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SENSORY HAIR CELL REGENERATION IN THE ZEBRAFISH LATERAL LINE

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Abstract

Damage or destruction of sensory hair cells in the inner ear leads to hearing or balance deficits that can be debilitating, especially in older adults. Unfortunately, the damage is permanent, as regeneration of the inner ear sensory epithelia does not occur in mammals. Zebrafish and other non-mammalian vertebrates have the remarkable ability to regenerate sensory hair cells and understanding the molecular and cellular basis for this regenerative ability will hopefully aid us in designing therapies to induce regeneration in mammals. Zebrafish not only possess hair cells in the ear but also in the sensory lateral line system. Hair cells in both organs are functionally analogous to hair cells in the inner ear of mammals. The lateral line is a mechanosensory system found in most aquatic vertebrates that detects water motion and aids in predator avoidance, prey capture, schooling and mating. Although hair cell regeneration occurs in both the ear and lateral line, most research to date has focused on the lateral line due to its relatively simple structure and accessibility. Here we review the recent discoveries made during the characterization of hair cell regeneration in zebrafish.

Keywords

neuromast; ear; Notch; Wnt/ β -catenin; transcriptomics

INTRODUCTION

Deafness or hearing deficits are most often caused by loss of sensory hair cells or defects in their function, which can be inherited or acquired. Postnatal hair cell death is induced by bacterial infections, damage from prolonged noise exposure, and treatments with certain ototoxic drugs such as aminoglycoside antibiotics or chemotherapy agents (Yorgason et al., 2006, Oishi and Schacht, 2011). Additionally, ageing is a prominent factor and the prevalence of hearing loss rises sharply as individuals get older (Bielefeld et al., 2010, Kidd Iii and Bao, 2012).

In all non-mammalian vertebrates sensory hair cells turn over during homeostasis and robustly regenerate after injury (Burns and Corwin, 2013, Rubel et al., 2013). In contrast, mammalian hair cells display a very limited regenerative ability around birth that is lost as the animals mature (Burns et al., 2012, Cox et al., 2014, Forge et al., 1993, Warchol, 2011,

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Warchol et al., 1993). The fact that non-mammalian vertebrates readily regenerate sensory hair cells is exciting, as it suggests that we should be able to trigger hair cell regeneration in mammals once we understand how regeneration is controlled in these other species.

Hair cell regeneration was first discovered in chicken, which has become a widely used model to study this process (Cotanche, 1987, Cruz et al., 1987). During recent years, the zebrafish has proven to also be an excellent model to study hair cell development and regeneration. Some of the characteristics that make zebrafish such a good model system are based on biological properties of these animals, whereas others are of a technical nature. Zebrafish not only possess sensory hair cells in the ear but also in the transparent skin. This superficial location of the sensory organs makes them experimentally accessible, enabling time-lapse imaging of the cellular behaviors that occur during hair cell regeneration in intact animals. In contrast, chicken or rodent sensory epithelia have to be cultured for any live observations. Also, the lateral line system develops rapidly and its sensory organs mature within the first week of development. Importantly, lateral line hair cells can be killed within 15–20 min of antibiotic exposure, whereas in chicken hair cell death occurs over a 24 hour time period (Cruz et al., 1987, Harris et al., 2003, Brignull et al., 2009). The synchronized death of lateral line hair cells greatly facilitates the identification of genes and pathways that are regulated during particular stages of hair cell regeneration (Jiang et al., 2014, Steiner et al., 2014). Efforts are also underway in several laboratories to identify regeneration specific genes in forward genetic screens, which is not yet possible in any other model system (Behra et al., 2009). In addition, a large number of mutations have been isolated in genetic screens all over the world and have been deposited in zebrafish stock centers. For example, the Sanger Institute aims to generate zebrafish mutations in all known genes over the next several years (www.sanger.ac.uk/Projects/D_zerio/zmp/). These mutants could be screened for regeneration defects. Additionally, the recent development of genome editing techniques such as Zinc finger, TALEN and CRISPR, now allow us to also test the function of candidate genes (Doyon et al., 2008, Huang et al., 2011, Hwang et al., 2013, Meng et al., 2008, Sander et al., 2011). Finally, the generation of fluorescent transgenic lines enables the in vivo visualization and tracking of specific cell types or sub-cellular compartments or serve as reporters for different signaling pathways, such as Wnt/ β -catenin and Notch (Dorsky et al., 2002, Parsons et al., 2009, Shimizu et al., 2012, Moro et al., 2012). All these recent technological advances greatly enrich the toolbox available to interrogate the process of sensory hair cell regeneration in zebrafish.

