Market size, basic research and a wealth of experimental techniques provide the platform for an unprecedented opportunity to take the fruits of hearing research into the clinic, where it could benefit millions of people worldwide

Keynote review:

The auditory system, hearing loss and potential targets for drug development

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There is a huge potential market for the treatment of hearing loss. Drugs are already available to ameliorate predictable, damaging effects of excessive noise and ototoxic drugs. The biggest challenge now is to develop drug-based treatments for regeneration of sensory cells following noise-induced and age-related hearing loss. This requires careful consideration of the physiological mechanisms of hearing loss and identification of key cellular and molecular targets. There are many molecular cues for the discovery of suitable drug targets and a full range of experimental resources are available for initial screening through to functional analysis *in vivo*. There is now an unparalleled opportunity for translational research.

There is a massive social and economic demand to develop therapeutic treatments for hearing loss. Deafness is one of the most widespread, costly and poorly understood disabilities in the world. It is also one of the most neglected. Its invisibility hides the suffering of many millions of people, who progressively lose their most important means of communication and who become socially isolated, especially in their later years.

In 2002, the World Health Organization (WHO) estimated that 250 million people have disabling hearing loss (www.who.int/pbd/deafness/en) and that two-thirds of them live in the developing world. The costs of communication disorders to the US economy have been estimated at US$ 154–186 billion per year [1]. In 1997 the cost of noise-induced hearing loss alone was estimated to be between 0.2% and 2% of the gross domestic product. In the UK in 2002 this fraction was equivalent to US$ 2.7–27 billion (Energy Information Administration, http://eia.doe.gov/emeu/international/other.html). The WHO described the scale of the problem, the primary causes and potential solutions in a series of conferences from 1994–1998 (www.who.int/pbd/publications/en/). It concluded that approximately 50% of hearing

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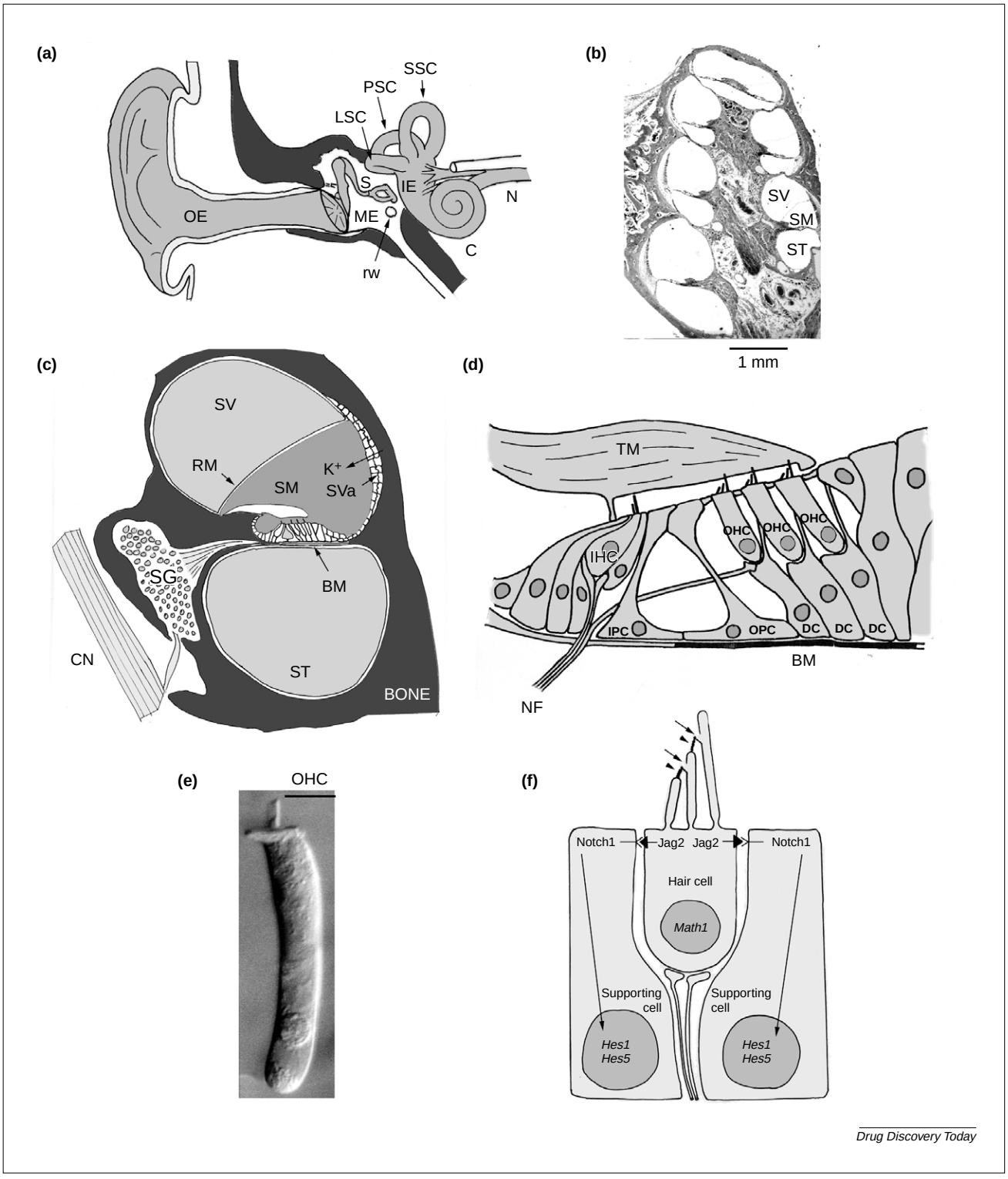

loss is avoidable through careful management of noise exposure and of the administration of prescribed, ototoxic drugs. One of the key recommendations was investment in research, including transitional research to bring some of the remarkable, recent developments in basic research closer to the clinic. Investment in hearing research has been extremely modest in comparison with the measured social and economic costs but it has yielded results to be envied by many other disciplines. Widespread research activity has been underpinned by organizations, such as the National Institute for Deafness and other Communication Disorders (NIDCD) in the USA (www.nidcd.nih.gov), and large European consortia, such a GENDEAF (www.miteuro.org/gendeaf.htm) and the very recently launched EUROHEAR (www.eurohear.org). The global demand for therapeutic treatments is increasing dramatically with industrialization and lifespan. In developed countries the appetite for leisure noise among the young is expected to

