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Regenerative medicine in hearing recovery

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Abstract

Hearing loss, or deafness, affects 360 million people worldwide of which about 32 million are children. Deafness is irreversible when it involves sensory hair cell death because the regenerative ability of these cells is lost in mammals after embryo development. The therapeutic strategies for deafness include hearing aids and/or implantable devices. However, not all patients are eligible or truly benefit from these medical devices. Regenerative medicine based on stem cell application could play a role in both improvement of extant medical devices and *in vivo* recovery of auditory function by regeneration of inner ear cells and neurons. A review of recent literature on the subject indicates that two promising approaches to renewal and differentiation of cochlear tissues are transplantation of stem cells and *in situ* administration of growth factors. Rather than directly regenerating dead cells, these procedures apparently induce, through various pathways, differentiation of resident cochlear cells. More studies on the possible adverse effects of transplanted cells and the recovery of tonotopic sensorineural activity or required. To date, no reliable clinical results have been obtained in the field of cochlear regeneration.

Key Words: *Cochlear stem cells, endogenous stem cells, growth factor supplements, hearing loss, hearing recovery, inner ear, stem cell transplantation*

Introduction

Worldwide, the majority of hearing disabilities (approximately 90%) result from the death of sensory cells, either hair cells (HCs) or spiral ganglion neurons (SGNs), thus leading to sensorial and/or neural hearing loss (SNHL). The etiology of SNHL includes mainly ototoxicity, deafening noise and presbyacusis [1,2]. Impairment due to SNHL has significant social and economic impact because it affects the ability to interact with people and the surrounding environment and, when it occurs early in life, causes language development delays and social integration problems [3,4].

The cochlear implant is the unique surgical option for people with severe-to-profound SNHL. This electronic high-technology device transforms sound waves into an electric stimulus, bypassing the damaged cochlear cells to directly stimulate the acoustic nerve. However, even with recent advances in engineering, surgery and pharmaceutical treatments that have improved the efficacy of cochlear implants and reduced electrode insertion trauma, normal auditory function cannot be completely restored [5,6]. New therapeutic strategies based on molecular, cellular and nanotechnological tools are aimed at regenerating and/or preserving sensory cells in the cochlea, contributing to improved cochlear implant outcomes [7–9]. Studies on nanotechnological tools involve the development of nanostructured electrodes and the improvement of drug delivery systems based on nanoparticles [10–12]. Regenerative medicine, an intriguing therapeutic strategy, has been successful in several research and clinical fields, such as dermatology, cardiovascular medicine and orthopedics [13]. Among regenerative medicine strategies, the use of stem cells (SCs) to restore damaged tissues is one of the most studied cell-based applications [14]. In otology, for example, SCs transplanted on synthetic scaffolds have recently been applied in tissue engineering for reconstruction of the human auricle [15,16]. The aim of SC-based therapy in SNHL is to replace lost HCs or SGNs, and the major challenge is to achieve this without affecting the complex cytoarchitecture of the cochlea and any residual hearing function [17,18].

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This review highlights the state of the art in regenerative medicine of inner-ear cells, discussing recent applications in hearing disabilities. Two general approaches are commonly used for tissue regeneration based on SCs: tissue-resident SCs or transplanted allogeneic SCs. The review focuses first on cochlear-resident putative SCs and then on *in vitro* production of HCs and SGNs. Finally, it considers *in vivo* tissue regeneration by transplantation of allogeneic SCs and/or growth factor supplementation for neuronal regrowth and protection.

Cochlear SCs

The identification of neural SCs (NSCs) in the adult central nervous system [19] and the well-known capability of injured non-mammalian adult vertebrates to restore damaged auditory sensory epithelium [20] encouraged researchers to investigate whether SCs could also be located in the mammalian inner ear and whether their regenerative ability could also be exploited in mammalian tissues.

In inner ear, a small number of SCs were isolated from adult mouse utricles, amounting to 0.025% of utricular cells [21]. Later, several studies identified and isolated SCs from mammalian cochlear sensory epithelium, spiral ganglion and stria vascularis of early postnatal mice, rats, guinea pigs and human fetuses [22–26]. These cells form self-renewing spheres in non-adherent cultures, but this ability weakens after several *ex vivo* passages [27]. When isolated from the cochlear sensory epithelium, these SCs are also able to differentiate into inner ear cell lineages, such as HC-like cells and sensory neurons, and into mature neurons and glia cells when isolated from the spiral ganglion [28,29].