ANATOMY OF THE LATERAL LINE

The functional organs of the lateral line system are called neuromasts and are positioned regularly along the head and trunk (Fig. 1A–B). Embryonic neuromasts are periodically deposited by migrating clusters of cells, called primordia, which form anterior and posterior to the otic vesicle (Ghyssen and Dambly-Chaudiere, 2007, Metcalfe, 1985). The posterior lateral line, which most studies focus on, forms from two primordia. Primordium 1 migrates first and deposits 6–7 neuromasts. Primordium 2 migrates along the same path beginning a day later and deposits neuromasts in between the previously deposited neuromasts. The molecular signals and cellular behaviors that regulate primordium migration and neuromast

deposition have been recently reviewed and will not be covered here (Aman and Piotrowski, 2011, Chitnis et al., 2012, Ma and Raible, 2009).

Each neuromast contains mechanosensory hair cells that extend their stereocilia past the epidermis and are deflected by water movement (Fig. 1C). Lateral line hair cells are functionally and morphologically very similar to the hair cells in the inner ear of mammals and mutations that affect the function of lateral line hair cells in fish also cause deafness in humans (Nicolson, 2005, Whitfield, 2002). Even though zebrafish do not possess a cochlea, sensory hair cells are also located in the maculae and cristae in the zebrafish ear (Nicolson, 2005, Haddon and Lewis, 1996). Similar to hair cells in the mammalian inner ear and vestibular system, hair cells within a neuromast exhibit a unique planar cell polarity with roughly half the hair cells oriented in opposite polarity to the other half (Lopez-Schier et al., 2004). This allows neuromasts to perceive water movement coming from opposing directions. The axis of hair cell polarity aligns with the body axis or is orientated perpendicular to it, depending on whether the neuromasts originated from the first or second primordium, respectively (Lopez-Schier et al., 2004).

Neuromast hair cells are surrounded by at least two distinct support cell populations: inner support cells situated underneath and adjacent to hair cells and mantle cells that form a ring of cells encircling the entire neuromast (Fig. 2D). Mantle cells are multipotent lateral line progenitor cells as they can migrate posteriorly and give rise to entire neuromasts on regenerating axolotl tail tips (Jones and Corwin, 1993). In zebrafish, mantle cells also seem to be involved in neuromast regeneration after tail fin amputation, but this has not been formally shown (Dufourcq et al., 2006).

Neuromast hair cells are innervated by axons whose cell bodies form the lateral line ganglia (llg) (Raible and Kruse, 2000). Neuromasts also receive inhibitory and excitatory efferent projections from the brain, although the functions have not been extensively studied (Metcalf et al., 1985). The anterior llg (allg) is located just anterior to the otic vesicle and innervates all neuromasts in the head. The posterior llg (pll) forms just posterior to the otic vesicle and innervates all neuromasts on the trunk. Neurons within the llg are bipolar with one axon extending into the periphery where it innervates neuromasts and the other projecting into the octavolateralis nucleus in the brain (Alexandre and Ghysen, 1999). Neurons can innervate more than one neuromast but they only synapse with hair cells of the same polarity (Faucher et al., 2009, Nagiel et al., 2008). How axons choose hair cells of the same polarity and avoid those of the opposite orientation is not well understood, however the pattern of axonal connections, with axons forming synapses onto cells of the same polarity, is re-established after hair cell regeneration (Faucher et al., 2009, Nagiel et al., 2008). Interestingly, axons of the pll are also capable of regeneration after injury (Graciarena et al., 2014, Villegas et al., 2012).