FIGURE 1

Structure of the inner ear and organ of Corti. (a) Sound is conducted via the bones of the middle ear to the oval window, which lies beneath the footplate of the stapes. Oscillations of the oval window generate displacements in the inner-ear fluids and the pressure is equalized by reciprocal displacements of the round window. The inner ear includes the vestibular apparatus. The dorsal, posterior and lateral semicircular canals are located dorsally, the utricle and saccule are located centrally and the cochlea is in the form of a spiral projecting ventrally. The VIIIth nerve includes both vestibular and cochlear innervation. The middle ear and inner ear are encased in bone. **(b)** Section through the cochlea of a guinea pig. **(c)** Diagram of a section through one turn of the cochlea. **(d)** The organ of Corti. The supporting cells form a single, continuous layer, in which every cell spans the epithelium from the basement membrane to the epithelial surface. IHCs are supported by phalangeal cells and along their lateral side, opposite the central axis of the cochlea, by a row of IPCs. IPCs form a continuous row and with a similar row of OPCs they form the tunnel of Corti, a long triangular shaped canal that runs along the length of the organ. Pillar cells are composed of tightly packed bundles of actin filaments and microtubules, embedded at each end in a dense actin mesh. They form a rigid frame, which is important for coupling the mechanics of the basilar membrane with the hair cell surfaces. Each row of OHCs is supported by rows of another type of supporting cell, called Deiters' cells. These cells sit beneath the OHCs and connect them to the basilar membrane but they have narrow microtubular processes that project up to the epithelial surface. Lying over the tops of the hair cells is the tectorial membrane, which is a finely cross-linked structure of collagen fibers and tectorins [184]. **(e)** Isolated outer hair cell from the low-frequency region of a guinea pig cochlea, viewed by differential interference microscopy. Scale bar = 10 μm. **(f)** Diagram of a hair cell flanked by two supporting cells. The hair cell bundle is composed of stereocilia, which regulate the flow of potassium ions (K^+) from the endolymph through transducer channels attached to apical tip-links (arrowheads). Notch ligands (only jagged 2 is shown) in the hair cell activate notch in the adjacent supporting cell, which upregulates *Hes* genes and suppresses hair cell differentiation. Release of this inhibition is likely to be necessary for regeneration. Notch signaling is reinforced by several other ligands that communicate between supporting cells and between supporting cells and hair cells (see [67]). Abbreviations: BM, basilar membrane; C, cochlea; CN, cochlear nerve; DC, Deiters' cell; IE, inner ear; IPC, inner pillar cell; LSC, lateral semicircular canal; ME, middle ear; N, VIIIth nerve; NF, nerve fibers; OE, outer ear; OPC, outer pillar cell; PSC, posterior semicircular canal; RM, Reissner's membrane; rw, round window; S, stapes; SG; spiral ganglion in Rosenthal's canal; SM, scala media; SSC, superior semicircular canal; ST, scala tympani; SV, scala vestibule; SVa, stria vascularis; TM, tectorial membrane.

have a substantial, deleterious impact on hearing loss in older generations in the future. The aims of this review are to introduce the auditory system with a summary of the nature and scale of hearing loss and then to review recent research with a focus on the most likely cellular and molecular targets for drug development.

The auditory system

Sound travels in air along the outer-ear canal to the ear drum and is then transmitted via the bones of the middle ear to the fluid environment of the inner ear, where the sensory organ resides (Figure 1a). The neural output is conducted along the auditory nerve to the hindbrain and ultimately to the auditory cortex via the central auditory pathways. Complexity increases from the outer ear to the cortex and is inversely proportional to our understanding of auditory processing and to our ability to treat hearing problems. There are numerous diseases of the ear [2] but most forms of hearing loss are sensorineural, involving loss of the sensory hair cells and primary sensory neurons in the inner ear [3]. In addition, approximately one in seven people suffer from tinnitus, a complex condition involving endogenous generation of noise from the inner ear and central auditory pathways [4,5]. This article focuses largely on the potential for drug development to treat sensorineural hearing loss (SNHL). Drug discovery is often an opportunistic process. However, knowledge of potential cellular and molecular targets greatly enhances the chances of success in terms of discovery and of the assessment of safety and specificity. Knowledge of the relevant anatomy and physiology is crucial to the development of drug delivery systems [6].

Conductive hearing loss

Conductive hearing loss involves the attenuation of sound conduction through the outer ear and middle ear (Figure 1a). The most common problems involve accumulation of wax and various forms of infection or skin disease [2]. The outer-ear canal leads to the eardrum and middle ear, which contains a series of three small bones named the malleus, incus and stapes. These bones couple the tympanic membrane to the oval window of the inner ear, focusing the sound energy so that it can be transmitted efficiently from air to fluid. Conductive hearing loss in the middle ear is caused by a wide variety of problems, such as infection and inflammation, otosclerosis, carcinoma, head injuries and sometimes genetic defects. Most of these conditions can be treated with drugs or surgery, including replacement of the ear ossicles, if necessary. Otitis media is one of the major causes of treatable hearing loss in children but, if it is ignored, it can have a serious impact on learning and social interactions [2]. With the exception of cochlear implants, which have now been fitted to more than 80,000 patients with severe or profound SNHL, otolaryngologists do not often venture beyond the oval window and into the inner ear, where the most important cellular and molecular targets for treatment of SNHL are located.

The inner ear

The inner ear contains six anatomically separate mechanosensory epithelia, which are adapted to interpret different forms of mechanical stimulus. Five of them are part of the vestibular system. The posterior, superior and lateral semicircular canals project from the dorsal region of the inner ear (Figure 1a). At one end of each canal there is a chamber that contains a small sensory epithelium, known as a crista ampullaris, and the three cristae detect angular acceleration of the head in three planes. Two separate macular epithelia located within the saccule and the utricle detect vertical and horizontal linear acceleration, respectively. All of these sensory epithelia are positioned centrally within the inner ear. The auditory epithelium is located ventrally and is coiled into the characteristic structure of the cochlea.

The cochlea

The human cochlea is a coiled tube 30–35 mm long containing a collagenous basilar membrane, which is relatively narrow and thin at the basal end and which increases

progressively in width and thickness towards the apex (Figure 1b). Sound energy is absorbed maximally at the part of the membrane that shares a similar resonant frequency and results in oscillatory motion of the basilar membrane. Thus, the mechanical properties of the basilar membrane determine the range of frequencies that we can hear, which is from ~18 kHz in the base to ~20 Hz in the apex. A cross-section of the cochlear tube shows the basilar membrane as an extension of the bony spiral lamina with the sensory epithelium, or organ of Corti, on top (Figure 1c). One of the most important specializations of the cochlea is its division into three parallel chambers, the scala vestibuli and scala tympani, which contain perilymph, and the scala media, which contains endolymph. The maintenance and circulation of these fluids are critical for cochlear function and the dynamics of drug delivery. Perilymph is similar to the cerebrospinal fluid (CSF), as it contains ~138 mM sodium and only ~7 mM potassium. In fact, it communicates with the CSF via the cochlear aqueduct, which is located in the scala tympani at the basal end of the cochlea. Endolymph contains ~1 mM sodium and ~154 mM potassium [7]. Potassium ions are pumped into the scala media by cells of the stria vascularis, which lies against the lateral wall of the cochlear duct. The ionic difference provides the driving force for mechanoelectrical transduction, because the electrical potential in the scala media is ~80 mV compared with 0 mV in the scala vestibuli and scala tympani. This endocochlear potential occurs across the epithelial boundaries between the scalae. Endolymph is separated from perilymph in the scala vestibuli by the Reissner's membrane and in the scala vestibuli by the organ of Corti and the adjacent non-sensory epithelium (Figure 1c). The endocochlear potential is essential for mechanoelectrical transduction in hair cells and its demise is a critical factor in many forms of hearing loss.

