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TREATING HEARING DISORDERS WITH CELL AND GENE THERAPY

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ABSTRACT

Hearing loss is an increasing problem for a substantial number of people and, with an aging population, the incidence and severity of hearing loss will become more significant over time. There are very few therapies currently available to treat hearing loss, and so the development of new therapeutic strategies for hearing impaired individuals is of paramount importance to address this unmet clinical need. Most forms of hearing loss are progressive in nature and therefore an opportunity exists to develop novel therapeutic approaches to slow or halt hearing loss progression, or even repair or replace lost hearing function. Numerous emerging technologies have potential as therapeutic options. This paper details the potential of cell- and gene-based therapies to provide therapeutic agents to protect sensory and neural cells from various insults known to cause hearing loss; explores the potential of replacing lost sensory and nerve cells using gene and stem cell therapy; and describes the considerations for clinical translation and the challenges that need to be overcome.

INTRODUCTION

Hearing loss is a significant health burden on society. The World Health Organisation estimated in 2012 that over 5.3% of the world's population (approximately 360 million people) have a disabling hearing loss. Given that a large proportion of the population is aging, the number of people impacted by hearing loss will continue to rise. Hearing impairment can reduce quality of life resulting in communication disorders that affect the development of language in children, and have educational, social and vocational ramifications throughout life. Hearing impairment is also associated with the onset and progression of dementia (Lin *et al* 2011).

Sensorineural hearing loss (SNHL) is the most common form of hearing loss and typically occurs as a result of the loss of functional sensory hair cells within the cochlea (Figure 1). The sensory hair cells convert the mechanical acoustic input into electrical stimuli that are conveyed to the auditory brainstem via the auditory neurons, which are commonly known as spiral ganglion neurons (SGNs). The sensory hair cells are highly susceptible to damage from ototoxic drugs and over-exposure to noise, and viral and bacterial infections, such as meningitis and rubella, can also damage the hair cells and SGNs (Gupta *et al* 2012). Sensorineural hearing loss can also have a hereditary cause with over 60 genes implicated in non-syndromic hearing loss (Angeli *et al* 2012). Finally, age-related hearing loss, or presbycusis, gradually occurs in most individuals as they grow older, with approximately 30% of adults between the ages of 65-74 years having some degree of hearing loss (Albers 2012).

For patients with severe to profound SNHL a cochlear implant can provide the pitch and temporal cues necessary for speech perception by electrically stimulating the remaining SGNs. Presently there are approximately 320,000 implant recipients in over 120 countries. Although initially intended for people with profound SNHL, there is an increasing trend to provide cochlear implants to people with useful residual hearing. These recipients integrate auditory information provided by both acoustic input processed by their residual hair cells and by electrical stimulation of the SGNs, and show improved speech perception, particularly in the presence of background noise (Gantz *et al* 2005, Kiefer *et al* 2005, Podskarbi-Fayette *et al* 2010). However, up to 50% of cochlear implant recipients with residual hearing experience a

progressive hearing loss (ie. loss of hair cell function) following cochlear implantation (Gstoettner *et al* 2006, Gantz *et al* 2009, Carlson *et al* 2012, Tamir *et al* 2012). This can occur shortly after implantation as a consequence of surgical trauma, or many months following surgery via mechanisms that are not well understood (Turner *et al* 2008). In addition, many other forms of SNHL are progressive over time, with remaining hearing continuing to deteriorate. Indeed, SGNs undergo progressive degeneration in SNHL (Figure 2), ultimately resulting in significant neuronal loss after long periods of deafness, and findings from animal studies suggest that this may compromise the efficacy of cochlear implants (Shepherd and Javel 1997, Hardie and Shepherd 1999). Significantly, results from a recent study indicate that for individual patients, a larger number of surviving SGNs results in better performance after cochlear implantation (Seyyedi *et al* 2014), highlighting the importance of maintaining a viable SGN population.

Therefore, there is an opportunity to provide a therapeutic intervention to prevent progressive loss of hair cells and SGNs after initial detection of SNHL or following cochlear implantation. Intracochlear drug delivery has the potential to meet these needs; however, current methods, including a systemic approach or diffusion across the round and/or oval windows, are limited by blood-brain permeability, high dose rates and ineffective uptake (Shepherd 2011). Furthermore, clinicians are reluctant to surgically open the cochlea for drug delivery alone for fear of losing residual hearing. Technological advances are therefore required to make drug delivery a viable therapeutic strategy for the treatment of SNHL.

The development of a technology for intracochlear drug delivery, that could be provided at the time of surgery and used in conjunction with a cochlear implant, would certainly be a major breakthrough in the cochlear implant field and enable many more people to benefit from this device. Such techniques may also promote SGN fibre outgrowth, and if such outgrowth can be controlled and guided this may be able to be harnessed to improve the electrode-nerve interface, which in turn may improve the efficacy of the implant. This paper will mainly focus on intracochlear drug delivery for the aim of protecting residual hair cells and SGNs. We will also discuss the potential of cell replacement therapies to replace lost function.

INTRACOCHLEAR DRUG DELIVERY STRATEGIES

Studies are currently investigating the most clinically translatable strategy for drug delivery to the cochlea. Experimental approaches, including the use of a cannula attached to an implanted drug reservoir, have been used to deliver compounds with protective effects on the SGNs and/or hair cells, such as neurotrophic factors, anti-oxidants and anti-apoptotic agents (Gabaizadeh *et al* 1997, Gillespie *et al* 2003, Wise *et al* 2005, Maruyama *et al* 2008, Shepherd *et al* 2008, Eastwood *et al* 2010). The neurotrophic factors brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) are produced by the hair cells and supporting cells (Stankovic *et al* 2004, Green *et al* 2012, Ramekers *et al* 2012) of the organ of Corti (Figure 1A), and are key to both the development and ongoing survival of the inner ear sensory cells. Loss of these endogenous neurotrophic factors, as occurs following a SNHL, contributes to SGN degeneration. In addition, numerous aetiologies of hearing loss occur as a result of the generation of reactive oxygen species and subsequent oxidative stress, or the initiation of other apoptotic pathways. For example, the formation of oxygen free radicals is hypothesised as the major cause of noise-induced hearing loss (Shoji *et al* 2000, Henderson *et al* 2006), and the ototoxic side effects of cisplatin and aminoglycoside antibiotics are reported to be due to the generation of reactive oxygen species and oxidative stress (Schacht *et al* 2012).