CELLULAR ASPECTS OF ZEBRAFISH HAIR CELL REGENERATION

Over the last several years, much work has gone into characterizing and establishing the zebrafish lateral line as a model for sensory hair cell regeneration. Hair cell death can be easily induced by treatment with various ototoxic chemicals (Fig. 1E–G). For example,

exposure of freely swimming zebrafish larvae to aminoglycoside antibiotics, heavy metals, such as copper, or chemotherapeutic drugs, like cisplatin, lead to hair cell death in the lateral line (Harris et al., 2003, Hernandez et al., 2006, Linbo et al., 2006, Ou et al., 2007, Seiler and Nicolson, 1999, Ton and Parng, 2005, Williams and Holder, 2000). How these treatments lead to hair cell death is not completely understood, but antibiotic treatment induces mitochondrial swelling and transient increases in calcium levels (Esterberg et al., 2013b, Owens et al., 2007). Subsequent cell death is initiated through apoptotic pathways that are unique for each treatment used (Coffin et al., 2013a, Coffin et al., 2013b). Complete regeneration of hair cell numbers is typically achieved within 72 hours, but can be more prolonged depending on drug dosage (Harris et al., 2003, Hernandez et al., 2006, Mackenzie and Raible, 2012). Hair cells within the zebrafish ear are protected from drug containing media but can be experimentally damaged by prolonged noise exposure, laser ablation or direct injection of gentamicin (Millimaki et al., 2010, Schuck and Smith, 2009, Uribe et al., 2013).

BrdU and time-lapse imaging experiments demonstrate that regeneration of lateral line hair cells is achieved through induction of proliferation and subsequent differentiation of inner support cells (Lopez-Schier and Hudspeth, 2006, Ma et al., 2008, Mackenzie and Raible, 2012, Wibowo et al., 2011). Blocking proliferation with pharmaceutical inhibitors results in neuromast hair cell regeneration failure. Therefore, support cell proliferation is the major mechanism for producing new hair cells in zebrafish (Mackenzie and Raible, 2012, Wibowo et al., 2011). This is in contrast to chicken and amphibian sensory ear epithelia that regenerate via proliferation as well as direct transdifferentiation of support cells into hair cells (Adler and Raphael, 1996, Baird et al., 1996, Roberson, 1996). Unlike injured lateral line neuromasts, an increase in BrdU incorporation was not observed in the developing zebrafish ear after laser ablation, but rather support cells transdifferentiated into hair cells (Millimaki et al., 2010). However, proliferation is increased in the adult zebrafish ear after noise induced hair cell loss (Schuck and Smith, 2009, Schuck et al., 2011). Further studies are needed to determine if these differences in cell behavior in the ear are age-related or due to technical differences, such as the method used to kill hair cells.

SUPPORT CELL CHARACTERIZATION

As support cell proliferation is vital for hair cell regeneration, a detailed understanding of their biology and behavior is needed. For example, it is not known if support cells are a homogeneous population or if only subsets of support cells contribute to regeneration. The ability to perform time-lapse analysis in zebrafish *in vivo* allows the direct assessment of the specific cell-type contributions to regeneration and stem cell self-renewal. Multiple transgenic lines have been generated which label specific cell types within neuromasts. Examples of these transgenic lines are illustrated in Fig. 2. *Tg(cldnB:lyngfp)* labels all cell types in the lateral line primordium and neuromasts and has been extensively used to study the early development of the migrating primordium (Fig. 2A) (Haas and Gilmour, 2006). Several lines were generated during GFP-based enhancer trap (ET) screens (Nagayoshi et al., 2008, Parinov et al., 2004). *Tg(SqET20:gfp)* labels all mantle cells and some inner support cells (Fig. 2B) (Hernandez et al., 2007, Jiang et al., 2014, Parinov et al., 2004). An additional support cell specific expressing line, *Tg(tnks1bp1:EGFP)*, has also been recently

described (Behra et al., 2012). *Tg(SqET4:gfp)* labels mature hair cells, as well as their immediate progenitors (Fig. 2C) (Go et al., 2010, Hernandez et al., 2007, Parinov et al., 2004, Lopez-Schier and Hudspeth, 2006). In HGn39D larvae, GFP is expressed in the pll_g and labels the afferent axons that synapse onto hair cells (Fig. 2D) (Faucherre et al., 2009, Nagayoshi et al., 2008).