The organ of Corti, as with all mechanosensory epithelia, is composed of supporting cells and hair cells (Figure 1d). There are major differences between mammalian and non-mammalian auditory epithelia, because mammalian supporting cells and hair cells are structurally adapted for a highly specialized mechanism of mechanical tuning [8]. This mechanism appears to allow mammals to hear much higher frequencies, but this might have come at a price in terms of the loss of regenerative capacity.

There are ~15,500 hair cells in each human cochlea. These include 3,500 inner hair cells (IHCs) and 12,000 outer hair cells (OHCs) (Figure 1d and 2a). The IHCs generally form one or two rows along the inner edge of the organ of Corti and are the primary sensory receptors, innervated by ~95% of the primary sensory afferent neurons in the spiral ganglion. Their hair bundles are composed of 50–100 stereocilia organized in two or more linear rows, which increase in height away from the cochlear axis. OHCs are cylindrical and usually organized into three rows along the outer edge of the organ of Corti. They receive only 5% of the afferent innervation but the majority of the efferent fibers, which originate from the superior olive in the brainstem [9].

Stereocilia resemble large microvilli ~300 nm in diameter and 2–5 μm long, the longer bundles being located at the apical low-frequency end of the cochlea. They are made of a semi-crystalline array of actin filaments cross-linked with fimbrin and a host of other actin-binding proteins. Neighboring stereocilia are connected by short extracellular links, which maintain the integrity of the bundle [10]. Transduction is thought to involve specialized tip-links that connect the tips of the shorter stereocilia to the shafts of longer neighbors (Figure 1f). Appropriate displacement of a hair bundle increases or decreases the stress on the tip-links and regulates the gating of a small number of mechanosensitive ion channels in the stereociliar membrane. The molecular anatomy of the hair bundle involves hundreds of different proteins [11]. Recent evidence suggests that the tip-link is composed of cadherin 23 [12] and that the mechanotransducer channel is TRPA1, a member of the transient receptor potential family of ion channels [13]. The transducer channel is a non-selective cation channel that regulates the flow of potassium ions into the cell. It also allows calcium entry, which is important for sensory adaptation and for active mechanical responses in the hair bundle [14]. Receptor potentials in IHCs regulate glutamate release at high-speed ribbon synapses in the basolateral membrane, thus modulating the activity of auditory nerve fibers [15]. Stimulus intensity is encoded by the number of channels activated, which influences the size of the receptor potential, and by firing rate in low- and high-threshold sensory nerve fibers. Each IHC receives ~20 afferent endings but each afferent fiber innervates a single IHC. OHCs respond quite differently to changes in membrane potential. Their membranes include a semi-crystalline array of a protein called prestin, which alters its conformation with the membrane potential and forces cell length changes at acoustic frequencies [16,17]. The hair bundles also generated mechanical forces [14] and the two mechanisms are thought to amplify and tune the mechanical responses of the basilar membrane. The responses of the OHCs are modulated by the efferent innervation, primarily via activation of acetylcholine receptors, which permit calcium entry and subsequent activation of calcium-activated potassium channels [18]. Thus, there is a clear division of labor between the two types of hair cell. Without IHCs one would be totally deaf but the amplification provided by OHCs is extremely important and enhances our hearing sensitivity by 40–60 dB [16]. The innervation to the hair cells passes along the boney spiral lamina and into Rosenthal's canal, where the spiral ganglion is located (Figure 1c). The spiral ganglion includes all the cell bodies of the primary sensory neurons, whose axons project via the VIIIth nerve to the cochlear nuclei in the brainstem.

The mechanical coupling between the basilar membrane, hair cells and hair bundles is highly specialized and places challenging constraints on regeneration. The tips of the OHC bundles are attached to a loosely woven, collagenous tectorial membrane. The two membranes are hinged separately at different levels so that vertical motion of the organ of Corti leads to shear displacements between the hair cell apices and the lower surface of the tectorial membrane. This causes oscillatory, planar displacements of the hair bundles and generates receptor potential modulation in the hair cells. IHC bundles are indirectly coupled to the tectorial membrane by fluid displacement in the restricted space between the tectorial membrane and epithelial surface.

Pathology and treatment of hearing loss

Treatments for sensorineural hearing loss can be divided into three categories, preventative, prosthetic and regenerative. Inexpensive drugs have already been tested to protect the auditory system from the deleterious effects of noise or prescribed, ototoxic drugs [19,20]. Protein kinase inhibitors that block apoptosis via c-Jun N-terminal kinases provide effective protection against acoustic trauma and ototoxicity [21,22]. Cochlear implants can partially replace the function of lost, auditory sensory cells and even the primary sensory innervation [23]. Regenerative treatments are not available. However, during the past few years our knowledge of the development and genetics of the auditory system has increased dramatically and research has revealed clear potential for gene and cell therapies [24]. These advances allow us to search more effectively for potential drug targets.

Noise and ototoxic drugs

Noise-induced hearing loss (NIHL) is the major cause of avoidable, permanent hearing loss, accounting in part for about a third of affected people in developed countries. Although protection from excessive noise is desirable, uncontrolled exposure will remain a serious problem for the foreseeable future. Despite the fact that the prevalence of hearing loss could be cut in half by responsible care within social and industrial environments, there remains a substantial need for curative as well as preventive treatments. Very recent warnings from the Royal National Institute for Deaf People (RNID) urge music fans to limit the maximum volume of their iPods, because the enthusiasm for MP3 players promises irreversible hearing damage for this generation of users (www.rnid.org.uk). In some circumstances, for example in the military, exposure to noise is hard to avoid. Thus, it is important to uncover the pathogenic mechanisms of NIHL and to develop effective preventive medications.

Prescribed, ototoxic drugs, principally the aminoglycoside antibiotics, account for ~3–4% of hearing loss in children and adults in developing countries and a significant number of adults in developed countries [25]. The use of ototoxic drugs is justified in the face of life-threatening conditions and, as with NIHL, greater knowledge of the molecular mechanisms will help in the discovery of effective preventive medications [19].

