Clinical Application of Human Stem Cells

Subjects: Otorhinolaryngology

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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 were 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 emerged 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.

Keywords: human-induced pluripotent stem cell ; inner ear disorders ; disease modeling

1. Introduction

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, accessed on 17 November 2022).

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 [1][2]. Monogenic SNHL are considered rare disorders [3][4]. There are around 126 genes associated with non-syndromic SNHL (https://hereditaryhearingloss.org/dominant, accessed on 10 November 2022) and many of them are related to inner ear homeostasis and mechano-electrical transduction [5][6][7][8].

Several otologic conditions may show fluctuating SNHL, including Meniere Disease (MD), a debilitating condition, characterized by episodes of spontaneous vertigo, tinnitus, and aural fullness [9][10]. It is associated with the progressive accumulation of endolymph in the cochlear duct [11]. The diagnosis is based on the characteristic presentation of the different clinical symptoms mentioned during the vertigo attacks [12]. 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 [13]. MD is known to have a genetic predisposition [10] and familial MD is found in around 8–10% of cases with several genes involved [14][15][16][17][18]. 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 [19][20]. 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 [21][22][23][24][25][26][27]. 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 [28]. These medical devices require the presence of functional auditory neurons in the inner ear. Therefore, in recent years, new studies focused on possibilities for neuronal replacement, including exogenous stem cell transplantation and endogenous cell source replacement. Several studies 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 [29][30][31]. 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 [32][33]. The

main genes involved in neurosensory development in the inner ear are *MYO7A*, *HES5*, *SOX2, NEUROG1, NEUROD1,* and *POU4F1* [16][34][35][36][37].

Since animal models are only able to represent the chronic end stages of the disease when permanent damage of sensory epithelia 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.

2. 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.

Nevertheless, extensive studies used animal models, including frog, zebrafish, chick, and several species of mammals that expanded knowledge of the human inner ear function and disease, although access to the inner ear in mammalian animal models is limited, slowing progress in the field. Additionally, these animal models are only able to represent the chronic end stages of disease with permanent loss of hearing and vestibular function [38].

Access to the human inner ear is also strictly restricted since tissue sampling is challenging and leads to irreversible damage. In addition, non-invasive clinical imaging techniques, such as computed tomography or MRI, do not provide enough resolution to investigate most pathologies of the inner ear at the cellular and molecular levels [38][39].

For this reason, the best option to study the inner ear development and disease is to generate human cell models. Several protocols were devised to direct hPSCs into inner ear HCs and neuron-like cells [40][41][42][43]. The efficiency, reproducibility, and scalability of these protocols were enhanced by incorporating knowledge of inner ear development [5][6][44][45][46][47][48]. Early studies on the transplantation of hPSCs-derived otic progenitors were successful in certain animal models [49], but the hearing was transiently restored, and long-term cell survival continues to be a major challenge. Understanding the complex sequence of transcriptional changes and signaling pathways in vivo in inner ear development is critical to the successful differentiation of hPSCs into inner ear tissues, such as HCs, SCs, and neurons in vitro.

Recent years saw a surge in the number of studies that were conducted in vitro 2D and 3D hiPSC models to study auditory and vestibular disorders.

2.1. Sensory Epithelia

Several induction protocols were developed to differentiate hPSCs into HC-like cells. Many of these protocols start with the generation of floating embryoid bodies (EBs) followed by the combination of small molecules and/or recombinant proteins that become adhesive 2D cell cultures after approximately 5 days and other protocols were carried out entirely in 2D, which can provide a homogeneous cell population [8][44][47][50][51]. In contrast, 3D organoid systems contain multiple cell types and more exactly recapitulate in vivo composition of an organ.

The initial steps of inner ear development require the formation of the ectodermal germ layer followed by the generation of the pre-placodal ectoderm (PPE). Protocols first inhibit transforming growth factor β and WNT signaling and activate BMP to promote non-neural ectoderm (NNE) development, while reducing mesoderm development [8][44][45]. Insulin-like growth factor 1 promotes the fate of the anterior ectoderm, where the PPE emerges [52]. The PPE gives rise to most of the cranial placodes, including the otic placode. Physical environmental signals provided by extracellular matrices, such as Matrigel, improve the efficiency of differentiation from hPSCs, as well as the resulting cellular assemblage [45][46][53]. The addition of Matrigel in the 3D inner ear organoid systems facilitates the formation of fluid-filled vesicles containing HCs and SC-like cells [45]. However, vestibular tissue-like organoids derived from hPSCs using the rotary cell culture system form HC-like cells on the surface of the organoids [46]. To date, hPSCs-derived HC-like cells display molecular markers and electrophysiological properties of vestibular HCs, not cochlear HCs. The discovery of alternative small molecules or culture conditions to improve the generation of cochlear HCs from hPSCs is still needed.

