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Novel approaches to treating sensorineural hearing loss. Auditory genetics and necessary factors for stem cell transplant

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Summary

Sensorineural hearing loss is a chronic disease, with a serious impact on human communication and quality of life. Exposure to various factors can lead to irreversible hearing impairment, as the auditory epithelium in humans comprises terminally differentiated cells. By contrast, the inner ear of lower vertebrates and invertebrates shows regenerative capacity. Efforts to regenerate the damaged human inner ear may involve renewed cell proliferation, or transplanting cells that can differentiate into sensory cells.
Literature review.
Animal studies, *in vitro* studies, retrospective-cohort studies, community-based case-controls, clinical guidelines, and review articles.
Embryonic stem cells, inner ear stem cells, and stem cells from other tissues (i.e., neural tissue, hematopoietic system) may be candidates for restoring the auditory epithelium. Transcriptional regulation of *p27kip1* is the primary determinant of terminal mitosis and the final number of postmitotic progenitors of hair and supporting cells. Basic helix-loop-helix transcription factor *Math1* was found to be necessary and sufficient for the production of auditory hair cells. *Notch* signaling seems to play a major role in the regulation of *Math1*, through lateral inhibition. *Brn3c*, *Gfi1*, and *Barhl1* are also specific transcription factors that have been implicated in hair cell maintenance and consequent survival.
Evidence concerning development, maintenance, and regeneration of hair cells is still at an embryonic stage. Combined data, as attempted in the present study, will lead to a more successful management of deafness.

key words: **inner ear • stem cells • embryonic • commitment • survival • *Math1***

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Background

Sensorineural hearing loss (SNHL) is one of the most prevalent chronic diseases with a serious impact on language development [1], human communication, and quality of life. Profound congenital SNHL, which is estimated to affect 1 to 2 of every 1000 newborns in western countries [2–7], or acquired SNHL that appears later in childhood with an overall incidence of 0.29% at the age of 15 [8], represent only a small proportion of people with serious hearing loss. Indeed, the overall prevalence of moderate to profound hearing loss for 2005 was estimated in approximately 4.3% of the human population [9]. Furthermore, about 10% of the adult population in a United Kingdom study reported bilateral hearing difficulty in a quiet environment [10], while moderate to profound hearing loss was estimated in approximately 3.5% of people aged 20 to 80 years in another study [11].

Exposure to a variety of factors, such as overstimulation by noise, ototoxic drugs, or infections, along with aging and/or potential genetic disorders, may lead to irreversible hearing impairment, as the auditory epithelium in humans comprises terminally differentiated cells. By contrast, the basilar papilla in the avian cochlea and the lateral line organs in fish and amphibians show a regenerative capacity, which is characterized by postembryonic production of specialized hearing receptors in a precisely controlled manner [12–18].

Based on investigations in olfactory neurons, which undergo permanent regeneration in humans also, it can be concluded that the basic mechanisms of neurogenesis are also established, and therefore may be applied, in the mammalian brain [19–22]. Furthermore, neuronal differentiation and morphologic integration of neural precursor cells also occur after transplant into the brain of animal models with neurodegenerative diseases (mimicking Alzheimer's, Huntington's, or Parkinson's diseases), or in highly specialized neural structures, such as the damaged retina [23–28].

Hence, on the basis of the evidence extracted from animal models, efforts to regenerate the damaged human inner ear may involve either renewed cell proliferation of the mitotically quiescent auditory epithelium or transplant of cells that can differentiate into the highly specialized sensory cells of that sensory epithelium. In this context, gene manipulation and stem cell therapy represent relatively new and exciting approaches for the treatment of SNHL. The milestones achieved in these fields during the last decades of the second millennium, have led the way to new research that promises "hearing" to patients who have lost it or never had it.

The aim of the present paper is to review the current knowledge on the various types of stem cells that can be used, and the manipulation of related genetic pathways that may be required, to restore cochlear function.

Material and Methods

An extensive literature search of the was performed in Medline and other available database sources using the key words "ENT," "inner ear," "stem cells," "precursor," "embryonic," "neural," "growth factor," "transcription factor," and "gap junction." The key word "stem cells" was considered primary and was either combined with each of the other key words individually, or used in groups of 3. Information from electronic links and related books also was included in the analysis of the data. In addition, reference lists from the retrieved articles were manually searched. Language restrictions limited the search to English-language articles only. The number of studies initially selected was 232.

Two main categories of outcomes were established: (1) classification of the main stem cell sources for the restoration of the auditory epithelium, and (2) recognition of the genetic pathways that play a key role in cell fate determination. The retrieved studies were critically appraised, according to evidence-based guidelines for categorizing medical studies (Tables 1–3) [29].

Using this qualitative framework, 2 secondary endpoints also were analyzed: identifying the factors implicated in hair cell differentiation and identifying the factors implicated in hair cell survival. As a result of these methods, the number of studies that were finally included in data synthesis was 114.

An area of difficulty regarding the research explored in this paper was that most of the surveys concerning auditory genetics and/or stem cell transplant investigate highly specific research areas. Therefore, the results obtained cannot be directly integrated into the broader spectrum of clinical hearing restoration. The ambitious objective of this pa-

Table 1. Evidence-based categorization of medical studies.

Category of evidence	Origin of evidence
Ia	Evidence from meta-analysis of randomised controlled trials
Ib	Evidence from at least 1 randomised controlled trial
IIa	Evidence from at least 1 controlled study without randomization
IIb	Evidence from at least 1 other type of quasi-experimental study
III	Evidence from nonexperimental descriptive studies, such as comparative studies, correlation studies, and case-control studies
IV	Evidence from expert committee reports or opinions or clinical experience of respected authorities, or both

Table 2. Study characteristics regarding stem cell transplant in the inner ear.