Noise and aminoglycoside antibiotics have been used to produce animal models of hearing loss for some time and now we have a reasonably clear picture of the immediate pathology. Excessive noise can cause structural damage to the hair bundles and can generate excitotoxic effects on the sensory nerve terminals [26]. Hair cells die by apoptosis and are removed as the apices of the surrounding supporting cells converge to seal the epithelium without compromising the composition of the endolymph [27,28] (Figure 2). Unlike supporting cells in non-mammalian epithelia, mammalian supporting cells in the organ of Corti do not proliferate to replace lost hair cells and they do not naturally change their phenotype. Loss of hair cells leads to loss of spiral ganglion neurons

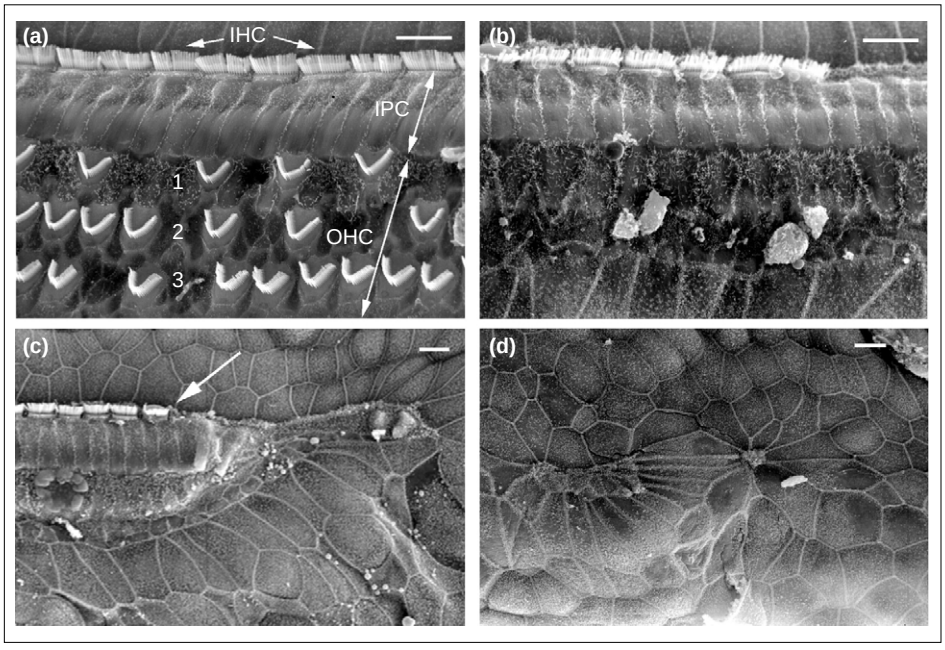

FIGURE 2

Scanning electron micrographs of the surface of a guinea pig organ of Corti, showing progressive loss of hair cells following treatment with the aminoglycoside gentamicin. (a) Hair cells seen from above the epithelial surface with the tectorial membrane removed. OHCs have a 'V' shaped organization and IHCs are linear. There are normally three rows of OHCs (1–3) and only a single row of IHCs. The 2 cell types are separated by the extended apices of the IPCs. At this stage several OHCs are missing, particularly from row 1, but the row of IHCs is complete. **(b)** At this stage the OHCs have been lost and the Deiters' cells have repaired the epithelial surface. Loss of IHCs is visible to the right hand side of the image. **(c)** Some IHCs remain (arrow) and the sensory epithelium appears to zip closed from the right. **(d)** All hair cells have been lost, with substantial changes in epithelial organization. It is not clear which of the remaining cells are Deiters' cells or pillar cells. Figure reproduced, with permission, from Ref. [62].

(SGNs) [29], which depend on hair cells for the production of survival factors such as the neurotrophin NT-3 and the brain-derived neurotrophic factor (BDNF). Ototoxic drugs can cause death of SGNs directly, although for aminoglycosides the effects appear to be indirect and related to loss of hair cells [30,31]. The degeneration of SGNs following hearing loss in humans is variable and rarely complete [32,33].

Surprisingly, little is known about the molecular events associated with hair cell degeneration in the longer term and it is not clear whether the failure to replace hair cells is predominantly a function of inhibitory signals, as occurs in the spinal cord [34], or a lack of regenerative potential in the supporting cells. Nevertheless, accumulation of free radicals, excitotoxicity mediated by glutamate receptors and activation of apoptosis are predictable players in the loss of cells. Animal experiments show that growth factors and drugs directed against apoptosis, excitoxicity and oxidative stress can provide valuable protection from hearing loss if applied during exposure [19]. For predictable exposure to noise or ototoxic drugs preventative treatments are already available and the market for them will remain substantial for the foreseeable future.

Age-related hearing loss

Age-related hearing loss (AHL or presbyacusis) is extremely complicated and includes the effects of both NIHL and ototoxic drugs [35]. Figures for the UK population reflect those for developing countries (www.rnid.org.uk). Moderate hearing loss, for which hearing aids are usually recommended, affects 1.6% of people from 16–60 years old, 16.5% of those between 61–80 years old and 57.9% of those over 80 years old. Combined with the effects of increasing lifespan and of leisure noise on younger generations, these numbers reveal the huge scale of the problem in the future.

AHL shares many of the features of classical neurodegenerative diseases, such as Parkinson's disease, motor neuron disease or Alzheimer's disease. Functional deficits are associated with irreversible losses of specific cell types. AHL is consistently associated with a loss of OHCs and a smaller decrease in the numbers of IHCs, which are lost progressively from the high-frequency end of the cochlea. These losses are usually associated with a decrease in the number of SGNs. As noted in the context of NIHL, these cells are susceptible to oxidative stress and they can be protected to some degree by antioxidants or possibly growth factors. However, the underlying causes of AHL are not known and the long timescales involved preclude the use of preventive drugs such as those used to treat NIHL.

The only way to treat AHL biologically is to replace lost hair cells and SGNs. This might be ineffective if the cause of cell death is indirect and has not been treated. Hair cells and SGNs often die first because they are the most vulnerable cells rather than because they malfunction [36]. AHL must certainly have a genetic component but is it simply a function of continuous accumulation of insults, such as noise, or part of a programmed decline? Intriguingly, most forms of inherited deafness are related to mutations in connexin genes [37,38]. Connexins are membrane proteins that provide low resistance electrical pathways between cells and permit exchange of small molecules, such a calcium ions, potassium ions and ATP. Hair cells do not express connexins but supporting cells do and they use them to form a functional syncytium, from which the hair cells are electrically isolated. One proposed function of supporting cells is to take up potassium ions that are pumped out across the hair cell basolateral membrane and recycle it back to the blood system or the scala media [39]. In mouse models of connexin mutations the hair cells die by apoptosis [40]. Replacing them or the SGNs would not solve the problem. Cochlear homeostasis is crucial and it is important to assess the contribution of other cells such as fibrocytes, which are distributed throughout inner-ear tissues [41,42]. Similarly, if the blood supply to the stria vascularis becomes less efficient with age, it can influence the endocochlear potential, which in turn will affect the hair cells. Ischemia is quite quickly followed by hair cell death and progressive degeneration of the stria could be an important factor in AHL [43]. However, despite suggestions that conditions such as atherosclerosis and hypertension cause hearing loss, the relationship might only be due to a shared association with ageing [44,45]. Bearing this issue in mind for future research programs, hair cell and SGN regeneration remains a potential option for AHL, which is a substantial, increasing cause of hearing loss worldwide and particularly in developed nations.