As yet, lineage analyses of all cells in regenerating neuromasts have not been performed. However, studies focusing specifically on cell divisions resulting in new hair cells by time-lapse analysis of the *Tg(SqET4:gfp)* line showed that asynchronous support cell divisions were symmetric, with divisions giving rise to two hair cells (Lopez-Schier and Hudspeth, 2006). It is not known if all inner support cells are able to respond to hair cell death, if any of the inner support cells are stem cells and self-renew or if mantle cells play a role during hair cell regeneration. Ma and Raible observed that cells located centrally within neuromasts show increased incorporation of BrdU after hair cell death (Ma et al., 2008). Similar results were seen in regenerating axolotl neuromasts using time-lapse analysis (Balak et al., 1990). Our own preliminary data shows that mantle cells do not increase their proliferation rate after hair cell death if inner support cells are still present. However, mantle cells function as progenitors for all cell types when regenerating entire neuromasts in axolotl and likely also in zebrafish (Jones and Corwin, 1993, Dufourcq et al., 2006).

The mirror symmetry of hair cell polarity is re-established during regeneration (Lopez-Schier and Hudspeth, 2006, Mirkovic et al., 2012, Wibowo et al., 2011). Mirror hair cell polarity arises because the two daughter cells of a dividing support cells acquire the opposite polarity as they differentiate into hair cells (Lopez-Schier and Hudspeth, 2006). The formation of a pair of hair cells with opposite polarity thus ensures that neuromasts maintain the same number of mirror-polarized hair cells after regeneration (Lopez-Schier and Hudspeth, 2006). How this mirror-symmetry is achieved is not completely understood. Axonal innervation by the pll_g is not required to establish correct hair cell polarity (Nagiel et al., 2008). Hair cell polarity is abnormal in zebrafish that have mutations in the planar cell polarity pathway member *vangl2* or decreased Notch signaling (Lopez-Schier and Hudspeth, 2006, Wibowo et al., 2011, Mirkovic et al., 2012). The molecular mechanism by which the Notch pathway affects hair cell polarity has not yet been determined. Time-lapse imaging during hair cell regeneration revealed that the majority of hair cell progenitors switch place through reorientation of their cell bodies immediately after division of the inner support cell (Wibowo et al., 2011, Mirkovic et al., 2012). This reorientation of hair cell progenitors is likely involved in setting up the opposing polarity of the two daughter cells, as Notch inhibition or mutations in *vangl2* cause fewer cell reorientations (Mirkovic et al., 2012).

In some regenerative systems the presence of axonal innervation is a prerequisite for normal regeneration (Kumar and Brookes, 2012). However, hair cell regeneration is normal in larval zebrafish that lack a posterior lateral line nerve and associated Schwann cells (Hernandez et al., 2007, Lopez-Schier and Hudspeth, 2006). Interestingly, posterior lateral line neuromasts do not survive in adult zebrafish in the absence of axonal innervation or Schwann cells (Honjo et al., 2011, Lush and Piotrowski, 2014, Wada et al., 2013a). This suggests that the long-term survival or maintenance of neuromast stem cells may depend on signals from axons or Schwann cells.

MOLECULAR ASPECTS OF ZEBRAFISH HAIR CELL REGENERATION

It is not known what signals trigger support cell proliferation in response to hair cell death. The presence of mature hair cells might block proliferation and differentiation of neighboring support cells via cell-cell adhesion or hair cells might express an inhibitory signal. When hair cells are lost due to turn over or damage, such inhibitory signals would disappear enabling support cells to generate new hair cells. Alternatively, dying hair cells may release a factor that induces support cell proliferation and differentiation. This type of behavior, termed apoptosis-induced compensatory proliferation, has been observed in a variety of regeneration paradigms in several species (Bergmann and Steller, 2010). Identifying the molecules required for hair cell regeneration in zebrafish and characterization of the cell types in which they are expressed should shed light on which model is more likely to be correct.