However, there are general concerns surrounding the longevity and risk of infection from such experimental delivery techniques, and such devices are therefore generally not considered suitable for clinical use to treat SNHL (Pettingill *et al* 2007). As a consequence, attention has focussed on other strategies to deliver therapeutics to the cochlea. These strategies are designed to target the hair cells, the SGNs, or both. Cell and gene-based therapies have the potential to be used to deliver therapeutic agents to support SGN and hair cell survival, and are the focus of ongoing research by numerous laboratories around the world.

Drug Delivery using Cell-Based Therapy

Cell-based drug therapy involves the use of cells that either naturally secrete therapeutic compounds or are genetically modified to over-express these compounds. Fibroblasts, Schwann cells and stem cells have all been used

successfully to express neurotrophic factors and have been reported to promote nerve survival, regeneration and recovery of function in various nerve degeneration models (Blits *et al* 2005, Hu *et al* 2005, Rejali *et al* 2007, Pettingill *et al* 2008, Makar *et al* 2009, Pettingill *et al* 2011, Zanin *et al* 2014). In addition, cell-based neurotrophin treatments have already been applied in clinical trials for diabetes (Calafiore *et al* 2006), retinitis pigmentosa (Sieving *et al* 2006), Alzheimer's disease (Wahlberg *et al* 2012) and Amyotrophic Lateral Sclerosis (Aebischer *et al* 1996), and in each of these clinical trials the implanted cells were well tolerated, and there were promising indications of efficacy [for review see (Zanin *et al* 2012)]. More recently, a Phase I/IIa clinical trial commenced using cell therapy for the treatment of Parkinson's disease (http://clinicaltrials.gov/show/NCT01734733).

The use of cell-based techniques to deliver therapeutic compounds to the cochlea is considered a clinically viable option and overcomes issues of infection that are problematic for pump-based devices (Shepherd 2011) and the issue of longevity of survival effects (Gillespie *et al* 2003, Shepherd *et al* 2005) associated with other experimental delivery methods. An important aspect for the clinical translation of cell-based therapies is the biocompatibility and safety of this approach, which can be facilitated through the use of cell encapsulation technology. Encapsulation of cells in a biocompatible, semi-permeable membrane provides an immunological barrier to protect the cells from the immune system of the host (Zimmermann *et al* 2007, Murua *et al* 2008, Thanos and Emerich 2008), and also acts to prevent cellular dispersal away from the site of implantation (Coleman *et al* 2006, Nayagam *et al* 2012). This approach has been used to encapsulate BDNF-expressing cells that were implanted into the cochlea in order to rescue SGNs from deafness-induced degeneration in the guinea pig (Pettingill *et al* 2011), providing survival effects for at least six months (Gillespie *et al* 2013). Furthermore, intracochlear implantation of encapsulated choroid plexus cells, which naturally secrete a host of neurotrophic factors and antioxidants, in conjunction with simultaneous electrical stimulation from a cochlear implant electrode array, enhanced SGN survival in the cat after eight months of deafness (Wise *et al* 2011a).

Furthermore, the use of biofunctionalised cochlear implant electrodes – whereby the electrodes are modified to incorporate a biological function – would combine the benefits of cell-based drug delivery with chronic electrical stimulation,

and has the potential to provide long-term drug delivery to enhance SGN survival and promote axonal regrowth. Indeed, enhanced SGN survival was observed in the basal region of the guinea pig cochlea following implantation of a modified cochlear implant electrode incorporating a coating of BDNF-expressing fibroblasts (Rejali *et al* 2007, Warnecke *et al* 2012). An advanced technique such as this for long-term drug delivery targeted to the inner ear is a promising option for the clinical application of therapeutic agents for the treatment of SNHL following cochlear implantation.

Drug Delivery using Gene Therapy

Gene therapy is a technique based on the introduction of a foreign gene into the body in order to synthesise a gene product that replaces a defective or missing gene, or to evoke a therapeutic response (Avraham and Raphael 2003). Gene therapy can therefore be an alternative method to deliver therapeutic compounds into the cochlea by the expression of the target therapeutic compound. The single inoculation eliminates the need for continuous infusion devices that have limited clinical viability. In addition, gene therapy enables control of therapeutic gene expression at the cellular and temporal levels, and by targeting gene expression to particular cells of the cochlea (Stone *et al* 2005, Liu *et al* 2007), may help to reduce the dose required to achieve a therapeutic effect. The advantages of gene therapy over other delivery systems are its ability to provide long-term expression of neurotrophic factors – a key requirement for SGN protection after hearing loss – and localised or cell-specific expression of genes, for guidance of nerve fibre regrowth, safety and improved therapeutic efficacy.

Gene therapy to provide neurotrophic factor genes has been used in the guinea pig cochlea and shown to promote protection of SGNs and hair cells from ototoxicity and noise exposure (Staecker *et al* 1998, Yagi *et al* 1999, Yagi *et al* 2000, Hakuba *et al* 2003, Nakaizumi *et al* 2004, Liu *et al* 2008, Shibata *et al* 2010). Gene therapy can be applied to the cochlea by direct injection into cochlear fluids via a cochleostomy (Stover *et al* 1999), indirect injection via the vestibular apparatus (Kawamoto *et al* 2001) or by diffusion through the round window membrane (Shibata *et al* 2012).