SCs play an active role in ion metabolism necessary for HCs' function. The connexin proteins are the most abundant gap junction proteins expressed in the SCs. Mutations in the *GJB2* gene, encoding connexin 26 (CX26), are the most common

cause of autosomal recessive non-syndromic SNHL [54]. An in vitro model for the homozygous 235delC mutation in *GJB2* was developed from hPSC to develop a therapy for deafness [55].

Frejo et al. generated a hiPSC line derived from an MD patient. This model was differentiated into HCs by a 2D protocol based on Boddy et al. [42]. This method consisted of the generation of otic epithelial progenitors (OEPs) and, consequently, differentiation to HCs-like cells.

Moreover, researchers started to generate inner ear organoids from the hiPSC-MD model derived from a patient with mutations in *DTNA* and *FAM136A* genes (https://hpscreg.eu/cell-line/GENYOi007-A, accessed on 15 March 2023) for studying the development of the inner ear in this patient. This model could explain how these mutations found in *DTNA* and *FAM136A* genes in a Spanish family with three affected women in three consecutive generations (autosomal dominant inheritance pattern) affect the development and functionality of the system itself when sensory organs mature [11][12][18][56][57]. Koehler et al. described the protocol [45] to generate inner ear sensory epithelium harboring HCs using an in vitro 3D differentiation system from hiPSCs. Cells were 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 underwent self-guided morphogenesis to form inner ear HCs and SCs.

2.2. Sensory Neuron

Early neural induction protocols from PSCs using stromal cells derived from skull bone marrow, resulted in efficient dopaminergic neuron production [58]. These protocols were modified to derive inner ear sensory neurons and glial cells from PSCs. However, many of the available protocols were tailored to generate hPSCs-dopaminergic neurons due to their role in neurodegenerative disorders, such as Parkinson's disease [59]. In contrast, glutamate was the main neurotransmitter for the synaptic transmission between HCs and afferent sensory neurons within the inner ear. So, the derivation of glutamatergic neurons was a key stage for recapitulating afferent neural transmission in the inner ear [60].

As with the sensory epithelia, inner ear sensory neurons are derived from the otic placode. Therefore, some of the early induction steps for hPSCs-derived sensory neurons are based on known developmental pathways of the otic placode. Small molecules, such as FGFs, BMP, SHH, and noggin, are used to support neuronal outgrowth from the otic placode [61][62][63]. POU4F1 and β-III tubulin are commonly used to verify neuron formation, with glutamate receptors and transporters subsequently used to confirm subtype-specific derivatives. Matsuoka et al. showed that ~90% of neuron-like cells were peripherin$^+$ and β-III tubulin$^+$, but only ~46% were POU4F1$^+$ [62]. To recapitulate the peripheral neural circuit in vitro, the model must contain both sensory epithelia and innervating neurons.

Two systems potentially can generate both tissues simultaneously, providing complementary research tools for disease modeling: 2D and 3D cell cultures.

The treatment of hESCs with FGF3 and FGF10 for 10–12 days in 2D culture gives rise to both otic epithelial progenitors (OEPs) and otic neural progenitors (ONPs), which can be distinguished based on their morphology [49]. HC-like cells can be derived from OEP after inhibiting Notch and supplementing with RA and epidermal growth factor (EGF) [7][47]. Sensory neuron-like cells can be derived from ONPs using bFGF and SHH followed by BDNF and NT3 supplementation [49]. Co-culture of these derived HCs and sensory neurons form neural connections in vitro. However, this method required separate induction protocols before co-culturing them [64].

The 3D inner ear organoids contain sensory epithelia and sensory neuron-like cells [45][53][65]. Furthermore, several studies replicated these protocols and used the systems for different applications, such as electrophysiological studies and disease modeling [66]. The inner ear organoid system is a powerful tool to study peripheral sensory neural networks in the inner ear in vitro. Human inner ear tissue derived from hPSCs also offers the chance to explore developmental biology and understand the 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 described a 3D protocol to form inner ear neurons from hPSC [67]. Other studies generated otic organoids with neuron-like cells from hiPSC models combining the 2D and 3D systems in the same differentiation protocol [41].

Stem cell science was also 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 [42]. 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.

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