In mice, the ability of sensory epithelial SCs to form spheres decreases about 100-fold during the second and third postnatal week, together with the expression of developmental and progenitor cell markers in the cochlea [21]. In contrast, utricle SCs maintain these characteristics into adulthood [20,21]. There is only one report concerning isolation of NSCs from adult human and guinea pig spiral ganglion [30].

Over the past few years, there has been evidence indicating that postnatal cochlear supporting cells also maintain SC-like characteristics in mammals [31] and are able to divide and trans-differentiate *in vitro* into HCs [32]. These abilities decline with age [33]: in cochlear SCs forming spheres, this decline is partially caused by changes in the expression of the cyclin-dependent kinase inhibitor (Cdkn1b), which plays a central role in regulating cell proliferation. After HC formation, the expression of Cdkn1b in the organ of Corti is restricted to non-sensory cells, preventing further divisions of HCs [33,34]. Moreover, p27-deficient mice exhibit hearing damage due to the over-proliferation and irregular positioning of both hair and supporting cells [35]. Another key gene involved in HC differentiation is *ATOH1* (atonal bHLH transcription factor 1), also known as *MATH1* (mouse atonal homolog 1) [36,37]. It has been shown that transfection of *ATOH1 in vivo* after acoustic trauma induces its over-expression in supporting cells, promoting their differentiation in HCs and hearing recovery [36,37]. A known marker of adult SC, Lgr5, has been used to verify that supporting cells are the progenitors of HC: $Lgr5^+$ supporting cells isolated from neonatal mice were able to form self-renewing neurospheres and differentiate into $myo7a^+$ HCs both *in vitro* and *in vivo* [38]. These observations are supported by those obtained on avian models, where adult supporting cells maintain their ability to differentiate into HCs, replacing them after a cochlear damage [39,40]. Other authors maintain that putative cochlear SCs may derive from the mesenchymal SCs (MSCs) reservoir located in the inner ear stroma underlying the epithelial tissue [41]. Although it remains unclear, some studies have shown that MSCs are able to differentiate *in vitro* in both HC-like cells and neurons [42,43].

In vitro regeneration by SCs

Transplantation of SCs to restore damaged inner ear cells and restore hearing function is an emerging field of research because mammalian HCs and SGNs are unable to regenerate after cell death resulting from trauma, disease or genetic mutation [7,44].

HCs and SGNs have been obtained *in vitro* from several types of SCs, including bone marrow MSCs (BM-MSCs), adipose-derived MSC (ASCs), olfactory precursor cells, embryonic SCs and adult brain germinal zone–derived cells (NSCs) from mice, rats and humans [45–52]. Despite these data, the ability of MSCs to differentiate into HCs and neurons is still controversial. Induced pluripotent SCs (iPSs) from mice were induced to become otic progenitors by exposure to growth factors, producing functionally active HCs [53]. Several authors investigated the possibility of obtaining new HCs and SGNs from endogenous sources and from putative cochlea-resident SCs. New functional sensory epithelia were obtained from endogenous avian inner ear cells by mesenchymal-to-epithelial transition after several culture freezing and expansion cycles, without co-culture with other tissues [54]. Isolated SGNs have been shown to able to survive *in vitro*, forming synapses with other neurons and HC in co-culture [48]. In *in vitro* co-cultures, also the neural progenitors derived from embryonic SCs were able to regenerate the complex neural network of the inner ear by producing neurites (positive to synaptic markers) elongating toward HCs [43,47,55,56]. These *in vitro*

data were supported by *in vivo* studies of embryonic SC transplantation after cochlea denervation [42,47,55–59].