The initial studies on gene therapy for SGN protection after hearing loss used direct injection of the gene therapy vector into the scala tympani cochlear compartment (Lalwani *et al* 2002, Nakaizumi *et al* 2004). Enhanced SGN survival

after hearing loss was reported in guinea pig cochleae expressing the BDNF gene from vectors such as herpes simplex virus and adenovirus. While direct injection of gene therapy vectors into the scala tympani resulted in a high level of neurotrophic factor production, the broad expression that was observed meant there was very little guidance control for the direction of SGN fibre regrowth (Wise *et al* 2010). Direct injection of the gene therapy vector into the scala media compartment of the cochlea, on the other hand, resulted in improved localisation of gene expression in the organ of Corti (Shibata *et al* 2010, Wise *et al* 2010) (Figure 3). Gene expression was detected in non-sensory supporting cells of the organ of Corti and was generally localised to the basal cochlear region. SGN survival from scala media injection of neurotrophic factor genes was only observed in the basal turn, consistent with the expression profile. The basal high frequency region of the cochlea is typically where hearing loss is most severe and the need for therapeutic intervention most critical. The low frequency apical cochlear region, where patients may have residual hearing, displayed no gene expression and low frequency hearing was unaffected by gene therapy when applied to normal hearing guinea pigs (Wise *et al* 2010). There was also evidence of local peripheral fibre resprouting towards cells expressing the genes (Shibata *et al* 2010, Wise *et al* 2010). These promising results show that targeting therapeutic drugs to the organ of Corti area via gene therapy is optimal for protecting SGNs and, importantly for restoration of hearing, that it is possible to control the pathway of resprouting fibres of the SGNs using gene therapy.

Gene therapy has also been used to protect hair cells from cochlear trauma. Neurotrophic factors such as glial cell-derived neurotrophic factor (GDNF) have been applied to the normal hearing cochlea up to seven days *prior* to an ototoxic insult such as ischemia or application of aminoglycosides, with reported hair cell and SGN protection in the following weeks (Yagi *et al* 1999, Hakuba *et al* 2003, Liu *et al* 2008). However, the ‘preventative’ approach, whereby gene therapy was provided before the insult, would not be suitable for clinical translation as people will not have a surgical treatment on a normal hearing cochlea. In a recent study, guinea pigs were treated with BDNF gene therapy seven days *after* noise exposure. Despite the fact that there was no detectable hearing loss at the time of injection, there were greater SGN densities and better hearing in treated cochleae compared to control (untreated) cochleae after eight weeks (Zhai *et al* 2010). The GDNF gene has also

been applied to the cochlea 4-7 days after a profound aminoglycoside-induced hearing loss and was shown to protect SGNs from degeneration (Yagi *et al* 2000). However, hair cell protection was not observed due to the rapid degeneration of the organ of Corti in this hearing loss model. Protection of residual hair cells with the GDNF gene following a detectable hearing loss is yet to be demonstrated, suggesting that the sensitive hair cells of the cochlea are difficult to protect once damage has been initiated.

REPAIR AND REGENERATION

In the mammalian cochlea, once the hair cells and SGNs are lost, they do not spontaneously regenerate. In addition to drug delivery, cell and gene therapies offer the possibility of repairing the damaged cells or possibly regenerating new cells in order to restore cochlear function.

Stem Cell Therapy

Stem cells offer the potential to replace damaged cells and tissues of the body, by providing a new source of healthy cells. In cases where there is a substantial loss of SGNs following SNHL, neural replacement with stem cell therapy would be suited. Neural stem cell therapy could potentially be combined with a cochlear implant to replace lost SGNs following significant nerve cell loss, where very few SGNs remain viable for electrical stimulation. Alternatively, in instances where a substantial proportion of hair cells remain functional, for example in cases of auditory neuropathy, then stem cell therapy may replace the lost neural link between the hair cells and the auditory brainstem.

The generation of functional neurosensory progenitors for cell replacement has been a major focus for stem cell researchers aiming to treat hearing loss. Encouragingly, cell phenotypes that strongly resemble hair cells (Oshima *et al* 2010, Chen *et al* 2012, Koehler *et al* 2013) and SGNs (Coleman *et al* 2007, Shi *et al* 2007, Martinez-Monedero *et al* 2008, Reyes *et al* 2008, Chen *et al* 2009, Chen *et al* 2012, Nayagam *et al* 2013) have been produced from stem cells, and more recently, from human stem cells *in vitro* (Shi *et al* 2007, Chen *et al* 2009, Chen *et al* 2012, Nayagam *et al* 2013). These differentiated stem cells express several of the key

biochemical markers typical for each cell type, are functional, and possess the overall cellular ultrastructure characteristic to hair cells and SGNs.

Stem cell-derived hair cells have been shown to co-express biochemical markers including *Atoh1*/MyosinVIIa (Oshima *et al* 2010), *Atoh1*/MyoVIIa/*Brn3c* (Chen *et al* 2012) and *Sox2*/Myosin VIIa (Koehler *et al* 2013), and possessed stereocilia rich in epsin (Oshima *et al* 2010, Chen *et al* 2012) and F-actin (Koehler *et al* 2013), with ultrastructural features closely resembling hair bundle protrusions (Oshima *et al* 2010, Chen *et al* 2012, Koehler *et al* 2013). In addition, *in vitro* studies have illustrated the potential to derive functional hair cells from various stem cell types, with patch-clamp electrophysiology demonstrating that stem cell-derived hair cells displayed functional calcium and potassium currents which were similar to those described for endogenous hair cells (Chen *et al* 2012, Koehler *et al* 2013), and the cells were sensitive to mechanical stimulation (Oshima *et al* 2010). Previous studies have attempted to replace degenerated hair cells *in vivo* using exogenous stem cells (Ito *et al* 2001, Tateya *et al* 2003, Sakamoto *et al* 2004, Hildebrand *et al* 2005, Parker *et al* 2007, Han *et al* 2010), and although the results of these studies have been variable, there are several consistent findings which are promising for future stem cell-based therapies. For instance, the survival of a range of exogenous stem cell types in the deafened cochlea for periods up to three months, the *in vivo* differentiation of cells into appropriate and relevant phenotypes, and the minimal tissue response observed following transplantation are encouraging findings for the potential clinical translation of this approach.

