Trends in Neurosciences

CellPress REVIEWS

Review

Challenges in Cell-Based Therapies for the Treatment of Hearing Loss

Hiroki Takeda,[1,2,3] Anna Dondzillo,[1,3] Jessica A. Randall,[1] and Samuel P. Gubbels[1,*]

Hearing loss in mammals is an irreversible process caused by degeneration of the hair cells of the inner ear. Current therapies for hearing loss include hearing aids and cochlear implants that provide substantial benefits to most patients, but also have several shortcomings. There is great interest in the development of regenerative therapies to treat deafness in the future. Cell-based therapies, based either on adult, multipotent stem, or other types of pluripotent cells, offer promise for generating differentiated cell types to replace lost or damaged hair cells of the inner ear. In this review, we focus on the methods proposed and avenues for research that seem the most promising for stem cell-based auditory sensory cell regeneration, from work collected over the past 15 years.

Highlights

Mouse and human pluripotent stem cells can be differentiated into an inner ear supporting cell and functional hair cell fate *in vitro* as well as into inner ear organoids.

Progenitor cells capable of differentiation into inner ear hair cells have been identified in the neonatal and early adult cochlea.

Transplantation of pluripotent stem cell-derived progenitors into the chick auditory sensory epithelium as well as the adult rodent auditory nerve leads to survival and terminal differentiation of grafted cells.

Transplantation of pluripotent stem cell-derived progenitors into the normal and lesioned rodent cochlea leads to survival, but not integration or terminal differentiation, of grafted cells.

Inner Ear Hair Cell Regeneration

Hearing loss affects 11% of the US population aged 50–59 years and 25% of the population at ages 60–69 years [1]. Globally, more than 360 million people have disabling hearing loss (according to WHO). Hearing loss is most commonly caused by aging, ototoxic medications, noise exposure, diseases of the inner ear, hereditary disorders, and congenital conditions. Most cases of acquired hearing loss are due to degeneration and subsequent loss of cochlear hair cells (HCs) located in the organ of Corti (OC; Figure 1).

The OC is the auditory sensory epithelium that consists of one row of inner and three rows of outer HCs (OHC) that rest upon a cohort of different types of supporting cells (Figure 1). The inner HCs (IHC) act as mechanosensors by converting sound-induced basilar membrane vibration into an electrical signal. Afferent spiral ganglion neurons receive input from the IHC and transmit this information to the cochlear nucleus in the brainstem followed by relay ultimately to the auditory cortex. The OHC are responsible for amplification of the incoming signal via somatic electromotility. The mammalian cochlea is tonotopically organized with high-frequency reception in the base progressing to low frequencies in the apex. The cochlea is encapsulated in particularly dense bone located within the temporal bone, one of the osseous structures that make up the base of skull.

Studies in the late 1980s revealed that the avian auditory sensory epithelium is capable of regeneration after lesioning, and subsequent similar findings were observed in rodents (albeit in more limited contexts). These results have piqued the interest of many auditory scientists in understanding inner ear HC and supporting cell development and regeneration, so that targeted biological therapies for hearing loss may be developed. Multiple approaches to HC regeneration have been proposed including gene transfer, pharmacotherapies, and stem cell-based approaches. In recent years there have been many studies investigating two stem cell-based approaches for hearing loss (Figure 2). The first approach is based on the regeneration of cochlear HCs from multipotent inner ear progenitor cells that reside in the OC. The second approach is based upon *in vitro* differentiation of pluripotent stem cells (PSCs) to an

[1]Department of Otolaryngology, University of Colorado Denver, Anschutz Medical Campus, Aurora, CO, USA
[2]Department of Otolaryngology-Head and Neck Surgery, Kumamoto University Graduate School of Medicine, Kumamoto City, Japan
[3]These authors contributed equally to this work

*Correspondence: samuel.gubbels@ucdenver.edu (S.P. Gubbels).

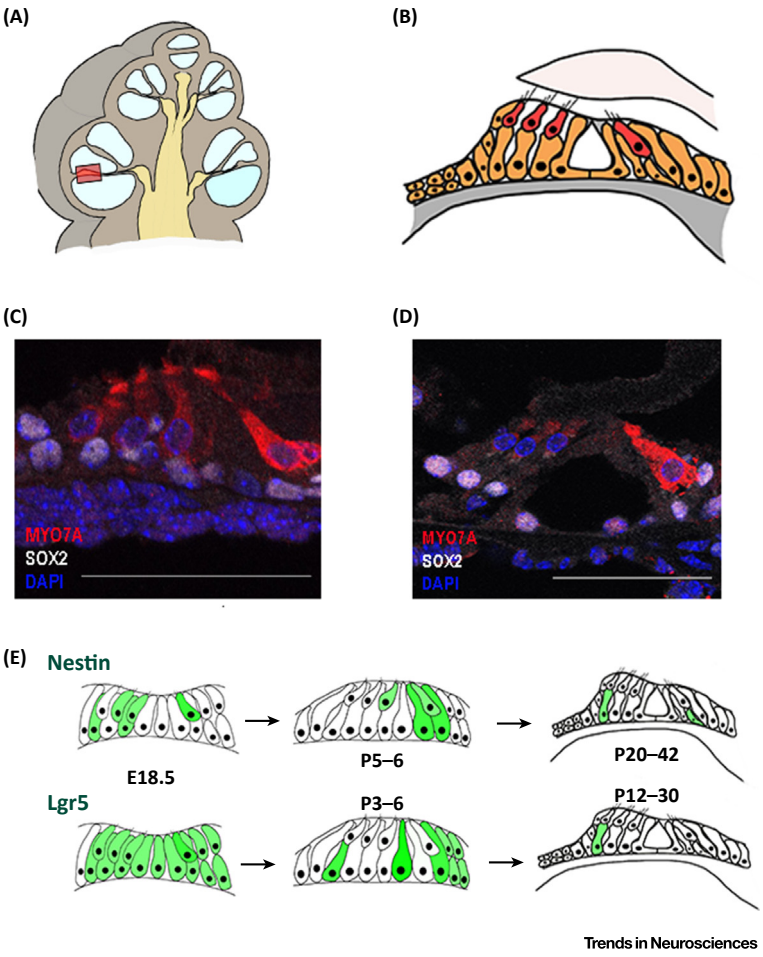


Figure 1. Localization of the Precursor and Sensory Cells within the Mammalian Cochlea in the Adult and Developing Organ of Corti. (A) Schematic of a mid-modiolar section through the cochlea, with the red-shaded box on the organ of Corti. (B) Magnified schematic of the organ of Corti with the hair cells in red and the supporting cells in orange. (C) Mid-modiolar section of organ of Corti of a postnatal day 5 (P5) mouse immunolabeled with hair cell marker MYO7A (red), supporting cell labeled with SOX2 (white), and nuclei labeled with DAPI (blue). (D) As in (C) but organ of Corti of P15 mouse. (E) Schematic of the organ of Corti with nestin-positive (top, green) and Lgr5-positive (bottom, green) cell distribution at different developmental stages. Scale bars (in C and D), 50 μm.

inner ear fate with subsequent transplantation to the inner ear. Here, we review the most recent literature describing both approaches and the inherent challenges of each to provide a framework for understanding of this rapidly evolving field.

