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Comparing Sensory Organs to Define the Path for Hair Cell Regeneration

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Abstract

Deafness or hearing deficits are debilitating conditions. They are often caused by loss of sensory hair cells or defects in their function. In contrast to mammals, nonmammalian vertebrates robustly regenerate hair cells after injury. Studying the molecular and cellular basis of nonmammalian vertebrate hair cell regeneration provides valuable insights into developing cures for human deafness. In this review, we discuss the current literature on hair cell regeneration in the context of other models for sensory cell regeneration, such as the retina and the olfactory epithelium. This comparison reveals commonalities with, as well as differences between, the different regenerating systems, which begin to define a cellular and molecular blueprint of regeneration. In addition, we propose how new technical advances can address outstanding questions in the field.

Contents

1. INTRODUCTION

Three hundred years after the first discovery of hydra regeneration in the eighteenth century (Lenhoff & Lenhoff 1991), the research community is just beginning to understand the genes and cellular signals involved in tissue regeneration. Two types of regeneration have been observed within the animal kingdom: (*a*) physiological regeneration that defines the regular replacement of cells during homeostasis and (*b*) reparative regeneration in response to injury (Hunt 1901). While mammals can still undergo some degree of physiological regeneration, such as in the epidermis, hair follicles, and intestinal crypts, most mammals have lost the capacity to regenerate complex organs after injury, with the exception of the fingertips and the olfactory neurons (Seifert & Muneoka 2018). Conversely, some teleosts (e.g., zebrafish) and urodeles (e.g., salamanders) have retained the ability to undergo reparative regeneration with an exquisite level of precision and throughout their lives (Brann & Firestein 2014, Gemberling et al. 2013, Nacu & Tanaka 2011, Pinto-Teixeira et al. 2015). Mechanosensory hair cells in the ears of nonmammalian vertebrates, such as birds, reptiles, amphibians, and fish, robustly turn over and regenerate throughout the life of the animal (Corwin 1981; Corwin & Cotanche 1988; Corwin & Warchol 1991; Cruz et al. 2015; Ryals & Rubel 1988; Stone 1933, 1937). In contrast, most mammals have lost this regenerative ability, and hair cell loss leads to deafness and vestibular problems. The main challenge in regenerative biology and medicine is thus to understand how nonmammalian vertebrates robustly regenerate many tissues and why most mammals have lost this capacity. Precise regeneration of a complex organ involves the tight regulation of the resident cells. Activation of the organ stem cells, proliferation of the progenitor pool, terminal differentiation of each cell population, and sometimes transdifferentiation of neighboring cells must be coordinated to achieve the regeneration of an identical structure in terms of numbers, proportions, and differentiation state of the cells. Injury also triggers an inflammatory response involving the immune system that has to be controlled to prevent more damage to the organ. What are the signals that trigger the tissue response after injury? What are the cells that respond to this signal to coordinate the regenerative program? How is the inflammation temporally controlled? We believe that hypotheses can be generated by a comparison of organ regeneration between species. While such comparison for epimorphic regeneration (organ regeneration involving the formation of a proliferative blastema) has been documented in detail (Seifert & Muneoka 2018), we focus in this review on sensory cell and neuron regeneration. We compare organs from the central nervous system (retina) and from the peripheral nervous system (mechanosensory hair cells and olfactory epithelium) to aid in establishing a cellular and molecular

blueprint of sensory organ regeneration. The lateral line is a sensory system that detects water motion and allows the animals to orient themselves and detect prey. It consists of sensory organs, termed neuromasts, that contain mechanosensory hair cells. Lateral line hair cells are homologous to inner ear hair cells but are located in the skin and accessible to experimental manipulations. While the molecular triggers and the stem cells involved in the retina and olfactory epithelium have been identified by transcriptome analyses and lineage tracing, they are less well defined in neuromasts. Identifying both the molecular signature of the lateral line stem cell populations and the signals that trigger their activation will facilitate the development of more targeted strategies to unleash the regenerative potential of mammalian hair cells.

2. CELLULAR BASIS OF HAIR CELL REGENERATION

2.1. Heterogeneity of Sensory Organ Cells

A comparative approach to fully decipher the road map of regeneration should include the identification of different cell types that need coordination to achieve precise cellular replacement in respective organs. The roles of most of the signaling pathways have been assessed at global organ scales over the past decades. Classical histology, gene traps, and functional analyses allowed for broad cellular and molecular descriptions of each sensory organ (Kniss et al. 2016, Wan & Goldman 2016, Yu & Wu 2017). However, a description of the cell types and pathways involved at single-cell resolution is just beginning to emerge.

Zebrafish neuromasts, which derive from an embryonic placode [the peripheral nervous system (Piotrowski & Baker 2014)], consist of three major cell types: hair cells, support cells, and mantle cells. Hair cells are mechanosensory cells that possess stereocilia and kinocilia to detect movement, vibration, and pressure changes in the water (reviewed in Lush & Piotrowski 2014). Support cells are located around and underneath the hair cells and are a source of progenitors that replace hair cells during homeostasis and regeneration. Mantle cells are the outermost cells of the neuromast, are ring shaped, and are connected to interneuromast cells in the anteroposterior (A-P) direction (**Figure 1**).

The olfactory system develops from an olfactory placode (the peripheral nervous system) and consists of sensory olfactory neurons, glial-like sustentacular cells, and two different types of stem cells: horizontal basal cells (HBCs) and globose basal cells (GBCs) (**Figure 1**; reviewed in Yu & Wu 2017). HBCs are the injury-responsive stem cells (Iwai et al. 2008, Leung et al. 2007), while GBCs represent a pool of proliferative progenitors that replace the sensory neurons during homeostasis and support the continuous growth of the organ throughout the life of the animal (Caggiano et al. 1994, Graziadei & Graziadei 1979). The retinal epithelium forms from the neural plate and is part of the central nervous system. It comprises two types of sensory receptors (the rods and cones that are connected to the horizontal and bipolar cells) of supporting retinal pigmented epithelial (RPE) cells and of Müller glia cells (**Figure 1**) (Moshiri et al. 2004, Wan & Goldman 2016). Upon injury, the quiescent RPE cells or Müller glia reenter the cell cycle and are reprogrammed to a progenitor state (Powell et al. 2013). They then undergo a differentiation program to give rise to new retinal cells (Fausett & Goldman 2006). To support the continuous growth of the retina, another source of stem cells, located at the periphery in the ciliary marginal zone, differentiates into all retina cell types (Fischer & Reh 2000, Hollyfield 1968, Moshiri et al. 2004).