Stem cell-derived auditory-like neurons have also been derived from a variety of sources, including mouse embryonic stem cells (Coleman *et al* 2007, Reyes *et al* 2008), cochlear stem cells (Martinez-Monedero *et al* 2008), human embryonic stem cells (Shi *et al* 2007, Chen *et al* 2012, Nayagam *et al* 2013) and induced pluripotent stem cells (Gunewardene *et al* 2014). These neurons expressed a relevant cohort of neurosensory proteins and transcription factors (Nayagam *et al* 2013, Gunewardene *et al* 2014) and generated action potentials in response to intracellular current injection (Martinez-Monedero *et al* 2008, Chen *et al* 2012, Nayagam *et al* 2013, Needham *et al* 2014). Further studies have shown that stem cell-derived neurons are capable of forming synapses on early post-natal hair cells (Shi *et al* 2007, Matsumoto *et al* 2008, Nayagam *et al* 2013) with pre-synaptic terminals that

resemble those made by early post-natal SGNs with the developing sensory epithelium (Flores-Otero *et al* 2007). While the functional activity of these new synapses has not yet been demonstrated, recent findings suggest that there is potential for stem cell-derived neurons to make functional connections in the damaged auditory system (Chen *et al* 2012). *In vivo* studies have focussed on the delivery of stem cell-derived neurons directly into the cochlear modiolus or auditory nerve (Corrales *et al* 2006, Shi *et al* 2007, Chen *et al* 2012). Importantly, the most recent of these studies observed a recovery of auditory-evoked hearing thresholds following stem cell transplantation in a neuropathy model of deafness (Chen *et al* 2012). This study highlighted a number of advances in the field, which included a significant improvement in the neuronal density within the cochlea, expression of an afferent type I SGN marker (NKAα3) in the transplanted stem cells, and an elongation of peripheral fibres towards the hair cells and also a central projection of axons towards the auditory brainstem. Furthermore, expression of a synaptic marker was shown within stem cell-derived fibres contacting the hair cells, and a small number of central synapses were detected in the cochlear nucleus. Functional recovery of varying degrees was reported in all stem cell transplanted animals by using auditory evoked responses, which improved significantly from four until ten weeks post-transplantation (the end-point for the study). Interestingly, the authors noted that, in comparison to the number of transplanted cells, there were considerably fewer stem cell-derived central projections growing towards the cochlear nucleus. Moreover, functional improvement across all frequencies was reported, despite the observation that stem cells were detected only in the base of the cochlea. This suggests that further work is still required to fully elucidate the mechanism(s) by which function is restored in this model.

An alternative strategy for improved electrical hearing is being investigated for severe-to-profound SNHL, where there is extensive damage to hair cells and resulting progressive degeneration of SGNs (Coleman *et al* 2007, Gunewardene *et al* 2012). In these cases, stem cells may provide replacement SGNs for electrical stimulation with a cochlear implant. The new SGNs could potentially be encouraged to extend peripheral fibres in order to contact the electrode array. The axons of the endogenous SGNs may facilitate the formation of tonotopic connections in the cochlear nucleus by providing a scaffold and neuronal guidance upon which new

neuronal axons can grow and target the appropriate destination. Significant challenges still remain, including how to overcome the differences in tonotopic gradients present in SGNs (which are still being discovered (Flores-Otero *et al* 2007)) and encourage the growth of central axons through the glia limitans that may provide a cellular barrier limiting axonal regrowth. One possible approach to overcome this potential barrier is the application of repulsive guidance molecule inhibitors, which have been reported to improve axon regrowth through glial scar tissue after spinal cord injury (Hata *et al* 2006, Kyoto *et al* 2007). The observations that electrical stimulation can significantly enhance neurite outgrowth when combined with neurotrophin application (Evans *et al* 2009) are particularly encouraging as a combined therapy including neurotrophins and electrical stimulation may prove to be successful. Indeed, recent studies in the peripheral nervous system have illustrated the positive effect of electrical stimulation on the accelerated regrowth of endogenous sensory axons (Singh *et al* 2012). The delivery of neurotrophins could conceivably occur at the same time as stem cells were transplanted, and need only last long enough to allow for new axons to extend and synapse on central targets.

Although stem cell research is relatively new there have been some remarkable advancements including the recovery of hearing thresholds in a neuropathy model of deafness (Chen *et al* 2012) and the production of functional hair cells from an *in vitro* stem cell-derived otic placode (Koehler *et al* 2013). It is possible that stem cell therapy may, in the future, provide replacement cells to people with hearing loss and cochlear implant recipients.

Cochlear Gene Therapy

Similar to the potential of stem cells to replace lost cells in the auditory system, gene therapy also has the potential to restore hearing by replacing lost hair cells. However, in this circumstance, the new hair cells would be derived from existing cochlear cells, such as supporting cells in the organ of Corti. The *Atoh1* gene is a transcription factor that is important for the determination of hair cells in development (Bermingham *et al* 1999). The absence of Atoh1 results in a complete absence of hair cells and supporting cells (Pan *et al* 2011), while over-expression of Atoh1 results in ectopic hair cells in the organ of Corti (Izumikawa *et al* 2005). The

intracochlear delivery of the *Atoh1* gene transformed supporting cells of the organ of Corti into new hair cells (Kawamoto *et al* 2003, Izumikawa *et al* 2005). Kawamoto et al. (2003) reported the presence of ectopic hair cells following injection of the *Atoh1* gene into the scala media of normal hearing guinea pigs. These new hair cell-like cells were morphologically consistent with endogenous hair cells as evidenced by the formation of a stereocilia-like structure on their apical surface and biochemical features similar to hair cells with expression of 11 different hair cell proteins (Kawamoto *et al* 2003, Liu *et al* 2012). However, the transformed hair cells lacked expression of proteins normally found in mature hair cells, such as prestin and oncomodulin, and were generally considered to be “immature” hair cells. Importantly, new hair cells were only observed if the treatment was provided shortly following deafening. If there was severe degeneration such as the complete flattening of the organ of Corti epithelium after a profound hearing loss (Izumikawa *et al* 2008), then no new hair cells were found, indicating a short therapeutic window, as residual supporting cells were required for transformation into hair cells. Nevertheless, when the *Atoh1* gene therapy was applied shortly after deafening, improved hearing thresholds were reported (Izumikawa *et al* 2005), suggesting that the new hair cells were functional. However, if the lesion was too severe even at short time points, hearing could not be restored despite the presence of new hair cells (Atkinson *et al* 2014a). The implications of these studies are that the clinical translation of hair cell gene therapy would be limited to less severe forms of hearing loss such as noise-induced hearing loss. There is experimental evidence supporting this suggestion. When Atoh1 was introduced seven days after a damaging noise exposure, there was an overall improvement in hearing thresholds compared to controls, with the suggestion that stereocilia of hair cells near the regions of Atoh1 expression were repaired in these treated animals (Yang *et al* 2012).