Inner Ear Progenitor Cell-Based Strategies for Regeneration

Prior to 1980, there had been little to no evidence for inner ear HC regeneration in any species. This, in combination with the permanency that typifies most cases of hearing loss, led most to believe that the inner ear was a quiescent sensory organ lacking any regenerative ability. As such, the landmark discovery by two groups in 1987 and 1988 of the robust regeneration of HCs after ototoxic lesioning of the avian basilar papilla [2–5] (a homolog of the mammalian OC [6]) generated great enthusiasm among auditory scientists. Numerous studies in chicks, rodents, and other species since the initial discoveries have elucidated that inner ear HC regeneration occurs by two mechanisms: direct transdifferentiation of supporting cells into HCs

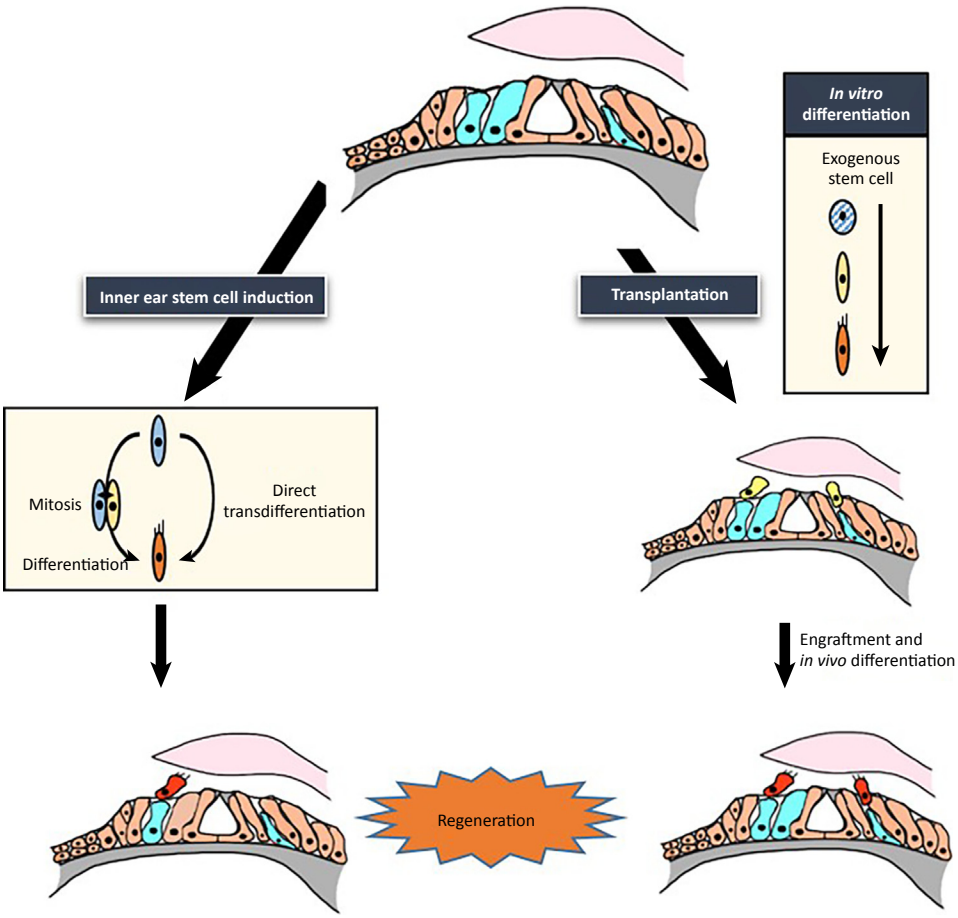


Figure 2. Stem Cell-Based Approaches for Auditory Regeneration. (Top) Schematic of deafened organ of Corti lacking both inner and outer hair cells. Multipotent stem/progenitor cells are highlighted in cyan. (Left) Induction of multipotent stem/progenitor cells existing among the population of supporting cells to undergo either direct differentiation or mitotic division leading to the generation of inner and outer hair cells (red). (Right) Transplantation of exogenous, pluripotent stem cells differentiated to otic progenitors (yellow) leads to engraftment in the organ of Corti and terminal differentiation into mature hair cells (red).

[7,8] and mitosis of supporting cells with the subsequent differentiation of daughter cells into one HC and one supporting cell [2,3]. Direct transdifferentiation replenishes lost HCs at the cost of the supporting cell population, whereas mitotic division maintains this cohort. Li and colleagues [9] investigated the regenerative potential of different subsites within the murine inner ear by using an *in vitro* sphere-forming assay. The ability to form self-renewing, floating spheres upon culture of dissociated cells was used as a proxy for the presence of a population of multipotent stem cells within the tissues of interest. Interestingly, they found that the mouse vestibular sensory epithelium retained the ability to form spheres up to 4 months postnatally. In contrast, the auditory sensory epithelium appears to lose sphere-forming ability by 2–3 weeks postnatally. Since this report, many groups have used cochlear organ culture and *in vivo* approaches to identify which cellular populations in the postnatal OC possess regenerative capabilities. To do so, investigators have focused primarily on the expression of protein markers associated with multipotency in other tissues known to have regenerative potential. Two such

protein markers, Nestin and Lgr5, have been studied extensively for the past decade, and we review the findings of these studies below.

Nestin

Nestin is a class VI intermediate filament protein associated with pluripotency in PSCs. In addition, nestin is expressed universally in the developing CNS and then becomes downregulated to proliferative areas of the dentate gyrus and subventricular zone in the adult [10]. Nestin expression is upregulated in the CNS and retina after lesioning, suggesting that nestin-expressing cells have a stem- or progenitor-like role in these tissues [11]. Given nestin's known role in the CNS and other tissues as a marker of multipotent cells, several groups have investigated the presence and localization of nestin-positive cells in the inner ear (Table 1). Lopez *et al.* [12], using a nestin-GFP mouse model, found widespread nestin expression at postnatal day 5 (P5) in border cells, inner phalangeal cells, Deiters' cells, and satellite cells and in the greater epithelial ridge as well as in the OHC. In older animals (P60), the nestin-GFP signal was observed at low levels only in the third row of Deiters' cells. In contrast, another study also using nestin-GFP mice found that in the P3 cochlea, the GFP signal localized to border and inner phalangeal cells and supporting cells near the IHC area and, to a much lower extent, in the IHC themselves [13]. In older mice (P56), the same study identified the nestin-GFP signal only within IHC afferent nerve fibers. Watanabe *et al.* [14] used nestin-β-gal mice and identified nestin-positive cells in the apical turn of the cochlea in the supporting cell region of the OC and in Rosenthal's canal during early postnatal development. In the adult mice, nestin expression was restricted to the third row of Dieter's cells in the apical turn. More recently, Chow *et al.* [15], using adult nestin-CreERT2/tdTomato-reporter mice, found that nestin-expressing cells were detected beneath and medial to the IHC layer in postnatal and young adult mice. Subsequently, Chow and colleagues [16] used a nestin-GFP mouse model and found that, similar to in the CNS, nestin was expressed widely throughout the developing OC, supporting its role as a progenitor cell marker. As development continued, nestin became downregulated to two discrete cell populations: the third row of Dieter's cells in a basal-to-apical gradient and in the area of the osseous spiral lamina area in an apical-to-basal gradient. Both of these gradients of discrete nestin expression persisted for as long as 1 year after birth. The differences among reports on nestin-positive cell types and localization in the mouse cochlea might be due to the different nestin constructs used in the transgenic mice generation. This explanation is supported by the rather consistent results obtained from rat studies that used only immunostaining. In these studies and similar to findings in mice, nestin expression in the postnatal rat OC has been found in the cells below and medial to the inner hair cells, and in the region around the basilar membrane [17–19].

