reported that 0.2 \(\mu\)M rapamycin relieved cell stressor-mediated cell death by activating autophagy in a high-stress/long-term model [12]. In this study, we demonstrate that rapamycin effectively decreased cell stress-mediated cell death in a high-stress/long-term model at concentrations as low as 0.01 \(\mu\)M in cells derived from H723R#16 line (Fig. 2 a-f). Next, we used a high-

![Fig. 1. Schema of acute stress models.](image)
stress/short-term model to analyze the effective lower concentration of rapamycin. In this model, rapamycin showed cytoprotective effects at 0.002 μM (Fig. 2 g–l). Finally, rapamycin showed cytoprotective effects at a concentration of 0.001 μM in a moderate-stress/long-term models (Fig. 3a–f). This cytoprotective effect was also observed in other two disease specific iPS cell lines, T410M#12 and M147V #18 (Fig. 3g and h).

3.2. Chronic stress model

Next, we developed a chronic stress model in the absence of a cell stressor by culturing cells long term (Fig. 4). To verify this model, we compared cell viabilities after 14 days of culture. Results showed that OSC-like cells derived from Pendred syndrome-specific iPS cells were less viable than control iPS WD39 cells (Fig. 5). This decrease in cell viability was not observed in cells derived from the site-specific gene-corrected GE21 line (Fig. 5e), which was previously established from the H723R #16 and in which the mutated SLC26A4 gene was corrected via gene editing. This decrease in cell viability in the diseased cells after long-term culturing was significantly attenuated by adding 0.002 μM rapamycin (Fig. 5e). This effect was confirmed after observing for a minimum of 42 days (Fig. 6). The survival of PENDRIN-positive cells derived from patient-specific iPS cells significantly decreased after culturing long-term; however, cell survival improved after treatment with low-dose rapamycin.
Finally, we compared the numbers of intracellular aggregations after culturing long-term with or without low-dose rapamycin. We did not observe significant differences between the groups (Fig. 7).

4. Discussion

Primary cultures of animal cochlea or cell lines derived from inner ear tissue have been used for screening drugs in vitro. Especially, HEI-OC1 cell line [16] has been widely used for this purpose [17–20]. In vitro drug screenings using these cells are useful for finding potential new drugs or oto-protective compounds to treat hearing loss. The effective concentration of compounds can also be determined by adding drugs at different concentrations. There are advantages associated with screening for drugs using such cell line-based in vitro methods compared to in vivo animal experiments. Particularly, in vitro methods do not require sacrificing animals and thus can be used to test multiple compounds at various concentrations. However, the cell lines used in this type of experiment are generally immortalized; thus, it is sometimes difficult to interpret data, especially when the immortalized cell lines are used for cell survival assays. Further, forced expression or knock down of targeting genes using targeting vectors is needed when using these cells as a disease model, and the procedure can interfere with interpreting the results.

In this report, we alternatively used OSC-like cells derived from human iPS cells (Fig. 1). We previously reported that these cells have the characteristic features of inner ear cells and show a transplantation affinity for inner ear cells in vivo [12,21]. Undifferentiated iPS cells have limitless proliferation ability, and we can induce inner ear cells from these cells without immortalization. Further, if we use disease-specific iPS cells, we can acquire disease-specific inner ear cells without forced expression or knock down of targeted genes. The induced human inner ear cell-based in vitro drug assay used in this report is a candidate approach for otoprotective drug discovery.

Previously, we showed that cell stress susceptibilities specifically increase in Pendred syndrome patient-derived OSC-like cells and proposed that sporadic and transient or partially reversible hearing loss that is progressive over the long-term may be attributable to increased susceptibility to cellular stress. Moreover, we
revealed that rapamycin is a potential therapeutic drug for treating Pendred syndrome, as it relieved this cell stress susceptibility. However, we did not previously evaluate the minimum effective concentration of rapamycin. For clarification, we used a two-step approach to address this issue, including an acute stress model and a chronic stress model.