Mouse models are helping us to understand the process of *Atoh1* conversion of non-sensory supporting cells into hair cells. It is becoming apparent the specific supporting cell types that are being converted to hair cells are pillar cells and Deiters’ cells of the organ of Corti (Liu *et al* 2012) and that the converted hair cells are predominantly outer hair cell in phenotype, even when their location within the sensory epithelium is in the inner hair cell sensory region (Mizutari *et al* 2013, Bramhall *et al* 2014). Pillar and Deiters’ cells have also been identified to

spontaneously differentiate into new hair cells in the neonatal mouse after ototoxic deafening (Bramhall *et al* 2014), a process that was enhanced by inhibition of Notch signalling (which in turn increases *Atoh1* expression) after damage (Bramhall *et al* 2014). Notch signalling can be inhibited pharmacologically via a γ-secretase inhibitor with resulting hair cell regeneration and partial recovery of hearing in mice (Mizutari *et al* 2013) which presents an interesting and potentially clinically relevant alternative method to gene therapy for hair cell conversion after hearing loss. Direct transdifferentiation of supporting cells to hair cells will of course deplete the sensory epithelium of supporting cells and some strategies are focussing on simultaneously expanding the supporting cell population with proliferative factors such as *Pax2* while converting supporting cells to hair cells with *Atoh1* via combinatorial adenoviral gene therapy (Chen *et al* 2013). Furthermore, under some circumstances, *Atoh1* expression in neonatal transgenic mice yielded hair cells with surrounding supporting cells resembling the normal sensory mosaic via a Notch-dependent mechanism, despite ubiquitous expression of Atoh1 in the sensory epithelium (Kelly *et al* 2012). However, in this and other transgenic studies, many of the findings relating to hair cell regeneration were in juvenile animals and the ability to convert supporting cells to hair cells in mammalian models decreases dramatically with age (Kelly *et al* 2012, Liu *et al* 2012). From these and many other transgenic mouse studies it is clear that the challenges that lie ahead for hair cell replacement will include converting supporting cells into functional, mature hair cell in adults via reliable and reproducible gene therapy techniques.

CLINICAL APPLICABILITY

A number of important considerations for both the delivery of therapeutic agents and the replacement or regeneration of cells must be made prior to clinical application of these therapies.

Cell-Based Therapy: Cell Types and Origins

Cell-based drug delivery therapies have typically used fibroblasts and Schwann cells due to the ease with which they can be harvested, their overall robustness, their ability to be genetically modified to express therapeutic agents, and the long-term expression profiles of these agents (Winn *et al* 1996, Rejali *et al* 2007, Pettingill *et al*

2008, Pettingill *et al* 2011). Long-term expression of the therapeutic drug is likely to be required to maintain the clinical benefits of this approach. For example, it has been reported that neural survival following the removal of cochlear treatment with neurotrophic factor was not sustained (Gillespie *et al* 2003, Shepherd *et al* 2005), and although another study reported continued neuronal survival for a short period after treatment (Agterberg *et al* 2009), it is considered that long-term therapies which result in long-term outcomes will be of greater benefit.

The source of the transplanted cells requires careful consideration in order to minimise potential rejection. Autologous cell transplantation – where the cells are harvested from the patient – would minimise the immune response and the risk of rejection. However, this form of customised cell therapy involves significant time, expertise and expense. An alternative approach would be the use of allogeneic strategies, whereby same-species cells could be pre-prepared and stored in a cell 'bank' for future use. This option would address some of the potential problems, but would be dependent on the availability of appropriate donor cells. The long-term viability of cell therapy remains a major factor to overcome and a potential problem that may limit the therapeutic benefit of this strategy.

A significant challenge to the successful clinical translation of stem cell therapy is the production of stable populations of the correct cell phenotypes for replacement, and to determine the specific stage of cell differentiation at which the optimal number of donor cells will incorporate effectively into the target tissue. There is a delicate balance to produce cells of a specified progenitor state where they are no longer dividing continuously (eg. tumorigenic), yet haven't reached terminal differentiation to the point that they lose their ability to functionally reconnect into the target system. As a consequence of concerns regarding the proliferative nature of cell lines and the potential for tumour formation, differentiated cells are the preferred cells for transplantation.

Most research undertaken on stem cells has been conducted using embryonic stem cells, which involve the use of embryos to derive stem cell populations. Following the successful isolation and culture of the first human embryonic stem cell lines (Thomson *et al* 1998, Reubinoff *et al* 2000), there have been ethical debates around the preferred use of adult-derived cells for clinical translation. Researchers have successfully reprogrammed adult somatic cells to a pluripotent state, from

which they can be differentiated into multiple lineages similar to embryonic stem cells (Takahashi and Yamanaka 2006). While the long-term genetic stability of these 'pluripotent' stem cells is currently unknown, they may potentially provide patient-matched cells which will also avoid the destruction of embryos (Fu and Xu 2011, Fu and Xu 2012).

Stem Cell Therapy: Cellular Integration

The ultimate challenge in stem cell therapy for hearing loss is the successful delivery and functional integration of these sensory progenitors into the deaf cochlea. For the replacement of hair cells, the stem cell-derived hair cells would need to migrate along the basilar membrane to the damaged region, which may vary depending upon the aetiology of the hearing loss. For instance, for noise-induced hearing loss hair cell degeneration typically occurs at a frequency-specific location. This poses several further complications for hair cell replacement, including differences in hair cell morphology and frequency tuning along the length of the cochlea. Furthermore, success depends upon the presence of at least a rudimentary structure of the organ of Corti, and would require the integration of new inner hair cells in the correct orientation, and into the appropriate regions of the organ of Corti. The stem cell-derived hair cells would also possibly need to overcome the phalangeal scar formed from the degeneration of the endogenous hair cells (Okano and Kelley 2012). Finally, the newly transplanted hair cells would need to reconnect with peripheral fibres of healthy SGNs in a tonotopic fashion and form functional connections. Considering recent findings illustrating ongoing degenerative changes to the hair cell-SGN synapse following noise-induced trauma (Kujawa and Liberman 2009), this is likely to be a significant challenge to overcome. Interestingly, the well-known observation that birds can successfully regenerate their inner ear hair cells (Cotanche *et al* 1994) potentially provides a framework whereby molecular mechanisms responsible in this species might be applied in the mammalian cochlea in future. For the replacement of SGNs, new cells must extend fibres peripherally and axons centrally, forming synapses that mimic the existing tonotopic organisation in both the cochlea and cochlear nucleus. In the event that both the SGNs and hair cells require replacement, then co-ordination of the above-mentioned events would need to occur. Despite these seemingly insurmountable challenges, progress has been made toward the use of stem cells for hearing loss, as described above.

