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Regenerating cochlear hair cells: *quo vadis* stem cell

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Abstract

Many elderly people worldwide lose the neurosensory part of their ear and turn deaf. Cochlear implants to restore some hearing after neurosensory hearing loss are, at present, the only therapy for these people. In contrast to this therapy, replacement of hair cells via stem cell therapies holds the promise for a cure. We review here current insights into embryonic, adult, and inducible stem cells that might provide cells for seeding the cochlea with the hope of new hair cell formation. We propose a two-step approach using a first set of transcription factors to enhance the generation of inducible pluripotent stem (iPS) cells and a second set of factors to initiate the differentiation of hair cells. Recent evidence regarding ear development and stem cell research strongly suggest that microRNAs will be an important new regulatory factor in both iPS cell formation and differentiation to reprogram cells into hair cells. In addition, we highlight currently insurmountable obstacles to the successful transformation of stem cells into hair cell precursors and their injection into the cochlear canal to replace lost hair cells.

Keywords

Hair cell loss; Stem cell therapy; iPS cells; miRNA; Transcription factors

Introduction

Hearing loss is the second most frequent debilitation of old age. Approximately 50% of the elderly, 65 years or older, will suffer from some degree of hearing loss affecting their social interactions. Hearing loss can be characterized as conductive hearing loss resulting from partial or complete loss of function of the middle ear or as neurosensory hearing loss resulting from an irreversible loss of hair cells in the cochlea. Environmental factors (i.e., loud noise), infection, inadvertent ototoxic treatments (e.g., aminoglycoside antibiotics), and genetic predisposition all contribute to hair cell loss. These insults are further compounded by age.

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In contrast to many non-mammalian vertebrates (Stone and Cotanche 2007), humans and other mammals cannot regenerate hair cells. Therefore, cochlear implants are the only treatment for neurosensory hearing loss (Roehm and Hansen 2005). Cochlear implants represent a technological breakthrough (Turner et al. 2007), but they still cannot match the innate ability of the fully functional organ of Corti to discriminate sound as a continuum of frequencies. The recent advancement of molecular and cellular therapy with multipotent or pluripotent stem cells sets the stage for the development of new solutions for hearing loss that have the goal of restoring the lost hair cells and thus natural hearing. Attempts at seeding the ear with pluripotent stem cells to form new hair cells or neurons are being pursued and have been successfully implemented in animal models for sensory neuron loss (Martinez-Monedero et al. 2006; Senn and Heller 2008). With our growing knowledge of the functional genomics of hair cell differentiation, we might soon be able to use regulatory genes for the transdifferentiation of supporting cells into hair cells (Raphael et al. 2007). However, the drawback to such an approach is that it would deplete our supply of supporting cells and cause structural disorganization within the cytoarchitecture of the organ of Corti (Fritzsch et al. 2006). Therefore, the formation of new hair cells must occur either through the proliferation of existing cells or through the introduction of stem-cell-derived new cells. Although stem cell technology is still in its infancy, the differentiation of stem cells might be possible through a well-designed series of treatments with regulatory factors, reflecting the native hair cell lineage developmental pathway, aimed at eventually replacing lost hair cells. Here, we will highlight the strengths and weakness of such approaches starting with the different cellular material.

Stem cells

Stem cells are characterized as undifferentiated, toti-, plurior multipotent, and self-renewing cells that have the capacity to differentiate into any cell type of the body given appropriate intracellular gene regulation, intercellular communication, and environmental cues. The promise that these cells hold is the ultimate replacement of lost cells and possibly organs. Work is currently being conducted on three distinct types of stem cells briefly introduced below.

Stem cells originating form the ear and stem-cell-derived cells implanted into the ear