Type of study	Authors	Level of evidence	Type of stem cells	Type of mammals	Reported advantages	Reported disadvantages	Remarks
Prospective control [19]	Hildebrand et al., 2005	IIa	Embryonic	Deafened experimental/ normal hearing controls	a) minimal mechanical trauma b) high survival rate c) NEM cells in aggregations	No integration into endogenous tissue	a) highly successful results b) xenotransplant
Prospective control [21]	Kojima et al., 2004	IIa	Embryonic	Damaged experimental IEs/normal hearing controls	a) survival in the IE b) differentiation into stem cells c) integration with stem cells	a) endocochlear duct could not be approached b) aggregation of cells on bony walls	IEs provide important cues for survival & differentiation
Prospective [22]	Sakamoto et al., 2004	IIb	Embryonic	Damaged IEs	Differentiation of transplanted cells into ECT cells	Characteristics of undifferentiated cells remained	Unsuccessful attempt
Prospective control [23]	Coleman et al., 2006	IIa	Embryonic	Deafened	a) minimal mechanical trauma b) no inflammatory response c) survival of implanted cells	a) cells decrease after 4 weeks b) dispersal to CSF c) not significant cell count into the Rosenthal canal	The deafened cochlea environment can support the survival of exogenous tissue
Prospective control [30]	Hu et al., 2005	IIa	Neural	Deafened & NGN treated experimental/ normal hearing controls	a) better survival in deafened animals b) better neuronal differentiation in NGN animals c) migration toward functionally relevant sites d) minimal mechanical trauma	a) poor survival after 2 weeks b) dramatic decrease of surviving cells in 4 weeks	a) injection site is functionally irrelevant & lacks growth factors b) xenotransplant
Prospective [20]	Ito et al., 2001	IIb	Neural	Normal hearing	a) development of HC b) integration into the OC c) migrational capacity d) wide adaptation	a) not high HC count b) new HC do not express all HC features	NSC migrate/ differentiate over a wider area than expected
Prospective [33]	Tamura et al., 2004	IIb	Neural	Deafened	a) robust survival in all experimental animals b) migration activity into the modiolus	Differentiation predominantly into glial cells	Relatively poor neuronal differentiation
Prospective [38]	Naito et al., 2004	IIb	Bone-marrow	Damaged IEs	a) robust survival in multiple regions of the cochlea in all animals b) neuronal differentiation c) migrational capacity	Paucity of cells in the scala media	Perhaps more appropriate for SGN regeneration
Prospective [39]	Matsuoka et al., 2006	IIb	Bone-marrow	Normal hearing	Neuronal differentiation	a) low survival rate b) few surviving cells in the scala media	a) scala media is a hostile environment b) modular approach is more appropriate for SGN restoration c) xenotransplant

IE – inner ear; OC – organ of Corti; HC – hair cells; SC – supporting cells; ECT – ectodermal; NEM – neuroectodermal; SGN – spiral ganglion neurons; NGN – neurogenic, CSF – cerebrospinal fluid.

per was to fill the related gaps and give a realistic estimate of the current knowledge in this area. Thus, nonvertebrate, avian, mammalian, and human evidence were combined, taking into account the specific differences between species and the fact that some of them may produce conflicting or ambiguous evidence.

Table 3. Study characteristics regarding cell fate determination in the inner ear.

Type of study	Authors	Level of evidence	Type of Intervention	Type of mammals	Reported advantages	Reported disadvantages	Remarks
Prospective control [19]	Chen et al.	IIa	Targeted gene disruption	*Ink4d*-null mutants experimental/ normal controls	a) embryonic patterning of the OC occurs normally in the absence of Ink4d b) hearing is significantly compromised c) CKIs are important for active maintenance of the postmitotic state	CKIs are crucial for maintaining cellular homeostasis in postmitotic cell populations	a) recessive type of mutation b) annulment of the postmitotic state results in HC death
Prospective [22}	Lee et al.	IIb	Gene modification	*p27kip1*/BAC transgenic mice	a) *p27kip1* expression is regulated at the transcriptional level b) *p27kip1* expression precedes the wave of cell cycle exit c) *p27kip1* expression is responsible for the temporal separation between cell cycle exit and cell differentiation	The reason for establishing a fixed number of postmitotic progenitors within the cochlear duct is not clear	a) regulated transcription is partially responsible for the high level of *p27kip1* expression in stem cells b) the persistence of high *p27kip1* levels in stem cells may be an obstacle to regeneration
Prospective control [21]	Lowenheim et al.	IIa	Targeted gene disruption	*p27kip1* heterozygous experimental/ *p27kip1*-wild-type & *p27kip1*-null controls	a) *p27kip1*-deficient mice demonstrate true hyperplasia based on increased proliferation b) *p27kip1*-deficient mice demonstrate degeneration & loss of HCs c) substantially elevated ABR thresholds for null vs controls across the entire examined frequencies	None reported	a) *p27kip1* expression is confined to the stem cells b) the disruption of the *p27kip1* gene promotes cell division in the postnatal & adult OC well after terminal mitosis
Prospective control [23]	Lanford et al.	IIa	*In situ* hybridization	*Jag2* mutants experimental/ wild-type controls	a) initiation of *Math1* expression is independent of *Jag2*-dependent Notch signaling b) the number of cells that maintain *Math1* expression is greater in *Jag2* mutants c) *Jag2* deletion results in a dramatic down regulation of *Hes5*	a) specific molecular relation between *Hes5* & *Math1* has not been demonstrated b) the function of *Hes5* as a repressor of *Math1* transcription is speculative	a) notch signaling acts at an early time point to regulate the number of cells that differentiate as HCs b) *Hes5* is a downstream regulator of Notch signaling
Prospective control [25]	Kiernan et al.	IIa	a) conditional gene inactivation b) targeted gene disruption	*Dl1*hypomorphic *Jag2* mutants experimental/ heterozygous controls	a) *Dl1* function synergistically with *Jag2* during HC differentiation b) supernumerary HCs are present in double mutants c) supernumerary HCs are not arising through continued proliferation	a) stem cells exhibit abnormal proliferation b) supernumerary HCs may lead to HC disorganization	a) pillar cells may have unique stem cell-like properties b) cell fate switch from Deiters cells to OHCs