Gene Therapy: Viral Vectors for Gene Transfer

There are a number of different vectors capable of delivering genes to cells in the cochlea, each with their own advantages and disadvantages. Most commonly, viral-based delivery strategies have been used experimentally to introduce a gene into the cochlea. These include adeno-associated virus (AAV), adenovirus, herpes simplex virus, lentivirus and vaccinia virus. Factors that will determine the clinical translation of this approach include safety, toxicity, immunogenicity, the duration of gene expression, cell specificity and ease of production (e.g. gene insertion capacity and the ability to generate a large titre). The most commonly used vector for gene transfer in the cochlea is the AAV. The AAV enables long-term gene expression, infects a wide variety of cells in the cochlea, has low toxicity and immunogenicity and is generally safe to use. However, a significant drawback of the AAV is its limited gene packaging capacity, as genes greater than 4-5 kB in size cannot be inserted into AAV vectors.

The newer generation of viral vectors have improved the safety of gene therapy. There are far fewer toxicity and immunological side effects than early first generation vectors. Viral based gene therapy is currently in phase I clinical trials for the treatment of Parkinson's disease with many reported improvements and very few reported side-effects (Kaplitt *et al* 2007, Eberling *et al* 2008, Muramatsu *et al* 2010, LeWitt *et al* 2011). A pre-clinical trial involving AAV injection into the nervous system demonstrated expression lasting for years (Bankiewicz *et al* 2006), and adenovirus gene therapy is also in phase I clinical trials for hepatocarcinoma. However, AAV has been associated with side effects such as fever, flu-like symptoms, pain at the injection site and a reduced blood cell count (Sangro *et al* 2010). Alternatively, electroporation might be a suitable approach for gene therapy in combination with a cochlear implant, whereby electrical stimulation delivered by the implant can be used to drive gene expression without necessitating the use of viral vectors (Pinyon *et al* 2014). Although it is encouraging that clinical trials have commenced, more controlled trials will be needed to prove the effectiveness of gene therapy for human use.

A fundamental advantage of gene therapy is that it involves a single treatment making it a safer alternative to therapies that require continual top-ups or re-implantations. Once injected, gene therapy vectors are taken up into cochlear cells

within minutes, with the duration of gene expression lasting at least six months (Atkinson *et al* 2014b) and in some cases for years (Hadaczek *et al* 2010, Nathwani *et al* 2011). Thus a single application of gene therapy can provide a long-term neurotrophic factor source for the survival of SGNs. Gene therapy also has the capacity to target specific populations of cells leaving other cells unaffected. For example, directed expression of the reporter gene green fluorescent protein could be achieved exclusively in SGNs, hair cells, supporting cells, blood vessels or cells of the spiral limbus using promoters specific for each cell type (Luebke *et al* 2001, Stone *et al* 2005).

However, caution must be made when targeting cell populations that may degenerate due to the ongoing pathology of the condition that is being treated. For instance, the sensory structures in the deafened cochlea degenerate over time, and the effectiveness of gene therapy has been shown to diminish if the targeted cells comprise these residual sensory structures (Wise *et al* 2011b, Atkinson *et al* 2014b). Therefore, gene therapy to non-degenerative cells in the scala tympani or to stable cells within Rosenthal's canal may be a more effective target population. Importantly, not all types of deafness result in organ of Corti degeneration and hence gene therapy targeting cells of the organ of Corti could be an alternative in these deafness aetiologies.

Surgical Considerations

The delivery of gene therapies to the scala media compartment is able to achieve localised gene expression in the organ of Corti by targeting the cells that are affected. However, this approach is surgically challenging as the scala media is small and difficult to access. The scala media also contains the very sensitive sensory elements of the cochlea that we are trying to protect and this surgical approach would potentially risk the viability of these cells. Alternatively, the simplicity of the scala tympani approach makes it more attractive for clinical translation. It could be performed at the time of cochlear implantation, for example, without any further changes to the surgical approach. The surgical approach used for gene therapies to treat hearing loss is therefore going to be dependent upon the status of the hair cells and SGNs within the cochlea and the desired outcome.

Similarly to the delivery of gene therapy, the transplantation of stem cells into the cochlea is not straightforward, requiring precise surgery to access the appropriate compartments – the scala media for hair cells and the auditory nerve/modiolus for SGNs – and therefore several major challenges remain to be overcome in terms of developing a clinically useful stem cell therapy to treat hearing disorders (Park *et al* 2014). Previous studies have used approaches including injection through the semi-circular canals and lateral cochlear wall (Iguchi *et al* 2004), into the endolymphatic space (Han *et al* 2010) and through the basilar membrane (Hildebrand *et al* 2005). Direct injection into the scala media is appealing since this can be used to deliver cells in close proximity to the organ of Corti. One potential problem arising from this delivery technique is a compromise of the electrochemical gradient that exists in the scala media compartment, which is essential for normal cochlear hair cell function. While these early studies delivered exogenous stem cells in close proximity to the organ of Corti (Iguchi et al., 2004; Hildebrand et al., 2005), the molecular characterisation and functional integration of these transplanted cells was not examined. The functional integration of stem cell-derived sensory hair cells remains a major challenge in the field.

Potentially compounding the problem of exogenous stem cell integration into the sensory epithelium, is the high concentration of potassium ions in the cochlear endolymph, which may be toxic to transplanted cells. Recent experimentation has attempted to address this problem by transiently reducing the potassium levels in the scala media and manipulating junctions in the sensory epithelium. In doing so, exogenous cell survival and integration in the mammalian scala media was improved (Park *et al* 2014). Although the exogenous cells used in the described experiments were not stem cells, these findings support the rationale that temporarily altering the cochlear environment can facilitate the survival of exogenous cells.

