Review

Application of Human Stem Cells to Model Genetic Sensorineural Hearing Loss and Meniere Disease

Mar Lamolda , Lidia Frejo * , Alvaro Gallego-Martinez , Jose Antonio Lopez-Escamez *

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Review

Application of Human Stem Cells to Model Genetic Sensorineural Hearing Loss and Meniere Disease

Mar Lamolda [1,2,3], Lidia Frejo [1,2,3,*], Alvaro Gallego-Martinez [1,2,3] and Jose A Lopez-Escamez [1,2,3,4,*]

[1] Otology and Neurotology Group CTS495, Department of Genomic Medicine, GENYO - Centre for Genomics and Oncological Research - Pfizer, University of Granada, Junta de Andalucía, PTS, Granada, Spain
[2] Division of Otolaryngology, Department of Surgery, Instituto de Investigación Biosanitaria, ibs.GRANADA, Granada, Universidad de Granada, Granada, Spain
[3] Sensorineural Pathology Programme, Centro de Investigación Biomédica en Red en Enfermedades Raras, CIBERER, Madrid, Spain
[4] Meniere's Disease Neuroscience Research Program, Faculty of Medicine & Health, School of Medical Sciences, The Kolling Institute, University of Sydney, Sydney, New South Wales, Australia
* Correspondence: lidia.frejo@genyo.es: jalopezescamez@ugr.es

Abstract: Genetic sensorineural hearing loss and Meniere disease have been associated with rare variations in the coding and non-coding region of the human genome. Most of these variants are classified as likely pathogenic or variants of unknown significance and require functional validation in cellular or animal models. Given the difficulties to obtain human samples and the raising concerns about animal experimentation, human induced pluripotent stem cells emerge as cellular models to investigate the interaction of genetic and environmental factors in the pathogenesis of inner ear disorders. The generation of human sensory epithelia and neuron-like cells carrying the variants of interest may facilitate a better understanding of their role during differentiation. These cellular models will allow us to explore new strategies for restoring hearing and vestibular sensory epithelia as well as neurons. This review summarizes the use of human induced pluripotent stem cells in sensorineural hearing loss and Meniere disease and proposes some strategies for its application in clinical practice.

Keywords: human induced pluripotent stem cell; inner ear disorders; disease modeling; sensorineural hearing loss; Meniere disease; biomedical applications

1. Introduction

Stem cell culture is essential in biomedical research to study cellular and developmental biology [1,2]. Several biological phenomena specific to humans, such as brain development and inner ear formation amongst others, are not reproduced in animal models. This limitation has promoted the generation of cellular models to reproduce more exactly human biology and development [1], leading to a better understanding of genetic disorders.

Stem cell science presents a highly promising direction in translational medicine, allowing the proliferation and differentiation of any cell type. Stem cell models are more clinically appropriate to study the molecular pathophysiology of certain diseases, and further develop better therapeutic strategies [2]. Therefore, human pluripotent stem cells (hPSCs) are progressively used to model rare diseases, as an alternative to animal models which may raise research costs and ethical issues [1]. Disease modeling of human disorders may allow the development of new therapies for rare diseases [1]. Henceforth, human organoids provide a unique opportunity to study them in a multicellular environment and complement animal models [3].

The most common sensory disorder is hearing loss, affecting around 15% of the population [4]. Sensorineural hearing loss (SNHL) and vestibular disorders are caused by damage to the sensory

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epithelia and neurons, which do not regenerate in humans. These could originate from a variety of causes, such as genetic and environmental factors, aging, and ototoxic drugs [5].

Extensive studies using animal models have expanded our learning about the human inner ear, although access into the inner ear is restricted in mouse models, slowing research advance in the area [6]. Access to human inner ear is also strictly restricted since tissue sampling may lead to irreversible damage and profound hearing loss or vestibular impairment. In addition, the non-invasive diagnostic imaging procedures such as magnetic resonance imaging or computerized tomography scan, do not supply enough resolution for the investigation of human inner ear disorders at the cellular and molecular levels [7].

Researchers have differentiated hPSCs into inner ear progenitors and sensory cells [8–11] using different induction protocols to form Hair cells-like cells. Many of these differentiation protocols are developed completely in 2D culture generating a homogeneous population of inner ear cells-like cells [12–14]. Instead, 3D cell culture presenting numerous cell types can better mimic the structure of the human organ in vivo [15–18]. Since Hair cells (HCs), supporting cells (SCs), and their associated neurons are damaged in SNHL, gene and cell-based therapies may restore hearing and vestibular impairment.

This scoping review describes the major achievements, gaps in research, and challenges to translating the application of hPSCs into clinical practice. Furthermore, we present their application in monogenic deafness and the potential use to model Meniere disease (MD).