Until recently, there was no evidence to indicate the existence of different support cell types in lateral line neuromasts. The first hint of heterogeneity within neuromast support cells originated from cell fate tracking using live imaging of neuromast-specific transgenic lines in zebrafish during homeostasis and regeneration (Romero-Carvajal et al. 2015). This study revealed that support cells, on the basis of their proliferative behavior and cell fates, can be divided into at least four

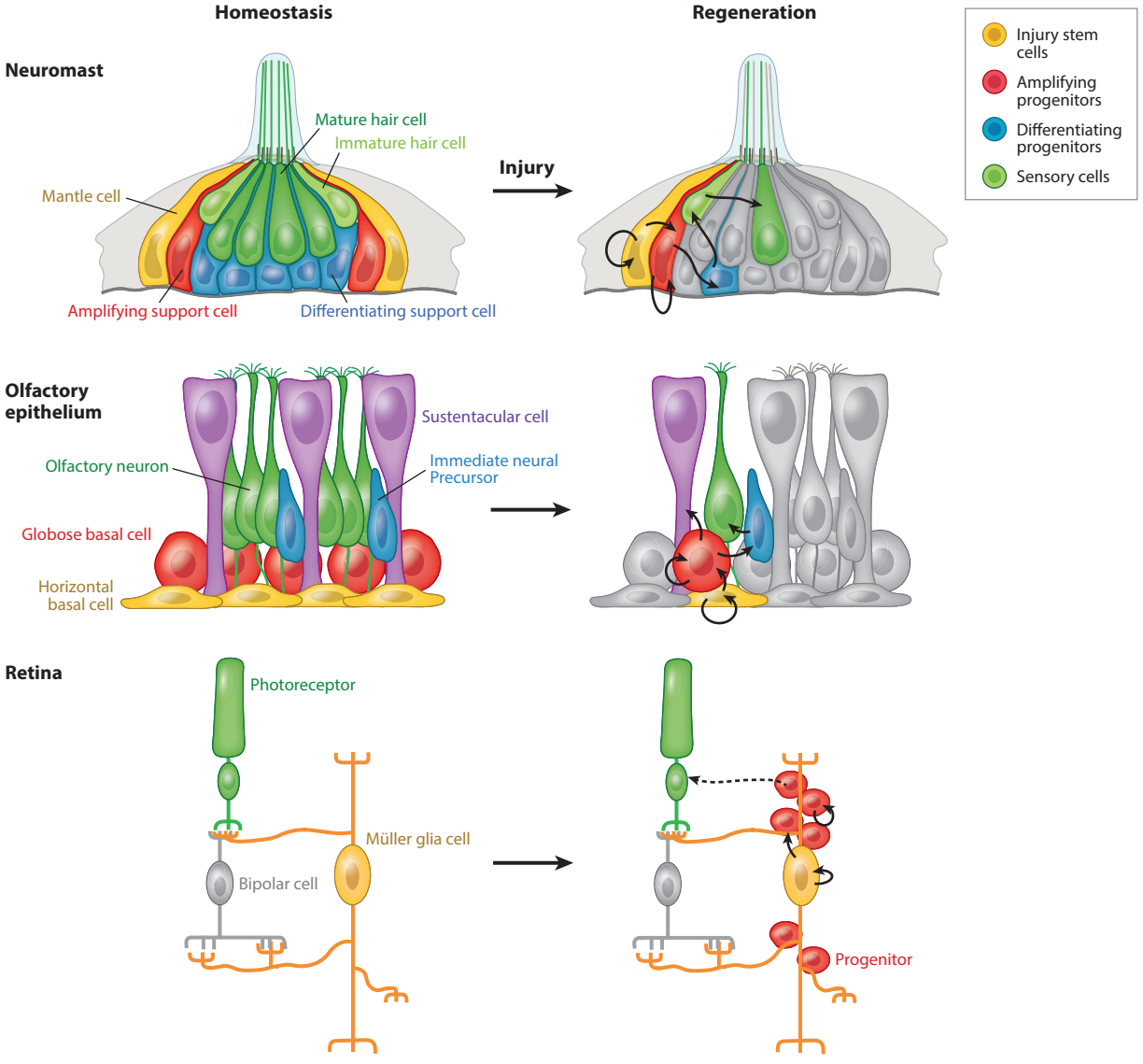

Figure 1

Cellular blueprint of sensory cell regeneration. Shown is a simplified cellular map of the neuromast (*top*; adapted from Lush et al. 2019), olfactory epithelium (*middle*), and retina (*bottom*) before (*left*) and after (*right*) injury. Arrows at right represent the cell fate path after injury. The dashed arrow represents a hypothesis. There is no direct evidence that shows that the progenitor cells (*red*) directly differentiate into photoreceptors. There may be intermediates, but those have not been found.

major groups: (*a*) quiescent mantle cells that do not respond to hair cell death; (*b*) quiescent cells in the A-P poles (Cruz et al. 2015); (*c*) amplifying support cells adjacent to mantle cells, in which one support cell divides into two daughter support cells; and (*d*) differentiating support cells, in which one support cell divides and subsequently differentiates into two hair cells.

BrdU-incorporation assays revealed that amplifying support cells are located in the dorsoventral (D-V) poles of neuromasts adjacent to mantle cells. However, the progeny of amplifying

support cells can differentiate into hair cells when they lose contact with mantle cells and are displaced toward the center of the neuromast. Differentiating support cells are located closer to the center without any axial bias (**Figure 1**). The most central support cells, located immediately beneath hair cells, divide only after hair cell death and not during homeostasis, suggesting that hair cells have an inhibitory effect on these support cells. The nature of this inhibition is still unknown. Similar findings have recently been reported by using photoconversion and lineage tracing of support cell subtypes (Thomas & Raible 2019, Viader-Llargues et al. 2018).

The presence of spatially distinct support cell types with different behaviors raises the questions of how molecularly heterogeneous these support cells are and whether additional support cell populations exist that were missed in these image-based analyses. The advent of single-cell transcriptomics (scRNA-seq) provides us with a powerful tool to molecularly describe all cell populations existing in sensory organs at a molecular level. To date, no comprehensive data set of mature retina scRNA-seq analysis in a regenerative species exists, and only one study reported the single-cell microarray analysis of 42 cells in the developing mouse retina (Trimarchi et al. 2008). In contrast, comprehensive scRNA-seq data sets were recently generated for the homeostatic and regenerating mouse olfactory system and homeostatic zebrafish lateral line neuromasts (Fletcher et al. 2017, Gadye et al. 2017, Lush et al. 2019). Fletcher et al. (2017) performed scRNA-seq from 687 homeostatic cells representing a pool of resting HBCs and the progeny of activated HBCs. This combined data set allowed these authors to identify six main cell types (13 cell clusters). They were able to bioinformatically align the cells along a developmental trajectory, revealing that decreasing Krt5 levels signify that HBCs are activated. During homeostasis, activated HBCs give rise either to sustentacular cells via transdifferentiation or to GBCs by proliferation. The proliferating, amplifying GBC population expresses *Ascl1* and *Kit1* and gives rise to three populations of immediate neural precursors (INP1, INP2, and INP3).