CONCLUSION

Cell-based therapies are capable of delivering therapeutic agents to the cochlea using cells that naturally secrete these agents, or, when combined with gene transfer technology, can be engineered to over-express these agents. Gene therapy can be used to introduce genes into a system in order to induce the expression of

genes for therapeutic agents, to replace defective genes, or re-program supporting or surrounding cells to acquire the phenotype of lost or damaged cells in order to repair or regenerate the damaged tissue. Stem cell transplantation is a further option to replace damaged or missing hair cells or SGNs with a new population of cells with the appropriate characteristics. These therapeutic approaches may therefore be used to protect the sensory elements in the cochlea required for hearing, to control the direction of re-growing SGN fibres, and to replace lost cells, providing a novel treatment for hearing loss.

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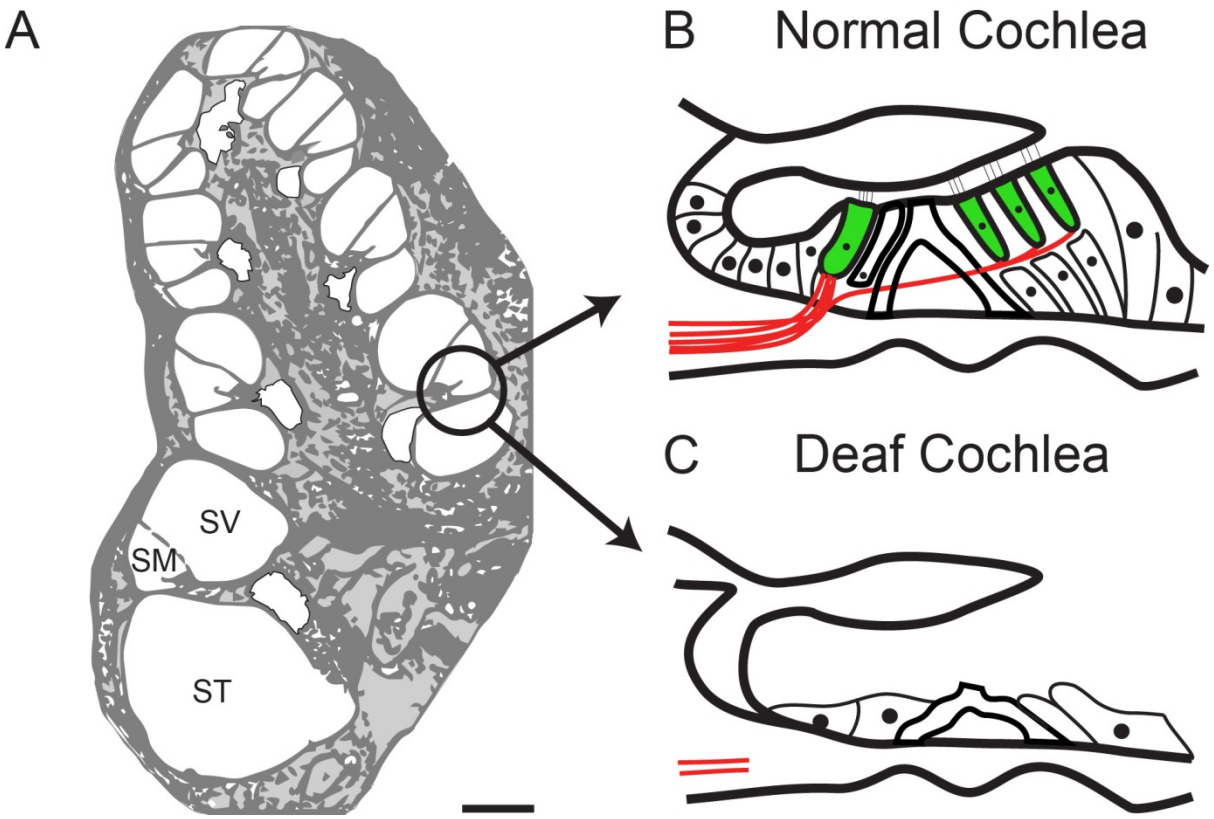

Figure 1. (**A**) Schematic image of a guinea pig cochlea in cross-section indicating the location of the sensory region (organ of Corti - circled), the scala vestibuli (SV), the scala tympani (ST) and the scala media (SM). (**B**) In an animal with normal hearing the organ of Corti contains sensory hair cells (green) and supporting cells that provide the structure. The peripheral fibres of the SGNs (red) form synaptic connections with the hair cells. (**C**) In a deafened cochlea, the sensory hair cells are damaged or lost and the organ of Corti losses the structural integrity provided by the supporting cells. The peripheral fibres of the SGNs retract and degenerate. (A) Scale bar = 500 µm.