Genetics of hearing loss

Studies on the genetics of deafness have had a huge impact on our understanding of the development and physiology of the inner ear [39,46,47]. Hundreds of genes are involved in syndromic and non-syndromic hearing loss. Van Camp and Smith created an extremely useful database entitled the hereditary hearing-loss homepage (http://dnalab-www.uia.ac.be/dnalab/hhh/), which provides key information about deafness loci, genes, markers, published references and some gene-expression patterns. During the past 10 years nearly 60 autosomal recessive genes and a similar number of autosomal dominant genes have been discovered and many more remain to be identified. Some late-onset or progressive-deafness genes have been identified recently and much greater attention is now being given to risk factors that underlie susceptibility to noise, ototoxicity and age [48–50]. In therapeutic terms there is unlikely to be a single solution for the many forms of inherited deafness. Apart from mutations in connexin genes, the numbers of people suffering from mutations in any specific gene are small, often involving only a few families.

Cellular targets for regeneration

To stimulate regeneration it is important to identify the potential source of new cells, not only in the healthy ear but also in ears that have degenerated over a period of time. Furthermore, we must consider where any new cells must be located, if they are to be functionally useful. Non-mammalian vertebrates, especially birds and amphibians, regenerate lost hair cells naturally [51,52]. Supporting cells and hair cells share a common progenitor during development [53] and supporting cells are the natural source of new hair cells, either by straightforward conversion [54–56] or by a single asymmetric cell division [57–59]. There is evidence that mammalian vestibular hair cells can be replaced relatively slowly [57,60–62] but no evidence for replacement in the organ of Corti [62,63]. Interestingly, the very limited regenerative capacity of the vestibular epithelia, which are structurally similar to non-mammalian auditory epithelia, suggests that the specialized mechanism of mechanical tuning in the organ of Corti is not the sole reason for its resistance to regeneration. Developmental and genetic studies have revealed a long list of regulatory genes that control morphogenesis, cell proliferation and cell differentiation in the mammalian inner ear [64,65]. It is logical to assume that knowledge of development will inform therapeutic approaches to regeneration. This must be true to some extent but there are differences between the two processes. Tissue environment, including molecular interactions with adjacent cells and connective tissue, has a substantial impact on cell identity and cell fate. Many regulatory genes are expressed transiently during development and the tissue environment changes dramatically with time, so the adult organ of Corti has a very different molecular profile to that of earlier developmental stages. This is reflected in response to extrinsic signals, for example retinoic acid and thyroid hormone [66,67]. Nevertheless, useful information for regenerative purposes is likely to emerge from studies on that period of development, when progenitors are being selected as hair cells, supporting cells or neurons.

The nature of cochlear degeneration and the molecular profile and developmental competence of the cells that remain after longer term loss of hair cells are important factors (Figure 2). This has been studied in the short term following acute insult [68,69] and in animals that suffer from AHL [42], although we still know very little about molecular changes that might influence regenerative responses. Another factor is coupling new sensory cells with the sensory input. Hair cells must be located above the basilar membrane with some mechanical coupling between their hair bundles and the tectorial membrane. Considering all of these issues, in the organ of Corti the target cell population must be the supporting cells, which include inner phalangeal cells, pillar cells and Deiters' cells.

Spiral ganglion neurons

Less attention has been given to neural regeneration because it tends to be viewed as secondary to the loss of hair cells. Spiral ganglion cell loss is variable and rarely complete in humans, even after long periods of deafness [33]. There is evidence that new hair cells can direct their own innervation from existing SGNs [70] but they are unlikely to be able to stimulate neuronal regeneration. Noise and prescribed drugs can damage SGNs directly and there is a therapeutic interest in regenerating auditory innervation alongside other treatments, such as cochlear implants [71,72]. It might be possible to stimulate proliferation and differentiation of replacement SGNs, either from existing SGNs or the glia but there is currently little experimental evidence to support this idea.

Stem cells

An excellent recent review describes the current state of research on inner-ear stem cells and their therapeutic potential [73]. There are three reasons for studying stem cells. The first is that they can potentially be transplanted into host tissue to replace lost cells. A surprisingly large number of exploratory cell transplantation experiments to the inner ear have already been carried out [74–82]. Cell transplantation is an unpredictable science, in which many experiments are conducted in a highly exploratory manner. The number of variables involved in a given experiment is so great that few studies can be compared directly. This has proved to be a major issue for analysis of cell therapies in Parkinson's disease [83]. Cells for transplantation include embryonic (ESCs) [78], neural (NSCs) [84], mesenchymal (MSCs) or hematopoietic (HSCs) stem cells [73,85]. There are numerous different stem-cell lines that are prepared and treated in different ways before transplantation. The procedures for transplantation and the state of the host tissue provide further variation. However, there are some excellent animal models for the auditory system, and studies show that transplanted cells can reach the critical areas for repair, particularly the spiral ganglion and the cochlear duct [75,86].

The second reason for studying stem cells is of greater interest in the context of drug discovery. It involves the activation of endogenous, tissue-specific stem cells to effect repair. Growth-factor treatment does appear to awaken dormant stem cells in the hippocampus, following ischemic injury [87]. Stem cells require controlled environments to ensure that they remain undifferentiated and multipotent [88]. Only recently have such cells in a defined 'niche', for example in the eye, been discovered [89]. The challenge is thus to uncover a potential niche in the ear and then to find ways of activating the cells within it. The only evidence so far has come from an analysis of cells from the mouse utricular macula [90]. Nothing similar has been discovered in the cochlea. The third reason for working on stem cells is to find out how to differentiate them into the target cell type. Mouse ESCs can be cultured *in vitro* and transferred into chick otocysts, where they subsequently differentiate as hair cells [91]. The conditions used to prepare these cells before transplantation