OC – organ of Corti; HC – hair cells; OHC – outer hair cells; SC – supporting cells; CKI – cyclin-dependent kinase inhibitor; Math1 – mouse atonal homologue 1; Hes5 – mammalian hairy & enhancer-of-split homologue 5; Jag2 – jagged 2; Dl1 – delta 1; BAC – bacterial artificial chromosome, ABR – auditory brainstem response.

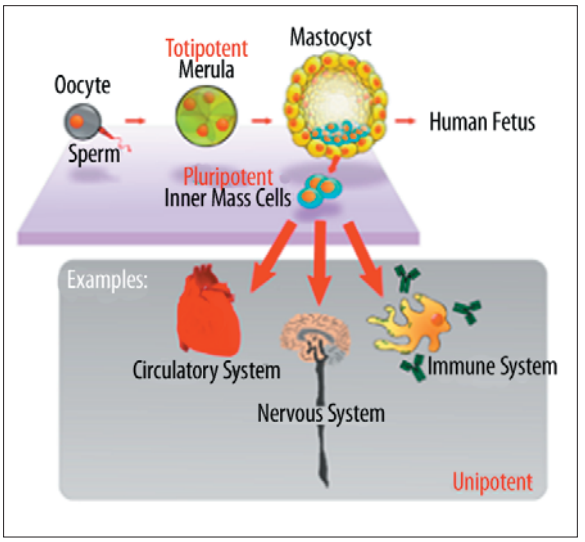

Figure 1. Pluripotency in the human fetus.

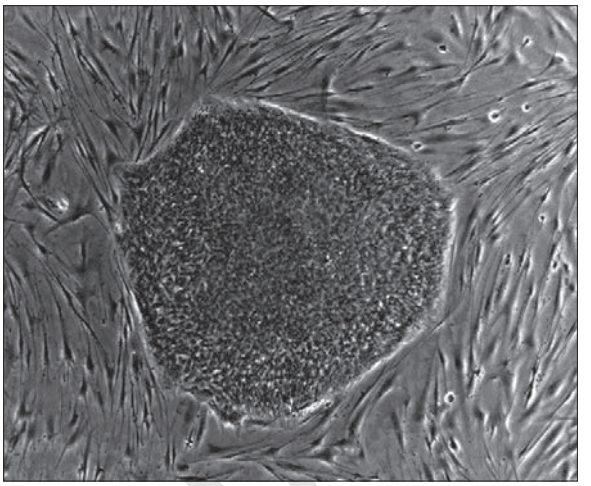

Figure 2. Human stem cells.

RESULTS

Seventy animal studies, 25 *in vitro* studies, 2 retrospective cohort studies, 2 community-based case controls, 1 clinical guideline, and 12 review articles met the defined criteria and were included in study selection.

Analysis of evidence

Potential stem cell types for inner ear restoration

Three main sources of stem cells may be considered as candidates for restoring the auditory epithelium: embryonic stem cells, stem cells and precursors isolated from the targeted organ (inner ear), and stem cells from other tissues (e.g., neural tissue or the hematopoietic system). In addition, a subset of stem cells called the *side population* has been identified in several mammalian tissues and is reportedly capable of differentiating into hair cell-like cells [30].

Embryonic stem cells (Estem cells)

Estem cells derived from the embryonic blastocyst are provided with the fundamental capacity of differentiating into all other cell types of the organism (pluripotency, Figure 1). This has been recently confirmed in practice by generation of inner ear progenitors from murine Estem cells *in vitro* [1]. However, the induction effect of Estem cells into ectodermal cells proved insufficient to ultimately induce formation of actual hair cells [31]. Nevertheless, transplanted Estem cells were found to survive in the damaged mammalian cochlea for at least 9 weeks; predifferentiation of these cells *in vitro* is a factor that may favorably influence the observed survival rate [32]. Furthermore, partially differentiated Estem cells also might assist the functional recovery of the spiral ganglion neurons (SGNs), if they are also affected by SNHL. It is well known that SGNs are dependent on factors secreted by sensory hair cells [33]. Thus, hair cell loss may result in a secondary degeneration of SGNs as a consequence of SNHL. However, the time range of secondary SGN degeneration is not yet known. In many cases of SNHL, spiral ganglion cells are intact in large numbers, even if there is a limited number of remaining hair cells.

Inner ear stem cells (IEstem cells)

Isolation of inner ear stem cells has been intensively pursued, as they seem most likely to differentiate more completely into hair cells, compared with other stem cell derivatives (Figure 2). However, the population of these cells may be different in mammal and nonvertebrate animal models. In birds, supporting cells within the sensory epithelia seem to be the cellular precursors for hair cell regeneration [34].