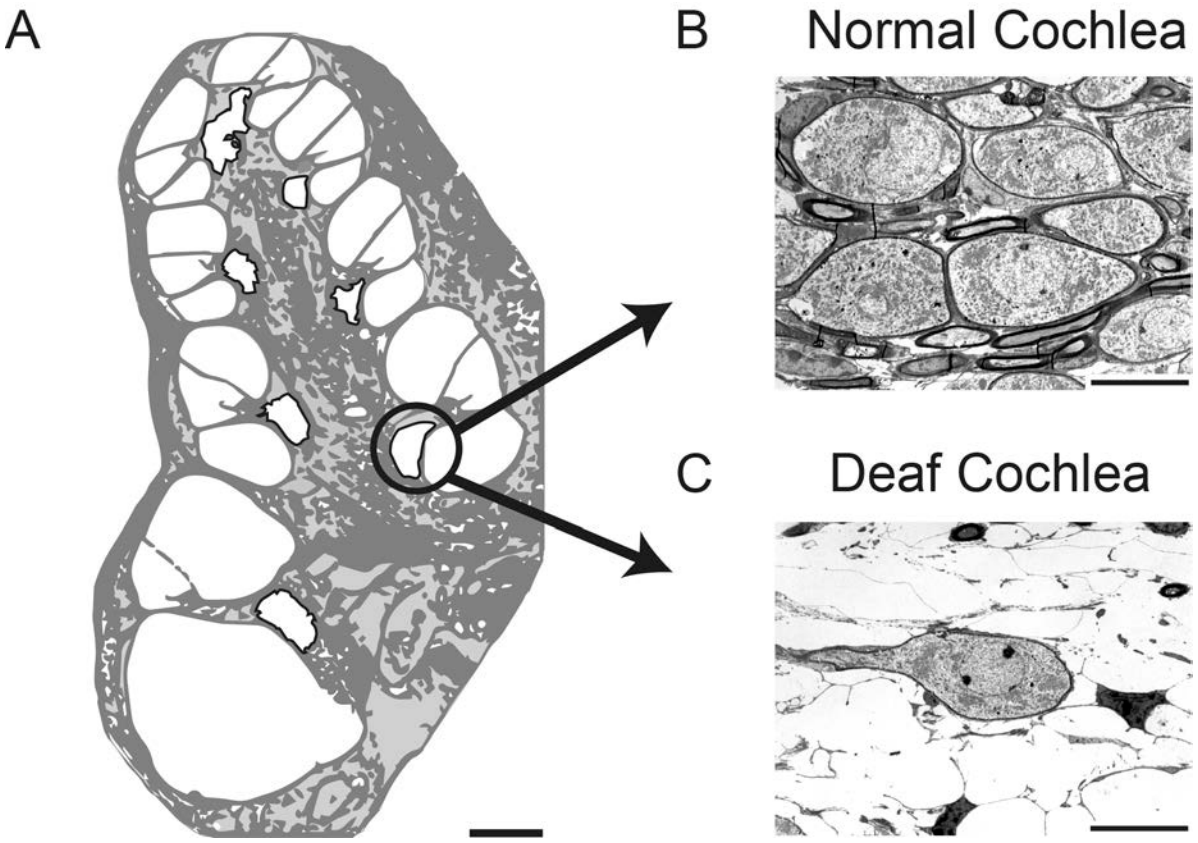

Figure 2.

(**A**) Schematic image of a guinea pig cochlea in cross-section indicating the location of the SGN cell bodies (Rosenthal's canal – white and circled). (**B**) Transmission electron microscope images of SGNs within Rosenthal's canal from a guinea pig with normal hearing. Rosenthal's canal contains a full complement of SGN cell bodies that are myelinated (**C**) In a deafened guinea pig cochlea, loss of the sensory hair cells can lead to degeneration of the peripheral fibres of the SGNs and, ultimately, death of the SGNs themselves. Scale bars, **A** = 500 µm, **B** and **C** = 10 µm.

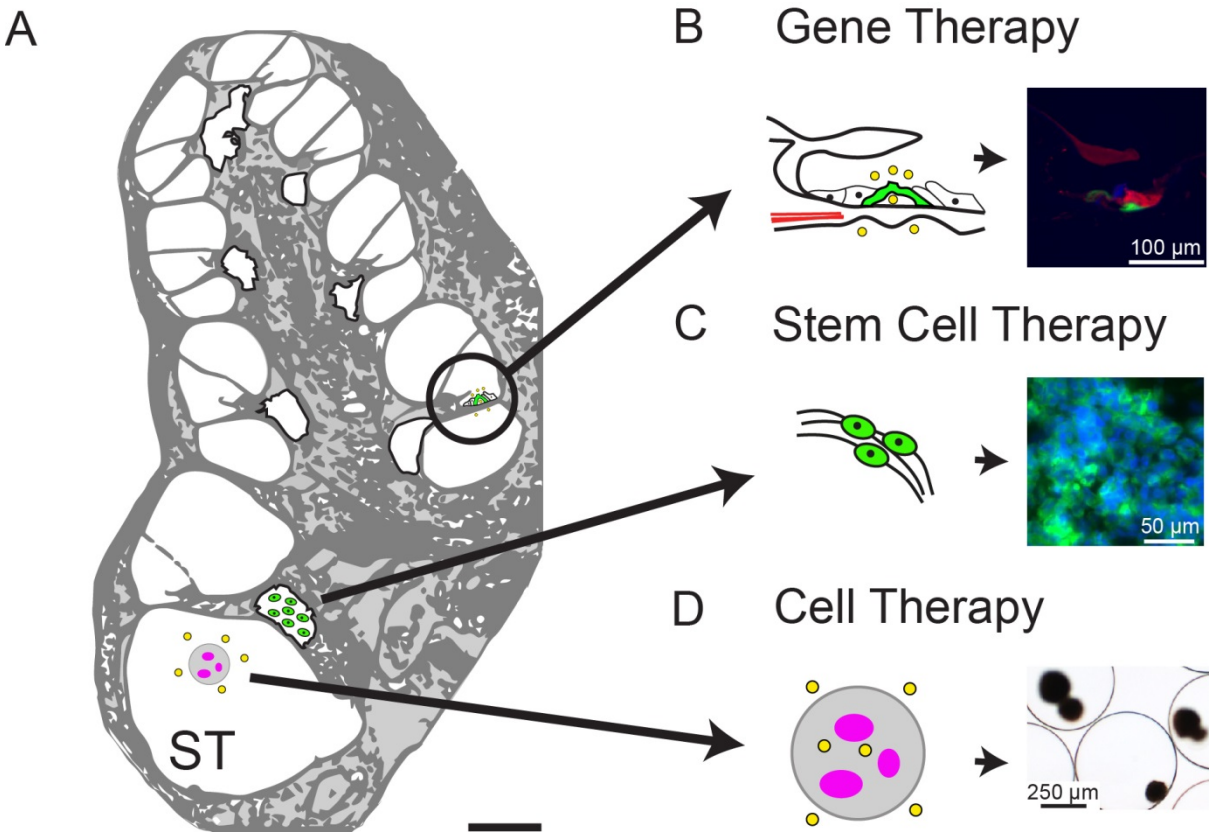

Figure 3.

(**A**) Schematic image of a guinea pig cochlea in cross-section indicating the regions of the cochlea targeted for gene, stem cell and cell therapies. (**B**) Gene therapy can be used to target residual supporting cells (green) so that they produce and release neurotrophic factors (yellow circles). A fluorescent image from a guinea pig cochlea following gene therapy shows a transfected supporting cell (green) and other supporting cells (red) of the organ of Corti. (**C**) In a deafened cochlea where extensive SGN loss has occurred, stem cell therapy may be used to replace lost SGNs (green). The fluorescent image shows neural stem cells (green). (**D**) Cell-based therapies, where neurotrophin producing cells are encapsulated and implanted into the cochlea, can be used to promote SGN survival. Scale bar **A** = 500 µm.