2. Generation of Human Pluripotent Stem Cells

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and hiPSCs, have emerged as a new model system that offers unique advantages for disease modeling [2].

hESCs are derived from the inner cell mass of the blastocyst of an embryo, while hiPSCs are derived from adult somatic tissues. Since their discovery in 2007 [19], hiPSCs have emerged as an alternative to hESCs, because they can be obtained from individual patients or healthy donors and immune rejection can be avoided when they are transplanted autologously [20,21]. Since then, researchers have shown the potential of reprogramming to convert a given somatic cell type to an iPSC state.

Reprogramming methods may be divided into two categories, integrative and non-integrative (Table 1). Integrative reprogramming approaches generate heterogeneous hiPSCs lines and present genomic mutation risks, which could obscure comparative analysis between lines. So, to avoid the risk of tumorigenesis, non-integrative reprogramming methods have been designed, including non-integrating viral vectors such as Sendai virus (SeV), adenovirus, and non-viral vectors like episomal DNA, Piggy-Bac transposons, modified synthetic mRNA (modRNA), microRNA and recombinant proteins.

Table 1. Reprogramming methods used in human somatic cells.

	Type of Vector	Integration	Factors	References
Viral	Retrovirus	Yes	OSKM	[19,22–24]
			OSK	[25,26]
			OK	[27]
			Hsa-miR-302[a]/b/c/d	[28]
	Lentivirus	Yes	OSKM	[29]
			OSNL	[30,31]
			OSKMNL	[32]
			OSN	[33,33]

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			O	[34]
	Bacteriophage	Yes	OSKM	[35]
	Adenovirus	No	OSKM	[36]
	Sendai Virus	No	OSKM	[37,38]
Non-viral	piggyBac	No	OSKM	[39,40]
	Plasmid	No	OSNL	[41]
	Episomal Vector	No	OSKMNL	[42,43]
			OSKM*L	[44]
	Minicircle vector	No	OSNL	[45]
	Protein	No	OSKM	[46]
	mRNA	No	OSNL	[47]
			OSKM (L)	[48]
	microRNA	No	miR-200c, 302ª/b/c/d, 369-3p/5p	[49]

O (OCT3/4); S (SOX2); K (KLF4); M (C-MYC); M* (L-MYC); N (NANOG); L (LIN28).

The most commonly used reprogramming methods to generate hiPSCs are SeV, synthetic mRNAs, and episomal vectors due to their high reprogramming efficiency and their wide application to different cell types [20,22–25]. In addition, SeV reprogramming is highly effective, with a lower workload and no non-appearance of viral sequences in most cell lines at higher passages in culture. Moreover, the combination of bFGF, ascorbic acid (AA), and Y-27632 dihydrochloride – ROCK Inhibitor in the culture medium increase the reprogramming efficiency [54–56] (Figure 1). However, in the last years, modRNA-based somatic reprogramming has become a more effective alternative to the SeV system, since the modRNA molecules encode multiple pluripotent factors and can be applied successfully in reprogramming somatic cells [24,25,29].

Selecting the most suitable reprogramming method and implementing good manufacturing procedures for hiPSCs generation is key to obtaining high-quality hiPSCs cell lines.

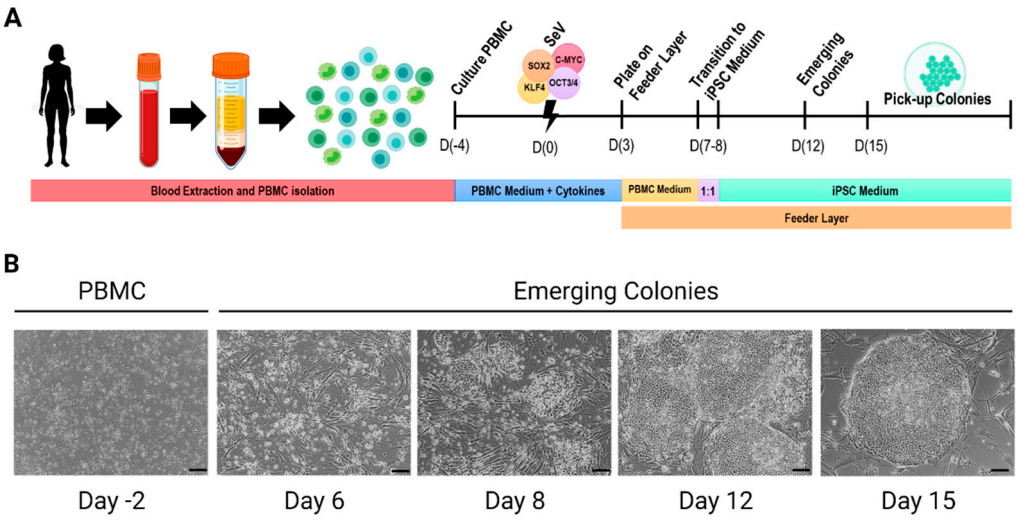

Figure 1. Cell reprogramming of human PBMC. A. Generation of hiPSCs lines by SeV cell reprogramming technology. B. Images of cell reprogramming procedure. Scale bar = 100µm. (Created with BioRender.com).