Taken together, these findings indicate that nestin is expressed widely in the developing OC and later becomes downregulated to two discrete populations wherein its expression persists well into adulthood. This, in combination with nestin's well-defined role as a marker for progenitor cells in other tissues, makes it an important candidate gene for continued characterization of any retained, quiescent stem-like capabilities in the OC. That said, the variability in reported expression patterns of nestin in the mouse cochlea call into question whether it is a reliable stem cell marker in the inner ear. Additional studies are required to resolve this question and to better understand any multipotent qualities harbored by nestin-expressing cells in the inner ear. For example, the *in vitro* assays for multipotency of nestin-positive cells purified from a transgenic mouse cochlea of different ages would confirm their stem cell character. Further experiments on *in vitro* differentiation of purified nestin-positive cells toward the hair/supporting cell fate could reveal factors involved in terminal differentiation of these cells. In addition, a nestin knockout transgenic strategy might further evaluate the role of nestin as a marker of a

Table 1. The Nestin-Positive Cells' Anatomical Location in the Cochlea of Transgenic Young and Adult Mice

≤P3	P5	P14	P21	P56–60	Construct	Refs
–	Border cells, inner phalangeal cells, Deiters' cells, satellite cells, grater epithelial region, and some OHCs	P15 similar expression to the P5 but no positive OHC	–	Only some Deiters' cells	Plasmid vector pNcoNesPlacZ/3 covering the 5′ upstream region of the translation start site and the 1.8-kb enhancer region of the third intron	[12]
Border cells, inner phalangeal cells, supporting cells near IHC area, some IHCs	–	–	–	Spiral ganglion cells, IHC afferent nerve fibers	Nestin Xh5 plasmid	[13]
Sensorineural epithelium in the apical and basal turns; among mesothelial cells under the basilar membrane	–	Sensorineural epithelium but restricted to the OHC region; among supporting cells lateral to OHC area	Narrow strip lateral to the last row of OHCs; only in the apical turn; lateral to the OHC area in the third row of Deiters' cells and/or Hensen cells	Up to P90 distribution of Nestin-positive cells like that observed at P21	Nestin second intronic/ promoter enhancer drives the expression of β-galactosidase	[14]
				Spiral ganglion cell region and multiple cell types in the organ of Corti, medial to and below the inner hair cells, and in Deiters' cells in the cochlear apex	Nestin Xh5 plasmid	[15]
E13.5: spiral ganglion region E14.5: basal region of sensory epithelium. Base to apex gradient until E15.5, then in all three turns. From E18.5 IHCs, supporting cells, osseous spiral lamina, and spiral ganglion, apical IHCs until P2	P6: border and inner phalangeal cells, osseous spiral lamina; Deiters' cells in the basal region	P12: Deiters' cells in the basal and midregion	P20–42: Deiters' cells in the basal, midregion and apex Cell population under and medial to IHCs	P63: mostly basal Dieters' cells, weaker expression in apical and midregion At 1 year of age only apical population of Dieters' cells	Plasmid vector pNcoNesPlacZ/3 covering the 5′ upstream region of the translation start site and the 1.8-kb enhancer region of the third intron	[16]

population of multipotent cells in the cochlea, as has been demonstrated in other organs systems. For example, multipotent neural stem cells isolated from a nestin knockout mouse had decreased survival and self-renewal capability [17], supporting a role on some level of nestin in the process of tissue regeneration. In summary, while reports to date support that nestin-expressing cells might have some progenitor or stem cell role during development and adulthood, respectively, additional *in vitro* and *in vivo* studies are necessary to definitively characterize this to be the case.

Lgr5

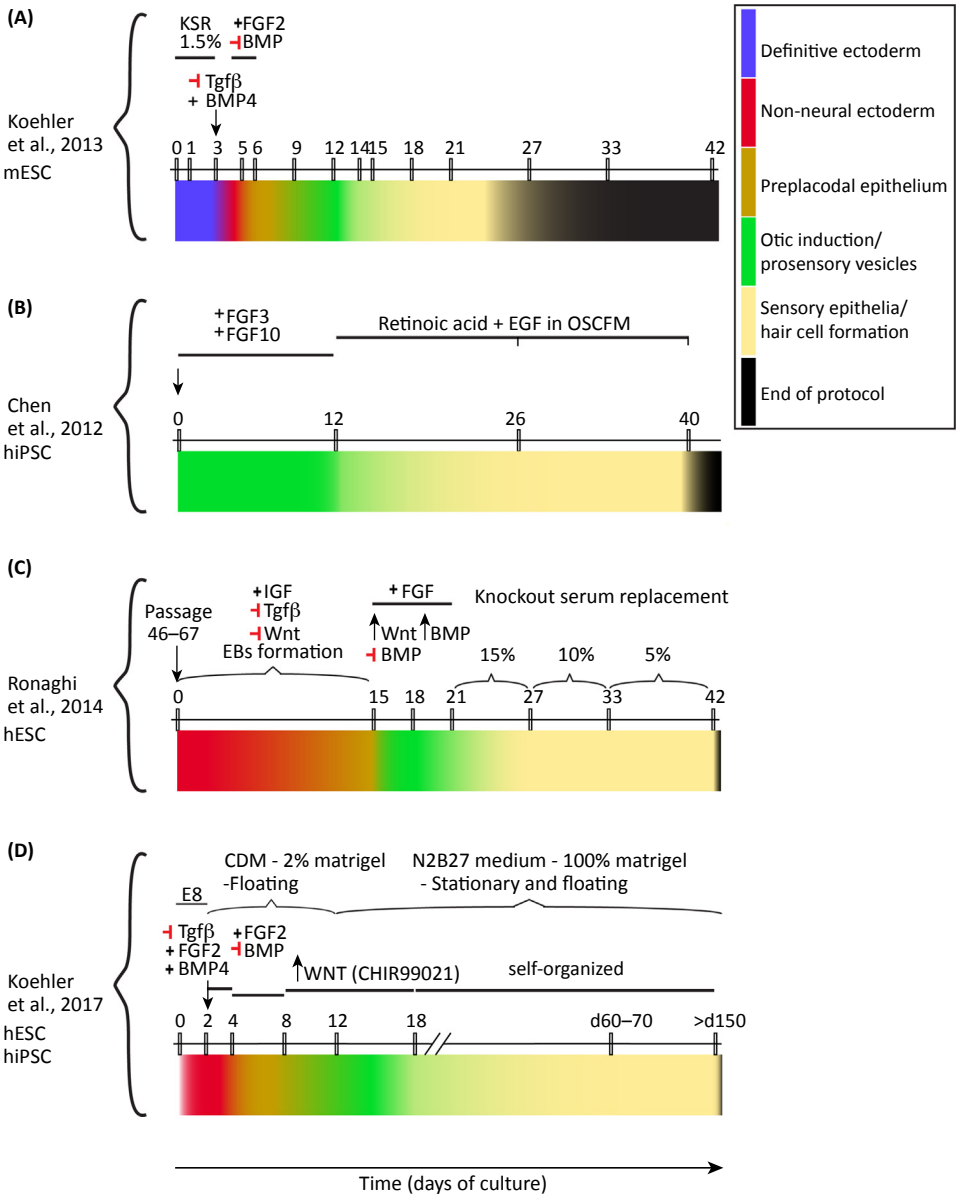
Lgr5, a Wnt target gene, has been well characterized as a stem cell marker in multiple proliferating adult tissues [18–21]. Given this role, Lgr5 has more recently emerged as a target gene of interest for inner ear HC regeneration [22–24]. In the inner ear of Lgr5-EGFP knockin reporter mice, Lgr5 is expressed in the floor epithelium at embryonic day 15.5 (E15.5) and HCs and supporting cells in the apical turn at E18.5 and is gradually downregulated thereafter [23]. In the young adult mouse (P30), only the third row of Deiters' cells remain positive for Lgr5. Multiple groups have performed analyses of early postnatal Lgr5-expressing cells and have demonstrated that these cells retain the ability to proliferate and form HCs and supporting cells *in vitro* and *in vivo*, thus establishing Lgr5 as a robust marker for cochlear progenitor cells [24–28]. Kuo and colleagues [29] showed that in neonatal mice the combination of overexpression of β-catenin and Atoh1 in Lgr5-positive cells leads to augmented HC generation with subsequent survival up to 21 days. Because of their ability to proliferate and form both HCs and supporting cells, Lgr5-positive cells, were they able to be targeted effectively, could potentially be used to repopulate the damaged or lost HCs in patients with hearing loss. That said, while Lgr5-expressing cells have been well characterized in the early postnatal mouse, the molecular mechanisms that control proliferation and direct differentiation of these cells into HCs remain poorly understood. Some *in vitro* studies report that Lgr5+ cells can proliferate and differentiate into hair cells, but some *in vivo* studies suggest that Lgr5+ cells can only temporarily proliferate in response to Wnt signaling [28], but not necessarily then differentiate into HCs or supporting cells. The fact that these observations are made using cells and tissue from the same genetic mouse model emphasize the importance of performing *in vivo* validation of *in vitro* findings. In addition, because the genetic constructs designed for lineage tracing are knocked in to one of two homologous sites, it is possible that this process can create a haploinsufficiency phenotype that confounds experimental interpretation. For example, use of Sox2-EGFP-CreERT constructs for supporting cells and Lgr5-EGFP-CreERT constructs for Lgr5-expressing cells might themselves produce a phenotype independent from that produced due to the intended experimental design and thus complicate interpretation of the results. Another consideration regarding the regenerative capabilities of Lgr5+ cells in the cochlea is whether the increased abilities of cell regeneration are due to this protein itself or rather to Wnt signaling. Recent findings in the intestinal stem cells show that Lgr5 as well as Lgr4 and Lgr6 function as receptors for R-spondins, which are involved in regulating Wnt signaling. Whether Lgr5 functions similarly in the cochlea has not yet been explored, but the mechanism observed in the intestinal stem cells suggests a molecular feedback between Lgr4-Lgr6 and Wnt/Frizzled receptor and functional separation of Lgr5-R-spondin and Wnt signals [30]. An improved understanding of the role of Lgr5, Wnt signaling in general, and other factors that render Lgr5 cells more able to regenerate in the postnatal cochlea will provide crucial insight to potentially design therapies targeted at promoting endogenous regeneration in the mammalian cochlea.