Several approaches have been utilized to discover the genes and signaling pathways required for hair cell regeneration. Candidate gene approaches examine if genes crucial for hair cell development are re-employed and needed for regeneration. Gene expression analyses of regenerating support cells, as well as genetic and chemical screens are unbiased and designed to identify genes previously not implicated in regeneration.

CANDIDATE GENE APPROACH

Studies in a variety of model organisms demonstrate that hair cell development is orchestrated by the interplay of several signaling pathways (Kelley, 2006, Munnamalai and Fekete, 2013, Fritsch et al., 2011, Wu and Kelley, 2012). These include the Notch, canonical and non-canonical Wnt, Fgf, Bmp and Hedgehog pathways. It is assumed that many of the genes and signaling pathways required for hair cell development will also be required during some aspects of regeneration. Two signaling pathways that have been studied during zebrafish hair cell regeneration are the Notch and Wnt/ β -catenin pathways, as well as the transcription factor Sox2 (Head et al., 2013, Jacques et al., 2013, Ma et al., 2008, Wibowo et al., 2011, Millimaki et al., 2010).

Notch signaling during development

Notch signaling serves several functions during hair cell development. During early development, Notch induces prosensory domains through lateral induction, but inhibits hair cell formation later in development through lateral inhibition (Kiernan, 2013, Neves et al., 2013). Mouse mutants in the Notch ligand *Jagged1* show a reduction in the size of the prosensory domain, and overall fewer hair cells (Kiernan et al., 2006, Brooker et al., 2006). In agreement with the *Jagged1* results, early overexpression of an activated form of Notch, NICD, is sufficient to induce ectopic prosensory regions containing hair cells and support cells in mouse and chicken (Hartman et al., 2010, Pan et al., 2010, Daudet and Lewis, 2005). In contrast, mouse mutants for other Notch ligands, such as *Jagged2* or *Delta1*, show increased hair cell formation, indicating a role for Notch signaling in lateral inhibition (Brooker et al., 2006, Lanford et al., 1999, Kiernan et al., 2005a). Similar results were seen after inhibiting Notch signaling with the gamma-secretase inhibitor DAPT at different time points in mouse cochlear cultures. DAPT treatment early, during the establishment of the

prosensory domain, leads to a decrease in hair cell formation, while treatment later, during proneural specification, induces an increase in hair cell production (Hayashi et al., 2008, Munnamalai et al., 2012). The transcription factor *Rbpjk* is thought to be the main effector of Notch signaling (Tanigaki and Honjo, 2010). However, the analysis of conditional mouse mutants for *Rbpjk* revealed prosensory regions still form, but cells within these regions eventually die (Basch et al., 2011, Yamamoto et al., 2011). This suggests that Notch signaling is either only required for the maintenance of prosensory patches and not their induction or that Jagged1 controls prosensory domain induction and size independently of *Rbpjk* (Kiernan, 2013, Okano and Kelley, 2012). As NICD overexpression induces ectopic sensory patches (Daudet and Lewis, 2005, Hartman et al., 2010), it might not act through *Rbpjk*. However, this has not been investigated.

In zebrafish ear and lateral line development, all research to date has only shown a role for Notch signaling in lateral inhibition as zebrafish with mutations that decrease Notch signaling cause the overproduction of ear hair cells at the expense of support cells (Haddon et al., 1998, Haddon et al., 1999, Riley et al., 1999). Likewise, loss of Notch signaling during development of the lateral line leads to disruption of primordium migration and neuromast deposition, as well as increased expression of the hair cell determinant *atoh1* (Itoh and Chitnis, 2001, Matsuda and Chitnis, 2010).