Past research has identified several sources of stem cells in the ear, most importantly also in human ears. For example, apparent neuronal precursors have been identified from spiral ganglion explants of human biopsies (Rask-Andersen et al. 2005). Others have identified various sources of apparent stem cells such as the stria vascularis and organ of Corti (Oshima et al. 2007) and the vestibular organs (Li et al. 2003; Oshima et al. 2007; Senn et al. 2007), even from postmortem specimens (Senn et al. 2007). Various attempts have been made to transform, through stepwise changes of culture medium allowing exposure to various factors, the potential of stem cells derived from several different sources into hair-cell like cells showing specific hair cell markers (Jeon et al. 2007; Li et al. 2004; Senn and Heller 2008). Stem cells derived from several sources such as bone marrow (Jeon et al. 2007), various neural tissues (Tamura et al. 2004; Tateya et al. 2003), or neurosensory precursors (Hu et al. 2005) have been tested for their capacity to develop into hair-cell-like cells and to survive when injected into the ear, generating a substantial body of literature indicating how best to implant cells for neuronal (Corrales et al. 2006; Martinez-Monedero et al. 2006; Tamura et al. 2004; Tateya et al. 2003; Ulfendahl et al. 2007) or sensory (Iguchi et al. 2004; Li et al. 2004; Naito et al. 2004; Nakagawa and Ito 2005) replacement. Overall, the data are encouraging as they indicate that the technical problem of how to implant properly primed precursors into the cochlea or modiolus for hair cell and sensory neuron replacement is solvable. The main obstacles seem to be the identification of a source that provides enough stem cells to allow such therapies to have a good chance of success. We therefore concentrate below on accessible sources of stem

cells and molecular pathways that should increase the likelihood of those cells to respond to the ear environment with proper differentiation into hair cells or sensory neurons.

Embryonic stem cells

Embryonic stem (ES) cells come from the inner cell mass (ICM) of the pre-implantation blastocyst and can be made to differentiate into virtually any cell as they are totipotent. ES cells have the capability of self-renewal or regeneration that can be attributed to the expression of a limited number of genes such as *OCT4*, *NANOG*, and *SOX2* (Boyer et al. 2005). Deregulation of any or all of these genes causes ES cells to lose pluripotency and to differentiate. Importantly, ES cells are subject to immune responses that might ultimately lead to the rejection of derivative cells by the host. Although immunosuppressive therapy can counteract ES cell rejection, it also causes the reduced ability to fight opportunistic infections and other side-effects such as kidney failure, osteoporosis, diabetes, and hypertension (Grinnemo et al. 2008). Nonetheless, ES cells have played a major role in helping to unravel the molecular signals that specify stem cells and help to make cell fate decisions (Losick and Desplan 2008). This is important for attempts to differentiate stem cells into the specific cell types that they are intended to replace (Bang and Carpenter 2008; Jaenisch and Young 2008).

Adult stem cells

An alternative to ES cells are pluripotent adult stem (AS) cells (Beites et al. 2005). AS cells have been discovered in several organs including the ear (Senn and Heller 2008) and can differentiate into a multitude of other cell types. However, ear stem cells are difficult, if not impossible, to retrieve without destroying the organ that they should help to restore and are thus an unlikely candidate for human therapies. Given that the ear derives from embryonic ectoderm through molecular transformations, we are beginning to understand (Fritzsch et al. 2006) that one logical approach is to isolate stem cells from easily accessible skin or skin-related tissue. Moreover, isogenic AS cells have the potential to evade immune rejection because they are isolated from the same individual that will later receive derivative cells for therapeutic treatment.

Three sources of AS cells have been identified in skin and related tissues. Limbal epithelial stem cells (LESC) reside at the junction of the cornea and the conjunctiva of the eye. Cultured LESCs are one of the few AS cell populations that have been successfully used for clinical treatment (Notara and Daniels 2008).

Stem cells have been isolated from the whisker follicles of mice and shown to continue to divide and develop into neurons, smooth muscle cells, Schwann cells, and melanocytes (Sieber-Blum et al. 2006). These cells have been used in spinal cord regeneration (Sieber-Blum et al. 2006). Whether such cells can be isolated from human hair is currently unknown but certainly worth exploring. If successful, the harvesting of these cells could be as simple as plucking hairs under sterile conditions.

In addition to the stem cells in whisker follicles, periodic reactivation of follicular stem cells is required for the cyclic involution and renewal of hair follicles for each round of hair growth (Alonso and Fuchs 2006). Follicular stem cells, localized at the lowest point of the permanent portion of the follicle, are also activated to repopulate the epidermis following injury (Taylor et al. 2000; Tumbar et al. 2004). Such cells might be transformed into neurosensory precursors in the presence of other critical ear-specific genes such as *NEUROG1* and *FOXG1* in tissue culture (Fritzsch et al. 2006). Although the vast majority of stem cells in the skin reside in the follicular bulge, the epidermis is also believed to contain stem cells that replace terminally differentiated keratinocytes (Panteleyev et al. 2001). Again, whether such cells can be isolated

and expanded to sufficient numbers and will retain the capacity to be reprogrammed into other cell types for cell therapy has not been explored.

