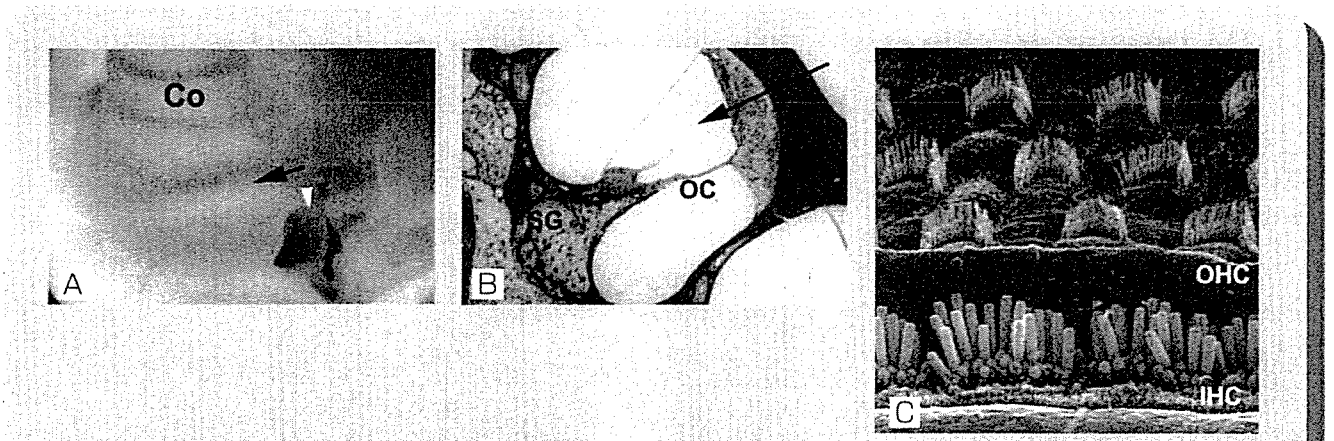


A：中耳からみたモルモットの内耳を示す。白矢頭は蝸牛窓，黒矢印は中央階を示し，黒矢印からアプローチすると，有毛細胞や支持細胞が存在するコルチ器まで到達可能である。
B：内耳の断面を示す。Aで示した黒矢印の部分を開放すると，中央階にあるコルチ器(organ of corti：OC)に到達することが可能である。SG：spiral ganglion，らせん神経節
C：コルチ器の有毛細胞を示す走査電子顕微鏡写真。内有毛細胞(inner hair cell：IHC)と外有毛細胞(outer hair cell：OHC)を示す。

図1　内耳の構造

単に述べると，通常，音(波)は外耳，鼓膜，中耳の耳小骨を振動として伝わり，内耳に到達する(図1)。内耳は蝸牛(聴覚系)と前庭(平衡覚)に分かれているが，中耳から来た機械的振動が蝸牛内の有毛細胞で電気的信号に変換される。その後，らせん神経節細胞から聴覚伝導路を伝わり大脳聴覚野に至る。内耳が障害されると，中枢に電気的信号が伝わらなくなる。

前述のごとく，臨床的に聴覚の再生は内耳の再生，すなわち有毛細胞とらせん神経節細胞がターゲットとなる[3)4)]。らせん神経節細胞は有毛細胞消失後に伴い変性するため，神経節細胞の変性予防あるいは再生が必要になる。

本稿では，有毛細胞の再生のメカニズムとして細胞周期について検討し，また，再生医療として，内耳における遺伝子治療の可能性やらせん神経節細胞の変性予防・防御に関して展望を述べたい。

内耳障害

内耳の再生医療が必要になる疾患として，以下に示すような有毛細胞の障害を伴う内耳性難聴が考えられる。ウイルス性内耳炎(ムンプスあるいはHunt症候群など)[5)]，老人性難聴[6)]，音響外傷[7)]，薬剤性難聴(アミノグリコシド系薬剤[8)]，抗癌剤[9)])，などがある。一部の遺伝性難聴にも有毛細胞の構成蛋白に異常が生じるものがある[10)11)]。また，病因は不明であるが，突発性難聴[12)]は内耳性難聴であると考えられ，予後が悪いものでは再生医療の適応となるだろう。

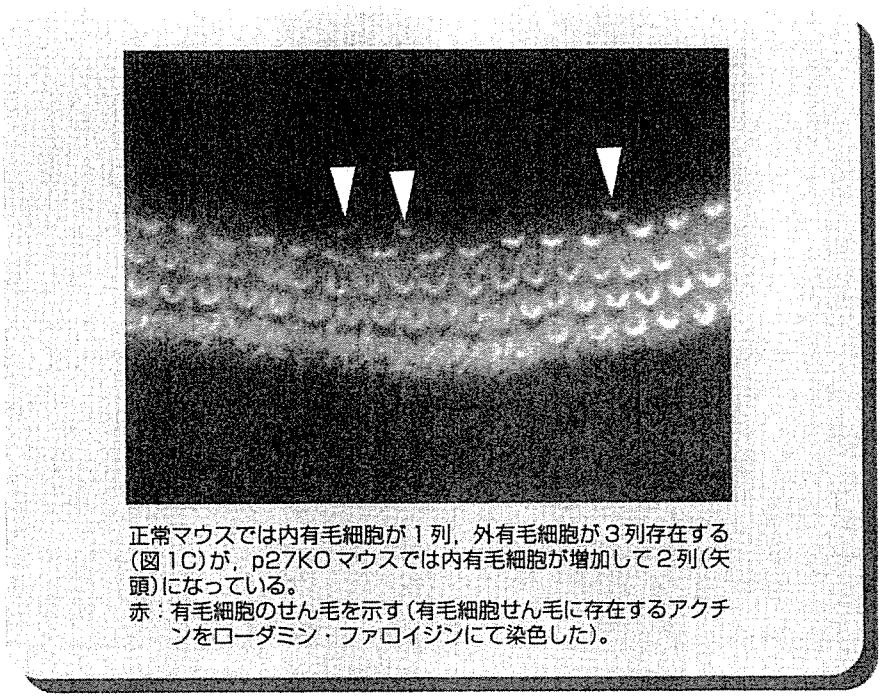
有毛細胞の再生

有毛細胞の再生は，鳥類では再生する[13)]が，哺乳類の蝸牛では再生しない[14)]。一方，哺乳類の前庭では，有毛細胞に一部再生能があると考えられる[14)15)]。哺乳類の蝸牛と前庭におけるこの差異は，内耳幹細胞の存在の有無に関連しているかもしれない。現在，成マウスの前庭(平衡器)では，幹細胞の存在が報告された[16)]が，蝸牛には存在の報告がない。