3. Disease modeling by human induced Pluripotent Stem Cells

The advantages of hiPSCs are well-known at many distinct levels such as their capacity to self-renew indefinitely in culture and differentiate to any cell type in the human body. So, hiPSCs are the ideal source for generating a patient-derived personalized disease model [1,2].

Experimental human modeling of inherited rare disorders provides insight into the cellular and molecular mechanisms involved in the disease. It is worth mentioning the early success history of disease modeling using hiPSCs, which was based on neurodegenerative diseases [58]. Neuronal differentiation was one of the initially differentiated target tissues from several sources of stem cells due to their potential future therapeutic usage [59–61]. Neurodegenerative disorders such as amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, and Alzheimer's disease are among the most studied ones [62–64].

Recent advances in organoid technology have revolutionized the *in vitro* culture tools for biomedical research by creating powerful 3D models to recapitulate the cellular heterogeneity, structure, and functions of the primary tissues, compared with 2D cell models. Hence, is a methodology with many translational applications such as regenerative medicine, drug discovery, and precision medicine. In 2011, the first organoids from hPSCs were generated to recreate intestinal tissue *in vitro* [65]. In 2012, the first retinal organoids were generated from hPSCs [66]. These human retinal organoids are larger than mouse organoids and can grow into multilayered tissue containing both rods and cones [66,67]. In 2013, Lancaster *et al*. further established the 3D cerebral organoids containing different brain regions within single organoids [68].

4. Clinical applications in genetic deafness and vestibular disorders

According to the World Health Organization (WHO), hearing impairment, the partial or total inability to hear sounds, is among the top 10 disabilities of today's society. (https://www.who.int/newsroom/factsheets/detail/deafness-and-hearing-loss).

Congenital SNHL is estimated to have a genetic origin in 70% of cases and, mutations can affect the organ of Corti, the spiral ganglion, and almost any part of the auditory pathway [41,42]. Monogenic SNHL are considered rare disorders [43,44]. There are around 126 genes associated with non-syndromic SNHL (https://hereditaryhearingloss.org/dominant) and many of them are related to inner ear homeostasis and mechano-electrical transduction [72].

Several otologic conditions may show fluctuating SNHL, including Meniere Disease (MD), a debilitating condition, characterized by episodes of spontaneous vertigo, tinnitus, and aural fullness [73,74]. It is associated with the progressive accumulation of endolymph in the cochlear duct [75]. The diagnosis is based on the characteristic presentation of the different clinical symptoms mentioned during the vertigo attacks [76]. MD prevalence is higher in European than Asian and African populations with a range of 10-225 cases/100,000 individuals. Most patients suffer from SNHL in one ear, but 25-40% of these patients with unilateral SNHL may develop hearing loss in the contralateral ear, after several years [77]. MD is known to have a genetic predisposition [74] and familial MD is found in around 8-10% of cases with several genes involved [78–82]. Some forms of monogenic SNHL and familial MD are rare diseases which may benefit from gene and cellular therapy.

Despite the extensive occurrence of genetic SNHL in the world, there are no Food and Drug Administration (FDA)-approved cellular or molecular therapies [4,50]. Current treatments for human SNHL and MD are medical therapy using steroids, hearing aids, surgery to correct the cause of the hearing loss, or cochlear implants [84–89]. Though these devices offer significant relief of the moderate and severe SNHL by amplifying sound or directly electrically stimulating the auditory nerve, they have significant limitations in terms of speech discrimination in complex acoustic environments [90]. These medical devices require the presence of functional auditory neurons in the inner ear. Therefore, in the last years, new studies focus on possibilities for neuronal replacement, including exogenous stem cell transplantation and endogenous cell source replacement. Several studies have proved that neural stem cell transplantation in the inner ear has an important therapeutic effect on the activation and regeneration of cells, restoring damaged neurons [91–93].

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However, more research is still needed to improve and standardize the protocol for differentiating stem cells into inner ear HCs and neurons.

Transcriptional networks are key in governing the regeneration or replacement of auditory neurons from stem cells. Development of the inner ear is an organized molecular transformation of a set of epidermal cells (the otic placode) into the fully developed ear with its neurosensory component, necessary for signal extraction and transmission, and the non-sensory component, forming the labyrinth necessary for directing sensory stimuli to specific sensory epithelia [6,94]. The main genes involved in neurosensory development in the inner ear are *MYO7A*, *HES5*, *SOX2, NEUROG1, NEUROD1,* and *POU4F1* [80,95–98].