Lush et al. (2019) performed scRNA-seq analyses using FAC-sorted cells from transgenics zebrafish larvae expressing EGFP in all neuromast cells. These researchers grouped 1,521 neuromast cells into seven major cell types (14 different clusters) by using t-distributed stochastic neighbor embedding and Seurat algorithms based on specific marker expression: (*a*) hair cell lineage, (*b*) central support cells, (*c*) amplifying cells, (*d*) D-V pole cells, (*e*) A-P pole cells, (*f*) mantle cells, and (*g*) cells arranged in a ringlike pattern that span several clusters. The position of these cell types in intact neuromasts was determined by in situ hybridization experiments. Pseudotemporal ordering of cells recapitulates cell state transitions during hair cell differentiation. This analysis indicates that central support cells are the immediate hair cell progenitors. Time-lapse analyses of central cells confirmed that they give rise to the hair cell population during regeneration. Confirming this analysis, the well-known cell fate specification and stem cell factors *gata2a* and *gata2b*, *prox1a*, and *isl1* are expressed in these central support cells (Ahlgren et al. 1997, Briegel et al. 1993, Cai et al. 2003, Dorfman et al. 1992, Escobedo & Oliver 2016, Karlsson et al. 1990, Kelley et al. 1994, Laugwitz et al. 2008, Oliver et al. 1993, Pfaff et al. 1996, Thor et al. 1991, Tremblay et al. 2018, Tsai et al. 1994, Wigle & Oliver 1999, Yamamoto et al. 1990). Moreover, cell cycle regression analysis confirmed the existence of two different major lineages, the differentiating hair cell lineage and the amplifying lineage, that had been previously identified by imaging-based experiments (Romero-Carvajal et al. 2015). Combining imaging-based data with scRNA-seq data provides us with a cellular and molecular map of neuromasts in a regenerative species. The identification of all neuromast cell types and their expression profiles now allows for more direct comparisons with nonregenerative species. Such a comparison will reveal whether mammalian support cells are similar to or more differentiated than support cells in a regenerative species and whether mammalian support cells mirror the heterogeneity of support cells in zebrafish neuromasts. A characterization of mammalian support cells will indicate whether the inability of mammals to regenerate is due

to a loss of competent progenitors or a lack of signals to activate them. Future scRNA-seq studies of mouse inner ear epithelia will be instrumental in answering these questions.

2.2. Regeneration and the Hunt for Lateral Line Stem Cells

Decades of work in the retina, olfactory system, inner ear, and lateral line aimed at elucidating the cellular mechanisms of sensory cell regeneration have revealed that sensory cell death triggers the activation of stem cells that proliferate to both self-renew and differentiate into new sensory cells. While the retina and olfactory organ stem cells have been identified, the nature of the lateral line stem cells is not well defined, although lineage-tracing analyses and scRNA-seq analyses have provided some tantalizing clues. Here, we compare the molecular underpinnings of regenerating neuromasts, the retina, and olfactory neurons to identify commonalities in cell behaviors and molecular pathways that will aid in building a road map to sensory organ and neuron regeneration. Organ-specific differences are expected, but some molecular mechanisms are strikingly conserved.

2.2.1. Different stem cells respond to different injuries. In the olfactory system and the retina, two populations of stem cells coexist: homeostasis stem cells (GBCs in the olfactory system and the stem cells in the CMZ in the retina) and injury response stem cells (HBCs in the olfactory system and Müller glia cells in the retina). Homeostasis stem cells cycle quickly, add new cells to the periphery of the organs, and replace sensory cells during tissue turnover. The injury response stem cells are relatively quiescent and respond only to severe injury to regenerate the damaged part of the organ (Wan & Goldman 2016, Yu & Wu 2017).

On the basis of lineage-tracing analyses, we believe that similar populations exist in neuromasts. During homeostasis and after neomycin-induced hair cell death, the amplifying support cells replenish the progenitor pool but also give rise to mantle cells (Viader-Llargues et al. 2018) or to hair cells if the support cells are displaced into the center (Romero-Carvajal et al. 2015). Because the amplifying support cells replenish other cell types even during homeostasis, they appear analogous to organ growth and homeostasis stem cells in the olfactory system and the retina. However, long-term lineage-tracing analyses are needed to test whether amplifying support cells continuously self-renew or whether they are eventually depleted. We also have not identified the signal that activates amplifying support cells to proliferate.

Mantle cells, in contrast, do not respond to neomycin-induced hair cell death (Romero-Carvajal et al. 2015, Thomas & Raible 2019, Viader-Llargues et al. 2018) but robustly regenerate neuromasts if a larger number of support and hair cells are killed (Romero-Carvajal et al. 2015). Interestingly, in axolotls, mantle cells have the ability to give rise to neuromasts on regenerating tail tips (Jones & Corwin 1993), and long-term lineage tracing of mantle cells in medaka demonstrated that they constitute a slow-cycling stem cell pool that over time gives rise to all cell types in a neuromast (Seleit et al. 2017). In addition, mantle cells postembryonically give rise to neuromasts that bud off older neuromasts, a process termed stitching (Ledent 2002, Nunez et al. 2009). All these findings suggest that mantle cells possess the characteristics of injury-responsive stem cells. Questions that remain unanswered are the nature of the signal that activates mantle cells after injury and whether mantle cells undergo a dedifferentiation process upon activation, as mantle cells express mantle cell type–specific genes, indicating they have already differentiated (Lush et al. 2019).

We suggest that, as in the retina and the olfactory epithelium, two different populations of stem cells exist in neuromasts to regulate physiological or reparative regeneration. Also, even though neomycin treatment leads to the depletion of most, if not all, hair cells, it does not constitute a

major injury, since no other cell types in the neuromast are damaged and quiescent mantle stem cells do not respond. Thus, the antibiotic-induced hair cell loss paradigm likely represents an extreme case of physiological regeneration.

To better characterize potential stem cell populations in neuromasts, the authors of a recent study ablated different support cell populations and tested the effect on regeneration (Viader-Llargues et al. 2018). Even though some of the results support the findings that mantle and amplifying support cells are stem cells, laser ablations are not precise enough to exclusively kill one specific population, and robust conclusions cannot be drawn. The most conclusive and elegant results could be achieved by cell type–specific genetic ablations. The recent scRNA-seq data sets identified sets of marker genes for each cell population, which can now be used to build cell type–specific tools (Lush et al. 2019).

Interestingly, manipulations of the Notch and Wnt pathways suggest that, depending on the signaling pathways the cells are exposed to, amplifying support cells can differentiate into hair cells and differentiation of central support cells can be inhibited, making the latter population behave like amplifying support cells. This finding suggests that all lateral line support cells can act as stem cells, depending on the signals they receive.