感覚上皮細胞には，有毛細胞と支持細胞がある。支持細胞は，いわゆる有

毛細胞を解剖学的・機能的にも支持している。発生学的には有毛細胞と支持細胞が，共通の前駆細胞をもつと報告[17]されている一方，支持細胞から有毛細胞に分裂・分化することを示唆する報告[3,14]があり，内耳再生を考える上では重要である。内耳の発生過程では感覚上皮層からp27が発現し，増殖が停止してから，一部の細胞に転写因子である*mammalian atonal homolog 1* (Math1)が発現し，前駆細胞が分裂し，支持細胞と有毛細胞に分化するのではないかと考えられている[18,19]。上述した鳥類や哺乳類前庭で観察された報告から，有毛細胞の再生には大きく分けて，①前駆細胞が支持細胞と有毛細胞に分裂・分化する，②支持細胞が有毛細胞に分化する(transdifferentiation)，③自己修復する(self repair)という3つのコンセプトが考えられている。はたしてどれが正しいのか，あるいはすべて正しいのか，現時点では結論は出ていないが，ここでは①のコンセプトにおいて，支持細胞(あるいは前駆細胞)を分裂させる上で必要な細胞周期について述べる。

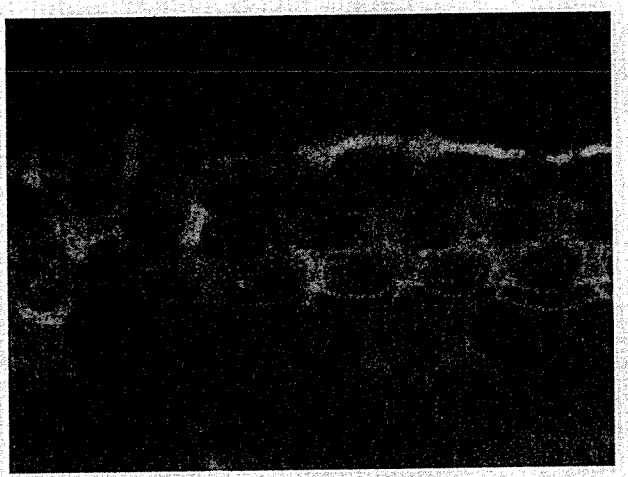
成体蝸牛に存在する有毛細胞や支持細胞は,休止期にあるため分裂しない。したがって，増殖させるためには細胞周期を制御し，増殖させることが必要となる。細胞周期を制御しているcyclin dependent kinase(cdk)の阻害因子であるp27は，細胞を休止期にとどまらせる。**図2**のごとく，p27ノックアウトマウスでは支持細胞や有毛細胞が増加している。ホモ，ヘテロ，野生型の順でこれらの細胞が増加していたが，同時に有毛細胞の増加数に比例して難聴も高度であったことから，有毛細胞が単純に増加するだけでは機能回復につながらないことが示唆された。増加した有毛細胞に神経線維の連絡がないことも考えられる。また，有毛細胞自体の機能の検討も必要であろう。実際，新生した有毛細胞の細胞質には空胞化が観察され，これも一因と考えられる。むしろ，p27は細胞を休止期におくことで，内耳特有の複雑な構造の維持を行っていると考えられる。しかしながら，支持細胞を分裂させる戦略が，有毛細胞消失後に必要であることには異論がない。また実際，高度内耳性難聴では有毛細胞が消失していると考えられるため，残された支持細胞を標的として内耳再生医療を考えることになる。



正常マウスでは内有毛細胞が1列，外有毛細胞が3列存在する(図1C)が，p27KOマウスでは内有毛細胞が増加して2列(矢頭)になっている。
赤：有毛細胞のせん毛を示す(有毛細胞せん毛に存在するアクチンをローダミン・ファロイジンにて染色した)。

図2　p27ノックアウト(KO)マウスにおける内・外有毛細胞
(➔巻頭Color Gravure参照)

筆者らは，生後1～5日目のマウス内耳器官培養下[20]において，アデノウイルスベクターを用いて高率に支持細胞に遺伝子導入が可能であることを報告した(**図3**)。さらに動物モデルの検討では，筆者らの開発した蝸牛中央階を経由(**図1 A, B**)した投与法にて，モルモット内耳で多くの支持細胞にアデノウイルスベクターを感染させることができた[21,22]。しかしながら，投与時に機械的障害を起こしてしまうことで，有毛細胞を障害する可能性は否定



赤：アデノウイルスベクター（β-ガラクトシダーゼを発現する）に感染した支持細胞を示す。
緑：有毛細胞のせん毛を示す（有毛細胞せん毛に存在するアクチンをファロイジンにて染色した）。
（文献20より許可を得て転載）

図3　アデノウイルスベクターによる支持細胞の感染（生後5日目のマウス蝸牛器官培養）（➔巻頭Color Gravure参照）

できない。したがって，臨床応用をふまえれば，すでに大部分の有毛細胞が消失した高度難聴例に絞って検討することになるだろう。この知見に基づき，転写因子であるMath1遺伝子を組み込んだアデノウイルスベクターを支持細胞に感染させたところ，異所性有毛細胞が観察された[23]。しかし，現時点では，異所性に新生した有毛細胞であったためか，聴覚機能の回復は不完全である。Math1の発現をコントロールし，有毛細胞を適切な部位に再生させた上で，らせん神経節に向かう求心性神経線維とのネットワークを構築できれば，臨床応用に近づけるだろう。さらに，遺伝子治療と細胞治療を組み合わせた治療戦略は，今後の検討課題である。神経幹細胞の内耳投与により，ごく一部の細胞が有毛細胞に分化し，着床したという報告がある[24]。細胞移植させた後に，いかにして有毛細胞へ分化誘導させるかが課題である。

らせん神経節細胞の変性予防

次に，らせん神経節細胞の治療について述べる。有毛細胞が消失するに伴い，二次的にらせん神経節細胞が変性してくることが知られている[24]。らせん神経節細胞を生存させる意義は，臨床上二つある。一つは，有毛細胞の再生と同時にらせん神経節細胞の生存維持を考慮し内耳機能を獲得させなくてはならない[25]。もう一つの意義は電気刺激を受けるらせん神経節細胞を生存維持させて人工内耳の治療成績を向上させることにある[26]。両側高度内耳性難聴で補聴器を使用できない高度難聴者では，有毛細胞がすでに消失していると推測されるため，人工内耳の手術として一側の内耳に直接電極を挿入する。外部から入ってくる音エネルギーを電気的エネルギーに変換し，挿入した電極を通じてらせん神経節を直接刺激することにより聴力を獲得させる治療である[27]。

らせん神経節細胞の変性が進行している例（難聴を発症してから長期間経過した場合）では，細胞数の減少から，人工内耳の治療成績も不良であると推測されており[24][26]，神経節細胞変性の進行を予防したり再生させたりすることは，これらの治療成績向上に寄与すると考えられる。動物実験を用いた報告では，神経節細胞の変性予防としてbrain derived neurotrophic factor（BDNF），glial derived neurotrophic factor（GDNF）などの神経栄養因子は効果があるとしている[28][29]。筆者も，モルモットを用いて，人工内耳（動物の内耳に電極を挿入し，電気刺激する）とアデノウイルスベクターによるGDNF（遺伝子）投与の併用によって，人工内耳単独に比してらせん神経節細胞の変性をより強く予防し，機能回復につながることを示した（図4）[30]。このデータに基づき，ドイツでは電極と薬剤を投与できる人工内耳が開発された[31]。

