Symposium

Regenerative Medicine for the Special Senses: Restoring the Inputs

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Our senses connect our brains to the world: communicating with others depends on our auditory system, and navigating through space relies heavily on our visual system. And what would life be like without our sense of smell that mediates much of our ability to appreciate flavors of food and alert us to dangers in our environment? It is therefore not surprising that degenerative diseases of the special sense organs can have a devastating impact on the quality of life. Many of the conditions that lead to sensory impairment are age related and are therefore on the rise as the population ages. However, early-onset forms of sensory impairment can be even more devastating, since loss of sensory inputs during critical periods of development can lead to permanent disruptions in brain maturation. With the exception of the olfactory epithelium, the sensory cells of the special sensory organs are like most of the neurons in the brain: they are not replaced after they are lost to disease. Therefore, the loss of these cells leads to permanent sensory impairment. However, in recent years, many laboratories have focused the tools of regenerative medicine and gene therapy on diseases of sensory systems. In many ways, the special sensory organs provide highly amenable targets for regenerative approaches in the nervous system, due in part to their accessibility and the rigorous methods for characterization of functional restoration. The work in this field is already providing a "proving ground" for gene therapy and stem cell therapy with some of the first successful clinical trials. In this review, some of the key approaches will be discussed, and the successes highlighted. In addition, we will review some of the critical challenges that lie ahead in the application of gene therapy and stem cell approaches to sensory organ disorders, with the hope of further stimulating research in this area. This article is not meant to be a comprehensive review of this area, but rather to highlight a Symposium at the Society for Neuroscience Annual Meeting (for a recent more thorough review, readers are referred to Bermingham-McDonogh and Reh, 2011).

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Successful regeneration in the olfactory epithelium: implications for other sensory systems

The mammalian olfactory system is particularly susceptible to environmental insults, pathogenic exposure, and traumatic injury. In this sensory tissue, unlike hearing and vision, the primary sensory cells that transduce external stimuli, the olfactory sensory neurons (OSNs) directly contact the outside world and project axons directly into the CNS. Two important consequences of this organization have been recognized. First, OSNs, as well as other cells within the epithelial layer, have a remarkable ability to regenerate and establish new functional connectivity with the brain after extensive damage. Second, this inherent regenerative capacity appears tightly controlled, such that, under some modes of damage, proliferation is rapidly reinitiated to re-establish a normal epithelium. In contrast, lesions induced by other kinds of damage to the tissue result in prolonged proliferative suppression followed by subsequent regeneration. Recent studies have revealed new details of the molecular, genetic, and cellular basis for the initial establishment of the olfactory sensory epithelium and its robust regeneration upon damage.

The pseudostratified olfactory epithelium (OE) consists of four major cell types. The OSNs, residing in the middle layer of the OE, extend a short dendrite to the luminal surface terminating in the specialized cilia containing the odor transduction components and project a single unmyelinated axon through the cribriform bone and into the olfactory bulb at the front of the brain. Sustentacular cells reside in the most apical epithelial layer and provide barrier and support functions for the epithelium. The ability of the OE to undergo regeneration resides within a population of transit-amplifying and multipotent stem cells comprised of globose basal cells (GBCs) and horizontal basal cells (HBCs) that lie near the basal lamina.

Experimental lesioning paradigms have revealed two distinct mechanisms for neuronal replacement. Axotomy or re-

moval of the olfactory bulb leads to rapid and selective loss of the OSNs, a proliferative burst of *mash1*+ GBCs, and the subsequent repopulation of the neuronal layer within 14–30 d. In contrast, exposure of the OE to methyl bromide gas produces free radical damage from the apical surface and loss of sustentacular, neuronal, and globose cells. Cell fate mapping studies in adults suggest that the origin of the regenerated cells lies within the *mash1*+ population (or their immediate GBC precursors) in the first paradigm, and within the normally quiescent HBC population in the latter scenario (Leung et al., 2007). The HBCs pass through a brief proliferation phase that generates new GBCs and then become quiescent (Fletcher et al., 2011). Together, these observations suggest two distinct reservoirs of stem cells that differentially contribute to robust regeneration in the olfactory system.

Disease-induced inflammation from pathogens and other agents represents an additional insult that produces olfactory tissue damage and neuronal loss (Lane et al., 2010). In contrast to the situation in acute traumatic lesions when the progenitor proliferation occurs simultaneous with neuronal loss, persistent inflammation results in a depleted neuroepithelium as well as suppressed proliferation of progenitors. When the inflammation resolves, progenitors rapidly re-establish the epithelium. The molecular mechanisms controlling the cellular loss, maintenance of the depleted state, and subsequent neuronal repopulation are not known. It is interesting to speculate that the olfactory system has developed processes that promote rapid re-establishment of OSNs and their connections to the brain when the damage is traumatic, and transient other processes that suppress these connections when injury arises from pathogenic organisms that might use these pathways in gaining direct access from the outside world to the brain.