Notch signaling in regeneration

Because of its role in hair and support cell specification, Notch signaling was one of the first signaling pathways to be interrogated during lateral line hair cell regeneration. Notch signaling components, including target genes, are expressed in homeostatic neuromasts suggesting that it could be involved in specifying support cells or inhibiting support cell proliferation in the presence of hair cells (Jiang et al., 2014, Ma et al., 2008, Wibowo et al., 2011). Blocking Notch signaling with DAPT in an uninjured neuromast does not increase support cell proliferation rates nor does it induce the formation of new hair cells (Ma et al., 2008, Wibowo et al., 2011). This suggests that, even though Notch components are expressed in homeostatic neuromasts, Notch is not actively inhibiting hair cell formation in undamaged tissue. Similarly, DAPT treatment did not produce more hair cells in undamaged chicken auditory epithelia (Daudet et al., 2009). A caveat of these experiments is that the DAPT treatments were only performed for two to three days. It is possible that in homeostatic neuromasts not enough hair cells turn over to see a significant shift from support cells to hair cells. Additionally, DAPT treatment is not sufficient to block all Notch signaling in the primordium, therefore some Notch signaling may still be active in DAPT treated neuromasts (Matsuda and Chitnis, 2010).

In contrast to homeostatic neuromasts, blocking Notch signaling after antibiotic treatment produces an excess of hair cells during regeneration in zebrafish (Ma et al., 2008, Wibowo et al., 2011). In accord with these findings, neuromast hair cell regeneration is inhibited by overexpression of the intracellular domain of Notch (NICD), which constitutively activates Notch (Wibowo et al., 2011). Combined these experiments illustrate that Notch signaling is involved in lateral inhibition during regeneration as it is during development, acting to maintain the correct number of support and hair cells (Ma et al., 2008, Wibowo et al., 2011).

The majority of hair cells that formed during DAPT treatment were BrdU positive, suggesting that excess hair cell formation seen after Notch inhibition is not due to transdifferentiation of support cells, but an increase in their proliferation rate (Ma et al., 2008). Because downregulation of Notch signaling during homeostasis does not trigger hair cell formation (Ma et al., 2008, Wibowo et al., 2011), an additional mechanism that is activated by hair cell loss must trigger the regenerative response upstream or in parallel to inhibition of Notch signaling. The identity of the hair cell death-induced signal is currently unknown.

Notch inhibition with DAPT also leads to increased hair cell numbers during regeneration in the adult chicken auditory epithelium and mouse utricle in vitro and induces the regeneration of functional hair cells in the adult mouse cochlea (Mizutari et al., 2013, Daudet et al., 2009, Lin et al., 2011). However, the increased hair cell regeneration seen after downregulation of Notch signaling in both chicken and mouse occurs via direct transdifferentiation of support cells in the absence of proliferation (Daudet et al., 2009, Mizutari et al., 2013). As such, even though blocking Notch activity could be a universal and promising mechanism for inducing hair cell formation after injury, it has to be combined with manipulations that allow support cells to re-enter the cell cycle, such that they self-renew but also generate new hair cells. Therefore studies have focused on the identification of injury-induced mitogenic signals (Burns and Corwin, 2013, Corwin and Oberholtzer, 1997, Schimmang and Pirvola, 2013). An additional caveat of Notch inhibition is that defects in hair cell polarity are observed in developing and regenerating zebrafish (Mirkovic et al., 2012, Wibowo et al., 2011). Hair cell polarity has not been characterized in the mouse or chicken after Notch downregulation (Mizutari et al., 2013, Daudet et al., 2009), but it would be very interesting to test if Notch signaling is also required for the establishment of hair cell polarity during regeneration in these other model systems. If downregulation of Notch signaling indeed results in polarity defects of regenerating hair cells, then manipulation of Notch signaling might not be the best avenue to regenerate a functional sensory epithelium in mammals.

Wnt/ β -catenin signaling during development

Wnt/ β -catenin signaling regulates stem cell proliferation and differentiation of multiple tissues (Clevers and Nusse, 2012), including aspects of ear development, such as early axis formation, otic induction and hair cell polarity (Groves and Fekete, 2012, Munnamalai and Fekete, 2013). Additionally, increased Wnt/ β -catenin signaling increases support cell proliferation and hair cell production in the mouse ear both in vitro and in vivo (Chai et al., 2012, Jacques et al., 2012b, Jan et al., 2013, Shi et al., 2013, Shi et al., 2012). Likewise, during early lateral line development, Wnt/ β -catenin signaling regulates proliferation of neuromast progenitors within the primordium and interneuromast cells (Aman et al., 2011, Lush and Piotrowski, 2014).