Two precursor cell populations with a regenerative potential are currently discussed. For inferior sensory epithelial damage, cuboidal or hyaline epithelial cells appear to serve as precursors for the regeneration of both hair cells and supporting cells. For the repair of isolated superior damage, supporting cells may be the effective precursor population [35]. With regard to fish, embryoniclike neuroepithelial cells, with elongated nuclei and processes extending basally and apically, have been identified as the immediate source of new hair cells and supporting cells. Basally located S-phase cells, with small nuclei and little surrounding cytoplasm, are thought to be Schwann cell precursors [36]. In the postembryonic fish inner ear, hair cell precursors and supporting cells are obviously closely related, if not the same cell type [37]. A large number of nonsensory supporting cells are capable of entering the cell cycle, after experimental elimination of the normal population of S-phase cells by antimitotic agents [16].

In mammals, adult IEstem cells were initially isolated from the sensory epithelium of the mouse utricle [38]. The unexpected regenerative capacity of mammalian hair cells previously reported [39,40], may thus be attributed to the presence of these stem cells. Recent findings not only concur with the regenerative potential of the mammalian inner ear, but further suggest that the mouse neonatal cochlea harbors cells capable of forming spheres [41], and cells from these spheres express genes that are indicative of inner ear progenitor cells [42,43].

Other sources of stem cells

Neural stem cells (Nstem cells)

Neural stem cells are multipotent progenitor cells, characterized through the potential of self-renewal [44] and a high

plasticity to differentiate into several neuronal cell types and other germ layer tissue-specific cell lineages [45]. Implanted Nstem cells have been shown to survive in mature cochleae of animal models and to migrate into functionally relevant regions after experimental damage to the inner ear [46,47]. However, the survival of these cells in the inner ear decreases dramatically after a relatively short period [46]. Moreover, the morphology of the implanted cells is also considered to be a critical issue. In this context, the well-established integration of transplanted Nstem cells into the organ of Corti of newborn rats, and the adoption of the morphologic phenotypes of outer or inner hair cells (as demonstrated by phalloidin labeling), represent promising results, with regard to the main objective of hair cell restoration [48].

In addition, *in vitro* studies using immature neural progenitors have also established the potential of the latter to differentiate into hair cell immunophenotypes, as was demonstrated by the expression of both hair cell markers Brn-3c and myosin VIIa [45].

Furthermore, NSC transplanting to the mammalian inner ear may be a source of SGN regeneration as mentioned before [49]. However, neuronal differentiation of the former is predominantly driven toward glial cell fate, rather than neurons [49].

Bone-marrow stem cells (BMstem cells)

BMstem cells have shown a decent plasticity [50,51] with the capacity to differentiate into a variety of specialized cells [50]. Indeed, neurons and glial cells have been identified in central nervous structures of rodent models and human patients after transplanting BMstem cells [52–54]. In addition, mesenchymal cells in the adult inner ear (fibrocytes) may be continually derived from hematopoietic stem cells [50]. Thus, the differentiation of autologous bone marrow cells in damaged cochleae, along with their survival capacity and migrational mobility, may be exploited for the treatment of various degenerative inner ear diseases [55]. However, because most of the transplanted cells eventually evolve into nonneuronal cells [52,53], additional studies are required to identify factors that promote the differentiation of BMstem cells into distinct neural cell types, and provide adequate numbers of cells that could actually enhance cochlear function.

The discovery of cells displaying neuronal phenotypes in the area around the spiral ganglion is also clinically important, and suggests that marrow cell transplanting could increase the number of SGNs [55].

Potential targets of gene modification for inner ear restoration

Developmental genetics of the auditory epithelium

The highly orchestrated processes that generate the vertebrate inner ear from the otic placode, and the strictly ordered patterned mosaic of sensory hair cells and nonsensory supporting cells in the mammalian auditory sensory epithelium, result in the presence of 4 rows of mechanosensory hair cells in the cochlea—a single row of inner hair cells (IHCs) and 3 rows of outer hair cells (OHCs) – and the separation of each hair cell from the next, by an interceding supporting cell (Figure 3). As the sound vibrations are transduced into the inner ear, the hair cells convert the motion of the cochlear fluids (traveling wave) into electrical signals, which are conducted along the auditory nerve toward the central nervous system. Inner hair cells are basically responsible for the auditory function, and provide the main neural output of the cochlea. Outer hair cells, on the other hand, amplify and sharpen the traveling wave, thus extending the hearing range and improving frequency discrimination, and facilitate the optimal function of IHCs.

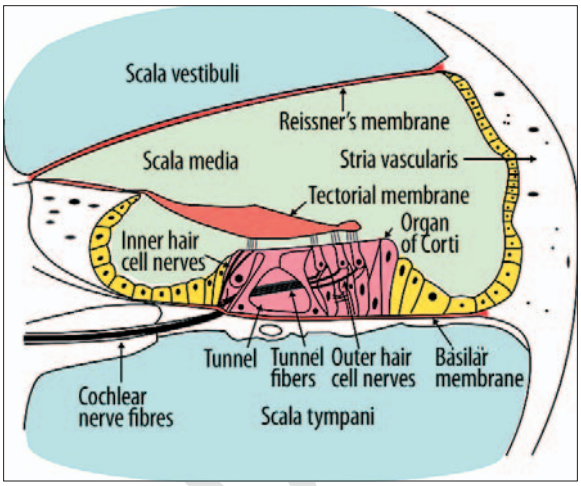

Figure 3. Inner ear structure.