Since animal models are only able to represent the chronic end stages of the disease when permanent damage of sensory epithelia has occurred, understanding, and identifying the transcription factors involved in the development and survival of auditory neurons is vital for the generation of disease models and the identification of more effective treatments for hearing loss in the future.

4.1. Modeling inner ear disorders: 2D and 3D cell culture

The inner ear is highly complex; both the anterior and posterior labyrinth are connected, and the extent of involvement in each organ may vary, resulting in hearing or vestibular disorders. Consequently, inner ear disorders may be caused by damage to sensory epithelia and neurons, which do not regenerate to any clinically relevant extent in humans. Since the pathophysiology of certain types of SNHL and MD have not yet been explained at cellular and molecular levels, it is difficult to generate an animal model that accurately reflects these diseases.

Several animal models, including zebrafish, bullfrog, chick, and several species of mammals have improved our knowledge of the biology of the inner ear. In addition, these animal models are only able to represent the chronic end stages of disease with permanent loss of hearing and vestibular function [5].

For this reason, the best option to study the inner ear development and disease is to generate human cell models. Several protocols have been devised to direct hPSCs into inner ear HCs and neuron-like cells. The efficiency, reproducibility, and scalability of these protocols are enhanced by incorporating knowledge of inner ear development [13,17,18,67–70]. Early studies on the transplantation of hPSCs-derived otic progenitors have been successful in certain animal models [103], but the hearing was transiently restored, and long-term cell survival continues to be a major challenge. To differentiate successfully hPSCs to HCs, SCs, and inner ear neurons in vitro is critical to know the transcription factors and signaling pathways involved in the inner development *in vivo.*

The last years have seen a surge in the number of studies that are conducted *in vitro* 2D and 3D hiPSCs models to study auditory and vestibular disorders.

4.1.1. Sensory Epithelia

Most of the induction protocols towards HCs-like cells derived from hPSCs are developed completely in 2D culture and others combined the embryoid bodies (EBs) formation with cytokines during 5 days until a 2D cell culture is finally obtained [12–14,67,72].

The first stages of the human inner ear development are the generation of the ectodermal germ layer followed by the pre-placodal ectoderm (PPE) formation. Differentiation protocols inhibit TGFβ and WNT signaling while activate BMP signaling to carry out non-neural ectoderm (NNE) generation [13,17,72]. The PPE generates the most of cranial placodes, including the otic placode. Physical environmental signals supplied by Matrigel, an extracellular matrix, enhance the hPSCs-differentiation protocols efficiency and the cellular assemblage [15,17,18]. The addition of Matrigel to the inner ear organoid cultures promotes the generation of fluid-filled vesicles which hold HCs and SCs-like cells [17]. Nevertheless, the protocols to generate organoids containing vestibular tissue from hPSCs use the rotary cell culture system (RCCS), a new 3D cell culture technology for culturing suspension cells and anchorage-dependent cells. This procedure allows to obtain HCs-like cells on the surface of the organoids generated [18]. To date, HCs-like cells from hPSCs show

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electrophysiological characteristics and molecular markers of vestibular HCs, but not cochlear HCs. So, it is necessary to identify new small molecules or culture methods to improve the generation of cochlear HCs from hPSCs.

SCs perform a key role in the ionic homeostasis, critical for the function of HCs. The gap junction proteins with higher expression in SCs are connexins. The autosomal recessive non-syndromic SNHL is commonly caused by mutations in *GJB2* gene which encodes for connexin 26 (CX26) [105]. An *in vitro* model for the homozygous 235delC mutation in *GJB2* has been developed from hPSC to develop a therapy for deafness [106] (Figure 2).