2.2.2. Molecular triggers of regeneration and the activation of stem cells. Despite decades of studies, there is an outstanding question: Which signal triggers hair cell regeneration? One hypothesis is that dying hair cells secrete molecules such as cytokines and that surrounding cells respond to these molecules by triggering an inflammatory response, resulting in cell regeneration. In the zebrafish retina, proinflammatory cytokines such as tumor necrosis factor α (Tnfα) are necessary to trigger Müller glia proliferation after injury (Nelson et al. 2013). Some Tnfα is released by the dying retina cells, most likely to attract tissue-resident macrophages, which in turn secrete large amounts of Tnfα to the injury site to attract more macrophages (**Figure 2**). Tnfα is also produced de novo by dying hair cells upon cisplatin injection both in vitro and in the rat's organ of Corti (So et al. 2007). Other potential molecular triggers are reactive oxygen species (ROS) (**Figure 2**). For example, ROS are generated in the mammalian cochlea after exposure to noise (Henderson et al. 1999) and after administration of ototoxic drugs such as cisplatin (Clerici et al. 1996, Kopke et al. 1997) and aminoglycoside antibiotics (Clerici et al. 1996). In the *Drosophila* imaginal disc, macrophages are recruited to the injury site by ROS and secrete TNF to activate JNK and induce proliferation of the neighboring tissue (Fogarty et al. 2016). Interestingly, JNK signaling is required for regenerative proliferation in the avian inner ear (Alvarado et al. 2011), and several genes involved in ROS production, such as *nox1*, *noxo1a*, and *noxa1*, are strongly upregulated immediately after hair cell death in zebrafish neuromasts (Jiang et al. 2014, Kniss et al. 2016). It is not known whether the signal from dying cells is an instructive signal directly affecting the stem/progenitor cells or whether it is a passive signal to recruit immune cells that then trigger inflammation and proliferation. While the identification of the signal from the dying cells remains technically challenging, determining whether immune cells are acting as intermediaries can be experimentally addressed by depleting immune cells and assessing the subsequent degree of regeneration.

In the fish retina, Müller glia cells (the injury stem cells) are activated upon major injuries such as mechanical insult and intravital injection of toxins (Maier & Wolburg 1979) that likely trigger inflammation or ROS production. The activation of Müller glia cells leads to their dedifferentiation to a stem cell–like state (Powell et al. 2013, Ramachandran et al. 2010). Müller glia cells start to express critical factors for their autoactivation, such as heparin-binding epidermal growth factor (*hbegf*), cytokines such as *tnfa* and *interleukin11* (*il11b*), the RNA binding protein *lin28a*, and signal transducer and activator of transcription factor 3 (*stat3*) (**Figure 2**) (Ramachandran et al. 2010,

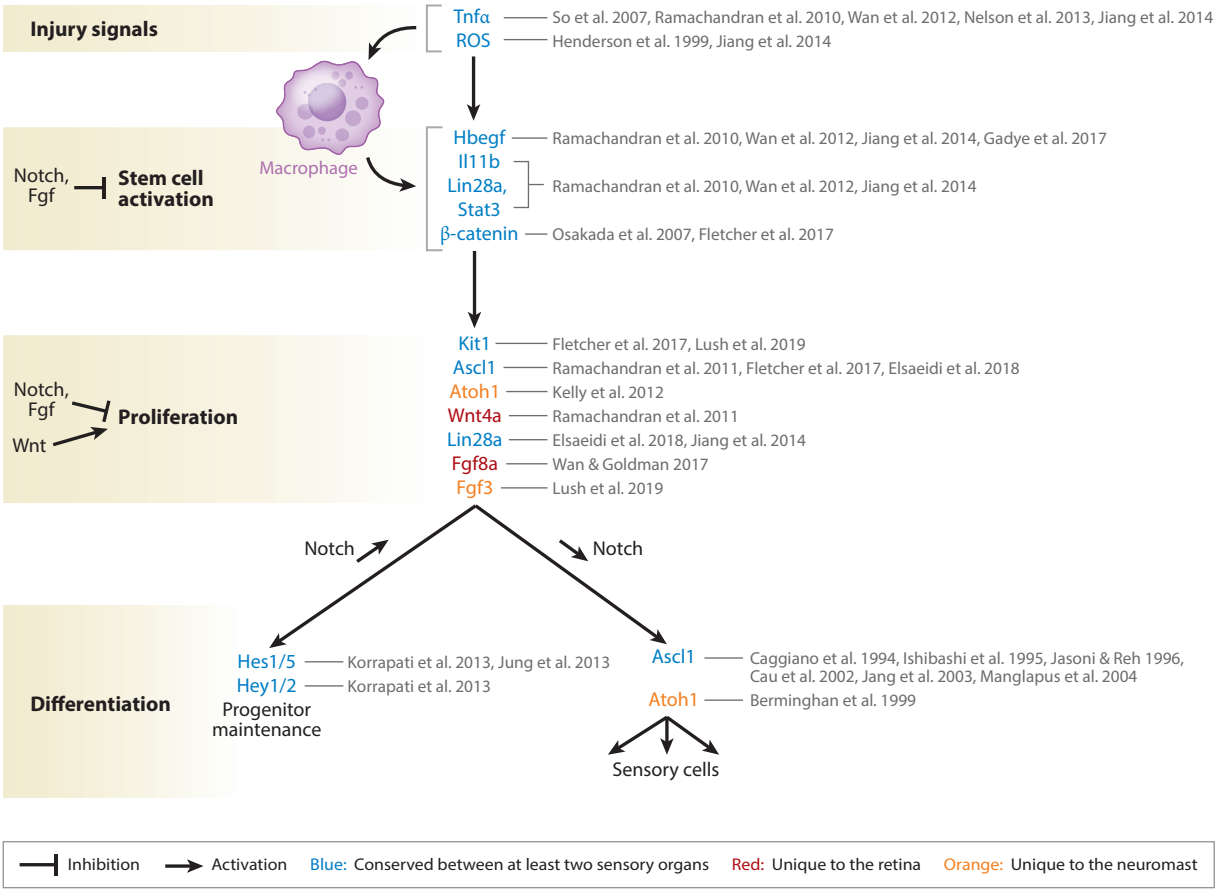

Figure 2

Molecular blueprint of sensory cell regeneration. Shown are genes conserved between at least two sensory organs (*blue*) or unique to the retina (*red*) or the neuromast (*orange*).

Wan et al. 2012). Interestingly, *hbegfb* is specifically expressed in central support cells in homeostatic neuromasts and is upregulated immediately after hair cell depletion with neomycin (Jiang et al. 2014). Likewise, *stat3* is enriched in mantle cells in homeostatic neuromasts and is upregulated shortly after neomycin treatment (Jiang et al. 2014, Liang et al. 2012). *tnfa*, *il11b*, and *lin28a*, which are not expressed in homeostatic neuromasts, are also strongly induced immediately after injury (Jiang et al. 2014). While we do not know in which neuromast cells these factors are activated, these results suggest that the activation signature is likely conserved between the zebrafish retina and neuromasts.

In the mouse homeostatic olfactory epithelium, immediately after a severe injury of the whole epithelium from administration of methimazole, quiescent HBCs are activated (Gadye et al. 2017). Expression of *Trp63* decreases and HBCs start to express factors linked to wound healing, such as *Hbegf* (as in Müller glia cells and neuromasts) (Gadye et al. 2017). The homeodomain-only protein homeobox (*Hopx*), a well-known key regulator of cardiac myoblasts (cardiac progenitors) and quiescent stem cell marker in hair follicles and the intestine (reviewed in Mariotto et al. 2016), is also expressed in activated HBCs. Interestingly, the scRNA-seq analysis of neuromasts shows

that *hopx* is highly enriched in mantle cells, A-P cells, and amplifying support cells (which are located adjacent to the mantle cells) (Lush et al. 2019). *hopx* is slightly upregulated immediately after neomycin treatment (Jiang et al. 2014). This suggests that the mantle cells are primed for activation and that an injury signal will increase the dose of *hopx*, leading to full stem cell activation. Altogether, this molecular signature supports the hypothesis that the molecular response that drives stem cell activation is similar across the different sensory organs.