The identification and consequent interpretation of the genetic pathways that play a key role in determining cell fates and cellular patterning in the cochlear mosaic might be a decisive step toward renewing the damaged epithelium.

Determining cell fate

Determining cell fate is the result of a cascade of events that includes commitment of the various cell lineages, exit from the mitotic cycle, and cell differentiation. Terminal mitosis and hair cell commitment are sequential and even partially overlapping events [56]. The former is triggered at the molecular level by the expression of specific cyclin-dependent kinase inhibitors [57], predominantly *p27kip1* and adjuvantly *ink4d*. Although these inhibitors regulate essentially redundant genetic pathways in the mature neurons, as the postmitotic state is maintained, regardless of which one is experimentally deleted [58], targeted deletion of *ink4d* in postnatal mice is sufficient to disrupt the maintenance of the postmitotic state of the organ of Corti. Thus, hair cells are observed to reenter the cell cycle and undergo apoptosis and death [58]. In addition, *p27kip1* expression is induced between embryonic days 12 and 14 in the primordial organ of Corti, correlating in this way with the cessation of cell division of hair and supporting cell progenitors [59]. Thus, the transcriptional regulation of *p27kip1* during inner ear development is the primary determinant of a wave of cell exit that dictates the number of postmitotic progenitors of both hair and supporting cells [60]. This wave reportedly advances in an apical to base direction [56].

In the adult organ of Corti, *p27kip1* expression persists at high levels in the supporting cell population [59,61]. As de-

letion of this gene leads to the observation of mitotically active cells in the postnatal period, its expression is thought to contribute to the maintenance of quiescence and possibly to the inability of regeneration [59]. Furthermore, age-dependent changes in the proliferative capacity of the supporting cells can be partially attributed to changes in the ability to down-regulate *p27kip1* [62]. With regard to hair cells, targeted deletion of the *p27kip1* gene in mice leads to the appearance of supernumerary hair cells (and supporting cells) [59] Interestingly, *p27kip1* heterozygotes only have additional IHCs [63].

It should be noted that *p27kip1* determines a specific territory within the inner ear prosensory domain – the region of the cochlear duct that will evolve into the organ of Corti – which Chen and Segil denominate as the zone of non-proliferation (ZNP). The entire cell population within the ZNP is also positive for the transcription factor *Sox2*, which actually precedes the appearance of *p27kip1*. Cells located within the ZNP begin to express the basic helix-loop-helix (bHLH) transcription factor *Math1*. *Sox2* seems to upstream of that expression [64]. *Math1*, which is encoded by the gene *Atoh1*, is specifically expressed in the developing auditory hair cells (AHCs) [63], and was found to be both necessary and sufficient for the production of hair cells in the mouse inner ear [65,66].

Indeed, it has been shown that absence of *Math1* in mice results in the complete disruption of the epithelial formation of the cochlea, including the development of hair cells and of the associated supporting cells. In addition, ectopic expression of *Math1* in nonsensory regions of the cochlea is sufficient to induce the formation of sensory clusters that contain both hair cells and supporting cells [67]. Overexpression of *Math1* in postnatal rat cochlear explant cultures also results in extra hair cells. The source of these ectopic hair cells was columnar epithelial cells located in the greater epithelial ridge (GER), which normally generate cells of the inner sulcus [65]. However, even though Chen and associates also report that in *Math1* mouse mutants, the generation of hair cells is blocked, the authors argue that *Math1* is not required for establishing the postmitotic sensory primordium, but instead, its role is limited to the selection and/or differentiation of sensory hair cells within the established primordium [68].

Whether the expression of *Math1* initially occurs in the cochlear duct and progressively becomes restricted to the hair cells of the sensory epithelium [69], or it is limited to a subpopulation of cells within the sensory primordium, which appear to differentiate exclusively into hair cells, as the sensory epithelium matures [68], is still not fully clarified. However, that expression appears to be negatively influenced by the bHLH genes *Hes1* and *Hes5* (mammalian Hairy and Enhancer-of-Split homologues) [69–71].

Hes1 is widely expressed in the GER and in the lesser epithelial ridge (LER), while *Hes5* is predominantly expressed in the LER, in a narrow band of cells within the GER, and in supporting cells [69–71]. Targeted deletion of *Hes1* leads to the formation of supernumerary hair cells in the cochlea [71], specifically IHCs [70], whereas cochleae from *Hes5* mutant mice show a significant increase in the number of OHCs [70].

The regulation of *Math1* expression in the developing cochlea is also influenced by other bHLH-related proteins that inhibit differentiation and DNA binding (*Ids*) [72,73]. In particular, the expression of *Ids* and *Math1* overlap in cochlear progenitor cells before cellular differentiation, and a specific down-regulation of *Id* expression was observed in individual cells that differentiated as hair cells. Meanwhile, the progenitor cells, in which the expression of *Ids* was maintained during the time period for hair cell differentiation, were inhibited from developing as hair cells [72].

The *Notch* signaling cascade seems to play a major role in tying together the above-mentioned bHLH positive and negative transcription factors, through inhibitory interactions between adjacent progenitor cells (lateral inhibition). *Notch* signaling defines an evolutionary ancient cell interaction mechanism, in which signals exchanged between neighboring cells through the *Notch* receptor can amplify and consolidate molecular differences, which eventually dictate cell fates [74]. Recent studies, mostly based on loss-of-function experiments that target the role of *Notch* signaling and bHLH genes in inner ear development, have indicated that they can regulate hair cell fate specification and their initial differentiation [75].