Another surgically accessible source for AS cells is the olfactory epithelium. Olfactory epithelia are the only mammalian sensory system that retains the capacity to regenerate neurons. Most importantly, the olfactory system utilizes many genes that are also employed for the developing inner ear such as *NEUROG1*, *NEUROD1* and *FOXG1* (Beites et al. 2005; Fritzsch et al. 2006; Kawauchi et al. 2005). Comparing the molecular basis for the continued replenishment of olfactory cells through regulation of cellular proliferation and differentiation with those processes of the inner ear could help reveal the molecular obstacles to natural regeneration of sensory hair cells in the mammalian ear.

Inducible pluripotent stem cells

The recent excitement about the induction of pluripotent stem (iPS) cells from fibroblasts (Wernig et al. 2007) and their successful use to correct disease (Hanna et al. 2007) marked 2007 as the year in which gene therapy was truly born (Jaenisch and Young 2008). iPS cells may have less transformation potential than ES cells, but given that iPS cells are reprogrammed adult cells, they might overcome the problem of histoincompatibility. Essentially, iPS (and AS) cells allow for isografting, whereas ES cells will be, in most cases, an allograft variably related to the molecular profile of the recipient.

The enormous potential of induced hematopoietic progenitors from iPS cells derived from skin fibroblasts has been demonstrated by the curing of a humanized form of sickle cell anemia in a mouse (Hanna et al. 2007). This “proof of principle” experiment coupled with the successful induction of pluripotent stem cells from fibroblasts and various epithelial tissues (Bang and Carpenter 2008) generates hope for the use of iPS cells for cellular gene therapy. Despite this proof of principle, optimism should be restrained. Some problems that affect other potential therapeutic approaches with ES and AS cells similarly apply to iPS cells, for which the efficacy of the procedure and serious risk of tumor formation make iPS cells currently unsuitable for human trials (Holden and Vogel 2008). More recent schemes to generate iPS cells have partially overcome tumor formation (Hanna et al. 2007; Jaenisch and Young 2008; Lengner et al. 2007; Takahashi et al. 2007). As with AS cells, the yield of skin-derived iPS cells is low (Bang and Carpenter 2008; French et al. 2008) leaving open the question as to which cells yield positive results upon treatment with combinations of transcription factors. Indeed, the potential to be reprogrammed may vary with the tissue involved and regenerative phase, as observed for hair growth (Bang and Carpenter 2008). Despite these apparent limitations, the data show that, beyond somatic therapy, genetically modified iPS cells are also suitable for germline correction and therapeutic cloning, thus providing the potential of correcting inherited diseases in future generations (Lanza 2007).

Clarifying the relationship of AS and iPS cells

One of the unanswered questions is why do only some cells respond to reprogramming with the various genes used to produce iPS cells? Future research can take two directions in order to understand this issue. One direction is to modify the set of genes used to induce reprogramming. It appears that the only genes consistently used thus far are *OCT4* and *SOX2* (Bang and Carpenter 2008; Jaenisch and Young 2008). Another direction is highlighted by the recent molecular understanding of the function of one reprogramming factor, *Lin28*, in regulating microRNA (miRNA) function (Viswanathan et al. 2008). Thus, the inclusion of miRNAs or properly designed short interfering RNAs (siRNAs) might enhance the yield of iPS cells. Although the importance of miRNAs in development is unquestionable (Amaral et al. 2008; Makeyev and Maniatis 2008; Ventura et al. 2008), identifying the best suited miRNAs

to enhance iPS cell yield in combination with specific transcription factors will require substantial additional investigation.

As outlined above, previous work has shown that the skin contains at least three kinds of AS cells with low frequency: stem cells within the basal layer of the epidermis that continuously replenish keratinocytes lost to terminal differentiation, hair follicle stem cells that are the basis for the cyclic involution and regrowth of hair follicles, and pluripotent stem cells, possibly of neural crest origin, that have previously been used in spinal cord regeneration (Alonso and Fuchs 2006; Chikh et al. 2007; Kaufman et al. 2003; Rhee et al. 2006; Sieber-Blum et al. 2006). In addition, blood will also carry bone marrow stem cells at a low frequency, and such cells will contaminate the samples. Whether any of these cells are more likely to be reprogrammed into iPS cells remains an open and important question. Here, we suggest increasing the propensity of iPS cell reprogramming through the use of the previously identified ubiquitous stem cell marker genes *OCT4*, *SOX2*, *NANOG*, and *Lin 28* (Viswanathan et al. 2008; Wernig et al. 2007) by increasing the numbers of existing AS cells through proper tissue culture approaches (Sieber-Blum et al. 2006).