このように，治療成績の向上を目的

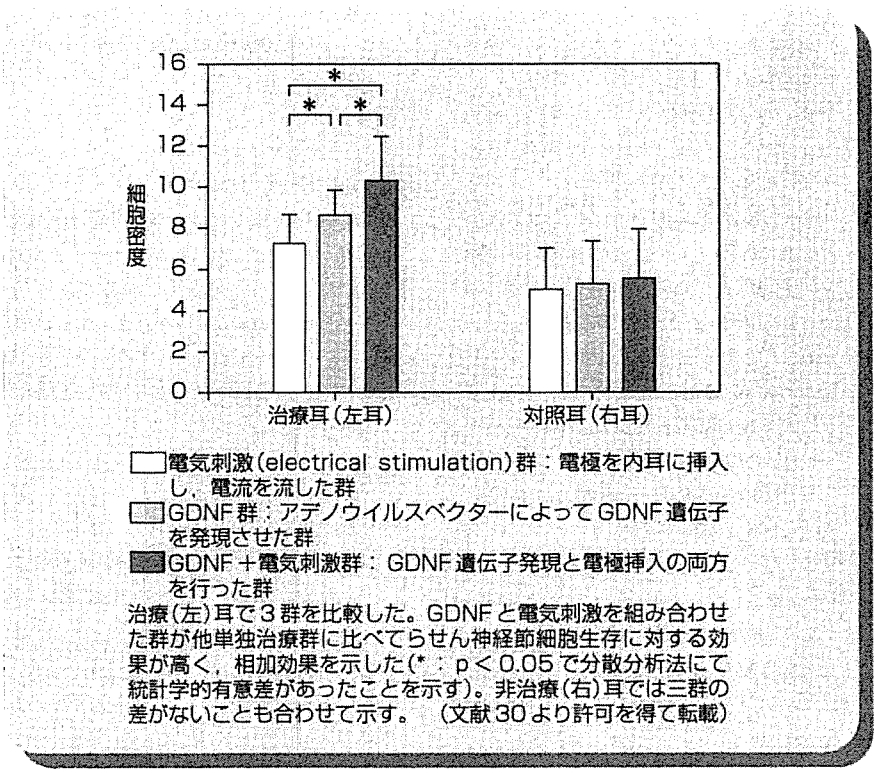


図4　らせん神経節細胞に対する各治療群の予防効果

として，人工内耳に新しいドラッグデリバリーシステムを加味した方法も検討されており，臨床応用は，らせん神経節細胞に対する治療のほうが有毛細胞より早期に実現するだろう。また，らせん神経節細胞の再生に関しては，神経幹細胞を内耳に投与する方法[25]も今後期待できるであろう。

内耳へのアプローチ

人工内耳に伴う技術開発のように，内耳有毛細胞の再生医療を行う上で，いかに簡便に内耳へ薬剤などを到達させるかを考える必要がある。

生理的な聴覚の獲得を目指す意味で，有毛細胞の再生が最も理想的な治療法である。内耳に到達するアプローチとして，①蝸牛窓(図1 A，B)，②蝸牛・中央階外側壁(図1 A，B)，③内リンパ嚢の3つを経由した方法が考えられる。①と③のアプローチは，すでに臨床応用されている。内耳はリンパ液で充満された液体空間であり，比較的拡散しやすいという特徴がある。一方で，液体空間に孔を開けて投与すると，内耳恒常性の破壊や薬液投与時の内耳に対する加圧による機械的障害が生じるため，中等度難聴のように有毛細胞や支持細胞が残存している場合，これらの細胞に障害を与える可能性がある。したがって，①のような投与経路で蝸牛窓を壊さず，蝸牛窓上に薬液などを留置し透過させることが可能であれば，高度難聴のみならず軽度の難聴にも治療適応を拡大させることができる。幸いにも，内耳は外耳道から鼓膜経由してアプローチ可能な部位である。現在，我々は新しい内耳用内視鏡を開発中であるが，内視鏡下で明視しながら正確に内耳へ局所投与を可能にすることを目的としている。本法により，安全性を高めて，薬剤を効率よく蝸牛窓上に留置できる。これらの医療機器・技術の開発は，内耳の再生医療にも将来的に十分貢献できると考えている。

おわりに

有毛細胞再生のメカニズムとらせん神経節変性予防について述べた。さらに遺伝子治療の可能性や内耳のドラッグデリバリーについても今後の展望を示した。内耳再生のメカニズムや非侵襲的な薬剤投与法に関して，更なる検討が望まれる。

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29頁

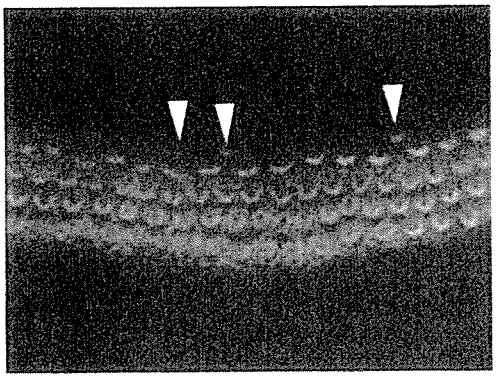

図2 p27ノックアウト(KO)マウスにおける内・外有毛細胞

正常マウスでは内有毛細胞が1列，外有毛細胞が3列存在する(図1C)が，p27KOマウスでは内有毛細胞が増加して2列(矢頭)になっている。

赤：有毛細胞のせん毛を示す(有毛細胞せん毛に存在するアクチンをローダミン・ファロイジンにて染色した)。

30頁

図3 アデノウイルスベクターによる支持細胞の感染(生後5日目のマウス蝸牛器官培養)

赤：アデノウイルスベクター(β-ガラクトシダーゼを発現する)に感染した支持細胞を示す。

緑：有毛細胞のせん毛を示す(有毛細胞せん毛に存在するアクチンをファロイジンにて染色した)。

(文献20より許可を得て転載)