In summary, the regeneration of olfactory sensory neurons occurs continually throughout adult life, primarily from a pool of committed progenitors that also serve as the reservoir for replenishment after acute neuronal injury. Upon more significant damage, HBCs provide a quiescent stem cell population that re-establishes the progenitor pool and gives rise to both neuronal and non-neuronal populations. The origin of HBCs and GBCs during embryonic development is poorly understood but recent cell fate mapping studies in the embryo and analysis of genetic knock-out mice suggest that a common GBC-like cell may directly generate HBCs during embryonic development (Packard et al. 2011) and subsequently form the GBC population. The simplicity of the olfactory system has made it particularly amenable to modeling the dynamics and homeostasis of cell types in this tissue and to manipulating these pathways in the embryo (Gokoffski et al., 2011) and the adult (Beites et al., 2005). The identification and characterization of the molecules and cells that create and regulate the robust regenerative capacity of this tissue should provide insights that may unlock regenerative processes in the other sensory organs.

Inner ear cells from scratch: implications for regenerative medicine

The ~15,000 sensory hair cells of the human cochlea provide sound sensitivity that underlies our speech communication, and hair cells in five vestibular epithelia sense linear and angular accelerations triggering reflexes that are critical to gaze stability and balance. Unfortunately, loud sounds, infections, and certain antibiotics and chemotherapeutics can kill hair cells, causing sensory deficits that are widespread and permanent. The human ear does not produce hair cells after birth, but the situation is quite different in nonmammals. Fish, amphibians, and birds produce hair cells throughout life and recover sensory functions within weeks of experiencing the same forms of hair cell damage that cause permanent deficits in humans. Recovery in these species depends on glia-like epithelial supporting cells that produce replacement hair cells, which become innervated and restore sensory function. In birds, for example, the supporting cells of the cochlear sensory epithelia respond to hair cell loss with two types of regenerative processes: cell division and direct transdifferentiation (Cafaro et al., 2007). Asymmetric divisions are the signature mechanism of somatic stem cell activity, and they result in new hair cells with preservation of the supporting/stem cell pool. The process of transdifferentiation is the direct conversion of supporting cells into hair cells. Mammalian supporting cells, on the other hand, lose the ability to regenerate lost hair cells. Nevertheless, recent findings suggest that the mammalian inner ear bears rudimentary regenerative capacity. Some vestibular supporting cells of rodents proliferate *in vitro*, and in neonates the progeny of such cells are able to give rise to new hair cells (Li et al., 2003; Burns et al., 2012). Even cochlear supporting cells display regenerative capacity *in vivo*, but only during the first neonatal weeks (White et al., 2006; Oshima et al., 2007; Sinkkonen et al., 2011).

So what accounts for the failure of regeneration in mammals? Greater cellular differentiation may be the answer. At least five highly differentiated and specialized types of supporting cells are present in the precisely ordered architecture of the organ of Corti auditory epithelium. This organization is only found in the mammalian cochlea, making it a much more complex sensory epithelium that harbors more specialized cell types with unique cytomorphologies when compared with nonmammalian cochlea or vestibular organs. The degree of differentiation of the supporting cells and the important functional consequences of individual cell positions in the organ of Corti may present substantial challenges for efforts to stimulate the functional regeneration. For this reason, the mammalian balance epithelia may hold greater potential for regeneration research. While the vestibular organs of very young mice can regenerate new hair cells, the tissue rapidly loses this ability as it matures (Burns et al., 2012). The loss of hair cell regeneration in mice occurs contemporaneously with unique thickening of F-actin belts that bracket intercellular junctions in all epithelia, but which become so thick that they fill 89% of the average adult vestibular supporting cell at the level of its intercellular junction in mice (and apparently in humans as well; Burns et al., 2008). In contrast with this, the F-actin belts in the supporting cells of chickens, sharks, zebrafish, and bullfrogs remain thin even in adulthood, and all those species readily regenerate hair cells throughout life. The 13-fold thickening of F-actin belts as mice mature from E18 to adulthood is closely paralleled by declines in supporting cell spreading ($r = -0.99$) and cell proliferation near sites of wounding ($r = -0.98$). In contrast, the F-actin belts in the supporting cells of chickens, sharks, zebrafish, and bullfrogs remain thin even in adulthood, and all these species readily regenerate hair cells throughout life. Cadherin proteins that adhere supporting cells to their neighbors, also differ between intercellular junctions in the ears of humans and mice and those of sharks, bony fish, amphibians, and birds (Hackett et al., 2002; Warchol, 2007; Collado et al., 2011). These and other findings appear consistent with the notion that specialized characteristics of the mature mammalian inner ear may limit effective regeneration rather than the absence of essential molecular circuitry found in nonmamma-