The *Notch* signaling components that seem to play a role during cochlear morphogenesis and hair cell differentiation include the *Notch1* receptor, the *Notch* ligands *Jagged1* (*Jag1*) (or *Serrate1*), *Jagged2* (*Jag2*) and *Delta1* (*Dl1*), and the bHLH transcription factors *Hes1* and *Hes5*. These components seem to intersect in various levels of the lateral inhibition; for instance, the activation of *Notch*, via *Jag2*, inhibits the expression of *Math1* in cochlear progenitor cells, possibly through the activity of *Hes5* [69].

With regard to the *Notch1* receptor, experiments conducted in mice suggest that its activation may initially demarcate a prosensory region in the cochlear epithelium, and then inhibit progenitor cells from becoming hair cells, via classic lateral inhibition [76]. Post-hatch chicks, on the other hand, show *Notch1* expression, which is limited to the supporting cells of the quiescent auditory epithelium; however, that expression is increased in postmitotic cell pairs, during hair cell regeneration [14].

A positive feedback loop appears to bind the expression of activated *Notch1* and *Jag1* [76]. Furthermore, a decrease of either *Notch1* or *Jag1* expression, by antisense oligonucleotides in cultures of developing mammalian sensory epithelium, results in an increase in the number of hair cells [77].

In general, *Jag1*-mediated *Notch* signaling is considered essential for establishing the prosensory regions during early development of the mammalian inner ear, possibly by maintaining the normal expression levels of both *p27kip1* and *Sox2*, because *Jag1* mutant mice show partial sensory development in the cochlea and utricle, and complete lack of cristae [78]. The expression of *Jag1* becomes progressively restricted to the supporting cells of each sensory patch [79]. *Jag1* is also an early marker in the chick inner ear, during the diversification of sensory epithelial cells into a mixed population of hair and supporting cells [80]; nevertheless, its expression is not altered, during the course of drug-induced hair cell regeneration [14].

The pivotal role of the *Notch* signaling pathway in the differentiation of hair and supporting cells, however, is mainly maintained through the ligands *Jag2* and *Dl1* [78]. Thus, genetic deletion of *Jag2* leads to 2 rows of IHCs and 4 rows of OHCs [81]. *Dl1,* on the other hand, is expressed in nascent hair cells, disappearing as they mature, and may act at each developmental branchpoint to drive neighboring cells, along different developmental pathways [80]. Findings in zebra fish mutant embryos indicate that this action is mediated through a RING ubiquitin ligase, encoded in the *mib* gene, which interacts with the intracellular domain of *Dl1* [82]. *Dl1* expression is absent in the mitotically quiescent avian basilar papilla. Following hair cell injury, however, *Dl1* mRNA levels are elevated in progenitor cells during DNA synthesis, and that expression is maintained in both daughter cells immediately after mitosis. *Dl1* expression remains up-regulated only in differentiating hair cells and returns to normal 10 days after the initial injury [14].

There is also evidence that *Dl1* and *Jag2* function synergistically to regulate hair cell differentiation in the cochlea, probably through the Notch1 receptor. Furthermore, the supernumerary hair cells in *Dl1/Jag2* double mutants seem to arise primarily through a switch in cell fate, rather than through excess proliferation [83].

Finally, the fact that the activated Notch inhibited neuronal differentiation in the wild-type, *Hes1*-null, and *Hes5*-null mouse embryos, but not in the *Hes1-Hes5* double-null background, demonstrated that the bHLH transcription factors *Hes1* and *Hes5* serve as essential Notch effectors in the regulation of neuronal differentiation [84].

Another signaling pathway, which has been identified to play a role in promoting hair cell differentiation within the developing sensory epithelia, includes the bone morphogenetic proteins (*BMPs*), which are members of the transforming growth factor beta (*TGF-β*) gene family [85]. Two *BMPs, BPM4* and *BMP7,* seem to play a role during cochlear patterning. Although *BMP4* homozygous null mice die as embryos, they are viable in the heterozygous state, and exhibit structural and functional deficits in the inner ear [86]. However, *BMP4* is not considered to sufficiently induce the development of the auditory epithelium [87]. In the developing avian auditory organs, *BMP7* gene expression becomes restricted to the sensory tissue over time and is eventually concentrated in supporting cells, whereas *BMP4* gene expression is localized in the hair cells [88]. It has been proposed that at the stage of terminal division, the balance between *BMP* and *BMP*-inhibitory signals regulates survival and specification of hair cell precursors [89], and may thus be implicated in inducing the switch from proliferative sensory epithelium progenitors to differentiating epithelial cells [85]. In addition, excess levels of *BMPs* may limit the final number of sensory hair cells [89]. Therefore, an autoregulatory loop seems to exist, between *BMP4* and its antagonists (namely noggin) [89]. In support of that, hair and supporting cell generation was remarkably reduced, when *BMP* signaling was blocked either with noggin, or by using soluble *BMP* receptors. However, an increase in the number of hair cells was observed, when cultured avian otocysts were treated with exogenous *BMP4* [85]. Interestingly, the latter also led to the reduced proliferation of progenitor cells [85].

The *BMP* signaling activities also may integrate with neural cell responses to the Sonic hedgehog (*Shh*) protein [90], a key regulator of vertebrate organogenesis, which is also essential during development for auditory cell fate determination and inner ear dorsal/ventral patterning. *Shh* accelerates the proliferation of inner ear progenitor cells, and actually increases the number of hair cells in cultures of inner ear progenitor cells [91]. Thus, *Shh* may serve as a regulator of inner ear progenitor growth and hair cell generation.