In summary the mammalian cochlea contains progenitor-like cells capable in some contexts of replenishing both HCs and supporting cells. The regenerative potential of these cells is less clear however due to the decreasing size of their populations and loss of progenitor-like abilities in adulthood. Furthermore, since the postnatal cochlea of the mouse is generally equivalent to the early prenatal cochlea of the human, the relevance of these early inner ear progenitor cells to future meaningful regenerative therapies in humans is less clear. Regardless, the study of multipotency in the cochlea using these cells types has yielded, and will likely continue to yield, an improved understanding of processes that could be relevant to future targeted regenerative therapies.

Cell Transplantation-Based Strategies for Regeneration

In Vitro Differentiation of PSCs to HCs

PSCs are self-renewing and have the ability to make all cell types found in the body. Examples of PSCs include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Li and colleagues [31] were the first to report on the successful generation of inner ear HC-like cells from mouse ESCs (mESCs) that they characterized using gene expression and immunostaining for HC-specific proteins. Oshima and colleagues [32] reported successful *in vitro* differentiation of mouse PSCs to mechanosensitive HC-like cells using a step-by-step differentiation protocol based on the principles of early otic development. The HC-like cells that were generated displayed stereociliary bundles resembling those of postnatal mouse vestibular HCs and were found to have an electrophysiological signature consistent with immature yet functional HCs. The authors used an *in vitro* feeder layer of chick utricular stromal cells to enable terminal differentiation and functional maturation of the stem cell-derived HCs. Since this landmark report, others have used similar feeder layer-based *in vitro* strategies in combination with transfection of the pro-hair cell transcription factor Atoh1 for differentiating mESCs to inner ear sensory HCs [33]. In another landmark study, Koehler and colleagues [34] developed a feeder layer free *in vitro* differentiation protocol for mESCs to generate inner ear organoids including supporting cells, mechanosensitive HC-like cells, and neurons that established synaptic connections with the sensory cells (Figure 3). A novel protocol in which stepwise differentiation is circumvented by doxycycline-inducible simultaneous expression of three genes (Gfi1, Pou4f3, and Atoh1) has been recently proposed by Costa *et al.* [35]. Here, the authors used mESCs to generate HC-like immunophenotypes with protrusions reminiscent of stereociliary bundles in a short 12-day protocol. All of the studies focusing on PSC differentiation to inner ear lineages described above were done using mouse ESCs and/or iPSCs. Studies on this topic using human cells are more limited. The protocols of two such reports on generating HC-like cells from human PSCs are shown in Figure 3. Chen and colleagues [36] used a monolayer human (h)ESC system to differentiate hESCs into either otic neural progenitors (ONPs) or otic epithelial progenitors (OEPs). Subsequent differentiation of OEPs generated cells expressing HC proteins with immature-appearing stereociliary bundles and some electrophysiological features suggesting a functional HC fate. Differentiation of ONPs generated cells with a protein expression and electrophysiological profile suggesting an auditory neuronal fate. Ronaghi and colleagues [37] developed a differentiation protocol for hESCs to sequentially generate cells expressing non-neural ectoderm, preplacodal ectoderm, otic placode and inner ear HC immunophenotypes. The HC-like cells resulting from this protocol did express multiple HC markers as identified with immunohistochemistry, and some had rudimentary stereociliary bundles; however, the efficiency of HC generation was limited. Using published HC differentiation protocols, two groups have recently demonstrated functional and morphological rescue of deafness-causing Myo7a and Myo15 mutations in human patient-derived iPSCs *in vitro* [38,39]. Both teams generated disease-specific iPSC lines, edited the mutated genes, and then differentiated the induced pluripotent stem (iPS) cells to HC-like cells by using the protocol of Chen *et al.* [36]. In doing so, both groups demonstrated the feasibility of gene correction via advanced gene editing techniques with subsequent HC generation from patient-specific iPSCs resulting in morphological and functional restoration. Most recently, Koehler and colleagues [40] successfully demonstrated the feasibility of generating human inner ear organoids from hESCs that contained neuronal, supporting cell, and functional HC-like cells in a cytoarchitecture suggestive of inner ear sensory epithelium (Figure 3). The organoid culture approach in this study offers the ability to study development and model disease by using human tissue on molecular and cellular levels not previously possible.