2.2.3. Control of stem and progenitor cell proliferation. As proliferation is the basis of regeneration, understanding its regulation is crucial for devising strategies to trigger cell division in mammals. After activation, the stem cells in all sensory organs undergo proliferation to self-renew or to differentiate into sensory cells. In the zebrafish retina, progenitor cells undergo asymmetric divisions, with one daughter cell differentiating into a Müller glia cell and the other daughter cell differentiating into a sensory cell (Nagashima et al. 2013). In contrast, clonal lineage tracing in the mouse olfactory system and live imaging of zebrafish neuromasts revealed that, in these sensory organs, stem cells undergo mainly symmetric divisions (Gadye et al. 2017, Romero-Carvajal et al. 2015).

The Wnt, Notch, and Fgf pathways either activate or inhibit proliferation, and recent data show that they interact (**Figure 2**). In the zebrafish retina, pharmacological and genetic inhibition of Notch signaling after mechanical injury enhances the proliferation of Müller glia cells (Conner et al. 2014, Wan et al. 2012), suggesting an inhibitory role in proliferation. Interestingly, this antiproliferative effect of Notch signaling is conserved in neural stem cells (NSCs) and lateral line neuromasts. Pseudo–time analysis from scRNA-seq in hippocampal quiescent NSCs of adult mice showed that Notch signaling–related genes are decreased in the transition from quiescence to activated NSCs (Shin et al. 2015). Likewise, blocking of Notch signaling by the γ-secretase inhibitor DAPT promotes cell proliferation and protein synthesis in quiescent NSCs, which in turn proportionally increases activated cells (Llorens-Bobadilla et al. 2015). These data suggest that downregulation of Notch signaling is required to trigger the activation of quiescent NSCs. In neuromasts, Notch signaling is also immediately downregulated after neomycin treatment (Jiang et al. 2014, Romero-Carvajal et al. 2015), and pharmacological inhibition of Notch signaling promotes cell division and increases the total number of hair cells (Romero-Carvajal et al. 2015, Thomas & Raible 2019). Similarly, in the postnatal adult mouse cochlea and utricle, downregulation of Notch signaling leads to the formation of new hair cells (Burns et al. 2012, Jung et al. 2013, Korrapati et al. 2013, Li et al. 2015, Lin et al. 2011, Slowik & Bermingham-McDonogh 2013). Inhibition of Notch alone leads to modest hair cell regeneration and recovery of hearing; however, these cells fail to fully mature (Maass et al. 2015, Mizutari et al. 2013). Surprisingly, manipulation (pharmacological inhibition and overexpression of NICD) of Notch signaling during homeostasis in the absence of an injury signal has very little to no effect on proliferation in zebrafish neuromasts (Romero-Carvajal et al. 2015). This finding suggests that Notch signaling may regulate the competence of stem cells to respond to an injury signal. Alternatively, Notch signaling requires an injury signal to trigger proliferation. Identifying the injury signal as well as the receptors on Notch-responsive cells will be instrumental in deciphering the precise mechanism of Notch signaling in regulating proliferation.

Like Notch signaling, Fgf signaling affects proliferation (**Figure 2**). Even though Fgf signaling has a positive effect on proliferation in many contexts, such as in zebrafish fin regeneration, mouse hearts, and primary cardiomyocyte cultures (Lee et al. 2005, Poss et al. 2000, Rochais et al. 2014), it inhibits proliferation in regenerating sensory organs.

For example, in the fish retina, *fgf8a* negatively regulates Müller glia cell proliferation (Wan & Goldman 2017). Three hours post–mechanical injury, *fgf8a* expression and expression of the

Fgf-responsive genes *dusp6* and *etv5b* are transiently suppressed. Forced expression of *fgf8a* after mechanical injury to the retina reduces proliferation of Müller glia cells. Interestingly, *fgf8* knockdown by morpholino injection is not sufficient to induce Müller glia cell proliferation in the uninjured retina. This result suggests that, in the fish retina, like for Notch signaling, the role of *fgf8a* is to control the competence of stem cells to the injury signal by locking them into a quiescent state or that an injury signal is required to trigger proliferation. In fish neuromasts, genetic knockout of *fgf3* in zebrafish is sufficient to induce a slight overproduction of hair cells due to a robust increase in progenitor proliferation. This mutant analysis established a role for Fgf signaling in inhibiting proliferation, in parallel with Notch signaling (Lush et al. 2019). Likewise, overexpression of Fgf20 inhibits support cell proliferation in the chick utricle, supporting an antiproliferative role for Fgf signaling in hair cell regeneration (Ku et al. 2014).

In zebrafish neuromasts, pharmacological upregulation of Wnt signaling stimulates cell proliferation of support cells, resulting in a proportional increase in the number of hair cells during both homeostasis and regeneration (Jacques et al. 2014, Romero-Carvajal et al. 2015). Likewise, in the olfactory epithelium, HBC-specific activation by constitutively active β-catenin is sufficient to trigger proliferation of the HBCs in the uninjured mouse olfactory epithelium (Fletcher et al. 2017). Even in the mammalian retina and inner ear, Wnt activation by GSK3β inhibition or stabilization of β-catenin promotes proliferation (Osakada et al. 2007). In contrast, genetic inhibition of the Wnt pathway in the zebrafish retina and in neuromasts blocks proliferation and regeneration (Ramachandran et al. 2011, Romero-Carvajal et al. 2015).

While the Wnt pathway is clearly involved in regulating stem/progenitor cell proliferation, what are the upstream and downstream factors? In the zebrafish retina, the bHLH transcription factor *ascl1a* acts upstream of Wnt signaling and promotes proliferation. Ascl1a represses the expression of the Wnt ligand antagonists *dkk1b*, *dkk2*, *dkk3*, and *dkk4* but also increases Wnt signaling directly by controlling *wnt4a* expression (Ramachandran et al. 2011) (**Figure 2**). Interestingly, activation of Wnt signaling by GSK3β inhibition is sufficient to dedifferentiate Müller glia cells and induces *ascl1a* expression, suggesting the presence of a feedback loop to further activate genes associated with Müller glia cell dedifferentiation and proliferation (Ramachandran et al. 2011). Simultaneous overexpression of *ascl1a* and *lin28a* in the uninjured zebrafish or the adult mouse retina is sufficient to promote a low level of proliferation of Müller glia cells (Elsaeidi et al. 2018). Simultaneous inhibition of Notch signaling dramatically increases the number of Müller glia cells proliferating in zebrafish, while it has no enhancing effect on the mouse retina, suggesting a different sensitivity for Notch signaling between the two species (Elsaeidi et al. 2018). Upregulation of Wnt signaling, in turn, causes an increase in *ascl1*, showing that Wnt and *ascl1* act in a feedback loop (Ramachandran et al. 2011).