lian ears; however, alternative hypotheses deserve consideration since a definitive test for causation remains to be conducted. Also, there is a continuing need for greater understanding of how specific subcellular mechanisms may restrict regenerative responses in mammalian ears (Chen and Segil, 1999; Lin et al., 2011; Loponen et al., 2011).

Strategies to entice adult supporting cells to show regenerative capacity include the forced expression of developmentally important transcription factors such as Atoh1 (Shou et al., 2003), deactivation of cell cycle inhibitors (Chen and Segil, 1999; Löwenheim et al., 1999; Sage et al., 2005), or cellular reprogramming toward a phenotype equivalent to progenitor (prosensory) cells that transiently exist during development. Forced expression of Atoh1, for example, is sufficient to entice some inner ear cells, such as young supporting cells to transdifferentiate into hair cells, whereas many other cell types are not responsive to Atoh1 expression. This observation suggests that Atoh1-induced transdifferentiation requires a form of cellular competence. In addition, *Atoh1* expression in competent cells such as adult supporting cells does not lead to cell division (Shou et al., 2003), although recent findings suggest that some neonatal supporting cells might respond to forced *Atoh1* expression with S-phase re-entry (Kelly et al., 2012). This still poses a problem with respect to using this transcription factor for regenerative therapy in the adult because turning the limited number of remaining cells in the hair cell-depleted organ of Corti solely into hair cells will deplete supporting cells, which are essential for inner ear function. As an alternative to the introduction of a single transcription factor, cellular reprogramming has been put forward as a potential strategy to convert adult organ of Corti nonsensory cells into prosensory cells. Prosensory cells remain committed to the otic lineage, and the prevailing hypothesis is that they will differentiate into hair cells and supporting cells. Researchers are actively investigating the molecular pathways required for reactivation of regenerative programs in the damaged mammalian cochlea.

Finally, promising experiments with embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have used knowledge of the cellular signaling steps that are active when the inner ear forms during embryonic development (Oshima et al., 2010). For example, inhibition of endoderm and mesoderm formation during the process of ESC and iPSC differentiation results in an enrichment of ectodermal cells, which are competent to respond to otic inducers such as FGF2. The resulting early otic cells, generated from either murine or human sources, can be further differentiated into inner ear cell types. Cytohistological, cytomorphological, and functional analyses revealed that *in vitro*-generated epithelial patches of inner ear cells display characteristics of nascent inner ear hair cells as well as surrounding supporting cells. The ability to generate sensory hair cells in culture combined with iPSC technology provides an unprecedented opportunity to study the cellular phenotypes caused by inner ear disorders directly. For cases of hereditary human hearing loss, this might be done without generating animal models. Stem cell guidance technology can also be used to study inner ear developmental processes, which might lead to identification of signaling pathway combinations that could be used to stimulate regenerative processes in mammalian cochlear supporting cells. Since, cell-based assays can be scaled up for high-content screening approaches, it is also conceivable that stem cell-generated inner ear cell types can be used for drug discovery. Future screening technology could specifically be used to gain novel insights into ototoxicity, otoprotection, as well as regeneration of sensory hair cells and potential novel treatments.

Gene therapy for blindness: moving into the clinic

Stem cells and regenerative medicine are also beginning to show promise in the repair of the retina. Over the past 10 years, several groups have developed protocols for directing embryonic stem cells to form retinal cells *in vitro* (Lamba et al., 2006, 2009; Meyer et al., 2009), and in some cases these cells can even form organized retinal structures (Eiraku et al., 2011; Phillips et al., 2012). The ability to generate human retinal cells in large quantities has led to attempts to transplant these into animal models of retinal degenerations, and human ESC-derived photoreceptor cells have even been shown to restore light responses to congenitally blind mice. To date, the most success has been obtained with a non-neural cell in the retina, the pigmented epithelial cell. These cells form a layer adjacent to the rods and cones of the neural retina, and are important for the phagocytosis of the outer segment disc material from the photoreceptors. In age-related macular degeneration (AMD), a common cause of visual impairment in individuals over the age of 60 years, the pigmented epithelial cells become atrophic, particularly those underlying the fovea. One of the first clinical trials using human embryonic stem cell derivatives was initiated last year by advanced cell technology, in which pigmented epithelial cells derived from human ESCs were transplanted into the subretinal space to replace the atrophic pigmented epithelial cells in patients with AMD (Schwartz et al., 2012). An early report on this Phase I trial suggests that the cells have not caused any immediate problems, e.g., teratomas, but careful follow-up will be needed to determine whether the cells will survive and benefit the patient.