*In vitro* drug screening with cell stressors or toxins is widely used for otoprotective compounds. For example, cisplatin- or gentamicin-induced cell damage models using HEI-OC1 cells have been used to evaluate the effects of potential compounds [17,19,20]. These assays are usually performed over the course of 2–3 days, and this short-term screening is suitable for easily testing multiple compounds or various concentrations. In this report, we first used an acute stress model and found that rapamycin has model-dependent cytoprotective effects at 0.001 μM.

*In vitro* models using cell stressors are useful for investigating the effectiveness of compounds; however, the results are obtained under artificial or non-physiological stress conditions. Thus, we established a chronic stress model that is not dependent on artificial cell stressors. As such, the iPSC-derived cells differed from general cell lines in that they were not immortalized.

Long-term culturing of neural cells induced from neurodegenerative disease-specific iPSCs has been used to model chronic disorders *in vitro* by mimicking the aging process or the progression of neurodegeneration in patients [22]. In this report, we applied this strategy toward Pendred syndrome (Fig. 4). In the chronic stress model, inner ear cells derived from Pendred syndrome patients were more vulnerable to long-term culturing, and this weakness was attenuated by treatment with low-dose rapamycin. Our results suggest that this vulnerability during long-term culturing of patient cells may account for the slow progression of hearing loss in patients. Further, rapamycin could attenuate the progression of symptoms in Pendred syndrome patients.

Rapamycin has been clinically used as an immunosuppressive drug during organ transplantations. The immunosuppressive effects of rapamycin occur at 16–24 ng/mL in the blood after renal transplantation [23]. Rapamycin is also used as a treatment for lymphangioleiomymomatosis (LAM) [24,25], in which it is used at 5–15 ng/mL in the blood. In this report, rapamycin showed cytoprotective effects at a concentration of 1.828 ng/mL (0.002 μM), which is approximately 1/5-1/10 the clinical concentration used for these diseases. Our result suggest that low-dose rapamycin therapy may relieve the symptoms of Pendred syndrome without the accompanying immunosuppressive effects, although the concept should be practically proven in clinical trials.

Our results showed that the effect of low-dose rapamycin would be different between the patients. As shown in Fig. 6, the protective effect is most significant on M147V #18 line, while the effect is less significant in T410M #12. We think there are two possibilities. First, difficulties of breakdown of the misfolding protein aggregation by autophagy mediated by rapamycin administration would be different between genotypes of the SLC26A4 mutations. Second, the degree of activation of autophagy mediated by rapamycin would be different between the patients depending on their ability of drug metabolisms at cellular level, regardless of genotype of SLC26A4 mutations (probably due to the genetic backgrounds). More detailed analysis targeting larger number of patients including multiple genotypes should be awaited for clearing these possibilities.

In our previous report, we showed that rapamycin via cytoprotective effect on Pendred syndrome patients’ cells via activation of autophagy [12]. We also reported that the cite-specific correction

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*Fig. 4.* Schema of chronic stress model. In this study, we established a chronic stress model independent of a cell stressor. We compared cell viability after long-term culturing with or without rapamycin. Cell viabilities were estimated at a maximum of 42 days. PDS, Pendred Syndrome; RAP, rapamycin.
of mutation of H723R in SLC26A4 gene may decrease number of intra-cellular aggregations. In the presenting paper, we showed survivals of patients’ cells after culturing long-term were significantly improved with low-dose rapamycin. However, we could not observe decreases of the intra-cellular aggregations in rapamycin treated cells in this condition, while previously it was reported that gene correction of SLC26A4 mutation by genome editing on Pendred syndrome patients’ cells reduced intracellular aggregation.
This result indicates two possibilities. First, the cells with obvious aggregations were more easily to die and we could not detect the increasing of intracellular aggregations in control group. This could explain that no significant decreasing of the ratio of cells with intracellular aggregations was observed in the rapamycin treated group while cell survival was improved. Secondly, protein aggregation itself would not be cytotoxic and rapamycin might show its effect without resolving the completely structured aggregations. Now protein aggregations are known to be common pathological features of neurodegenerative diseases, however some recent studies suggested that highly aggregated proteins could be dissociated from neuronal cell toxicity. These reports suggested that protein aggregation intermediates, which were formed before the development of insoluble inclusion body, could be more neurotoxic. For example, Aβ oligomers, α-synuclein oligomers and prion oligomers have all been linked to its toxicity before forming visible intracellular aggregations [26–33] and clearance of misfolded protein oligomers by activating autophagy is still one of the therapeutic targets for neurodegenerative diseases [34]. This hypothesis would explain rapamycin showed cytoprotective effect with activation of autophagy without reducing cellular aggregations, while the possibilities that rapamycin acts via other pathways still remained. A more detailed pharmacological study will need to be carried out in near future.