Trends in Neurosciences

Figure 3. Comparison of Four Feeder Layer-Free Protocols for Otic Lineage Differentiation of mESCs, hESCs, and human induced pluripotent stem cells (hiPSCs). Koehler and colleagues used an inner ear organoid culture approach for mouse embryonic stem cells (mESCs) [34] and human embryonic stem cells (hESCs) [40] to recapitulate inner ear development. The Ronaghi *et al.* [37] and Chen *et al.* [36] studies used a monolayer cell culture strategy for otic lineage differentiation also based upon developmental principles. Protocols are in the same time scale. Colors represent stages of differentiation. In the Chen's protocol OSCFM stands for otic stem cell full medium. The assessment of a hair cell fate after terminal differentiation included (A) immunohistochemistry (IHC): Myo7a, Brn3c, cD1, Calb2, Espin, Ctbp2; electron microscopy (EM) confirmation of hair bundles and electrophysiology (e-phys) [34]; (B) IHC: Myo7a, Atoh1, Brn3c, Espin; EM confirmation of hair bundles and e-phys [36]; (C) IHC: Myo7a, Atoh1, Myo6, Espin; quantitative RT-PCR: *MYO7A*, *ATOH1*, *MYO6*, *ESPN*; single-cell analysis; *MYO7A*, *ATOH1*, *MYO7A*, *MYO15A*, *OTOF*, *ESPN*; EM confirmation of hair bundles and e-phys [37]; and (D) IHC: Myo7a, Espin, TubA4A, F-actin, Anxa4, Pcp4, Calb2 and in the ATOH1-eGFP cell line: Gfp, Brn3c, Espin, Ctbp2, Nefl, Nefh, Syp; and e-phys [40]. Abbreviations: BMP, bone morphogenetic protein; CDM, chemicallydefined medium; CHIR99021, acts as Wnt activator; E8, E8 media; EBs, embryonicbodies; EGF, epidermal growth factor; FGF, fibroblast growth factor; KSR, knockout serum replacement; OSCFM, otic stem cell full medium; Tgfβ, transforming growth factor beta; WNT, Wingless-relatedintegration site.

In summary, significant progress has been made in generating inner ear cell lineages *in vitro* with both mouse and human PSCs. Nevertheless, additional studies are needed to refine techniques for the generation of enriched and consistent populations of supporting and HC-like cells from human PSCs. Doing so will enable the use of PSC-based strategies to elucidate mechanisms underlying inner ear development, model deafness using human tissues, identify novel deafness-causing mutations, screen drugs to treat deafness, and potentially allow for transplantation-based approaches for hearing loss.

Defining a Functional Regenerated HC

One challenge to all stem cell-based approaches (as well as any HC regenerative approach) aimed at inner ear HC regeneration lies in how best to define a functional regenerated HC. Lower levels of evidence of a HC fate commonly used include the expression of Atoh1, Myo7a, or other HC-specific gene and protein products yet do not speak to functionality. The demonstration of a characteristic stereociliary bundle atop the apical surface of HCs also suggests, but does not define, functionality. Uptake of the styryl dye FM1-43 or fluorescently labeled gentamicin, which are selectively taken up by the stereocilia mechanotransducer channels located on the bundles, are a pseudo-functional assay that can support a functional hair cell fate yet are not definitive in doing so. While technically challenging, demonstration of characteristic electrophysiological signatures of inner and outer HCs using mechanotransduction approaches is the most definitive means for assessing a HC fate in regenerative studies. Currently, the literature on regenerating functional HCs, regardless of the approach taken for doing so, has not definitively demonstrated an ability to direct regeneration specifically toward an IHC or OHC fate, rather only a more general HC fate commitment. Perhaps using novel technologies such as single-cell RNA sequencing to study inner ear HCs during development will provide novel insight into key regulators of inner versus outer HC differentiation that can be applied to further refine our ability to direct regeneration in a more targeted manner in the future.

Cochlear Transplantation

Over the past 15 years, many groups have evaluated the feasibility of transplanting stem cells into the inner ear in hopes of developing a novel method for treating hearing loss [36,41–58]. Li and colleagues [31] were among the first in this regard when they transplanted mESC-derived otic progenitor cells into the nascent chick basilar papilla. They found that the grafted cells integrated into the sensory epithelium and terminally differentiated into mature HC-like cells. Jeon and colleagues [59] later reported similar findings using the same xenotransplantation model and grafting of mouse bone marrow-derived mesenchymal stem cell-derived otic progenitors. Lang and colleagues [42] demonstrated that mESC-derived neural progenitors injected via the round window survive longest in Rosenthal's canal and the scala tympani, but not scala media, if the injection was made soon (1–3 days) after lesioning of the spiral ganglion. The longest reported survival of stem cell-derived progenitors grafted to the inner ear was reported by Chen *et al.* [36], who transplanted hESC-derived neural progenitors into the ouabain-lesioned adult gerbil modiolus via the round window membrane. The transplanted cells survived for 10 weeks and were found to have engrafted and differentiated into mature-appearing neurons that extended peripheral processes toward the OC. Auditory brainstem response assessment showed improvement in the hearing thresholds of grafted ears after transplantation. While these findings were encouraging, the experience with transplantation of stem cells into the adult mammalian cochlea (rather than the auditory nerve) has been mixed (reviewed in Table 2).

Review of these studies reveals that cellular engraftment is most successful when the grafted cells are delivered to the scala tympani either through the lateral wall [51] or posterior

Table 2. The Animal Models of Cochlear Cell Transplantation

Author	Year	Animal	Deafening	Cell type	Differentiation of cells	Cite of transplantation	Engraftment in the organ of Corti	Cell survival	Immunosuppression	Refs
Kamiya *et al.*	2007	Adult rat	3NP	Rat mesenchymal stem cell	–	Lateral semicircular canal	–	11 days	–	[43]
Lang *et al.*	2008	Adult gerbil	Ouabain	Mouse embryonic stem (ES) cell	Neural induction	Rosenthal's canal/scala tympani/scala media via round window	–	4 weeks	–	[44]
Ahn *et al.*	2008	Adult mouse	Drug (cisplatin and kanamycin)	Mouse ES cell	–	Posterior semicircular canal	+ (Anatomically)	4 weeks	–	[45]
Nishimura *et al.*	2009	Neonatal mouse	–	Mouse iPS cell	Neural induction	Scala tympani via round window	–	1 week	–	[46]
Han *et al.*	2010	Adult guinea pig	–	Atoh1-expressing mouse neural stem cell	–	Scala media via lateral wall	+ (Anatomically + Myo7a)	2 weeks	–	[47]
Sullivan *et al.*	2011	Adult mouse	Noise	Mouse tongue-derived stem cell	–	Scala tympani/ scala vestibuli via lateral wall	–	4 weeks	–	[48]
Pettingil *et al.*	2011	Adult guinea pig	Drug (kanamycin and frusemide)	Brain-derived neurotrophic factor-expressing schwann cells	–	Scala tympani via lateral wall	–	4 weeks	–	[49]
Nishimura *et al.*	2012	Neonatal mouse	–	Mouse ES/iPS cell	Neural induction	Scala tympani via round window	–	4 weeks	–	[50]
Nayagam *et al.*	2012	Adult guinea pig	Drug (kanamycin and frusemide)	Mouse ES cell	Neural induction	Rosenthal's canal via lateral wall	–	1 week	–	[51]
Choi *et al.*	2012	Adult rat	Noise/drug (neomycin)	Human mesenchymal stem cell	–	Intravenous	–	4 weeks	–	[52]
Chen *et al.*	2012	Adult gerbil	Ouabain	Human ES cell	Neural induction	Modiolus via round window	–	10 weeks	+ Cyclosporine	[38]
Zhao *et al.*	2012	Adult rat	Drug (amikacin)	Mouse ES cell	–	Scala tympani via lateral wall	+ (Anatomically + Myo7a)	6 weeks	+ Cyclosporine	[53]
Zhang *et al.*	2013	Adult rat	Ouabain	Mouse olfactory neural stem cell	–	Scala tympani via lateral wall	+ (Anatomically)	1 week	+ Cyclosporine	[54]