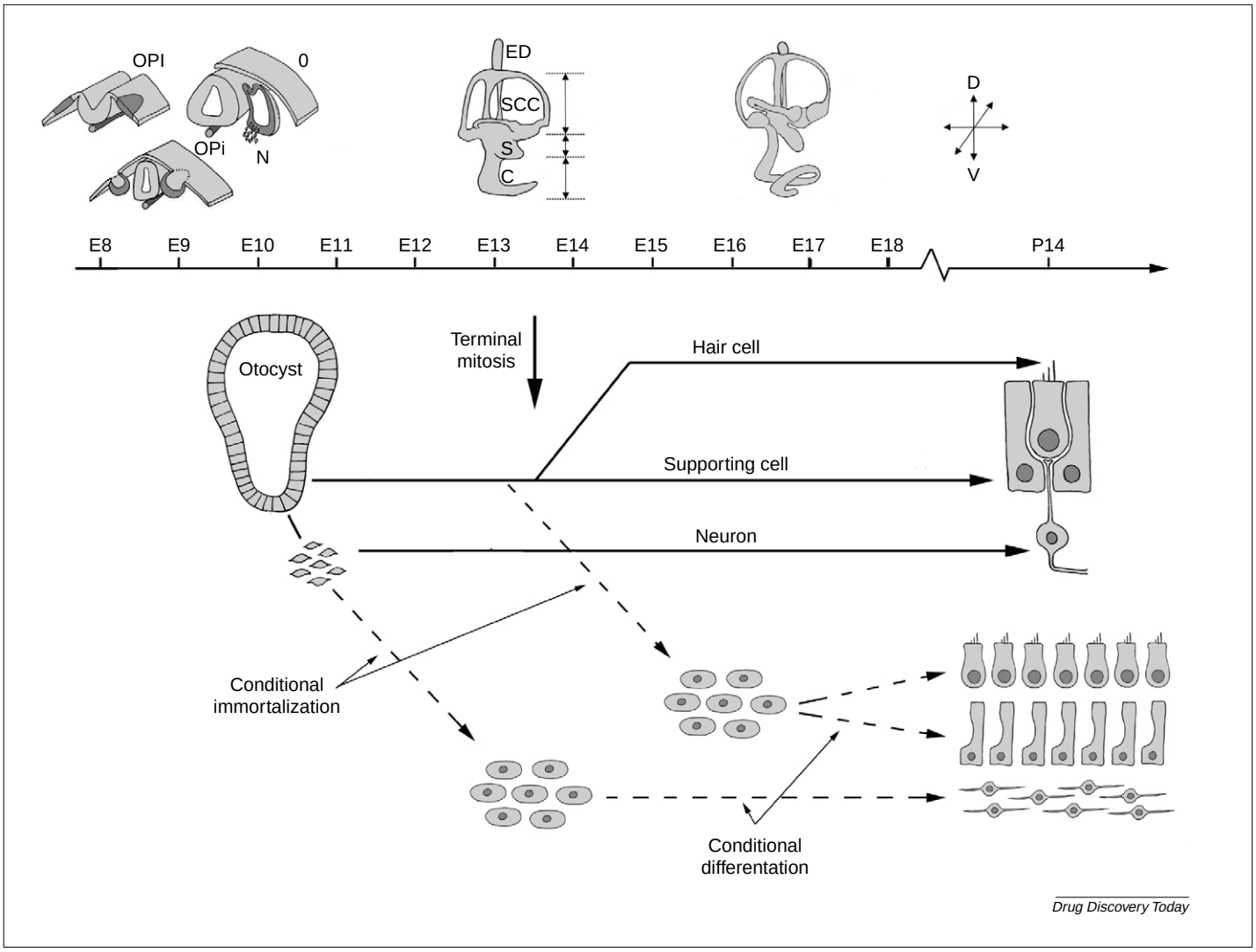

FIGURE 3

Development of a mouse inner ear from embryonic day E8 to post-natal day P14 when the ear becomes fully functional. The top row of figures shows the formation of the darkly shaded otic placode (OPI), a patch of cells in the neural ectoderm that are destined to form the inner ear. These cells invaginate to form an otic pit (OPi) and then an enclosed otocyst (O). At E9–E10 the sensory neuroblasts (N) delaminate from the anteroventral region of the otocyst. At E13 the structures of the adult ear are apparent, including the endolymphatic duct (ED), semicircular canals (SCC), saccule (S) and cochlea (C). The lower row of figures shows the otocyst at E10 with delaminated neuroblasts. Hair cells, supporting cells and neurons cease proliferation at E12–14 and start to differentiate. Note that the SGNs migrate away from the tissue in which they are specified to form separate ganglia. The dashed lines in the lower part of the figure indicate the time and location from which some conditionally immortal cell lines have been derived. Lines derived from the locations indicated can be used as *in vitro* models for the differentiation of neuroblasts and sensory epithelial cells.

are based upon knowledge of early development and are designed to induce gene-expression profiles similar to those of the early otic vesicle. It is not clear how important this conditioning process is or in what way the otocyst influences the ESCs but the preparations should allow these questions to be addressed. Most recently, progenitors for SGNs have been isolated from adult guinea and human auditory tissue and, if they can be cultured reliably and repeatedly, they should provide excellent material for transplantation as well as for studies on neuronal differentiation [92].

Molecular targets for regeneration

Impressive progress has been made in uncovering the genes that regulate proliferation and differentiation in auditory sensory cells. However, many of these regulatory molecules are not suitable as drug targets, which normally include membrane receptors, ion channels, proteases and other enzymes. Nevertheless, they do indicate the relevant molecular mechanisms and provide clues to the signaling pathways, within which suitable drug targets might be identified.

Proliferation of hair cells and supporting cells

In mammalian ears, the hair cells, supporting cells and sensory neurons differentiate during embryonic development (Figure 3). The most detailed studies come from the mouse [93], which has a gestation period of 19–21 days. At embryonic day E9.5, a patch of cells in the neural ectoderm has invaginated to form the otocyst (Figure 3),

a ball of epithelial cells that quickly establishes dorsoventral, mediolateral and anteroposterior axes [65,94]. Cochleovestibular neurons are selected from the anteroventral region from E9.5 and are among the first cells to differentiate. The cochlea forms from a tubular, ventral projection of the otocyst from E10.5–11.5. The first molecular marker for the organ of Corti in the ventral otic epithelium is the cyclin-dependent kinase inhibitor $p27^{kip1}$ [95]. Precursors of hair cells and supporting cells at the tip of the cochlear projection exit the cell cycle at about E12.5 and become located in the apical low-frequency region of the cochlea. Proliferation continues at the base and the epithelium elongates until E14.5, when the last cell precursors exit the cell cycle at the basal end.

Cells that differentiate as hair cells selectively downregulate $p27^{kip1}$ but they subsequently express another cell cycle inhibitor, $p19^{ink4d}$ [96] and the retinoblastoma protein (pRb) [97,98]. In the null mouse for $p27^{kip1}$ the organ of Corti develops with a few extra rows of hair cells and supporting cells, as if the proliferation of progenitors has been able to overrun for a short time before being inhibited [99]. Supporting cells normally maintain expression of $p27^{kip1}$, so it was thought that if the protein could be inactivated in adult cells it might induce proliferation followed by differentiation of a daughter cell as a hair cell. $p19^{ink4d}$ is coexpressed with $p27^{kip1}$ in the sensory precursors and persists in differentiating hair cells. Mice lacking $p19^{ink4d}$ do not form an abnormal epithelium but, within the first few weeks of birth, hair cells enter the cell cycle and die by apoptosis [96]. The pRb, encoded by the gene *Rb1*, also regulates the exit of hair cells from the cell cycle [97,98]. Related members of the same family include p107 and p130, which are encoded by *Rbl1* and *Rbl2*, respectively. All three genes can cause cell-cycle arrest if they are overexpressed. Through the critical period of hair cell differentiation in the mouse utricle, from E12.5 to full functional maturity at post-natal day P12, *Rb1* is expressed constantly, *Rbl1* is upregulated and *Rbl2* is downregulated. Hair cells express *Rb1* but when this gene is deleted they can re-enter the cell cycle and produce new, functionally mature hair cells. It might be possible to manipulate these cell cycle regulators therapeutically, although it could be hard to produce a coherent effect by targeting them individually. We need to know more about cell-cycle regulation in supporting cells. The function of *Rb1* could be therapeutically valuable but the main caveat is that the cellular targets are more likely to be supporting cells, because there is probably less inclination to stimulate regeneration before the hair cells have been lost.