Investigations of stem cell repair of the retina are still in their infancy, but gene therapy for retinal disease has already produced breakthroughs. Most impressive have been the results in patients with Leber congenital amaurosis type 2 (LCA2). LCA2 causes childhood blindness due to a visual cycle defect in the *Rpe65* gene responsible for isomerizing the bleached visual pigment all-*trans*-retinal back to its active 11-*cis*-form. LCA2 had been considered incurable. Preclinical efficacy and safety basis for LCA2 was established and an adeno-associated virus (AAV) vector clinical trial for LCA2 initiated ~4 years ago (Hauswirth et al. 2008). Each of the first three LCA2 patients received 150 μl (5.9×10^{10} vector genomes) of GMP grade AAV2-CBA-hRPE65 vector subretinally at a single site, and their visual function was followed periodically (Hauswirth et al., 2008; Cideciyan et al., 2009a). No adverse events were noted for any patient and all patients tolerated the procedure without incident. All three patients also exhibited substantial and significant improvement in light sensitivity, but only in the area of retina that received the vector, as documented by high-density threshold perimetry. After correcting for the fraction of photoreceptors lost in each patient before treatment, two of the three patients experienced full recovery of retinal function within the vector-treated area.

At 1 year post-treatment, but not before, one patient reported new visual perceptions that mapped to the treatment area (Cideciyan et al., 2009b). When asked to detect a very bright target that she could see before treatment, she continued to use her fovea, her only area of useable, although poor, vision. If, however, she was asked to detect a dimmer target that she could not detect at pretreatment baseline, she could not see it at any time up to 9 months post-treatment. However, at 12 months the patient reported seeing the dim object for the first time. When detecting this target, the patient now shifted

her locus of visual perception away from her central fovea to the treated retinal area. In effect, the patient had developed a second fovea or "pseudo-fovea" that was used only when the target was too dim to be perceived by her anatomical fovea. Subsequently, five other treated patients have developed a pseudo-fovea after a significant delay. This slow emergence of a pseudo-fovea suggests that cortical "learning" is possible but slow in children or young adults.

A longer-term study (Jacobson et al., 2012) of all 15 patients currently treated (3 months to 3 years of follow-up) showed the following: (1) all patients gained in full-field light sensitivity from 10-fold to 10,000-fold; (2) all patients gained in local light sensitivity in the retinal region treated from 200-fold to 60,000-fold; (3) 13 of 15 patients gained in pupillary light reflex; (4) 5 of 6 patients gained in maze mobility performance; (5) 12 of 15 patients gained in visual acuity, with 4 of those patients gaining more than three lines; and (6) 6 of 15 patients have presently developed a pseudo-fovea at their retinal treatment locus.

What has not worked? Subfoveal vector delivery, which necessarily detaches the fovea from the underlying retinal pigment epithelium cell layer, seems to cause more damage than benefit for vision. Five of the 15 LCA2 patients received subfoveal vector, and 3 of those experienced loss of foveal cones. Of those 3 patients with foveal thinning, none experienced improvement in visual acuity, the only patients of the 15 whose visual acuity did not benefit.

We conclude that AAV-mediated gene therapy for LCA2 is safe, effective at restoring useful vision, and stable for at least 3.5 years. All patients gained significant vision benefit except those experiencing a foveal detachment during vector delivery. Moreover, vitreal injection of novel mutant AAV vectors that can nontraumatically deliver DNA to foveal cones is showing promise.

Conclusions

The degeneration of the special sensory systems is a growing societal problem, and there is an urgent need for advances in the treatment of these disorders. While there are ongoing efforts to intervene early in the course of sensory cell degeneration, millions of individuals continue to suffer sensory impairment; frequently, patients only seek treatment once a significant number of sensory cells are already lost. The developing fields of gene therapy and regenerative medicine are beginning to provide some hope for both slowing the sensory cell loss and potentially even for restoring the input function. Moreover, success in these approaches in the sensory receptor organs, where the effects of treatment can be more rigorously monitored, will likely aid other clinical neuroscientists in the design of similar studies in other less tractable regions of the nervous system. Neuronal degenerations are complex disorders, but perhaps the relative simplicity of the sensory receptor organs will make them more amenable to these promising new therapies.

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