**Fig. 6.** Low-dose rapamycin (RAP) improved cell viability after long-term culturing of diseased cells. In the chronic stress model, RAP (0.002 μM) significantly improved cell viability of outer sulcus cell (OSC)-like cells derived from disease-specific pluripotent stem (iPS) after 42 days of culture (N = 4). The nuclei were counterstained with Hoechst (blue). Scale bar: 200 μm in (a–f). Data are represented as mean ± SEM. *: P < 0.05.
Fig. 7. Low-dose rapamycin (RAP) did not reduce visible intracellular aggregations. In the chronic stress model, RAP (0.002 μM) did not significantly reduce visible intracellular aggregations of outer sulcus cell (OSC)-like cells derived from disease-specific pluripotent stem (iPS) after 28 days or 42 days of culture (N = 4). Data are represented as mean ± SEM.

5. Conclusions

Our results suggest that low-dose rapamycin not only decreases acute symptoms but may prevent progression of hearing loss in Pendred syndrome patients without the accompanying immunosuppressive effects.

Conflicts of interest

H.O. is a founding scientist and a paid member of the Scientific Advisory Board of San Bio Co. Ltd.

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References

[1] Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–72.
[2] Okita K, Yamakawa T, Matsumura Y, Sato Y, Amano N, Watanabe A, et al. An efficient nonviral method to generate integration-free human-induced pluripotent stem cells from cord blood and peripheral blood cells. Stem Cell 2013;31:458–66.
[3] Okano H, Yamanaka S. iPS cell technologies: significance and applications to CNS regeneration and disease. Mol Brain 2014;7:22.
[4] Consortium H D i. Induced pluripotent stem cells from patients with Huntington’s disease show CAG-repeat-expansion-associated phenotypes. Cell Stem Cell 2012;11:264–78.
[5] Kondo T, Asai M, Tsukita K, Kutoy Y, Ohsawa Y, Sunada Y, et al. Modeling Alzheimer’s disease with iPSCs reveals stress phenotypes associated with intracellular Abeta and differential drug responsiveness. Cell Stem Cell 2013;12:487–96.
[6] Yamashita A, Morioka M, Kishi H, Kimura T, Yahara Y, Okada M, et al. Statin treatment rescues FGFR3 skeletal dysplasia phenotypes. Nature 2014;513:507–11.
[7] Hiroe K, Horigome K, Nishio M, Komura S, Nagata S, Zhao C, et al. Activin-A enhances mTOR signaling to promote aberrant chondrogenesis in fibrodyplasia ossificans progressiva. J Clin Invest 2017;127:3339–52.
[8] Pendred V. Deaf-mutism and goitre. Lancet 1896;i:5332.
[9] Scott DA, Wang R, Kreman TM, Sheffield VC, Karniski LP. The Pendred syndrome gene encodes a chloride-iodide transport protein. Nat Genet 1999;21:440–3.
[10] Everett LA, Glaser B, Beck JC, Idol JR, Buchs A, Heyman M, et al. Pendred syndrome is caused by mutations in a putative sulphate transporter gene SLC26A4. Nat Genet 1997;17:411–22.
[11] Fraser GR. Association of congenital deafness with goitre (Pendred’s syndrome): a study of 207 families. Ann Hum Genet 1965;28:201–49.
[12] Hosoya M, Fujioka M, Sone T, Okamoto S, Akamatsu W, Ukai H, et al. Cochlear cell modeling using disease-specific iPSCs unveils a degenerative phenotype and suggests treatments for congenital progressive hearing loss. Cell Rep 2017;18:68–81.
[13] Imaiuni Y, Okada Y, Akamatsu W, Harada M, Copper induces hepatocyte injury due to the endoplasmic reticulum stress in cultured cells and patients with Wilson disease. Exp Cell Res 2016;347:192–200.
[14] Kalinec CM, Webster P, Lim DJ, Kalinec F. A cochlear cell line as an in vitro system for drug ototoxicity screening. Audiol Neurotol 2003;8:177–85.
[17] Chang J, Jung HH, Yang JY, Choi J, Im GJ, Chae SW. Protective role of antidiabetic drug metformin against gentamicin induced apoptosis in auditory cell line. Hear Res 2011;282:92–6.