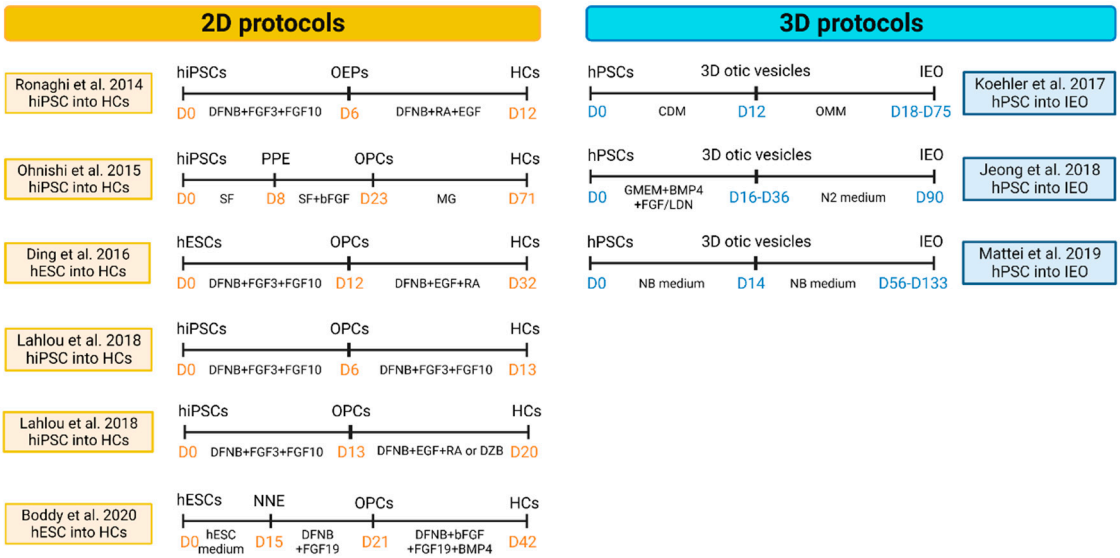

Figure 2. 2D and 3D hPSCs-derived inner ear hair cells protocols. (Created with BioRender.com).

Frejo *et al.* have generated a hiPSC line derived from an MD patient. This model has been differentiated into HCs by a 2D protocol based on Boddy et al.[101]. This method consists of the generation of otic epithelial progenitors (OEPs) and consequently differentiation to HCs-like cells (Figure 3).

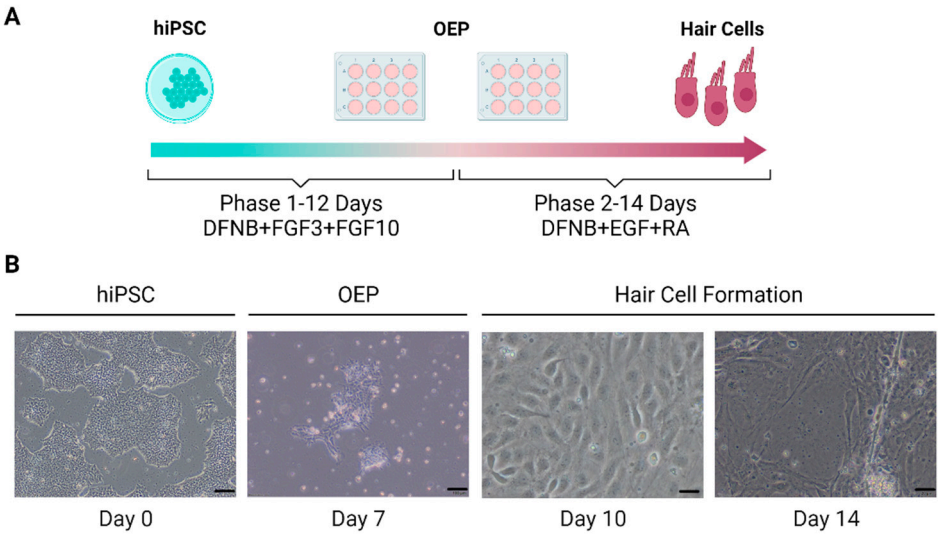

Figure 3. Schematic representation of 2D differentiation protocol of hPSCs-derived hair cells. A. Protocol of the generation of inner ear hair cells from hPSCs. B. Images obtained by microscopy of hair cell formation. Scale bar at day 0 and 7 = 100µm and at day 10 and 14 = 20µm. OEP: Otic epithelial progenitor. (Created with BioRender.com).

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Moreover, we have started to generate inner ear organoids from the hiPSC-MD model derived from a patient with mutations in *DTNA* and *FAM136A* genes for studying the development of the inner ear in this patient as well as how mutations found in *DTNA* and *FAM136A* affect the development and functionality of the system itself when sensory organs mature [75,76]. Koehler *et al*. described the protocol [17] to generate inner ear sensory epithelium harboring HCs using an *in vitro* 3D differentiation system from hiPSCs. Cells are treated with recombinant proteins that modulate BMP, FGF, and WNT signaling pathways to induce the sequential formation of NNE, otic-epibranchial progenitor domain (OEPD), and otic placodes. The otic placodes subsequently undergo self-guided morphogenesis to form inner ear HCs and SCs (Figure 4).