scRNA-seq analysis of the adult mouse subventricular zone reveals that *Ascl1* has low expression in quiescent NSCs and differentiated neurons but is highly upregulated in activated NSCs (Zywitza et al. 2018), suggesting a conserved role for *Ascl1* in positively regulating proliferation in the brain and the retina. In zebrafish neuromasts, *ascl1a* is not expressed but *lin28a* is highly upregulated immediately after neomycin treatment (Jiang et al. 2014). The bHLH transcription factor *atoh1* plays an analogous role in the mouse cochlea to *ascl1* in the central nervous system. Prolonged and specific overexpression of Atoh1 in the postnatal mouse cochlea leads to cell proliferation of the sensory epithelium in regions that are otherwise postmitotic (Kelly et al. 2012). Moreover, the cyclin-dependent kinase inhibitor Cdkn1b is strongly downregulated in Atoh1-induced cells (Kelly et al. 2012), strongly suggesting that Atoh1 positively regulates proliferation in addition to its role in specifying the hair cell progenitors in the mouse cochlea (Bermingham et al. 1999).

In the olfactory epithelium, Ascl1 has been traditionally associated with controlling differentiation (Caggiano et al. 1994, Cau et al. 2002, Jang et al. 2003), but recent studies show that it is expressed in proliferating GBCs (Fletcher et al. 2017). However, the role of Ascl1 in proliferation has not been tested. It is tempting to hypothesize that the bHLH transcription factors Ascl1 and Atoh1 have a conserved role in regulating proliferation downstream of Wnt signaling in all sensory organs (**Figure 2**). A potential role for Atoh1 in upregulating Wnt signaling in neuromasts and the inner ear, analogous to the feedback mechanism between *ascl1* and Wnt in the fish retina, remains to be investigated.

2.2.4. Regulation of differentiation and cell fate choice. The differentiation of stem cells into a particular type of sensory cell is a multistep process that needs to be tightly regulated. What are the molecular regulators of this process?

2.2.4.1. Differentiation of the sensory epithelia. Sensory cell differentiation must be balanced with the maintenance of a progenitor pool during both development and regeneration. Which pathways and effectors control this initial cell fate choice? In the retina, the inner ear, and the olfactory epithelium, Notch signaling drives this binary lineage decision by positively regulating bHLH transcriptional repressors from the Hes/Hey family, which maintain progenitors (Hatakeyama & Kageyama 2004, Jarman & Groves 2013, Manglapus et al. 2004, Yu & Wu 2017, Zine et al. 2001). These repressors inhibit the expression of bHLH activators (Ascl1 in the retina and olfactory epithelium and Atoh1 in neuromasts and the cochlea), precluding differentiation into sensory cells (Abdolazimi et al. 2016, Ishibashi et al. 1995, Manglapus et al. 2004). Thus, Ascl1 and Atoh1 not only are involved in proliferation but also are crucial for cell fate specification (Bermingham et al. 1999, Cau et al. 2002, Jasoni & Reh 1996).

2.2.4.2. Neuronal subtype cell fate decisions. The retina, the inner ear, and the olfactory neurons are composed of various neuronal and glial cell types (**Figure 1**). What factors promote one fate over the other? In the retina, genetic removal of Notch1 in postmitotic progenitors leads to almost all these cells becoming rod photoreceptors, showing that Notch1 inhibits the rod receptor fate (Mizeracka et al. 2013). Similarly, during hair cell regeneration in neuromasts, treatment with a low dose of a γ-secretase inhibitor affects only hair cell differentiation, whereas high doses also inhibit support cell proliferation (Romero-Carvajal et al. 2015). Notch signaling components are heterogeneously expressed in the neuromasts (Lush et al. 2019, Ma et al. 2008, Romero-Carvajal et al. 2015, Wibowo et al. 2011). *dla* is expressed in the D-V poles of support cells, while ligand-encoding *dlb*, *dlc*, and *dld* are strictly expressed in hair cell progenitors (Ma et al. 2008, Romero-Carvajal et al. 2015). Likewise, the Notch receptors are expressed in different support cell populations (Romero-Carvajal et al. 2015, Wibowo et al. 2011). While there is only one sensory cell type in neuromasts (hair cells), hair cells differ in their polarity (López-Schier et al. 2004, Navajas Acedo et al. 2019, Platt 1977, Rouse & Pickles 1991). Cells expressing Notch1a repress Emx2, a transcription factor involved in polarity specification (Jiang et al. 2017), causing these cells to adopt a rostral orientation, while Emx2-positive cells adopt a caudal orientation (Jacobo et al. 2018). In the adult zebrafish brain, several Notch receptors are expressed in different cell types, and *notch3* is involved in the maintenance of NSC quiescence, while *notch1b* regulates NSC differentiation (Alunni et al. 2013). A similar heterogeneity of expression of Notch components is conserved in the adult olfactory epithelium. Notch1 is expressed specifically in Bowman's gland, while Notch2 is expressed in the sustentacular cells (Carson et al. 2006). Altogether, this heterogeneity of expression and the dependence of different cell fates on Notch signaling make it tempting to hypothesize that different combinations of ligands and Notch receptors drive cell-specific fates in sensory organs.

An equivalent mechanism occurs in chick somites, in which brief activation of the Notch1 receptor by Delta1 triggers a muscle differentiation program by activating Hes1, while prolonged interaction with Delta4 inhibits myogenesis by activating Hey1 (Nandagopal et al. 2018).

Altogether, these data show that the nature and expression pattern of Notch receptors and their ligands are crucial for cell fate choice. This effect on cell fate choice may be due to the differing strengths of interaction between receptor and ligand pairs, leading to short or prolonged activation of the pathway (Andrawes et al. 2013, Fryer et al. 2004, Housden et al. 2013, Ilagan et al. 2011). Thus, the dosage of Notch signaling is important for cell fate decisions. These data also show that, while Notch signaling must be inhibited to trigger proliferation of stem and progenitor cells during regeneration, Notch signaling needs to be reactivated both to maintain the progenitor pool and to later induce different cell types in the sensory organs. The repeated requirement and different functions of Notch signaling in different stages of the regenerative process require tight and dynamic regulation for the successful regeneration of hair cells in mammals.

Wnt signaling has different effects on sensory cell differentiation, depending on the organ. As discussed above, Wnt signaling needs to be inhibited for photoreceptor differentiation (Ramachandran et al. 2010), whereas in neuromasts, Wnt signaling activation does not affect hair cell differentiation (Romero-Carvajal et al. 2015). Wnt activation causes a proportional increase in the number of lateral line hair cells, but only because more support cells are proliferating. In contrast, in the developing mouse inner ear, Wnt/β-catenin signaling has been reported to regulate not only proliferation but also hair cell differentiation. Indeed, the *Atoh1* enhancer possesses β-catenin binding sites, although it is unclear whether this enhancer is used in vivo (Shi et al. 2010).