In vivo regeneration by SCs

Cochlea-resident endogenous SCs may represent the main source for inner ear cell repair, but no evidence of their ability to generate new HCs or neurons as physiological responses to auditory injuries is currently available. The use of exogenous sources for SC transplantation in SNHL has been established, however. Several studies have investigated transplantation of SCs isolated from various tissues into the damaged cochlea to increase HC and/or SGN number and function, thus restoring auditory capability. In some of these studies, NSCs or *in vitro* neural-differentiated exogenous SCs (ExSCs) were transplanted via topic administration into cochleae with pharmacological or physical damage; results indicated survival of injected cells, an increased number of target cells, partial SC differentiation into neurons and/or glial cells, and, in some cases, improved hearing function [60–65]. Sakamoto *et al.* [66] transplanted undifferentiated mouse ExSCs into neomycin-treated mouse and embryonic chicken inner ears. Some ExSCs developed into ectoderm cells but not into HCs in mice, and in chickens, these cells were driven into neural crest cells, next to the otic vesicles. However, Zheng *et al.* [67] isolated cochlear SCs from newborn rats and transplanted them into the scala tympani of gentamicin-deafened rats, showing a migration of transplanted HC-like cells from the injection site to the basal membrane and organ of Corti with hearing recovery. Other studies showed that transplanted SCs could survive at the injection site, regardless of the presence of an external acoustic damage [68–70]. Kasagi *et al.* [68] investigated the ability of young and old mice to accept mouse BM-MSC transplantation into the perilymph, showing a positive engraftment only in young individuals, with no adverse effects on auditory brainstem response (ABR) test in both groups. The recovery of auditory function in mice with age-related hearing loss by transplantation of human adult olfactory SCs into the cochlea was investigated by Pandit *et al.* [69], who found that transplanted cells could survive in cochlear tissue and improve hearing function, although they did not integrate in the tissue [69]. Embryonic rat NSCs transfected with *ATOH1* gene were transplanted into normal guinea pig cochleae by Han *et al.* [70]. All NSCs survived; approximately 10% differentiated into HCs in the cochlear epithelium and more than 10% into neurons in the endolymphatic space.

Another target for SC therapy in SNHL is the cochlear lateral wall because hearing loss is also known to be caused by mutations of genes encoding for gap-junction proteins [71–75]. These molecular alterations have been studied in the stria vascularis [71], in otic fibrocytes [72] and in age-related or damage-induced fibrocyte degeneration in the spiral ligament [73,74]. Kasagi *et al.* [68] and Kamiya *et al.* [75] reported transplanted rat BM-MSCs in the perilymph of a SNHL rat model with a lateral wall fibrocyte dysfunction not associated with changes in the organ of Corti. Transplanted cells found in the injured area could express connexin 26 and connexin 30, indicating a reactivation of gap junction between neighboring cells. Moreover, the transplanted rat group showed a higher hearing recovery ratio than controls [75]. Other studies have shown that intravenously transplanted BM-MSCs and hematopoietic SCs (HSCs) in mice were able to integrate in the cochlea and differentiate into mesenchymal cells, including fibrocytes, suggesting a continuous turnover from the HSC reservoir [76].

Despite these encouraging results in SC transplantation for SNHL therapy, several issues are still unresolved. First, only a few studies have evaluated the functional recovery associated to the engraftment of transplanted SCs and the repopulation of damaged tissues, and in some of theses, there were no significant differences compared with control groups [61,77]. These results could be partially ascribed to the insufficient formation of functional synapses between the new HC population and the surviving neurons [61,77]. On the basis of SC transplantation studies, cell regeneration appears unrelated to a direct ExSC differentiation into the depleted cell population, but rather to damage recover by activation of the reservoir of unaffected endogenous SCs through release of cytokines and growth factors by ExSCs [78–80]. Because the use of ExSCs and NSCs entails relevant issues concerning ethics and availability, some studies investigated the use of MSCs or SCs from other sources for inner ear cell therapy, as previously described. Mesenchymal and epithelial SCs have been shown to be recruited, survive and engraft into the injured area, in some cases expressing specific differentiation markers [75,77,81]. Cho *et al.* [82] used human BM-MSCs *in vitro* neural-differentiated to treat a guinea pig animal model with ouabain-induced auditory neuropathy. By injecting BM-MSCs in the scala tympani, they showed an increase in SGN number, some of them human-derived, associated with the improvement of hearing function [82]. Similar results were obtained by human embryonic SCs induced to differentiate *in vitro* into hair cells and auditory neurons: when transplanted into an auditory neuropathy model, these cells significantly improved hearing recovery [50]. Other authors used HSCs to treat HC death after transient cochlear ischemia in gerbils. These cells prevented HC degeneration and improved hearing function but did not transdifferentiate or fuse with endogenous cells, suggesting a paracrine

effect as the mechanism of action in HC repair [82]. Revoltella *et al.* [17] transplanted human umbilical cord blood (UCB) CD133$^+$ hematopoietic SCs intravenously into oto-injured mice and observed the recruitment of these ExSCs to the damaged ear tissue, morphologic recovery of the organ of Corti and a small number of heterokaryons, probably derived from fusion events between donor and endogenous cells. These heterokaryons could play an active role in the repair process [17]. Based on this study, a phase 1/2 clinical trial was initiated in 2014 in the United States on children with acquired hearing loss, who received a single dose of autologous UCB SCs via intravenous infusion (ClinicalTrials.gov identifier: NCT02038972). The clinical trial is still in process, but the main expected outcome is an evaluation of safety and feasibility of UCB transplantation, possibly with improvement of inner ear function, audition and language development as secondary effect [83].