[18] Kabeya Y, Kobayashi Y, Suzuki H, Itoh J, Sugita M. Transcription of plastid genes is modulated by two nuclear-encoded alpha subunits of plastid RNA polymerase in the moss Physcomitrella patens. Plant J 2007;52:730–41.

[19] So HS, Park C, Kim HJ, Lee JH, Park SY, Lee JH, et al. Protective effect of T-type calcium channel blocker flunarizine on cisplatin-induced death of auditory cells. Hear Res 2005;204:127–39.

[20] Kalinec G, Thein P, Park C, Kalinec F. HEI-OC1 cells as a model for investigating drug cytotoxicity. Hear Res 2016;335:105–17.

[21] Takeda H, Hosoya M, Fujioka M, Saegusa C, Saeki T, Miwa T, et al. Engraftment of human pluripotent stem cell-derived progenitors in the inner ear of prenatal mice. Sci Rep 2018;8:1941.

[22] Sanchez-Danes A, Richard-Patin Y, Carballo-Carbajal I, Jimenez-Delgado S, Caig C, Mora S, et al. Disease-specific phenotypes in dopamine neurons from human iPSC-based models of genetic and sporadic Parkinson’s disease. EMBO Mol Med 2012;4:380–95.

[23] Sehgal SN. Rapamune (RAPA, rapamycin, sirolimus): mechanism of action and safety of sirolimus in lymphangioleiomyomatosis. N Engl J Med 2011;364:1595–606.

[24] Ando K, Kurihara M, Kataoka H, Ueyama M, Togo S, Sato T, et al. Efficacy and safety of low-dose sirolimus for treatment of lymphangioleiomyomatosis. Respir Investig 2013;51:175–83.

[26] Conway KA, Lee SJ, Rocchet JC, Ding TT, Williamson RE, Lansbury Jr PT. Acceleration of oligomerization, not fibrilization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson’s disease: implications for pathogenesis and therapy. Proc Natl Acad Sci U S A 2000;97:571–6.

[27] Goldberg MS, Lansbury PT. Is there a cause-and-effect relationship between alpha-synuclein fibrillation and Parkinson’s disease? Nat Cell Biol 2000;2:E115–9.

[28] Lashuel HA, Hartley D, Petre BM, Walz T, Lansbury Jr PT. Neurodegenerative disease: amyloid pores from pathogenic mutations. Nature 2002;418:291.

[29] Walsh DM, Kryabin I, Fadeeva JV, Cullen WK, Amory R, Wolfe MS, et al. Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature 2002;416:535–9.

[30] Sharon R, Bar-Joseph I, Frosch MP, Walsh DM, Hamilton JA, Selkoe DJ. The formation of highly soluble oligomers of alpha-synuclein is regulated by fatty acids and enhanced in Parkinson’s disease. Neuron 2003;37:583–95.

[31] Cleary JP, Walsh DM, Hofmeister JJ, Shankar GM, Kuskowski MA, Selkoe DJ, et al. Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function. Nat Neurosci 2005;8:79–84.

[32] Lesne S, Koh MT, Kotilinik L, Kayed R, Glabe CG, Yang A, et al. A specific amyloid-beta protein assembly in the brain impairs memory. Nature 2006;440:352–7.

[33] Novitskaya V, Bocharova OV, Bronstein I, Baskakov IV. Amyloid fibrils of mammalian prion protein are highly toxic to cultured cells and primary neurons. J Biol Chem 2006;281:13828–36.

[34] Menzies FM, Hourez R, Iamariso S, Raspe M, Sadiq O, Chandraratna D, et al. Puromycin-sensitive aminopeptidase protects against aggregation-prone proteins via autophagy. Hum Mol Genet 2010;19:4573–86.