2.2.5. Possible mechanisms of sensory organ size control. What regulates the cessation of proliferation and therefore the total number of sensory cells? Molecular regulation of mechanosensory cells would be a powerful way to prevent crowding and an excess of sensory cells and to maintain a proper balance between the progenitors and the sensory cells. In the olfactory epithelium, a competition between the Tgfβ pathway inhibitor *Follistatin*, expressed in progenitors, and the Tgfβ agonist *Gdf11*, expressed in sensory neurons, controls the total number of sensory cells formed (Gokoffski et al. 2011). When enough sensory cells are produced and a sufficient amount of Gdf11 is secreted, Gdf11 will outcompete the inhibitor Follistatin and activate Tgfβ receptors to induce the cell cycle inhibitor $p27^{kip1}$ in progenitors (Wu et al. 2003). This ligand-versus-inhibitor competition is a very efficient and reversible mechanism for controlling the total number of sensory cells and maintaining the proper balance between sensory and progenitor cells. In homeostatic zebrafish neuromasts, *follistatin* is specifically expressed in the amplifying progenitor, while *gdf3* is expressed in hair cells (Lush et al. 2019), suggesting that a similar mechanism may be at play in fine-tuning the number of hair cells. Alternatively, the Hippo pathway has been implicated in regulating organ size in many species and contexts (Yu et al. 2015). Indeed, molecular regulation of mechanosensing would be a powerful way to prevent crowding and thus an excess of sensory cells and to maintain a proper balance between progenitors and sensory cells. Interestingly, in the zebrafish neuromast, most of the core components of the Hippo pathway, such has *sav1*, *lats1/2*, *mob1a*, *mob1ba/b*, and *yap1*, are expressed in support cells, while the upstream acting mechanosensory protocadherins *fat1b* and *fat2* are enriched in mantle cells (Lush et al. 2019). The progressive production and crowding of hair cells may increase the tension felt by mantle cells, which above a certain threshold could instruct support cells to stop proliferating by activating the Fat/Hippo pathway. The abovementioned mechanisms present future areas of investigation in regenerating sensory organs.

3. POSSIBLE DIFFERENCES BETWEEN REGENERATING AND NONREGENERATING ANIMALS

3.1. Regulation of Inflammation

An inflammatory response is proposed to be essential for proper regeneration (Eming et al. 2017). For example, treating zebrafish embryos with the immunosuppressant dexamethasone (Dex) prior to injury causes delays in Müller glia cell proliferation and in retina cell replacement (White et al. 2017). Conversely, application of Dex immediately after injury leads to accelerated regeneration, suggesting that regulating the timing of inflammation may be essential for proper regeneration (White et al. 2017). Similarly, when treated with Dex prior to injury, brain-injured zebrafish show decreased radial glial cell proliferation and decreased neurogenesis, suggesting that inflammation is required for brain regeneration (Kyritsis et al. 2012). Indeed, the proinflammatory cytokines *interleukin 8* (*il8*), *il1b*, and *tnfa* are upregulated immediately after injury.

While initially necessary, a persistent inflammation process can also be detrimental and must be tightly controlled. In the mouse brain, neuroinflammation caused by radiation injury blocks NSC differentiation (Monje et al. 2003). In addition, differentiation of cultured NSCs is reduced by 50% in the presence of either of the proinflammatory cytokines IL6 and Tnfα (Monje et al. 2003), suggesting that prolonged exposure of NSCs to proinflammatory cytokines is detrimental. Could the regulation of inflammation be the key difference between regenerative and nonregenerative species? A recent study shed light on this process by comparing the regenerative spiny mouse (*Acomys cahirinus*) to the nonregenerative house mouse (*Mus musculus*) (Simkin et al. 2017). Following ear punch injury in the mouse, Simkin et al. (2017) observed that both spiny mice and house mice exhibited acute inflammation. The main differences between the two species were the dynamics and the identity of the immune cells regulating this acute inflammation. In the house mouse, neutrophils were recruited early after injury and stayed in the injury site for several days, while macrophages were recruited later and for a shorter period of time. The prolonged myeloperoxidase activity of neutrophils is associated with fibrosis and scarring (Simkin et al. 2017). In the spiny mouse, neutrophils were also recruited to the site of injury but were cleared rapidly, while macrophages accumulated at the injury site for several days. Chemical ablation of macrophages in the spiny mouse abolished ear regeneration, showing that macrophages are necessary for regeneration. The comparison between the two species suggests that the difference between scarring and regeneration may lie in the nature of the immune cells that regulate inflammation and in the dynamics of immune cell recruitment. Rapid clearance of the neutrophil and the prolonged persistence of macrophages may be signatures of regenerating tissues, as these characteristics have also been observed in the regenerating zebrafish fin and the axolotl limb (Godwin et al. 2013, Petrie et al. 2014).

3.2. Macrophages Play Critical Roles in Regeneration

Macrophages are essential for axolotl limb and zebrafish tail and heart regeneration in both embryos and adults (Godwin et al. 2013, Huang et al. 2013, Petrie et al. 2014). If regulation of inflammation is a key process of regeneration, and macrophages are the main immune cells involved in the process, what happens to hair cell regeneration when macrophages are not active or are absent? After hair cell damage in chick or axolotl lateral line neuromasts, macrophages are recruited to the lesion site in vitro and in vivo (Bhave et al. 1998, Warchol 1997). Subsequent studies revealed that chemically induced blockade of macrophage activation in cultures of chick sensory epithelia results in decreased hair cell regeneration (Warchol 1999). In zebrafish, macrophages are recruited to the site of hair cell–damaged neuromasts, and pharmacologically induced or morpholino-based

ablation of macrophages delays the progress of hair cell regeneration (Carrillo et al. 2016). These findings suggest that macrophage-dependent inflammation is necessary for hair cell regeneration and beg the question of what the nature of the molecular interactions between dying hair cells, macrophages, and support cells is. Global transcriptomic analyses in zebrafish neuromasts revealed that proinflammatory genes such as *tnfa*, *il6r*, *il6st*, and *il11b* are rapidly upregulated following hair cell damage (Jiang et al. 2014). The upregulation of *il6r* is interesting, as *il6* is a classical secreted marker of proinflammatory macrophages (Nguyen-Chi et al. 2015). By transiently expressing the receptor (*il6r*) and the transducer of the pathway (*il6st*), the regenerating tissue could control the timing of the inflammatory response.

Are macrophages solely professional phagocytes, or do they play an instructive role during hair cell regeneration? The growing literature on the role of macrophages in regulating stem cells (Naik et al. 2018) makes it tempting to speculate that macrophages play an instructive role in activating neuromasts or inner ear stem cells. In addition to controlling inflammation through cytokine signaling, macrophages secrete growth factors such as PDGF, TGFB1, IGF-1, VEGF, and WNTs to promote proliferation (see Chakrabarti et al. 2018, Wynn & Vannella 2016). High-temporal-resolution transcriptome analyses during inflammation and regeneration will help to decipher the roles of macrophages by elucidating the progression of growth factors and cytokines secreted during the regeneration process. The main caveat of such studies is the need to dissociate tissues and sort cells (using fluorescence-activated cell sorting), which induces a stress response generating potential false-positive candidates. Blocking transcription using actinomycin D or fixing cells prior to dissociation could circumvent this challenge. Identifying the macrophage transcriptome would also provide new tools for controlling the inflammatory response by transiently inhibiting or activating growth factor and cytokine combinations. Finding the precise cocktail of factors and the precise timing of action that support regeneration would be transformative for regenerative medicine.