36頁

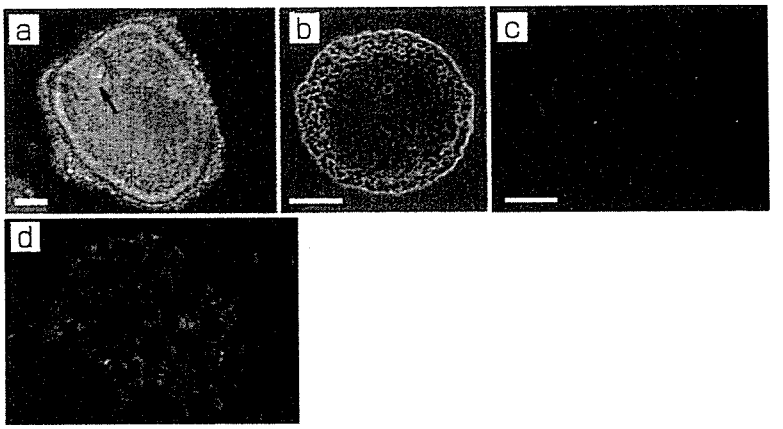

図2 耳胞細胞培養系を樹立

a：胎生12日ラット耳胞，Bar＝100μm，b：増殖する耳胞細胞，Bar＝100μm，c：緑；BrdUを取り込んだ耳胞細胞の核，青；DAPIで対比染色された核，Bar＝50μm，d：ネスチン(赤)を発現する耳胞細胞，青；DAPIで対比染色された核

(文献14より引用改変)

36頁

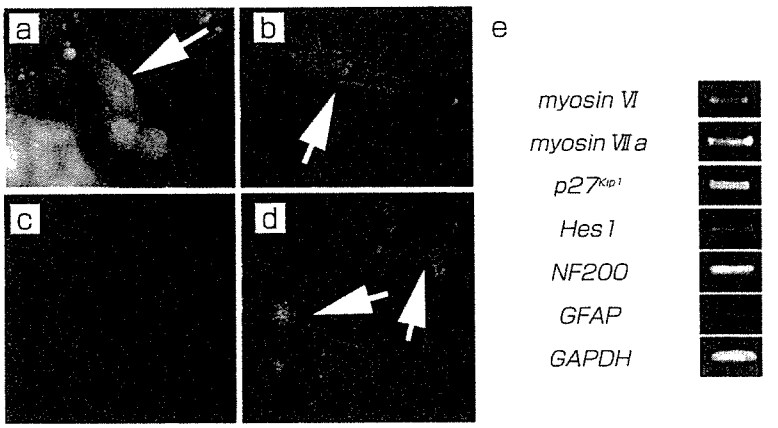

図3 耳胞細胞の分化誘導

a：有毛細胞のマーカーmyosin VIIa(赤)を発現する細胞(矢印)，b：支持細胞マーカーであるcytokeratin(緑)とp27^{Kip1}(赤)を共発現する細胞(矢印)，c：neuron specific enolase(神経のマーカー)を発現する細胞，d：支持細胞マーカーであるGFAPが陽性の細胞(矢印)，e：RT-PCRはそれぞれの細胞マーカーが発現していることを示す。

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Neural degeneration and regeneration in the auditory and vestibular systems

Sho Kanzaki
Department of Otolaryngology, Head and Neck Surgery, Keio University School of Medicine, 35 Shinanomachi Shinjuku, Tokyo, Japan 160-0082

Abstract

The most common type of hearing loss in humans results from damage to the inner ear, including trauma to hair cells (HCs) and spiral ganglion neurons (SGNs). In mammals, cochlear HC loss causes irreversible hearing impairment because this type of sensory cell cannot regenerate; interestingly, in avians, HCs can regenerate. The protection of SGN degeneration following HC insult has implications for cochlear prostheses implanted to alleviate deafness. This review details the events and mechanisms of mammalian HC damage and regeneration based on experimental observations. We also discuss how these affect SGN degeneration and describe potential new therapeutic interventions to reduce hearing loss

Correspondence/Reprint request: Dr. Sho Kanzaki, Department of Otolaryngology, Head and Neck Surgery Keio University School of Medicine, 35 Shinanomachi Shinjuku, Tokyo, Japan 160-0082
E-mail: skan@sc.itc.keio.ac.jp

through the regeneration of HCs and through the prevention of neural degeneration.

Abbreviations

AP-1; activator protein-1
BDNF; brain derived neurotrophic factor
CNTF; ciliary neurotrophic factor
ES; electrical stimulation
FGF; fibroblast growth factor
GDNF; glial cell derived neurotrophic factor
HC; hair cell
Hes; hairy and enhancer of *split*
JNK; bc-Jun N-terminal kinase
Math1; mammalian *atonal* homolog 1
NMDA; N-methyl-D-aspartate
ROS; reactive oxygen species
RNS; reactive nitrogen species
SGN; spiral ganglion neuron
SC; supporting cell
TGF a; transforming growth factor alpha

1. Introduction

Loss of hair cells (HCs) in mammals can occur as a result of viral infection, ischemic change, genetic mutation, disease, exposure to noise and toxins, and aging. HC-associated hearing loss and vestibular impairment are usually permanent, but progress is being made in preventing HC damage and diminishing hearing loss. In mammals, auditory or cochlear HCs cannot regenerate, whereas balance sensor or vestibular HCs can partially regenerate [1]. In a effort to gain insight to possible therapeutic targets to prevent or lessen hearing loss due to HC damage, this review also endeavors to describe the molecular mechanisms underlying regeneration in the cochlea and the future directions of animal-based experiments.

1.1 Anatomy of the auditory system

The human ear is comprised of three components: outer, middle, and inner ear (Figure 1A). Sound is transduced in the inner ear via three ossicles located in the middle ear. The mammalian inner ear contains the cochlea and the vestibule, which are responsible for hearing and balance, respectively. The cochlea, a coiled bony canal, is one of the most important structures in the auditory system (Figure 1A, B). The sensory epithelium of the cochlea is comprised of a highly ordered cellular mosaic of HCs and supporting cells