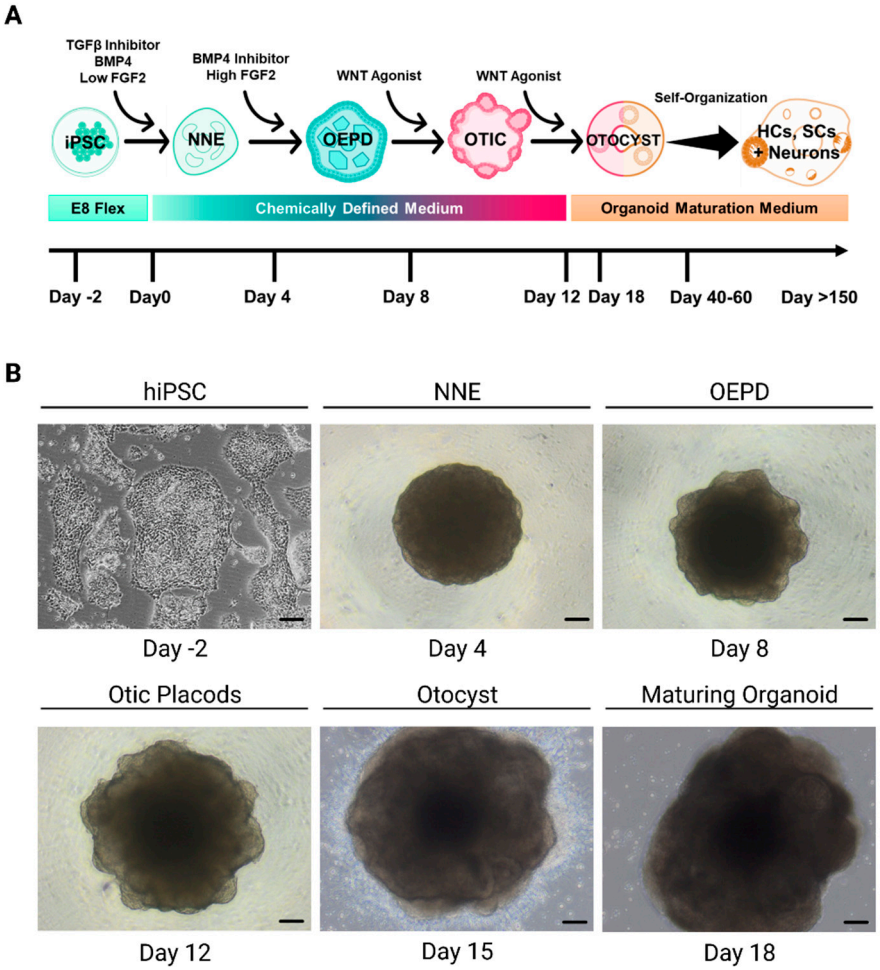

Figure 4. Generation of inner ear organoids from hPSCs (modified from Nie and Hashino 2020 [109]). A. 3D differentiation protocol of hPSCs into inner ear organoids from Koehler *et al.*[17]. B. Images of inner ear organoids generation. NNE: Non-neural ectoderm. OEDP: Otic-epibranchial progenitor domain. OTIC: Otic Placodes. (Created with BioRender.com).

4.1.2. Sensory Neuron

The first neural differentiation protocols from hPSCs were performed in stromal cells of skull bone marrow and generated dopaminergic neurons in an efficient manner [77,78]. These induction protocols have been remodeled to generate inner ear sensory neurons and glial cells derived from hPSCs. However, many of the culture protocols have been adapted to form hPSCs-dopaminergic neurons by their function in neurodegenerative diseases, including Parkinson disease [112]. The generation of glutamatergic neurons was a critical stage to recapitulate the afferent neural

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transmission in the human inner ear, since glutamate is the principal neurotransmitter of the synaptic transmission between HCs and the afferent sensory neurons in the inner ear [113].

The inner ear sensory neurons are obtained from the otic placode. Consequently, some of the first differentiation protocols steps to form sensory neurons have focused on the application of the otic placode developmental pathways. Some cytokines and growth factors like FGFs, BMP, SHH, and noggin protein are employed to aid neuronal growth from the otic placode [114–116]. BRN3A and β-III tubulin are regularly employed to confirm neuron formation. Matsuoka et al. differentiated hESCs to otic neuronal progenitors (ONPs) and spiral ganglion neurons (SGN)-like cells obtaining a 90% of neuron-like cells positives for peripherin and β-III tubulin, but only a 46% of cells were BRN3A^{+} [115]. So, to recreate in vitro the peripheral neural circuit by differentiating hPSCs into neuron-like cells is essential obtain sensory and innervating neurons.

Two methodologies can develop both tissues at the same time, providing complementary research tools for disease modeling: 2D and 3D cell cultures (Figure 5).