4. ENHANCER STUDIES AND PERSPECTIVES

Mouse sensory epithelia show some regenerative capacity at the neonatal stage, but this capacity is lost rapidly as the animal matures (Burns et al. 2012, Cox et al. 2014, Forge et al. 1993, Warchol et al. 1993). What influences this loss of regenerative ability? One possibility is the above-discussed differences in the immune response observed in regenerative and nonregenerative species. Another interesting hypothesis is that chromatin accessibility changes and particular enhancers are more accessible or are closed to transcription factor binding (Stojanova et al. 2016). Alternatively, the transcription factors that bind enhancers during regeneration may not be expressed in nonregenerative species. To shed light on these questions, it is essential to identify the enhancers used in regenerative species during homeostasis and regeneration. The identification of enhancers has been greatly aided by the development of the assay for transposase-accessible chromatin using sequencing (ATAC-seq) (Buenrostro et al. 2013), a method that identifies open chromatin regions, coupled with ChIP-seq of chromatin marks such as H3K27 acetylation. This technological advance allows for interrogation of the entire genomic landscape and accelerates the identification of regeneration enhancers. Together with transcription factor binding motif searches, the combination of ATAC-seq and ChIP-seq will help reveal transcription factor binding signatures within enhancers. For example, two studies have identified regeneration-specific enhancers. In *Drosophila*, *wg* is involved in wing imaginal disc regeneration. This enhancer drives regeneration in young but not older larvae (Harris et al. 2016). The *wg* tissue damage–responsive enhancer consists of one module that drives *wg* expression independently of the developmental stage. However, next to this module, another silencing element recruits increasing amounts of Polycomb group proteins that

promote trimethylation of H3K27, resulting in the progressive and specific silencing of the *wg* tissue damage–responsive enhancer in older larvae, inhibiting regeneration. Likewise, a zebrafish enhancer that drives the expression of *leptin b* only during regeneration in different tissues was recently identified (Kang et al. 2016). Within this enhancer, one element drives expression during fin regeneration, and another element drives expression during heart regeneration. Importantly, even though there is no sequence similarity with the mouse, these zebrafish enhancer elements are sufficient to drive expression of a variety of murine promoters, making these enhancers potentially suitable for activating gene expression after injury (Kang et al. 2016). These enhancers are activated in mouse after injury of both nonregenerating digit tips (Simkin et al. 2013) and regeneration-competent neonatal ventricles (Porrello et al. 2011). These findings suggest that the gene regulatory network responsible for the activation of these regeneration enhancers is present in regenerating and nonregenerating tissues. These findings also suggest that the difference between the regenerating and nonregenerating tissues may be the accessibility of the regenerating enhancers. The lack of sequence similarity associated with the capacity of these regeneration enhancers to respond to injury in mouse suggests that, while the transcription factors binding these enhancers are present at the injury site, the endogenous enhancers may be repressed or lost.

Indeed, while sequence alignment of enhancers between different species is almost always unfruitful, comparing transcription factor motifs will allow for the discovery of potentially repressed regeneration enhancers, or the lack thereof, in nonregenerative species. Importantly, in addition to identifying regeneration-specific enhancers, ATAC-seq and ChIP-seq can identify potential cell type–specific enhancers (Heinz et al. 2015, Zhou et al. 2017). A major drawback, when one is studying the role of a signaling pathway in a specific group of cells at a specific time, is the lack of tools with which to perturb the signaling pathway with enough spatiotemporal precision. Pharmacological treatments or conditional knockouts affect more than the tissue of interest and make interpretation of the role of the pathway challenging. Identifying cell-specific enhancers will be valuable in precisely deciphering the role of multiple signaling pathways during regeneration.

5. SUMMARY AND OPEN QUESTIONS

While different in some aspects, regeneration in sensory organs, such as the retina, ear, and lateral line, seems to follow a similar general blueprint. While inflammation appears to be a trigger for sensory cell regeneration, the precise machinery for controlling inflammation is poorly understood. Likewise, the role of macrophages in sensory cell regeneration is conserved in different sensory organs, but it is unclear whether macrophages function only as phagocytes or also play an instructive role.

Within sensory progenitor/stem cells, the sequence of downregulation of Notch and Fgf signaling, the upregulation of a bHLH transcription factor to allow progenitor proliferation by Wnt signaling, and the subsequent cell fate decision via Notch are conserved. In the mouse field, recent attempts to induce hair cell regeneration have focused on the manipulations of Notch and Wnt signaling, sometimes in combination with upregulation of Atoh1 (Burns et al. 2012, Jung et al. 2013, Korrapati et al. 2013, Kuo et al. 2015, Li et al. 2015, Lin et al. 2011, Mizutari et al. 2013, Shi et al. 2013, Slowik & Bermingham-McDonogh 2013). However, overexpression of Atoh1 leads to the formation of immature hair cells in mouse and humans (Izumikawa et al. 2005, Taylor et al. 2018, Zheng & Gao 2000). Proliferation in the mouse inner ear sensory epithelia can also be triggered by inhibiting negative regulators of the cell cycle, such as $p27^{Kip1}$ (Cdkn1b) and Rb1 (Sage et al. 2005, Walters et al. 2014). Support cells do reenter the cell cycle and produce a modest number of hair cells. However, these hair cells do not survive. A recent study may explain why the ability of Wnt and Notch manipulations to induce proliferation and hair cell differentiation

diminishes with the age of the mouse pup, even though the pathways are still active (Geng et al. 2016, Samarajeewa et al. 2018). Geng et al. (2016) and Samarajeewa et al. (2018) discovered that additional downstream pathways are required for the proliferative response to Wnt signaling and that these pathways are likely epigenetically silenced in postnatal stages. Indeed, Wnt activation coupled with a histone deacetylase inhibitor increases the proliferative response of support cells in culture (McLean et al. 2017).

Another, likely related, reason for the difficulties faced in regenerating mammalian hair cells with the current approaches could be that mammalian support cells are more differentiated than support cells in regenerating species. Attempts at first reprogramming adult support cells to an embryo-like state may be proven more successful in inducing the proliferation and differentiation program, leading to hair cell regeneration at a larger scale. Ongoing scRNA-seq experiments of mammalian support cells will allow for comparison of these cells and support cells in regenerating species and will identify factors required for their maintenance. Some efforts are already under way to reprogram mammalian support cells to a more stem cell–like state by using transcription factors such as Gfi1 and Pou4f3 (Costa et al. 2015, Ikeda et al. 2015, Walters et al. 2017).

The recent establishment of single-cell transcriptomics, techniques to interrogate the chromatin landscape, and interspecies and interorgan comparisons at the single-cell level will facilitate the elucidation of the molecular road map of regeneration that could be applied to unleash the regenerative capacities of human sensory organs.

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