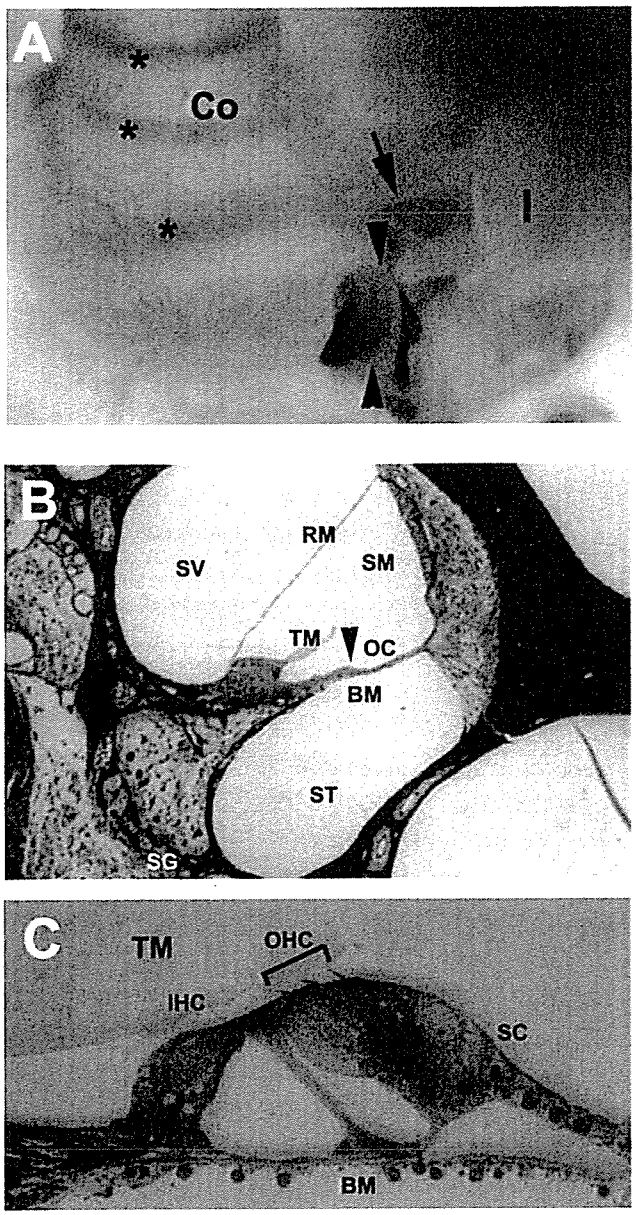

Figure 1. Photomicrograph of middle and inner ear in guinea pig.
(A) Middle and inner ear in guinea pigs
Co; cochlea, I; incus, *:stria vascularis
arrow heads; round window, arrow; oval window
(B) The organ of Corti and SG of a deafened guinea pig treated by kanamicin and ethacrinic acid.
BM; basement membrane, OC; organ of Corti, SG; spiral ganglion, TC; tectorial membrane
(C) HCs and SCs from the inner ear of a normal guinea pig
IHC; inner hair cell, OHC; outer hair cell, Bracket indicates OHC.
BM; basement membrane, TM; tectorial membrane.

(SCs). One row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) lie adjacent to the SCs (Figure 1C). Each HC is separated from adjacent HCs by an interceding SC, forming an invariant and alternating mosaic that extends the length of the cochlear duct. Sound vibrations are transduced to electrical signals by mechanosensory receptors or cochlear HCs. These electrical signals are then relayed to cochlear nuclei in the brainstem through spiral ganglion neurons (SGNs), whose axons comprise a portion of the nerve fibers of the VIIIth cranial nerve (auditory nerve).

2. HC degeneration and regeneration

2.1. HC damage

Recent studies have revealed part of the mechanism underlying HC damage (Figure 1B). Ototoxic drugs and noise exposure paradigms have been used to model various hearing loss diseases and have been used to develop animal models of deafness. One class of ototoxic drugs is the aminoglycosides, highly efficacious antibiotics frequently used for treating infections. Chronic injections of aminoglycosides produce reactive oxygen species (ROS) and results in glutamate-like excitotoxicity [2]. Accumulated damage stemming from aminoglycoside treatment initiates apoptotic HC death exemplified by DNA fragmentation and chromatin condensation. The first pathological sign of aminoglycoside ototoxicity is OHC loss mediated by increased intracellular calcium and c-Jun N-terminal kinase (JNK) activity; JNK is a stress activated protein kinase that induces neuronal cell death [3]. JNK activates cytochrome c and caspases that are integral components of the mitochondrial death pathway [3].

Acoustic trauma may also result in ROS production and activate transcriptional factors such as activator protein 1 (AP-1) [4-6], which can consist of either a Jun homodimer (c-Jun, JunB, JunD) or a Jun and Fos heterodimer. ROS can damage cells directly by reacting with proteins, lipids, and DNA. In response to ROS-associated cellular injury, JNK-dependent phosphorylation of c-Jun occurs, which in turn causes AP-1-DNA binding to take place through production of caspase 3 [7] and may induce expression of killer genes [8].

2.2. HC protection

Numerous substances have been found to attenuate HC damage. Among these agents are neurotrophic factors, N-methyl-D-aspartate (NMDA) antagonists, and free radical scavengers. Chronically exposing the cochlea to glial cell derived neurotrophic factor (GDNF), either via osmotic pump [9] or via transgene overexpression, effectively protects the inner ear from noise- or drug-induced hearing loss [10,11]. The NMDA antagonist MK801 similarly protects HCs against noise-induced hearing loss [12]. Interestingly, when combined with

neutrotrophin 3 (NT3), MK801 potently preserves both HC physiology and morphology after exposure to toxic levels of aminoglycoside [12].

A number of free radical scavengers have been shown to protect cochlear HCs from acoustic trauma [13-15]. As alluded to above, HC loss has been linked to the build up of ROS and reactive nitrogen species (RNS) in the inner ear after aminoglycoside treatment [2,16] and after exposure to noise [17]. ROS appears rapidly and transiently in the inner ear following damage, reaching a maximum level 7-10 days before declining. The finding that ROS and RNS build up gradually after noise exposure explains why HC loss progresses over time and stabilizes two or more weeks after the initial insult [15]. While HC damage is ultimately more related to ROS and RNS buildup, mechanical factors cause the initial damage soon after noise exposure. The delay and gradual free radicals may permit intervention using ROS and RNS scavengers if done soon after exposure to noise [15].

2.3. HC regeneration

Following inner ear damage in adult birds, regeneration of HCs proceeds through a series of processes not observed in mammals. Death of all or even some HCs triggers regeneration. First, nonsensory neighboring SCs act like stem cells, re-entering the cell cycle and transdifferentiating directly into HCs. Some SCs also proliferate. After transdifferentiation, the newly produced HCs then pass through normal developmental stages, similar to that seen during embryogenesis. Importantly, these new HCs appear to be functional, as the chickens appear to regain their hearing [18]. HCs that regenerate, should have appropriate ion channels for transduction, be re-innervated by nerve fibers of the VIIIth cranial nerve, and make appropriate connections in the cochlear nucleus [18].

HC regeneration is not limited to the auditory sensory epithelia of avians. In mammals, spontaneous HC regeneration has been observed to a limited extent but only in the vestibule [19,20]. Mammalian SCs are thought to play a role similar to that in the developing chicken cochlea.

Based on several studies of the avian and mammalian vestibular organ, three patterns of regeneration have been identified: regenerative proliferation of SCs, transdifferentiation of SCs, and self-repair of damaged HCs [21] (Figure 2). The regenerative proliferation of SCs is supported by developmental studies showing that HCs and SCs of the avian cochlea arise from a common progenitor and are produced differentially via asymmetrical cell divisions [22]. The direct transdifferentiation of SCs into HCs in the mammalian cochlea is consistent with the demonstration of transitional cells with stereocilia in the inner ear of acoustically damaged chicks [23,24]. These ciliated transitional cells possess several morphological characteristics of SCs.