Table 2. (continued)

Author	Year	Animal	Deafening	Cell type	Differentiation of cells	Cite of transplantation	Engraftment in the organ of Corti	Cell survival	Immunosuppression	Refs
He *et al.*	2014	Adult rat	Ouabain	Neural stem cell from olfactory bulb	–	Scala tympani via lateral wall	–	1 week	+ Cyclosporine	[55]
Tan *et al.*	2014	Adult guinea pig	Autoimmune	Interleukin-4-expressing bone marrow mesenchymal stem cell	–	Scala tympani via lateral wall	–	1 week	–	[56]
Fetoni *et al.*	2014	Adult guinea pig	Noise	Adipose tissue stem cell	–	Scala tympani via round window	–	1 week	–	[57]
Ishikawa *et al.*	2015	Adult guinea pig	–	Human iPS cell	Neural induction	Scala tympani via lateral wall	–	2 weeks	+ FK506	[58]
Xu *et al.*	2016	Adult rat	Noise	Rat olfactory stem cell	–	Scala tympani via lateral wall	–	5 days	–	[59]
Takeda *et al.*	2017	Prenatal mouse	–	Mouse iPS cell	–	Otocyst via uteral wall	–	4 days	–	[60]

semicircular canal [43]. However, it is challenging to make definitive conclusions given inconsistencies among reports in variables such as pre-transplantation deafening, use of immunosuppression, grafted cell type, stage of differentiation, and site of injection. In the future, a more systematic approach to evaluate the relative contribution of these variables toward a successful transplantation would be helpful in establishing reproducible protocols for cochlear cell transplantation. Below we examine these variables individually in the context of the reported literature.

Site of Injection

The most direct way of transplanting cells to the OC is through fenestration of the bony otic capsule or round window membrane so as to access either the perilymph of scala tympani, and vestibuli, or endolymph of scala media. Puncture of the round window membrane or semicircular canal delivers cells to the perilymph, while a lateral wall cochleostomy can provide access to both the endo- and perilymph (Figure 4). Cells delivered via the scala tympani or vestibuli, which contain perilymph, would have to cross the basilar or Reissner's membranes to engraft in the OC. Cellular delivery directly to scala media has a theoretic advantage of closer placement of grafted cells to the OC; however, the high K^+ concentration of the endolymph makes grafted

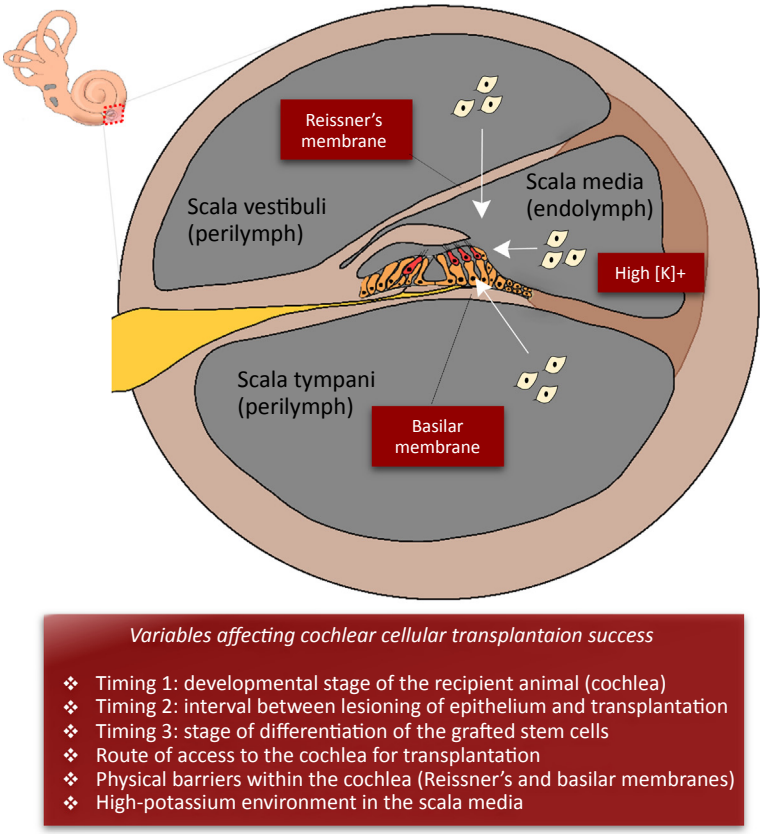


Figure 4. Routes for the Local Administration of Stem Cells into the Cochlea for Engrafting Procedures. Schematic drawing of the inner ear and the cochlea cross section. Reissner's and basilar membrane physically separate scala vestibuli and tympani from scala media, respectively, possibly impeding stem cell targeting toward the organ of Corti. To overcome these physical barriers, injection might be targeted directly to the scala media; however, the high-[K^+] endolymph in this chamber presents a potentially toxic environment for grafted cells.

cell survival less likely as shown *in vitro* and *in vivo* using HeLa cells [60]. An additional challenge of intracochlear cellular delivery lies in the lack of any significant fluid flow within the cochlea. Thus, it becomes very difficult for cells delivered to the basal portions of the cochlear duct (the area most cochleostomies and round window injections target) to gain access to more apical regions. In contrast to local administration, systemic administration has been used in an attempt to circumvent the anatomical complexity of the cochlea. Systemic administration has the disadvantages that it requires delivery of a large number of cells to overcome the 'blood-labyrinth barrier', relies on the cells capability of honing in to the right organ, and poses a significant threat of off target grafting that can cause undesirable side effects [61]. For example, Choi *et al.* [50] reported that human mesenchymal stem cells injected intravenously to adult rats with noise or neomycin-induced hearing loss were ultimately detected in the lungs. In summary, while transplantation of cells directly into the cochlear scalar fluids places the cells close to the OC, thus increasing the chance for engraftment, the physical and cytotoxic barriers of the mammalian inner ear present significant challenges for cell migration, distribution, and survival.

Does Timing of Transplantation Matter?

There are two important time-related factors in any stem cell transplantation-based regenerative strategy. One factor relates to the time after injury/lesioning (deafening in the case of hearing loss) and the other factor to the stage of differentiation of the transplanted cells. When these time-related factors are combined at their most optimal values, the efficiency of engraftment should increase. Appropriate timing of transplantation after lesioning is essential for the survival of the transplanted stem cells in the injured nervous system [62]. A similar finding has been observed in the cochlea. Lang *et al.* [42] reported that the transplanted cells were observed to survive better in recently damaged inner ears, suggesting that an acutely injured OC might be a more permissive environment for transplantation. Another related variable is the stage of development of the recipient animal. Several studies have reported that stem cell transplantation at the early developmental stages is the most promising in terms of survivability and differentiation of the transplanted cells. Multiple experiments in which mouse stem cells were transplanted to the nascent chick otocyst have demonstrated successful engraftment into the auditory sensory epithelium and differentiation [9,31,59] into HC phenotypes. A similar phenomenon has been observed in the CNS where stem cells that were transplanted to the developing rat ventricle successfully engrafted, differentiated, and achieved long-term survival [63]. Unfortunately, a recent report found that stem cells transplanted into the mouse developing otocyst survived and differentiated *in vivo* but failed to engraft in the sensory epithelium [58]. As such, it remains to be seen whether transplantation of stem cells into the mammalian developing otocyst under any circumstances would be capable of producing similar results to those observed in the chick. Another time-related factor important to the success of any inner ear stem cell transplantation strategy is the stage of differentiation of the transplanted cells. If cells are in too early of a stage of differentiation upon transplantation, then teratoma formation may occur. On the other hand, if the transplanted cells have been differentiated extensively toward an HC or supporting cell fate *in vitro* before transplantation, then it would be unlikely that they would have retained the capability to allow for successful engraftment in the OC.