Drugs that influence the cell cycle are focused predominantly on cancer, where the aim is to inhibit rather than to induce cell proliferation, largely with cyclin-dependent kinase inhibitors (CDKIs) applied as antitumor agents [100–102]. To stimulate regeneration of hair cells it is necessary to release the inhibitory effects of endogenous inhibitors transiently and to allow limited proliferation of supporting cells. Organotypic cultures of mammalian inner-ear epithelia provide excellent models for investigating these questions and have been used to screen the proliferative effects of growth factors [103–106]. For example, in the mammalian utricle, forskolin activates adenylyl cyclase, increases cAMP and leads some cells to enter the cell cycle. This response is enhanced by human recombinant glial growth factor 2 (hrGGF2) and is blocked by inhibitors of membrane-receptor recycling [107]. Such preparations are suitable for larger scale screening to identify cell-specific targets that regulate the cell cycle. CDKIs are also involved in other cellular processes such as apoptosis, cell differentiation and transcription, so research in this area might have an impact beyond that of cell-cycle control [100,108].

Selection and differentiation of hair cells and supporting cells

Proliferation and differentiation are not necessarily separate processes, although hair cells and supporting cells can clearly differentiate in the absence of $p27^{kip1}$, $p19^{ink4d}$ and Rb1. The selection of neuroblasts from the early otic epithelium, the development of prosensory epithelial patches and the selection of hair cells and supporting cells from within a sensory patch are regulated by notch signaling [67,109–111]. It is possible that the key to regeneration lies in stimulating proliferation and relying on endogenous interactions between notch receptors and their ligands to select an appropriate pattern of hair cells and supporting cells. Notch signaling can instruct cell differentiation by influencing expression of the bHLH genes *Math1*, *Hes1* and *Hes5* [67,109,110,112] (Figure 1g). Numerous studies on mutant and null mice reveal that these genes regulate the numbers and pattern of hair cells and supporting cells within the sensory epithelium. Cell fate can potentially be modified by drugs targeted against the notch signaling pathway [113,114]. Mammalian hair cells express relatively low levels of notch1 receptor but the levels in supporting cells are much higher. If inhibition of notch signaling were enough, then loss of hair cells should be sufficient to trigger a fate change in the supporting cells. This appears to happen in birds [115] and it might be possible to trigger it therapeutically in mammals.

The discovery that the POU domain transcription factor Brn3c is necessary for differentiation of all hair cells in the inner ear presented some exciting possibilities [116]. Subsequent experiments revealed that Brn3c is actually a survival factor [117], which regulates expression of gfi (growth factor independent) [118] and BDNF [119]. Brn3c is not able to drive hair cell differentiation when transfected into sensory epithelia. However, the bHLH transcription factor Math1 (Atoh1), the mouse homolog of *Drosophila* 'atonal', is also required for hair-cell differentiation [120]. There is some debate about whether Math1 is functional in sensory progenitors [121] as opposed to nascent hair cells [122], which is relevant in terms of its influence on hair-cell differentiation during development.

Most exciting, however, is that if it is transfected into cochlear or vestibular sensory epithelia *in vitro*, then it can induce hair-cell differentiation [123,124]. More dramatically, gene transfection to the guinea pig cochlea *in vivo* stimulates hair-cell differentiation [70,125] and in adults it leads to measurable functional recovery [125]. Gene transfection is not without its problems therapeutically [126] but these results are extremely promising. New hair cells attract dendrites from existing neurons and thus have the potential to be wired up to the cochlear nerve [70]. Cells transformed in the organ of Corti appear to be derived from Deiters' cells and retain their contact with the basilar membrane [125]. Most studies to date have been carried out on animal models that have suffered acute loss through chemical or noise-induced damage and it will be important to try the same approach in animal models of longer term hearing loss [42].

Given the fact that *Math1* transfection can induce hair cell differentiation in adult ears [125], it is worth looking for drugs that might activate expression of this gene. There are some extremely important tools available to do this, including a *Math1* reporter construct that has been used to create transgenic mice [127]. The reporter can be incorporated into inner-ear cell lines [128,129] for HTS of drugs that might activate expression. Interestingly, Id proteins, which regulate various aspects of cell proliferation and differentiation, have been identified as potential drug targets for cancer therapy [130]. They are expressed in inner-ear epithelia and SGNs [131] and might interact with the function of *Math1* [132].

Although some functional recovery is possible with *Math1* transfection, existing supporting cells are converted into abnormal hair cells without proliferation and this could limit the potential therapeutic benefit. With this in mind, some argue that genes normally expressed earlier in development might have the potential to regenerate a complete sensory epithelium. Mice lacking the transcription factor Sox2 lack hair cells and, based on expression of $p27^{kip1}$, also supporting cells [133]. Sox2 is known for its expression in stem cells [133] and might be an important regulator of pluripotency in early sensory progenitors [134]. Whether its expression in adult epithelia, in a cellular environment quite different to that during embryonic development, can lead to regeneration of the whole epithelium remains to be seen. Interestingly, the Sox2-null phenotype in the cochlea is similar to that of a mutant for fibroblast growth factor receptor 1 (FGFR1), which is required for the production of the sensory precursor population [135], and FGFRs are upregulated in supporting cells during regeneration of the chick auditory epithelium [136].

Growth factors and other signaling molecules can clearly change cell fate decisions. The protein sprouty2 (Spry2) is a negative regulator of receptor tyrosine kinases and it appears to antagonize FGF signaling during development of the organ of Corti [137]. In the absence of Spry2 the first row of Deiters' cells develops as a row of pillar cells. Constitutive activation of the canonical Wnt/β-catenin signaling pathway during embryonic development can convert sensory epithelium in the chick from an auditory to a vestibular phenotype [138]. There is a considerable body of evidence concerning the roles of different growth factors during inner-ear development [139]. Some growth factors have protective effects against predictable hearing loss [140] and there is evidence that they can stimulate a limited regenerative response in mammalian vestibular epithelia [141,142]. Their effects are generally mediated by receptor tyrosine kinases, whose ligand-binding sites and kinase domains present potential drug targets [143]. It is thus important to study these receptors and their downstream signaling pathways in targeted cell types within the inner ear [144,145]. The therapeutic application of growth factors is complicated by widespread side effects and the need to deliver them locally and for long periods. In this context, the inner ear has the advantage of being a relatively enclosed system, even though there is a direct link to the CSF [6].