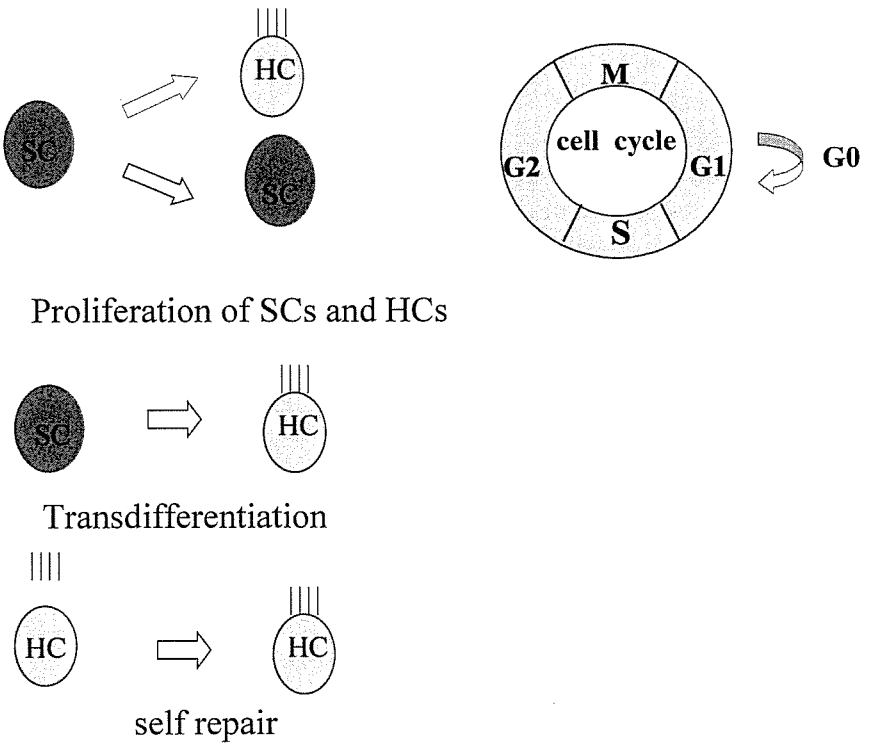

Figure 2. Schema of HC regeneration after damage.
Exposure to intense noise or ototoxins induce HCs to be extruded into the lumen and some SCs to divide. The M phase of the cell cycle takes place at the lumenal surface of the sensory epithelium.
(This schema was modified and adapted from Steacker et al., 1998 [21])

Evidence for self-repair of damaged HCs comes from the observation that ototoxin-damaged HCs of neonatal rats utricle can survive for prolonged times and can regenerate lost stereocilia in explant because cell division in HCs and SCs is limited and far below the number of repaired HCs [25]. These data suggest that the intracellular repair of partially damaged HCs can be an important contributor to spontaneous HC recovery in the mammalian inner ear [25].

It remains unclear why cochlear HCs are unable to regenerate. Hopefully, recent advances in molecular biological techniques can shed light on the molecular events underlying the inhibition of HC regeneration in the mature mammalian cochlea.

2.4. Molecular mechanisms mediating HC regeneration

The cochlea is comprised of a strictly ordered cellular array of HCs and SCs existing in the resting stage of the cell cycle. This organized array is very important for hearing function. Once HCs are injured, however, this arrangement may impede HC regeneration. To understand the molecular mechanisms

underlying HC regeneration, it is useful to consider the roles of growth factors, cell cycle regulation, and Notch signaling in regulating HC differentiation in the cochlea. The number of, as well as the timing of, HC and SC production is highly regulated. The progression of quiescent SCs into the G1 and S phases of the cell cycle plays a key role in initiating proliferation of the auditory sensory epithelium [26]. Recently, Notch signaling and basic helix-loop-helix genes (bHLH) have been found to regulate HC and SC cell-fate determination and their differentiation during inner ear development [27,28].

2.5. Role of growth factors in HC regeneration

Several signaling molecules and growth factors are necessary for vestibular HC regeneration. Insulin-like growth factor 1 (IGF-1) stimulates DNA synthesis in the chick vestibular sensory epithelium. Both IGF-1 and IGF-1 binding protein are upregulated in the damaged mammalian inner ear [29]. IGF-1 and FGF are important regulators of progenitor cell mitotic activity in other regions of the nervous system and similarly have been found to regulate HC development and regeneration [30]. Taken together, these results demonstrate that regeneration is likely to be regulated by the same factors normally involved in embryonic development. Indeed, transforming growth factor alpha (TGF alpha) and epidermal growth factor (EGF) in the presence of insulin stimulate cell proliferation of mature rat vestibular epithelium *in vitro* [31] and *in vivo* [32]. In addition, infusion of TGF and insulin directly into the inner ear of adult rats stimulates DNA synthesis in the vestibular sensory receptor epithelium [30].

2.6. Cell cycle regulation

In mammals, HC and SC proliferation in the inner ear has been reported to be regulated by certain cell cycle regulators [33, Lowenheim, 1999 #71]. One prominent cell cycle regulator is $p27^{Kip1}$, a member of the Cip/Kip family of cyclin dependent kinase inhibitors (CKIs). $p27^{Kip1}$ interacts with both CDK4/6 and CDK2 to arrest the HC cell cycle in prenatal mice [34]. $p27^{Kip1}$ expression occurs in the zone of non-proliferating cells (ZNPC) in the sensory epithelium between embryonic days 12.5 and 13.5 [33]. HCs continue to proliferate beyond postnatal stages in $p27^{Kip1}$-deficient mice [35]. These findings provide a basis for the use of cell cycle inhibitors, more specifically $p27^{Kip1}$ inhibitors, as possible therapeutics to stimulate HC growth in adults with hearing loss (Figure 2).

2.7. Notch signaling-mediated lateral inhibition

The members of the Notch signaling pathway and members of the bHLH family of transcription factors play key roles in mediating HC and SC differentiation in the developing cochlea [27,28] (Figure 3). In vertebrates, HC

production in the inner ear is regulated by the Notch signaling pathway in a process called lateral inhibition [27]. HCs typically express the Notch ligand Jagged2, which inhibits the differentiation of SCs to HCs [28,36]. Hence, Notch-mediated lateral inhibition is a key mediator of cell-fate determination within the array of HCs and SCs lining the cochlea, regulating the number of HCs in the inner ear by inhibiting HC proliferation [27].