Other data also suggest that fibroblast growth factor receptor 1 (*Fgfr1*) might have a distinct later role in intercellular signaling, within the differentiating auditory sensory epithelium. Indeed, loss-of-function *Fgfr1* mutations in mice caused dose-dependent disruption in the organ of Corti. Full inactivation of *Fgfr1* in the inner ear epithelium, by *Foxg1-Cre*-mediated deletion, led to an 85% reduction in the number of AHCs, possibly due to the reduced proliferation of cell precursors in the early cochlear duct [92].

Fgf signaling also plays a critical role in the commitment and differentiation of supporting cells, and is required for inner ear morphogenesis [93–95]. Results indicate that pillar cell differentiation is, in fact, dependent on the continuous activation of *Fgfr3*. Moreover, transient inhibition of *Fgfr3* does not permanently inhibit pillar cell fate, because *Fgfr3* reactivation results in the resumption of pillar cell differentiation [96]. The position of pillar cells appears to be determined by the activation of *Fgfr3* in a subset of progenitor cells that initially express this receptor [96]. Thus, the inner pillar cell never develops in *Fgfr3* mutant mice, while the outer pillar cell is stalled in its differentiation [97].

In addition to controlling fate decision between pillar cells and Deiters' cells, *Fgfr3* also regulates the width of the sensory epithelium; thus, an extra row of OHCs, and accompanying Deiters' cells, is found in the apical two-thirds of the organ of Corti, in the *Fgfr3* mutant [97]. Moreover, experiments in the avian basilar papilla indicate that Fgfr3 expression changes in an opposite way to that found in the mammalian cochlea, after drug-induced hair cell damage, and this may be involved in regulating the proliferation of supporting cells [98].

Other results in posthatch chicks suggest that *Fgf2,* which is a strong ligand for several *Fgf* receptors, may be involved in inhibiting cell proliferation, or stimulating precursor cell differentiation [99]. Furthermore, by exposing cochlear explants to *Fgf2,* a significant increase in the number of pillar cells and a small increase in the number of IHCs were observed. The fact that these results were not dependent on cellular proliferation indicates that the additional pillar and inner hair cells were a result of increased recruitment into the prosensory domain [96].

Hair cell survival

Following hair cell commitment and differentiation, the survival of hair cells is an essential requirement during cochlear development. Hair cell maintenance and preservation of the ionic equilibrium in the inner ear are key elements during this process.

Three specific transcription factors have been implicated in hair cell maintenance.

The *Pou*-domain class-IV transcription factor *Brn3c* (*Pou4f3*) is found in the auditory and vestibular hair cells and is required for proper development of the inner ear [100,101]. Mutation, or targeted deletion, of this gene in mice models results in complete deafness, which is attributed to a lack of hair cells in the inner ear with subsequent loss of cochlear and vestibular ganglia [101,102]. However, there appears to be no effect of *Brn3c* haplo-insufficiency on the mouse cochlea, implying that 1 intact copy of the gene is sufficient to maintain a normal cochlea [100]. Based on the observation that *Brn3c* proved capable of activating both brain-derived neurotrophic factor (*BDNF*) and neurotrophin-3 (*NT-3*) promoters in sensory epithelial lines of the inner ear, it has been suggested that 1 of the actions of *Brn3c* might be to regulate neurotrophin gene expression in the inner ear [103]. That expression is considered to be important for the development of the neuronal components of the inner ear [104].

Nevertheless, even limited expression of NTs in the cochlea of *Brn3c* null mice seems sufficient to sustain several sensory neurons (46%) until birth, despite the severe loss of hair cells (1% of the normal population) [105].

A comparison of inner ear gene expression profiles in wild-type and *Brn3c* mutant mice on embryonic day 16.5 revealed that *Brn3c* is also likely to down-regulate, *in vivo*, the gene that encodes the growth factor of independence 1 (*Gfi1*). This can be further validated by the fact that *Brn3c* deficiency leads to a statistically significant reduction of *Gfi1* expression levels, and that the pattern of expression for *Brn3c* correlates to the dynamics of *Gfi1* mRNA abundance [106].

Gfi1 is a transcriptional repressor expressed in the developing nervous system, which is also implicated in inner ear development [107,108]. Its properties in the inner ear are based on domain-dependent, cell-type-specific functions [107].

Although *Gfi1*-deficient mice initially specify inner ear hair cells, these cells are disorganized in the cochlea (and the vestibule). In addition, the OHCs are improperly innervated, and express neuronal markers that are not normally found in these cells. Thus, *Gfi1* seems to be required for hair cell survival, and its deficiency leads to the loss of all cochlear hair cells, just prior to, and soon after, birth, through programmed cell death [108].

The third transcription factor that is implicated in hair cell maintenance is *Barhl1*, a mouse homologue of the Drosophila *BarH* homeobox genes. *Barhl1* is expressed in all sensory hair cells 2 days after the onset of hair cell generation. The loss of *Barhl1* function in mice results in age-related, progressive degeneration of both inner and outer hair cells in the organ of Corti, following 2 reciprocal longitudinal gradients. In detail, OHCs are completely vanished from the apical and middle turn of the cochlea by postembryonic day 59, at a time point when IHCs appear normal in these areas. However, the latter also degenerate by postembryonic day 300, ultimately leading in severe to profound hearing loss. The fact that *Barhl1* mutant mice actually have relatively good hearing at the age of 3 months (which is far before day 300) indicates that *Barhl1* has a rather exclusive role in the long-term maintenance of cochlear hair cells [109].