We have previously pointed out that the use of such skin-derived AS cells could be particularly useful for replenishing lost cells in certain organs such as the ear (Fritzsch et al. 2006). For example, one could in principle reprogram such cells into organ-specific precursor cells by adding a combination of organ-specific early genes. Such genes for the developing ear include *Gata3*, *Eya1/Six1*, and *Fgf3/10* (Fritzsch et al. 2007). These genes have been shown to play important roles in inner ear development from a patch of ectodermal cells called the otic placode (Ohyama et al. 2007; Streit 2007). This patch of cells eventually gives rise to all the cells of the inner ear, producing the three-dimensional labyrinth structure and the neurosensory epithelia required for normal hearing and balance (Fritzsch et al. 2006; Kelly 2006). The use of such genes that, in development, guide the differentiation of ectodermal cells into ear neurosensory cells (Fritzsch et al. 2006; Kelly 2006) could significantly enhance the reprogramming of iPS cells.

Avoiding tumor formation through genetic approaches

Overcoming the potential transformation of iPS cells into cancer cells will require the use of a different approach for gene expression to achieve directed differentiation. Instead of the use of retroviruses to shuttle genes randomly into the genome, next-generation approaches are necessary to allow the stable insertion of genes into skin-derived cells without any risk for tumor transformation. One option that is being explored in several laboratories is the use of membrane-permeable small molecules that can localize to the nucleus to turn on specific genes (Holden and Vogel 2008). Another approach currently being explored is the use of bacteriophage-derived integrases (Calos 2006). These enzymes recognize only ~48 pseudo-integration sites in the human genome and would thus allow the stable insertion of “stem cell” transcription factors without tumor induction (Calos 2006). This approach would be particularly powerful in combination with appropriate genetically engineered expression systems that allow the activation of integrated genes “on command” (Weber and Fussenegger 2007). The employment of such synthetic gene regulatory circuits could possibly result in regulated gene expression without tumor formation.

Transforming otocyst-like iPS cells to hair cells

Cells, once seeded into the inner ear, might possibly pick up the proper cues to differentiate into hair cells. Thus, it may suffice to transfer otocyst-like iPS cells into the ear that has lost its hair cells. However, the adult ear might not provide enough intercellular contextual information (Losick and Desplan 2008) to induce differentiation, since requisite embryonic transcription regulators are no longer active. If so, we may of necessity have to drive otocyst-

like iPS cells to differentiate into hair cells. This can be accomplished through the use of transcription factors known to be essential for hair cell development, such as *ATOH1*, *POU4F3*, *GFI1* (Fritzsch et al. 2007; Hertzano et al. 2004), in conjunction with promoter elements capable of activation in the organ of Corti after the demise of hair cells. By analogy to the effect of multiple gene expression to reprogram AS cells into iPS cells (Wernig et al. 2007), we propose here that the co-expression of four or more factors will drive iPS cells to differentiate into hair cells in the ear. In order to achieve full differentiation of iPS cells into hair cells, it may be necessary to implant, into the cochlea, iPS cells that have been transiently treated with *SOX2*, *GATA3*, *OCT4*, and *NEUROG1* (and/or *EYA1*, *FOXG1*, *FGF*). Additionally, such hair-cell-specific genes might be regulated by using tamoxifen-inducible expression systems or less detrimental bacterial antibiotic-inducible expression systems (Tian et al. 2006). The success of such approaches will ultimately hinge on the availability of adequate promoter elements. With this caveat in mind, we want to explore here the potential role of miRNAs to induce hair cell differentiation.