In addition to the Notch-mediated regulation of HC production, two bHLH factors, mammalian *atonal* homolog 1 (Math1) and hairy and enhancer of *split* (Hes), also play a pivotal role in directing HC proliferation and differentiation [27]. Zheng and Gao (2000) showed that overexpression of Math1, a positive bHLH transcription factor, increased the number of HCs in the greater epithelial ridge, an area located outside of the sensory epithelium and an area that typically produces inner sulcus cells. Math1 also mediated the transformation of utricular SCs into HCs [37]. Interestingly, after adenovirus-mediated introduction of Hath1, a human homolog of Math1, into the rat utricle, bromodeoxyuridine (BrdU) failed to label newly generated HCs, indicating that Hath1-induced cell proliferation does not involve mitosis [38]. Taken together, these findings indicate that Math1 and Hath1 not only shape the production of HCs during postnatal development in the inner ears but also

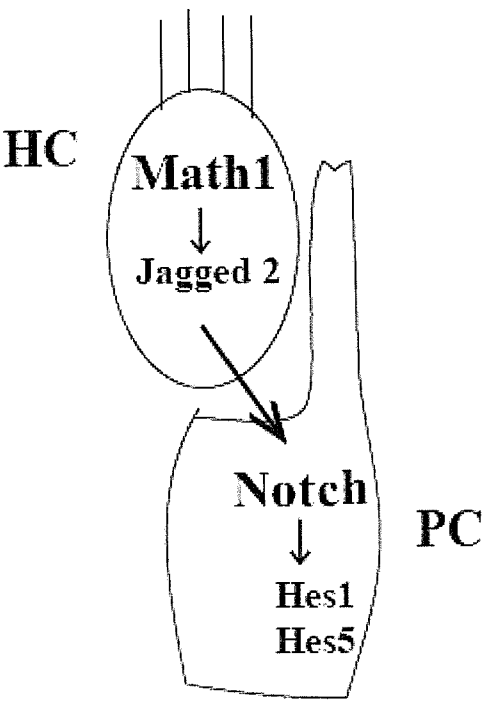

Figure 3. Schema of Notch signaling in the cochlear sensory epithelium.
Math1 regulates Jagged 2 activating Notch expressed in PC. Lateral inhibition was seen among HC and PC. HC; hair cell, PC; precursor cell

do so without inducing mitosis, suggesting that cells already present in the sensory epithelia possess the capacity to transdifferentiate in to HCs.

The counterparts of Math1 are the two negative bHLH transcription factors Hes1 and Hes5. In mice, deletion of Hes1 and Hes5 genes leads to an overproduction of HCs, suggesting that Hes1 and Hes5 also act as negative regulators of HC differentiation [39] (Figure 3).

2.8. Potential use of stem cells and gene therapy to aid regeneration

Stem cell therapy and gene therapy are two attractive tools to facilitate HC regeneration in mammalian systems. For endogenous HC regeneration to occur, however, it is critical to identify potential stem cells of the inner ear. Such identification can be achieved by screening for marker genes inherent to progenitor cells of the inner ear. Recently, it was discovered that mice have stem cells in the vestibular organ and that these form neurospheres expressing markers for SCs and HCs. These inner ear stem cells can assume a variety of cell fates of epidermal lineage [40]. Another putative marker for stem and progenitor cells of the inner ear is nestin, an intermediate filament originally found to localize in the developing central nervous system [41,42]. In the rat, HCs and SCs transiently express nestin late in embryogenesis, but not for very long after birth [43]. Another study showed that in postnatal transgenic mice containg a nestin promoter-GFP (green fluorescent protein) construct, from P0 to P5, the GFP signal appears in several types of SCs and OHCs but not in IHCs. At P15, OHCs do not express the GFP-fusion protein [44]. At P60, however, the stroma of the crista ampullaris and utricle, as well as a few Deiters cells, moderately express GFP [44]. Taken together, these findings suggest that nestin-expressing cells may also possess the potential for self-repair. Thus, a gene therapy that promotes nestin expression may be one way to drive HC regeneration.

Stem cell transplantation is another option. The finding that stem cells can be isolated from the CNS has lead to widespread speculation that they will lead to cures for many neurodegenerative diseases [45]. Moreover, recent studies have shown that neurons suitable for transplantation can be generated from cultured stem cells, and that the adult brain produces new neurons from its own stem cells in response to injury [46]. However, replacing lost neurons in the brain is a challenge, because transplanted neurons must synapse with the appropriate targets. Nonetheless, stem cell transplantation theoretically holds much promise as a potential treatment for hearing loss due to HC damage. Indeed, preliminary studies of stem cell transplantation into the injured inner ear have already proven to be quite promising. Hippocampal stem cells or neurospheres transplanted into the rat cochlea not only remain intact for up to 4 weeks after transplantation, but some of these cells also settle in to the

appropriate spatial arrangement, even taking on the morphology of HCs [47]. Autologous bone marrow stromal cells transplanted into the injured inner ear incorporate into various regions throughout the cochlea, as far as the spiral ligament [48]. Some of these grafted cells express certain glial cell markers, suggesting that these cells have the capability to differentiate into specific neural cell types. Moreover, rat fetal otocyst cells transplanted into the injured inner ear have been shown to migrate into the cochlea and differentiate into SCs [49]. To date, stem cells derived from bone marrow stroma may be best suited for transplantation into the injured inner ear, because these cells can be induced to differentiate into a variety of cell types, including neurons that form neurites [50]. The measured success of this technique suggests that individual stem cells may be easily obtained and implies that they may have real clinical use.

When considering the use of stem cells as a tool for biological repair, conventional wisdom dictates that it would be more practical to use stem cells that are isolated from the end organ in which they reside than to use stem cells from another source. Thus, stem cells to be used for treating hearing loss ideally should originate from the inner ear. Until a mammalian inner stem cell is identified, however, neural stem cells may provide the best alternative, since the inner ear and the brain are closely related. Additionally, *ex vivo* GDNF gene therapy as well as direct infusion of GDNF into affected areas of the brain, when implemented in conjunction with stem cell transplants, may prove to be useful to ameliorate hearing loss due to HC damage, because such therapy has already been shown to significantly ameliorate certain symptoms associated with Parkinson disease [45]. Before stem cell therapeutics can be used in the clinical sector, however, it is necessary to know how to direct proliferation and differentiation of stem cells into specific phenotypes, induce their integration into existing neural and synaptic circuits and optimize functional recovery in animal models closely resembling hearing loss in humans [51].

An alternative to using stem cells to treat hearing loss is to produce endogenous progenitor cells with the capacity to differentiate into HCs. One likely candidate cell type is the SC, which has the inherent capacity to regenerate [52]. A potential problem with using SCs as endogenous progenitors for HCs is that differentiation and proliferation within the sensory region of the cochlea are tightly regulated by the Notch signaling pathway, which mediates the lateral inhibition that prevents the *in vivo* differentiation of SCs to HCs [27]. One way to overcome this inhibition is for SCs to overexpress proteins, such as Math1, that can increase the regenerative properties of the sensory epithelium of the cochlea [53, 54]. Several workers have successfully used viral vectors to introduce transgenes into SCs for the purpose of overexpressing proteins that induce regeneration in the damaged inner ear [53-55]. Kawamoto et al. used this method to overexpress the Math1 transgene, a positive regulator of developing HCs not normally expressed by

SCs, in the endolymph of the mature guinea pig cochlea. In this system, auditory nerve axons extend toward some of the newly generated HCs, suggesting that these HCs attract auditory neurons [52]. Although it remains to be demonstrated whether these regenerated HCs can function normally, these data indicate that the SCs of the mature cochlea can potentially serve as progenitor cells for HCs.