Wnt/ β -catenin signaling in regeneration

The various roles of Wnt signaling during development have made it an attractive pathway to study during lateral line hair cell regeneration. Activation of Wnt/ β -catenin signaling, via chemically induced Gsk-3 inhibition, leads to increased proliferation within both homeostatic and regenerating neuromasts and increased hair cell production (Head et al.,

2013, Jacques et al., 2013). Increased neuromast size and elevated *atoh1* expression are also observed in zebrafish mutant for *apc*, a negative regulator of Wnt/ β -catenin signaling (Wada et al., 2013b).

Importantly, overexpression of the secreted Wnt/ β -catenin inhibitor *dkk1b* decreases proliferation in developing and regenerating neuromasts, suggesting that Wnt/ β -catenin signaling is indeed required for regeneration (Head et al., 2013). *dkk1b* itself is not expressed in neuromasts. However, another secreted Wnt/ β -catenin signaling inhibitor, *dkk2*, is expressed in maturing neuromasts and is involved in negatively regulating neuromast size and hair cell numbers (Wada et al., 2013b). The expression of *dkk2* is inhibited in mature neuromasts after antibiotic-induced hair cell death, which supports a model where the down regulation of *dkk2* after hair cell loss allows for Wnt/ β -catenin dependent support cell proliferation and differentiation (Wada et al., 2013b). As this work was performed in young larvae, while neuromasts were still developing, it would be interesting to test if this same pathway functions in mature, homeostatic neuromasts.

Two mechanisms seem to exist by which constitutively active Wnt/ β -catenin signaling leads to increased hair cell production. Developmental studies in mouse, chicken and zebrafish conclude that Wnt/ β -catenin signaling increases support cell proliferation (Head et al., 2013, Jacques et al., 2013, Jacques et al., 2012b). Jacques *et al.* also demonstrated that Wnt/ β -catenin signaling plays an additional later role in hair cell differentiation by upregulating *Atoh1* and the prosensory determinant *Sox2* (Jacques et al., 2012b).

In the lateral line primordium, Wnt/ β -catenin signaling induces Fgf signaling, which is required for *notch3*, *deltaA* and *atoh1a* expression (Aman and Piotrowski, 2008, Nechiporuk and Raible, 2008). Therefore, it is likely that in mature neuromasts and during hair cell regeneration Wnt/ β -catenin signaling affects the number of differentiated hair cells via upregulation of these genes, as well as by upregulating proliferation. Interestingly, even though Wnt/ β -catenin signaling clearly influences the number of regenerating hair cells it is not involved in triggering regeneration (discussed below).

As downregulation of Notch signaling alone in the mouse leads to transdifferentiation of support cells into hair cells, thereby leading to support cell depletion, it would be interesting to test if simultaneous upregulation of the Wnt/ β -catenin pathway would induce support cell proliferation and increase hair cells production by upregulation of *sox2* and *atoh1*. To better understand how the Wnt/ β -catenin pathway interacts with other signaling pathways during mouse development and zebrafish ear and lateral line hair cell regeneration, temporal expression profiles of hair cell regulatory genes such as *Atoh1*, Notch and Fgf pathways after Wnt/ β -catenin perturbation need to be performed.

Sox2, a member of the SRY-related HMG-box (Sox) family of transcription factors

Studies of mutant mice in which Sox2 expression is lost in the inner ear revealed a lack of hair cells (Kiernan et al., 2005b). Similarly, mutations in *Sox2* lead to hearing loss in humans (Hagstrom et al., 2005). Since these original phenotypic descriptions, a variety of studies have demonstrated that Sox2, like Notch signaling, serves several functions during hair cell development (Munnamalai and Fekete, 2013, Dabdoub et al., 2008).