In summary, both the timing after injury and the stage of differentiation of the transplanted stem cells are important factors to be considered in efforts to optimize the engraftment of transplanted stem cells into the auditory sensory epithelium. However, the ideal parameters for these variables are not well understood. Further research that would systematically analyze the effect of both of these variables on the efficiency of engraftment of transplanted cells would provide an improved understanding of how to optimize success for stem cell transplantation-based approaches for hearing loss.

Concluding Remarks

There are several approaches being taken in an effort to develop cell-based regenerative therapies for hearing loss. In this review, we focused on exogenous stem cell transplantation and endogenous pluripotent progenitor cells approaches. While significant knowledge about the developmental and genetic mechanisms involved in the differentiation of stem or progenitor cells has been gleaned from *in vitro* studies and research in the avian basilar papilla and murine cochlea, significant challenges remain in the pursuit of stem cell-based therapies for hearing loss (see Outstanding Questions). Continued research in this area may provide insight as to how to overcome these challenges and which particular approach has the most promise for future clinical translation in humans.

Outstanding Questions

Do inner ear multipotent stem cells persist into adulthood in the mammalian cochlea? If so, what are the most important factors inhibiting them from entering the cell cycle to generate new HCs and supporting cells over time or after injury?

How can the published protocols for the generation of inner ear hair and supporting cells from human pluripotent stem cells be streamlined to increase the efficiency and yield of terminally differentiated inner ear cell types *in vitro*?

How can the full length of the cochlear duct, from base to apex, be safely accessed to allow for successful regenerative therapies for hearing loss?

What factors in the developing auditory sensory epithelium and auditory nerve allow for survival, integration, and terminal differentiation of grafted pluripotent stem cell-derived progenitors? Can these factors be targeted in the adult mammalian organ of Corti to allow for successful transplantation of stem cells?

Acknowledgments

S.P.G. has grant support from the National Institutes of Health/National Institutes on Deafness and Other Communicative Disorders (R01 DC013912-01). S.P.G. has received an honorarium from Decibel Therapeutics for ad hoc consulting and receives consulting fees for Scientific Advisory Board Membership with Applied Genetic Technologies Corporation and Roche Pharmaceuticals.

References

1. Hoffman, H.J. *et al.* (2017) Declining prevalence of hearing loss in US adults aged 20 to 69 years. *JAMA Otolaryngol. Head Neck Surg.* 143, 274–285
2. Corwin, J. and Cotanche, D. (1988) Regeneration of sensory hair cells after acoustic trauma. *Science* 240, 1772–1774
3. Ryals, B. and Rubel, E. (1988) Hair cell regeneration after acoustic trauma in adult Coturnix quail. *Science* 240, 1774–1776
4. Cruz, R.M. *et al.* (1987) Light microscopic evidence of hair cell regeneration after gentamicin toxicity in chick cochlea. *Arch. Otolaryngol. Head Neck Surg.* 113, 1058–1062
5. Cotanche, D.A. (1987) Regeneration of hair cell stereociliary bundles in the chick cochlea following severe acoustic trauma. *Hear. Res.* 30, 181–195
6. Manley, G.A. (2017) Comparative auditory neuroscience: understanding the evolution and function of ears. *J. Assoc. Res. Otolaryngol.* 18, 1–24
7. Roberson, D.W. *et al.* (2004) Direct transdifferentiation gives rise to the earliest new hair cells in regenerating avian auditory epithelium. *J. Neurosci. Res.* 78, 461–471
8. Adler, H.J. *et al.* (1997) Further evidence for supporting cell conversion in the damaged avian basilar papilla. *Int. J. Dev. Neurosci.* 15, 375–385
9. Li, H. *et al.* (2003) Pluripotent stem cells from the adult mouse inner ear. *Nat. Med.* 9, 1293–1299
10. Lendahl, U. *et al.* (1990) CNS stem cells express a new class of intermediate filament protein. *Cell* 60, 585–595
11. Hombach-Klonisch, S., Panigrahi, S., Rashedi, I., Seifert, A., Alberti, E., Pocar, P., Kurpisz, M., Schulze-Osthoff, K., Mackiewicz, A. and Los, M. (2008) Adult stem cells and their trans-differentiation potential–perspectives and therapeutic applications. *J. Mol. Med.* 86, 1301–1314
12. Lopez, I.A. *et al.* (2004) Stem/progenitor cells in the postnatal inner ear of the GFP-nestin transgenic mouse. *Int. J. Dev. Neurosci.* 22, 205–213
13. Smeti, I. *et al.* (2011) Expression of candidate markers for stem/progenitor cells in the inner ears of developing and adult GFAP and nestin promoter-GFP transgenic mice. *Gene Expr. Patterns* 11, 22–32
14. Watanabe, R. *et al.* (2012) Nestin-expressing cells in the developing, mature and noise- exposed cochlear epithelium. *Mol. Cell. Neurosci.* 49, 104–109
15. Chow, C.L., Guo, W., Trivedi, P., Zhao, X. and Gubbels, S. (2015) Characterization of a unique cell population marked by transgene expression in the adult cochlea of nestin-CreERT2/tdTomato-reporter Mice. *J. Comp. Neurol.* 1, 1474–1487
16. Chow, C.L. *et al.* (2016) Evaluation of nestin expression in the developing and adult mouse inner ear. *Stem Cells Dev.* 25, 1419–1432
17. Park, D., Xiang, A.P., Mao, F.F., Zhang, L., Di, C.G., Liu, X.M., Shao, Y., Ma, B.F., Lee, J.H., Ha, K.S., Walton, N. and Lahn, B.T. (2010) Nestin is required for the proper self-renewal of neural stem cells. *Stem Cells* 28, 2162–2171
18. Barker, N. *et al.* (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449, 1003–1007
19. Barker, N. *et al.* (2010) Lgr5+ve stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell* 6, 25–36
20. Jaks, V. *et al.* (2008) Lgr5 marks cycling, yet long-lived, hair follicle stem cells. *Nat. Genet.* 40, 1291–1299
21. Ootani, A. *et al.* (2010) Sustained in vitro intestinal epithelial culture within a Wnt-dependent stem cell niche. *Nat. Med.* 15, 701–706
22. Shi, F. *et al.* (2013) Generation of hair cells in neonatal mice by β-catenin overexpression in Lgr5-positive cochlear progenitors. *PNAS* 110, 13851–13856
23. Chai, R. *et al.* (2011) Dynamic expression of Lgr5, a Wnt target gene, in the developing and mature mouse cochlea. *JARO* 469, 455–469
24. Shi, F. *et al.* (2012) Wnt-responsive Lgr5-expressing stem cells are hair cell progenitors in the cochlea. *J. Neurosci.* 32, 9639–9648
25. Wang, T. *et al.* (2015) Lgr5+ cells regenerate hair cells via proliferation and direct transdifferentiation in damaged neonatal mouse utricle. *Nat. Commun.* 6, 6613
26. Bramhall, N.F. *et al.* (2014) Lgr5-positive supporting cells generate new hair cells in the postnatal cochlea. *Stem Cell Rep.* 2, 311–322
27. Cox, B.C. *et al.* (2014) Spontaneous hair cell regeneration in the neonatal mouse cochlea in vivo spontaneous hair cell regeneration in the neonatal mouse cochlea in vivo. *Development* 141, 1599
28. Chai, R. *et al.* (2012) Wnt signaling induces proliferation of sensory precursors in the postnatal mouse cochlea. *PNAS* 109, 8167–8172
29. Kuo, B.R. *et al.* (2015) In vivo cochlear hair cell generation and survival by coactivation of β-catenin and Atoh1. *J. Neurosci.* 35, 10786–10798
30. Yan, K.S. *et al.* (2017) Non-equivalence of Wnt and R-spondin ligands during Lgr5+ intestinal stem-cell self-renewal. *Nature* 545, 238–242