We and others have shown that certain miRNAs (e.g., tandemly transcribed *miR-183*, *miR-96*, and *miR-182*) are expressed specifically in hair cells (Weston et al. 2006). Additionally, the *miR-183* gene is very highly conserved evolutionarily, demonstrating strong expression in the inner ear of the lamprey and hagfish and in putative sensory organs of the acorn worm and sea urchin (Pierce et al. 2008). This restricted expression together with high conservation suggests that *miR-183* is a critical player in inner ear hair cell specification. Indeed, our unpublished data strongly support the notion that specific miRNA expression is necessary for other transcription factors to achieve desired cell transformations. We presume that *miR-183* eliminates transcripts that might otherwise persist through cellular differentiation and thus shapes the landscape of those transcripts up-regulated by hair-cell-specific transcription factors so that they more effectively transform and maintain mature hair cells. As is now clear, the genes necessary for iPS formation, such as *OCT4* and *SOX2*, must ultimately be eliminated so that they do not interfere with the coordinated transformation of iPS cells into differentiated cells (Bang and Carpenter 2008); miRNAs play a critical role in this process (Viswanathan et al. 2008). As for the induction of iPS cells, elucidation of the roles of miRNAs in the differentiation process may prove to be an essential component for the therapeutic use of iPS cells to rebuild a functional organ of Corti.

Specifically, we suggest using the following genes for the initial transformation of skin-derived stem cells into more ear-like stem cells:

Oct4, *Sox2*, *Neurog1*, *Gata3*, *Eya1*, *Foxg1*, *Fgf3* Each of these genes has been demonstrated to be an early marker of the otic placode or causes specific changes in the neurosensory composition.

Although the molecular switches from proliferating precursors to differentiating hair cells remain unclear, the following set of genes should initiate the differentiation of hair cells based on their selective expression and function as transcription regulators or their need to maintain hair cells:

Atoh1, *Pou4f3*, *Gfi1*, *miR-183* These are minimally essential sets of candidate genes that will probably be refined with continued investigations.

Foreseeable obstacles

One concern for the use of iPS cells in the treatment of human diseases is the length of time required for the development of individual iPS cell lines. For acute illnesses such as spinal cord injury or myocardial infarction, it would not be practical to begin the generation of iPS cells following the injury (Holden and Vogel 2008). In contrast, adult-onset hearing loss is generally

a slowly progressing disease process. With early identification, possibly aided by screening for known and suspected genes that contribute to hearing loss, iPS cell lines could be initiated well before hearing loss progresses to a state requiring treatment. Patients with more rapid hearing loss, such as that induced by ototoxic agents, would still likely benefit from prospective iPS cell therapy because the sensory neurons survive long after the hair cells have died, and new hair cells might be able to induce the growth of neurons and their innervation into the organ of Corti through the production of neurotrophins (Fritzsch et al. 2006; Martinez-Monedero et al. 2006; Senn and Heller 2008).

Whereas the above-outlined processes might be able to generate hair cells in the diseased "flat" organ of Corti, some obstacles still must be addressed. Initially, it is critical that we not only find a way to turn on proliferation, but that we also have a means to turn it off. Without adequate cell cycle control, any of the techniques discussed above have the risk of forming tumors. Furthermore, the cochlea is an enclosed space surrounded by bone, in which an inner ear tumor would very rapidly destroy the delicate organ and the adjacent facial nerve. This is the case for Schwannoma of the VIIIth nerve. Another critical issue for the restoration of hearing is the rebuilding of the appropriate cytoarchitecture required for the organization and polarization of the hair cells within the organ of Corti. Any perturbation in the cytoarchitecture of the organ of Corti almost always leads to hearing loss. In a properly functioning cochlea, the outer and inner hair cells are organized in a three-to-one radial configuration. Additionally, each individual hair cell has a polarity defined by placement of the kinocilium and stereocilia pattern on the apical surface. In order for the hair cells to be stimulated by sound waves in the basilar membrane, they must all align perpendicular to the long axis of the cochlea. Although we know that genes such as *Foxg1* are involved in determining the number of rows of hair cells and the polarity of hair cells in the organ of Corti (Pauley et al. 2006), we do not yet have a firm grasp of the way in which these features are defined in the normal cochlea (Jones and Chen 2007). Likewise, the supporting cell distribution and differentiation is critical for hearing. For example, we do not understand what causes the formation of two rows of non-sensory cells to form that later differentiate into pillar cells that are essential components of the stiffness of the organ of Corti. Although we have made major inroads with regards to our understanding of the molecular basis of innervation, we do not yet know the molecular basis for outer and inner hair-cell-specific innervation. Clearly, the organization of cochlear hair cells, supporting cells, and their innervation will be, at least in part, governed by their surroundings. We have yet to determine how much influence the infrastructure (such as placement of the spiral artery) and gene expression from non-sensory cells adjacent to the organ of Corti will have on the developing hair cells, should attempts at regeneration as outlined above prove successful.

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