Spiral ganglion neurons

Numerous transcription factors regulate differentiation and survival of SGNs. These include the bHLH factors neurogenin 1 [146], which is equivalent to Math1 in hair cells [147], and NeuroD [148,149], the t-box protein Tbx1 [150], the LIM/homeodomain protein islet-1 [151], the POU-domain factor Brn3a [152] and the zinc finger factor Gata3 [153–155]. Furthermore, during early stages of cochlear neuroblast development, FGF1, FGF2 and the insulin-like growth factor 1 (IGF-1) are important for proliferation, differentiation and survival [156–160]. There is also a substantial literature on BDNF and NT-3 [161–164], which are secreted by hair cells and some supporting cells and which not only influence neuronal survival but also some of the more subtle electrical properties that differ between the apical and basal ends of the cochlea [165,166]. Applied together, the two factors can reduce SGN degeneration and enhance dendritic growth several weeks after deafening in adult guinea pigs [72]. This kind of treatment has potential applications for cochlear implants not only to enrich the interface between the dendrites and the implant electrodes but also to minimize surgical trauma during implant insertion. Survival of postnatal SGNs also depends upon neuregulin, which mediates reciprocal interactions between them and adjacent supporting cells [167].

Gene arrays and proteomics

Gene arrays and proteomics provide the opportunity to look for relevant signaling pathways and functionally related groups of molecules [168]. Affymetrix oligonucleotide arrays were used to profile gene expression during development of the mouse utricular macula and this provided clues to the recent work on retinoblastoma proteins

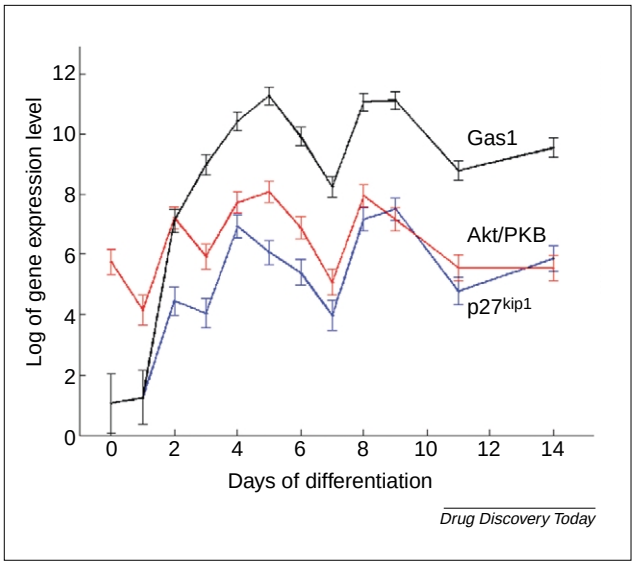

FIGURE 4

Temporal profiles for three genes clustered with Gata3 in an otic epithelial cell line. The genes are protein kinase B (Akt1/PKBα), $p27^{kip1}$ and growth arrest specific 1 (Gas1) [178]. Akt1 and $p27^{kip1}$ are known to be functionally linked. Gene expression is plotted against time of differentiation in days. Clustering of similar expression profiles provides a method of identifying functionally related genes that are expressed during specific cell behaviors. This approach can be used to identify signaling pathways linked to several regulatory genes.

as regulators of hair cell division [98]. Similar arrays were also used to identify the gene for growth factor independent 1 (gfi1) as a downstream target of the POU domain factor Brn3c [118]. cDNA arrays have been used to study gene expression in different development compartments of the early mouse otocyst [169]. Custom-made human cDNA arrays have been used to analyze differences in gene expression in the regenerating and quiescent chick cochlea [170]. Plasticity of the central auditory pathways has also been studied with cDNA arrays [171,172].

In complex tissues, these studies are challenging because many regulatory genes have different functions in different cells at different stages of development. It thus becomes difficult to look at specific processes in individual cell types. This issue can be addressed by establishing cell lines from the inner ear [129]. There are now many lines available and the majority of them are conditionally immortalized [128,173–176]. Conditional immortalization allows cells to be isolated and transformed from specific times and locations during development (Figure 3) [177]. The cells can be cloned and expanded but then 'differentiated' under controlled conditions after inactivation of the immortalizing gene. This approach has been used with affymetrix oligonucleotide arrays to plot temporal profiles of gene expression with time [178]. Genes that share similar temporal expression profiles are likely to be functionally related, even if they are not linked directly by transcriptional regulation. The transcription factor Gata3 is essential for the development of the mammalian ear [153,155] and is especially important for the formation of the spiral ganglion [154,155]. It is also upregulated during regeneration of the chick cochlea [170]. It has been functionally linked to the control of proliferation in hematopoietic cells and in a cochlear epithelial cell line it shares a close temporal profile to the CDKIs $p27^{kip1}$ (Figure 4) and p21. These cell lines can provide not only important information on gene networks but also the tools for studying *in vitro* interactions among proteins for undertaking large-scale drug screens [179]. Proteomics approaches are still in their infancy in the auditory system [180], although it is now a priority for funding at the National Institutes of Health [181].

Animal models and the move to human tissue

Hearing research is endowed with a wide range of animal models, which can be used to explore the nature of deafness and to assay the functional effects of experimental treatments, including gene transfection, drug delivery and cell transplantation. Rodents provide models for NIHL, drug-induced hearing loss, specific loss of SGNs, progressive and age-related hearing loss. Additional animal models such as the zebrafish [182] help us to understand genes involved in various forms of human deafness and this will be true for the worm, *Caenorhabditis elegans,* and *Drosophila*, which have provided clues to some of the most important developmental genes. The newt, a master of regeneration, offers preparations in which one can study the molecular mechanisms regulating cell proliferation and differentiation of new hair cells in adult vertebrates [55,183]. Nevertheless, there is a clear need to work with human tissue where possible. Human progenitors of SGNs have been cultured as neurospheres and can differentiate as neurons *in vitro* [92]. These cells will provide candidates for transplantation but will also be important for studies on proliferation and differentiation.

Conclusion

Deafness presents one of the largest global markets for drug development, and basic research has opened up many promising lines of research for preventive and regenerative treatment. This is backed by an impressive repertoire of experimental preparations from the most elementary *in vitro* models of cell proliferation and differentiation to functional analysis of the auditory system *in vivo*. Cell lines, stem cells, organotypic cultures and a wide range of animal models provide the key components for drug discovery and development. There is now an unparalleled opportunity to take the highly productive basic research of the past 10–20 years and focus very firmly on its translation to clinical application.

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