31. Li, H. *et al.* (2003) Generation of hair cells by stepwise differentiation of embryonic stem cells. *PNAS* 100, 13495–13500
32. Oshima, K. *et al.* (2010) Mechanosensitive hair cell-like cells from embryonic and induced pluripotent stem cells. *Cell* 141, 704–716
33. Ouji, Y. *et al.* (2017) Efficient induction of inner ear hair cell-like cells from mouse ES cells using combination of Math1 transfection and conditioned medium from ST2 stromal cells. *Stem Cell Res.* 23, 50–56
34. Koehler, K.R. *et al.* (2013) Generation of inner ear sensory epithelia from pluripotent stem cells in 3D culture. *Nature* 500, 217–221
35. Costa, A. *et al.* (2015) Generation of sensory hair cells by genetic programming with a combination of transcription factors. *Development* 142, 1948–1959
36. Chen, W. *et al.* (2012) Restoration of auditory evoked responses by human ES cell-derived otic progenitors. *Nature* 490, 278–282
37. Ronaghi, M. *et al.* (2014) Inner ear hair cell-like cells from human embryonic stem cells. *Stem Cells Dev.* 23, 1275–1284
38. Chen, J.-R. *et al.* (2016) Effects of genetic correction on the differentiation of hair cell-like cells from iPSCs with MYO15A mutation. *Cell Death Differ.* 23, 1347–1357
39. Tang, Z.-H. *et al.* (2016) Genetic correction of induced pluripotent stem cells from a deaf patient with *MYO7A* mutation results in morphologic and functional recovery of the derived hair cell-like cells. *Stem Cells Transl. Med.* 5, 561–571
40. Koehler, K.R. *et al.* (2017) Generation of inner ear organoids containing functional hair cells from human pluripotent stem cells. *Nat. Biotechnol.* 35, 583–589
41. Kamiya, K. *et al.* (2007) Mesenchymal stem cell transplantation accelerates hearing recovery through the repair of injured cochlear fibrocytes. *Am. J. Pathol.* 171, 214–226
42. Lang, H. *et al.* (2008) Transplantation of mouse embryonic stem cells into the cochlea of an auditory-neuropathy animal model: effects of timing after injury. *J. Assoc. Res. Otolaryngol.* 9, 225–240
43. Ahn, K.S. *et al.* (2008) Isolation of embryonic stem cells from enhanced green fluorescent protein-transgenic mouse and their survival in the cochlea after allotransplantation. *Cytotherapy* 10, 759–769
44. Nishimura, K. *et al.* (2009) Transplantation of mouse induced pluripotent stem cells into the cochlea. *Neuroreport* 20, 1250–1254
45. Han, Z. *et al.* (2010) Survival and fate of transplanted embryonic neural stem cells by Atoh1 gene transfer in guinea pigs cochlea. *Neuroreport* 21, 490–496
46. Sullivan, J.M. *et al.* (2011) Effect of epithelial stem cell transplantation on noise-induced hearing loss in adult mice. *Neurobiol. Dis.* 41, 552–559
47. Pettingill, L.N. *et al.* (2011) Enhanced auditory neuron survival following cell-based BDNF treatment in the deaf guinea pig. *PLoS One* 6, 2–9
48. Nishimura, K. *et al.* (2012) Fates of murine pluripotent stem cell-derived neural progenitors following transplantation into mouse cochleae. *Cell Transplant.* 21, 763–771
49. Nayagam, B.A. *et al.* (2012) Hydrogel limits stem cell dispersal in the deaf cochea: implication for cochlear implants. *J. Neural Eng.* 9, 1–16
50. Choi, B.Y. *et al.* (2012) Intravenous administration of human mesenchymal stem cells after noise-or drug-induced hearing loss in rats intravenous administration of human mesenchymal stem cells after noise-or drug-induced hearing loss in rats. *Acta Otolaryngol.* 132, 94–102
51. Zhao, L.-D. *et al.* (2012) Migration and differentiation of mouse embryonic stem cells transplanted into mature cochlea of rats with aminoglycoside-induced hearing loss. *Acta Otolaryngol.* 6489, 1–8
52. Zhang, P.Z. *et al.* (2013) Stem cell transplantation via the cochlear lateral wall for replacement of degenerated spiral ganglion neurons. *Hear. Res.* 298, 1–9
53. He, Y. *et al.* (2014) Wnt1 from cochlear schwann cells enhances neuronal differentiation of transplanted neural stem cells in a rat spiral ganglion neuron degeneration model. *Cell Transplant.* 23, 747–760
54. Tan, C.-Q. *et al.* (2014) Exogenous IL-4-expressing bone marrow mesenchymal stem cells for the treatment of autoimmune sensorineural hearing loss in a guinea pig model. *BioMed Res. Int.* 2014, 1–10
55. Fetoni, A.R. *et al.* (2014) Grafting and early expression of growth factors from adipose-derived stem cells transplanted into the cochlea, in a guinea pig model of acoustic trauma. *Front. Cell. Neurosci.* 8, 334
56. Ishikawa, M. *et al.* (2017) Transplantation of neurons derived from human iPS cells cultured on collagen matrix into guinea-pig cochleae. *J. Tissue Eng. Regen. Med.* 11, 1766–1778
57. Xu, Y.P. *et al.* (2016) Olfactory epithelium neural stem cell implantation restores noise-induced hearing loss in rats. *Neurosci. Lett.* 616, 19–25
58. Takeda, H. *et al.* (2017) Transplanting mouse induced pluripotent stem cells into mouse otocysts in vivo. *Neurosci. Lett.* 647, 153–158
59. Jeon, S. *et al.* (2007) Bone marrow mesenchymal stem cells are progenitors in vitro for inner ear hair cells. *Mol. Cell. Neurosci.* 34, 59–68
60. Park, Y.-H. *et al.* (2014) Conditioning the cochlea to facilitate survival and integration of exogenous cells into the auditory epithelium. *Mol. Ther.* 22, 873–880
61. Swan, E.E.L. *et al.* (2008) Inner ear drug delivery for auditory applications. *Adv. Drug Deliv. Rev.* 60, 1583–1599
62. Nishimura, S. *et al.* (2013) Time-dependent changes in the microenvironment of injured spinal cord affects the therapeutic potential of neural stem cell transplantation for spinal cord injury. *Mol. Brain* 6, 3
63. Munoz-Elias, G. *et al.* (2004) Adult bone marrow stromal cells in the embryonic brain: engraftment, migration, differentiation, and long-term survival. *J. Neurosci.* 24, 4585–4595