3. Spiral ganglion neuronal degeneration and regeneration

3.1. Spiral ganglion neuronal degeneration

Following significant hearing loss, severe atrophy occurs within the cochlea and other central auditory systems. SGNs degenerate following destruction of cochlear SCs. Also, cranial nerve degeneration secondary to denervation is a common finding in the nervous system [56]. Preservation of SGNs, their axons (i.e., VIIIth or auditory nerve), and their connections are necessary to restore auditory function. IHCs normally provide excitatory activation to the VIIIth cranial nerve, which carries auditory information. Destruction of IHCs due to trauma or disease leads to the degeneration of SGNs [57,58], which hampers the effectiveness of hearing aid devices, including cochlear prostheses. The clinical implications of these pathophysiological changes within the auditory pathway on cochlear implant function are discussed below.

3.2. Neurotrophic factors and electrical stimulation

Neurotrophic factors and maintenance of synaptic connectivity are important to prevent neuronal degeneration. Brain-derived neurotrophic factor (BDNF) and GDNF effectively promote SGN survival following exposure to deafening noise or ototoxic drugs [59]. Moreover, when administered in combination with BDNF, fibroblast growth factor (FGF) effectively induces the regrowth of neuronal processes [60]. Similar results have been reported for BDNF in combination with ciliary neurotrophic factor (CNTF). After deafening due to treatment with otoxins, infusion of BDNF and a CNTF analogue into the cochlea significantly enhanced the survival of SGNs [61]. Using electrically evoked auditory brainstem responses (eABR), the authors also showed that auditory function was significantly restored. These findings with neurotrophin-related enhancement of auditory function in deafness models should have great clinical significance for deaf patients with cochlear prostheses.

Electrical stimulation (ES) of SGNs has also been shown to enhance SGN survival [62]. Although ES is not always protective [63-65], many agree that stimulating the auditory nerve following IHC loss via chronic cochlear ES devices greatly reduces the deafness-related loss of SGNs [66-68]. How ES

enhances SGN survival is not completely understood. *In vitro* studies have shown that depolarization-induced enhancement of SGN survival is mediated by L-type voltage-gated Ca^{2+} channels [69]. Moreover, blocking L-type voltage gated Ca^{2+} channels eliminates the protective effects of ES *in vivo* [70]. Taken together, these findings indicate that L-type voltage-gated Ca^{2+} channels play a central role in mediating chronic ES-associated survival of SGN following hearing loss by maintaining intracellular Ca^{2+} levels within a physiological range. These findings provide a firm basis for the use of cochlear prostheses that deliver chronic electrical stimulation to the auditory nerve to increase SGN survival and decrease further hearing loss. Alternative therapies capable of maintaining Ca^{2+} homeostasis in SGNs by targeting L-type voltage gated Ca^{2+} channels may also prevent further hearing loss.

Another possible way to decrease irreversible damage to SGNs is to supplement chronic cochlear stimulation with the infusion of neurotrophic factors [71]. Additionally, treatment with GDNF and BDNF significantly enhance SGN survival compared to that in untreated deafened ears [59]. Combining ES with GDNF treatment protects against inner ear damage. In that study, the inner ears of auditory traumatized guinea pigs were inoculated with adenoviral vectors containing the GDNF transgene [72].

Soon thereafter, the cochlea overexpressing GDNF were electrically stimulated. This combined treatment prevented further damage to SGNs, thereby providing the impetus for using combined GDNF/ES therapies in cochlear implant recipients with the aim of improving auditory perception. ES of SGNs enhances their survival. The combined effects of the GDNF transgene delivered by adenoviral vectors (Ad-GDNF) and ES can provide significantly better preservation of SGN density than either treatment alone [72]. Even though ES parameters were optimized for maximal protection (saturated effect), the augmentation added by GDNF suggests that the mechanisms of GDNF- and ES-mediated SGN protection are, at least in part, independent [72]. GDNF/ES combined treatment in cochlear implant recipients can improve auditory perception. These survival factors that activate the auditory nerve fibers directly may be applied following deafness to maintain and enhance SGNs and to help the profoundly deaf receiving cochlear implants [60]. Pharmacologic intervention might also enhance cochlear implant performance. New modified electrodes could provide a safe and easy way to combine electrical stimulation with the beneficial effects of a local drug therapy applied to the inner ear [73].

3.3. Stem cell therapy

Neural stem cells have been successfully transplanted into the mouse inner ear, and a proportion of them differentiate into glia and other neural cells, some staining positively for HC biomarkers [40]. Importantly, the transplanted

cells arrange appropriately in the vestibular sensory epithelia. [74]. Bolstered by further research showing functionality, this study indicates that transplanting neural stem cells may be a promising strategy for replacing damaged HCs. As iterated above, it is logical to use stem cells derived from the same organ that will receive the stem cell transplant. Hence, because the cochlea contains nervous tissue, neural stem cells would be preferential for the treatment of auditory neuropathy and other VIIIth cranial nerve disorders. The use of neural stem cells is also a promising approach for replacing degenerated SGNs. Hu and colleagues report that neural stem cells harvested from adult mice were successfully transplanted into the inner ear of normal and experimentally deafened guinea pigs. They adopted the strategy of biasing the neural stem cells to assume a neuronal phenotype by first transducing them with neurogenin2. Transplanted cells that stained positively for neural class III beta-tubulin were observed close to the sensory epithelium, adjacent to SGNs, suggesting that adult neural stem cells can differentiate into an appropriate phenotype and arrange normally in the inner ear after damage. However, the above-mentioned caveat about functionality also holds here. [75]. These transplantation studies suggest that specific latent cues are present in the adult cochlea that can direct migration and differentiation of experimentally placed neural stem cells.

Finally, it may be possible to genetically engineer adult stem cells to develop into specific cochlear cell types. For instance, a neural stem cell could be developed that conditionally upregulates the Math-1 transcription factor, whose presence has been shown to be sufficient to produce HCs *in vitro* [37] and *in vivo* [52]. With exposure to the appropriate signal, Math-1 would be activated in such a stem cell, destining it to become an HC. Even if the cochlea is devoid of a stem cell source, it may be possible to use stem cells isolated from other tissues as tools for cochlear repair.

4. Conclusions and future directions

Studies on HC regeneration in the avian inner ear have begun to illuminate issues of functional recovery in mammals following HC damage. It has been confirmed that HC regeneration is possible in birds, and the molecular signals that stimulate and inhibit regeneration have begun to be identified. Limited postnatal proliferation and ectopic HC expression has been achieved in mammals by overexpressing Math1, applying cell cycle inhibitors, and transplanting stem cells. These studies may represent the beginnings of a major change in the field that will someday lead to therapeutic interventions for the hearing impaired. Although promising, it is still unknown whether these regenerated HCs make the correct functional connections with the spiral ganglion. With regard to this point, preservation of SGNs is very important and has implications for cochlear implants.

Based on results now being generated by molecular biologists, virologists and stem cell biologists, pure biological approaches to treatment of hearing loss may become ascendant as the choice of treatment in the future.

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