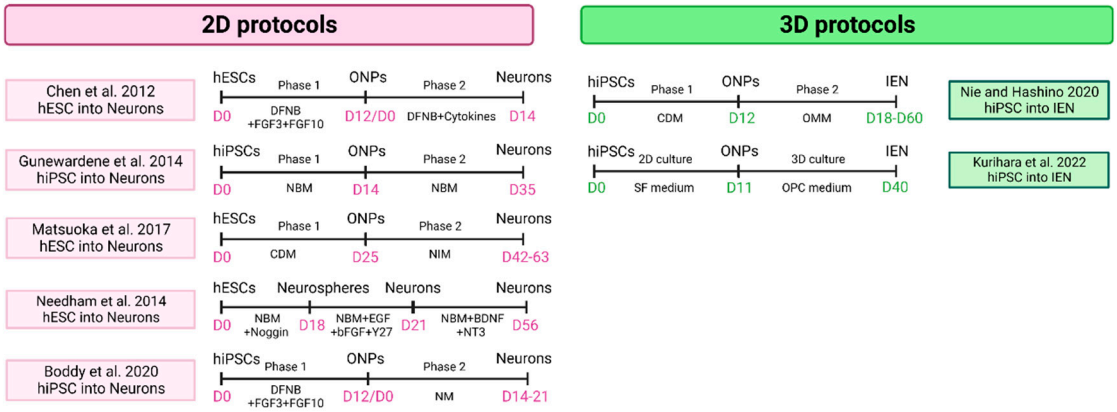

Figure 5. Cellular models of hPSCs-derived 2D and 3D inner ear neurons protocols. NBM: Neurobasal Medium. CDM: Chemically Defined Medium. NIM: Neural Inducing Medium. NM: Neuralizing Medium. (Created with BioRender.com).

To generate both otic epithelial progenitors (OEPs) and otic neural progenitors (ONPs) in 2D culture from hPSCs, cells are treated with FGF during 10-12 days [103]. HCs-like cells can be obtained from OEP following the inhibition of Notch signaling and supplementation with retinoic acid (RA) and epidermal growth factor (EGF) [67,71]. On the other hand, sensory neuron-like cells can be generated from ONPs by the addition to the cell culture FGF and SHH factors and a subsequent supplementation of BDNF and NT3 proteins [103]. The co-culture of HCs and sensory neurons generated from hPSCs form neural connections in vitro. Nevertheless, this system demands separate differentiation protocols before co-culture these cells [117].

The 3D culture of inner ear organoids show sensory epithelia and sensory neuron-like cells [15–17]. Several studies have reproduced these induction protocols to generate neuron-like cells for different applications, including electrophysiological studies and disease modeling [118]. The inner ear organoid methodology is an in vitro powerful tool to study the peripheral sensory neural system in the human inner ear. The generation of human inner ear tissue from hPSCs offers the potential to study the inner ear biology and understand differences between mice and human inner ear development. Moreover, it would enable both, *in vitro* screening of drug candidates for the treatment of hearing loss and balance dysfunction and a source of cells for cell-based therapies of the inner ear. Nie and Hashino describe a 3D protocol to form inner ear neurons from hPSC [109]. Other studies have generated otic organoids with neuron-like cells from hiPSC models combining the 2D and 3D systems in the same differentiation protocol [100].

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Stem cell science has also been applied to generate a hiPSCs model obtained from patients with severe tinnitus and rare variants in the *ANK2* gene, and differentiating them to inner ear neurons using a 2D system [101]. This protocol consists of two phases, a first phase in which hiPSC-derived otic neural progenitors (ONPs) are generated by inhibiting Wnt signaling, which is accompanied by subsequent activation of this pathway, and a second phase consisting of ONPs expansion and enrichment, which will later differentiate into inner ear neurons (Figure 6).