In vivo regeneration by growth factors

Another strategy for HC regeneration involves the administration of growth factors, which may protect from degeneration and/or restore HCs and SGNs. The growth factors most suitable for this task are the neurotrophic factors (NTFs), especially neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF).

During embryonic development in rats and mice, NT-3 was expressed in supporting cells at higher levels than in HCs [84]; after birth, it was higher in HCs but generally declined postnatally, although a partial expression remained in adult mice, mainly in inner HCs, but also in SGNs, glia and supporting cells [85,86]. NT-3 follows two expression gradients, one along the tonotopic axis (highest in the cochlear base, lowest in the apex), the other from the inner to the outer areas (higher in inner HCs, lowest in outer HCs) [86]. BDNF was also expressed in the organ of Corti during the embryonic development in rats and mice, but, in contrast to NT-3, its expression was highest in the cochlear apex, lowest at the base and equal in inner and outer HCs [87].

The BDNF expression declined prenatally and was at very low levels at postnatal day 1 (P1) but transitorily increased between P4 and P9. BDNF mRNA was also found in HCs in adult mice [87], although at low levels. Other neurotrophins were found to be expressed in the cochlea; for example, in HCs of adult mice, the mRNA levels of glial-derived neurotrophic factor (GDNF) was higher than those of NT-3, and ciliary neurotrophic factor (CNTF) was also expressed in the spiral ganglion [88]. Concerning their receptors (tropomyosin receptor kinases, Trk), the TrkB (a receptor of BDNF) and TrkC (receptor of NT-3) were shown to still be expressed in the postnatal SGNs, increasing neuronal metabolism and survival and promoting neurite outgrowth [89]. On the basis of these results, other groups investigated the ability of NTF to repair damages in the organ of Corti by cell preservation and neural network regeneration, thus preventing further degeneration and restoring the auditive function. The *in vitro* and *in vivo* treatment with NTF, supplemented with CNTF, GDNF, insulin growth factor-1 (IGF-1) or macrophage migration inhibitory factor, was able to promote SGN survival, neurite outgrowth, synaptogenesis and re-innervation of the sensory area [90–95].

The route of *in vivo* administration of growth factors is a highly debated issue. Most studies administered the growth factors locally, via mini-osmotic pumps, but a long-term delivery would be more suitable because neurotrophins have a short serum half-life. To ensure a long-term and more cell-targeted release, some studies used adenoviruses or viral vectors [96,97] or cell-based delivery [98–101]. To obtain a sustained topical release, another route of administration was devised by placing biodegradable hydrogels soaked with IGF-1 [33,102,103]. This protocol was also adopted for a clinical trial in which 25 patients affected by sudden sensorineural hearing loss and glucocorticoid resistance were treated with IGF-1-soaked gelatine hydrogels, intra-tympanically applied in the middle ear. The results showed that 48% and 56% of patients had hearing improvement 12 and 24 weeks after treatment, respectively, without adverse effects [104].

Perspectives

In conclusion, evidence of tissue-resident endogenous SCs in the inner ear raises the possibility of using these cells in SNHL by promoting their *in vivo* differentiation to replace damaged cells and restore cochlear morphology and function. At present, the *in vitro* and *in vivo* induction of resident cochlear cell differentiation is a promising procedure in regenerative therapy. More studies are nevertheless required to clarify the outcome of transplanted cells and avoid tumor development and to understand how to restore connections between the SGNs and HCs to recover the tonotopic sensorineural activity.

With regard to cochlear regeneration, significant progress has recently been made in understanding the molecular bases of inner ear development, and *in vitro* and *in vivo* models of inner ear regenerative medicine have been established. However, reliable results for cochlear regeneration have not yet been attained in clinical practice.

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