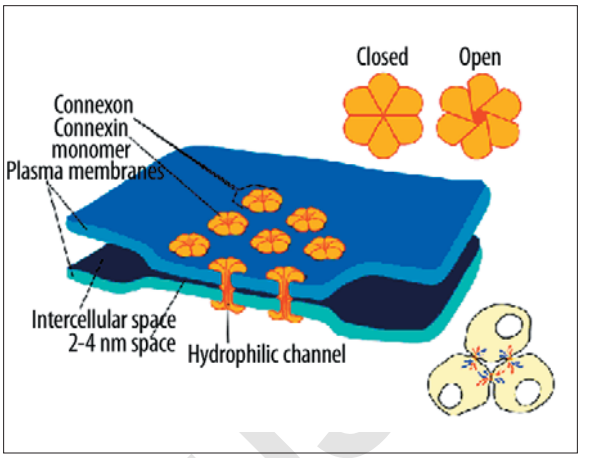

Figure 4. Schematic structure of mammalian gap junctions.

Intercellular communication through gap junctions, on the other hand, plays a major role with regard to maintaining the ionic equilibrium, and, consequently, the proper function of the inner ear. This is possibly achieved through the rapid removal of ions from the sensory cell region during transduction, which is essential for restoring the endocochlear potential. In addition, separate medial and lateral buffering compartments seem to exist in the hearing cochlea, which are individually dedicated to the homeostasis of the inner and outer hair cells [110].

Recent evidence is also supportive of the appearance of signal-selective gap junctions around the onset of hearing, with specific properties that are required to support auditory function [110]. This evidence also suggests that connexin 26 (*Cx26*) and *Cx30* are the major constituent proteins of the cochlear gap junction channels, possibly in a unique heteromeric configuration [110]. Furthermore, fluorescence recovery after photobleaching in the avian inner ear revealed asymmetric communication pathways among supporting cells in the basilar papilla, suggesting that this communication might mediate potassium cycling, and/or intercellular signaling (Figure 4) [111].

Cx26 is the predominant connexin isoform in the organ of Corti. Even though the *Cx26*-containing epithelial gap network is considered essential for cochlear function and cell survival, the determination of its exact role in the inner ear is difficult, because of the embryonic lethality of the *Cx26* knockout mice. However, this obstacle has finally been overcome by generating either homozygous *Cx26* mutant mice (through targeted ablation of *Cx26* [OtogCre]) [112] or transgenic mice (which express a mutant *Cx26* that inhibits the gap channel function of the coexpressed normal *Cx26* in a dominant-negative fashion [R75W]) [113].

The OtogCre mice initially showed normal development of the inner ear; however, on postnatal day 14, cell death appeared and eventually extended to the cochlear epithelial network and sensory hair cells. Cell death primarily affected only the supporting cells, suggesting that it could be triggered by IHC response to sound stimulation [112]. The R75W mouse model, on the other hand, showed deformity of the supporting hair cells, failure in the formation of the tunnel of Corti, degeneration of the sensory

hair cells, and consequent severe to profound hearing loss. Nevertheless, the high resting potential of the cochlear endolymph, which is essential for hair cell excitation, was normally sustained. Therefore, it was concluded that the homeostasis of the cortilymph, and not of the endolymph, was disturbed from the impaired potassium transport by the supporting cells [113].

By contrast, even though homozygous *Cx30* mutant mice also show severe hearing impairment, they lack a difference in the electrical potential, between the endolymphatic and perilymphatic compartments of the cochlea. In addition, the cochlear sensory epithelium in these mice starts to degenerate after postnatal day 18, by cell apoptosis. Hence, *Cx30* seems to play a critical role in generating the endocochlear potential, and in the survival of the AHCs after the onset of hearing [114].

CONCLUSIONS

Taking into account the devastating results of deafness to humans, efforts to regenerate the damaged inner ear and restore hearing remain one of the most challenging objectives in the third millennium. This may be accomplished either by stem cell transplant, or renewed cell proliferation of the mitotically quiescent auditory epithelium.

The generation of inner ear progenitors from murine embryonic stem cells *in vitro* and the survival, migrational activity, and integration of neural stem cells in the mammalian cochlea have yielded promising results about their successful use. Moreover, the functional relevance of inner ear stem cells to the cochlear structure and the autologous nature of bone-marrow stem cells, which theoretically bypasses potential immune barriers, give additional options to the related transplant armamentarium.

Transcriptional regulation of *p27kip1* is the primary determinant of terminal mitosis and the final number of postmitotic progenitors of hair and supporting cells. *Notch* signaling plays a pivotal role in the differentiation of these cells, by tying together basic helix-loop-helix positive and negative transcription factors through lateral inhibition. Other signaling pathways, such as *BMP* and *Fgf*, are also associated with the commitment and differentiation of hair and supporting cells, respectively.

Basic helix-loop-helix transcription factor *Math1* was found to be both necessary and sufficient for the production of auditory hair cells. In addition, *Brn3c*, *Gfi1*, and *Barhl1* are specific transcription factors, which have been implicated in hair cell maintenance and consequent survival, and intercellular communication through *connexins* plays a major role in maintaining the ionic equilibrium of the cochlear compartments, and the proper function of the inner ear.

The evidence presented in this paper suggests that development, maintenance, and regeneration of mammalian cochlear hair cells are feasible, although they remain at an early stage. However, mounting data, derived from numerous experimental and clinical surveys, approach a tremendous bulk, at a pace that the human mind may find difficult to conceive. Therefore, systematic reviews, such as the present one, are very important at certain stages of research, to combine the recent advances, regarding auditory genetics and stem cell transplant, and lead the way to more successful management of deafness in terms of either preventing, or restoring hearing.

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