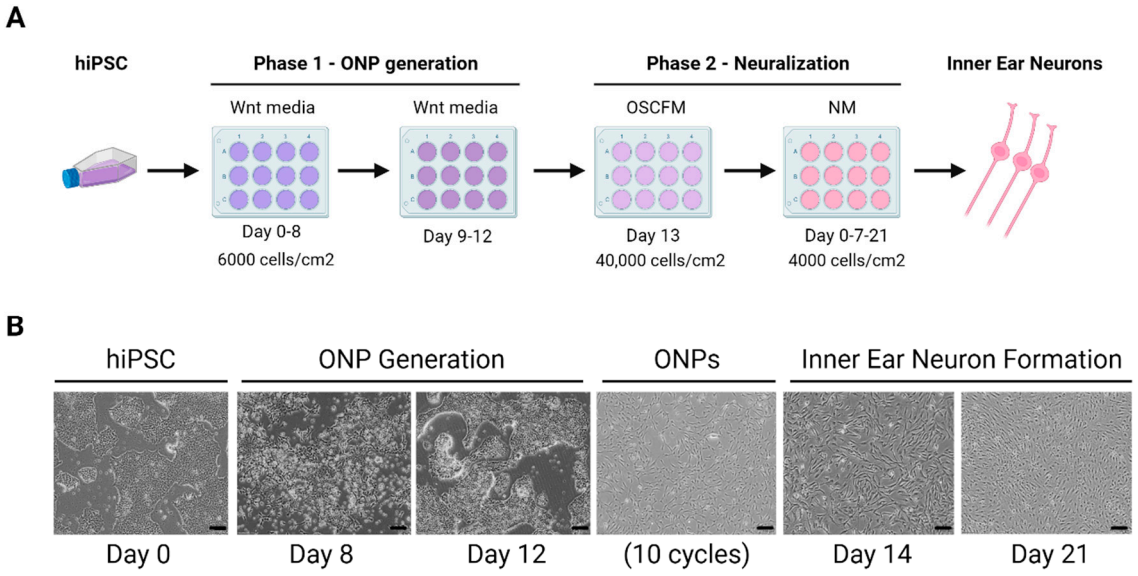

Figure 6. Inner ear neurons generation from hiPSCs. A. Differentiation protocol of hPSC-derived inner ear neurons in 2D culture from Boddy et al[101]. B. Images of inner ear neurons culture derived from hiPSC. Scale bar = 100µm. ONP: Otic Neural Progenitor. OSCFM: Otic Stem Cell Full Media. NM: Neuralizing Media. (Created with BioRender.com).

5. Clinical Trials in Inner Ear Disorders

Several clinical trials have been performed using human stem cells to treat SNHL. Autologous human umbilical cord blood stem cells have been used for over twenty years, with excellent safety records. A study in 2015 used umbilical cord blood stem cells to improve inner ear function, audition, and language in children [119]. The potential of human mesenchymal stem cells has been evaluated for years for regenerating inner ear HCs [120].

The major achievements in disease modeling using a hiPSC-derived inner ear for genetic SNHL include the genes *MYO7A, MYO15,* and *MERRF* in HCs [70,89,90], and *GJB2* and *SLC26A4* (Pendred syndrome) in SCs [106,123–125] (Table 2). These studies have defined the molecular mechanisms involving each gene and showed the cellular effects of each mutation. Recently, a clinical trial using iPSC derived from patients with Pendred syndrome to generate cochlear cells has allowed to study the effects of low-dose oral administration of sirolimus for fluctuating and progressive hearing loss [126].

Table 2. Genetic mutations causing inner ear disorders modeled using hPSCs.

Gene	RefSeq	Variant	Type of inheritance	Model	References
MYO7A	NM_000260.4	c.1184G>A c.4118C>T	Compound heterozygous	Hair Cells	[121]

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MYO15A	NM_016239.4	c.4642G>A c.8374G>A	Compound heterozygous	Hair Cells	[102]
MERRF/MT-TK	NC_012920.1	c.8344A>G	Mitochondrial	Hair Cells	[122]
SLC26A4	NM_000441.2	c.439A>G c.1229C>T c.2168A>G	Autosomal recessive	Supporting Cells	[124,125]
GJB2	NM_004004.6	c.235DelC	Autosomal recessive / Digenic	Supporting Cells	[106,123]

However, many research gaps have not been addressed and more than 150 genes in SNHL and familial MD remain to be modeled (https://deafnessvariationdatabase.org/). Some of the limitation of hPSCs to investigate human inner ear diseases involve time-consuming culture protocols that sometimes are not reproducible, with variable efficiency of tissue derivation, and restricted differentiation and integration in host tissues. Deciphering the disease molecular mechanisms is the first step to finding a drug target that may offer new opportunities for therapy.

6. Conclusions

Human iPSCs are a reliable model to study the functional consequences of rare variants in early stages of inner ear disease development and design novel gene therapies to restore the phenotype. The use of 3D models to generate patient-specific organoids allows us to elucidate the effect of rare variants in genetic deafness and vestibular disorders, investigate the molecular mechanisms underlying the disease, and analyze the effects of genetic mutations on the phenotype at the cellular and organoid levels. Modeling inner ear diseases by differentiating hiPSCs is a promising tool for designing novel therapies for the treatment of SNHL and MD.

Author Contributions: Mar Lamolda: paper search, literature review, writing and review. Lidia Frejo: design and search methods, literature review, writing and review. Alvaro Gallego-Martinez: Literature review, writing, and review. Jose Antonio Lopez-Escamez: Design and search methods, literature review, writing and review.

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ORCID:

Mar Lamolda https://orcid.org/0000-0002-2506-1120
Lidia Frejo https://orcid.org/0000-0002-6104-0941
Alvaro Gallego-Martinez https://orcid.org/0000-0002-6068-8965
Jose A. Lopez-Escamez https://orcid.org/0000-0002-8583-1430

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