Overexpression of Sox2 induces ectopic sensory regions in the chicken and mouse sensory epithelia (Neves et al., 2011, Pan et al., 2013a), suggesting that Sox2 is both necessary and sufficient for prosensory domain formation. Sox2 also plays a role during hair cell specification as it directly activates the expression of *Atoh1* in hair cells, which is important for their differentiation. However, the upregulation of *Atoh1* is transient, as *Atoh1* expression is not maintained after long-term overexpression of Sox2 (Neves et al., 2012, Ahmed et al., 2012). The eventual loss of *Atoh1* could be due to the fact that *Atoh1* and Sox2 antagonize each other, as is the case during development. Accordingly, after *Atoh1* activation, Sox2 becomes restricted to support cells where it inhibits hair cell differentiation and maintains support cells (Dabdoub et al., 2008). In summary, like Notch signaling, Sox2 has a prosensory function early in development but inhibits hair cell formation at later stages (Neves et al., 2013, Okano and Kelley, 2012, Munnamalai and Fekete, 2013). This similarity in function is likely a reflection of the dependence of Sox2 expression on Notch signaling (Dabdoub et al., 2008, Neves et al., 2011).

Sox2 hypomorphic mice, in opposition to complete null mutants, show increased hair cell numbers suggesting that the function of Sox2 is dosage dependent (Dabdoub et al., 2008). Munnamalai and Fekete proposed that during proneural specification, intermediate levels of Sox2 likely specify a prosensory fate, whereas high levels of Sox2 induce and maintain support cells (Dabdoub et al., 2008, Munnamalai and Fekete, 2013)

In zebrafish, *sox2* is expressed in support cells of the ear and lateral line (Hernandez et al., 2007, Millimaki et al., 2010), however the regulation and role of *sox2* has only been investigated in the ear. In the ear *sox2* expression depends on both Fgf and Notch signaling and overexpression of *sox2* induces an increase in hair cell numbers if induced early in development (Millimaki et al., 2010). Sox2 likely increases the size of the prosensory domain in the zebrafish ear, as co-overexpression with *atoh1a* enhances the number of ectopic hair cells when compared to *atoh1a* overexpression alone (Sweet et al., 2011). In contrast to what has been observed in the mouse, morpholino mediated knockdown of *sox2* in zebrafish does not affect initial hair cell development (Millimaki et al., 2010). The absence of an early hair cell phenotype in *sox2*-depleted zebrafish ears could be explained by the presence of other redundant Sox family members acting during hair cell development, such as *sox3* (Padanad and Riley, 2011), which needs to be tested.

Even though initial hair cell formation is unaffected, morpholino-mediated knockdown of *sox2* causes a decrease in the number of later forming hair cells (Millimaki et al., 2010). It is unclear what causes the reduction of hair cell formation after *sox2* knockdown. The expression of *atoh1a* is not affected, but an increase in cell death in support and hair cells occurs after *sox2* knockdown (Millimaki et al., 2010). This suggests that *sox2* might be required for cell survival in the zebrafish ear, however an increase in cell death has not been described in Sox2 mouse mutant ears or in chicken explants overexpressing a dominant negative Sox2 protein (Kiernan et al., 2005b, Neves et al., 2012). As increased cell death is a frequently observed artifact of zebrafish morpholino knockdown experiments (Robu et al., 2007), the generation and characterization of *sox2* mutant zebrafish will be important.

In contrast to initial hair cell development in the zebrafish ear, hair cell formation during regeneration is inhibited by *sox2* knockdown (Millimaki et al., 2010). The regenerative failure could be due to increased cell death (Millimaki et al., 2010), however, no gene expression studies have been performed yet that might help explain why hair cell regeneration fails.

GENETIC AND CHEMICAL SCREENS FOR HAIR CELL REGENERATION GENES

Mutagenesis screens in zebrafish have proven extremely effective in uncovering genes required for embryonic development (Driever et al., 1996, Haffter et al., 1996). Fortunately, screening for mutations that affect hair cell function is also relatively easy to execute. A lack of mechanotransduction in the ear is easily detectable because larvae or adult fish show a characteristic circling swimming behavior (Nicolson et al., 1998). Defects in the function of lateral line hair cells can be readily observed by lack of mechanotransduction-dependent uptake of various fluorescent dyes (Santos et al., 2006). Both of these straightforward techniques have been used to perform large-scale genetic screens to uncover new genes required for ear and lateral line development (Malicki et al., 1996, Nicolson et al., 1998, Whitfield et al., 1996). Since the initial large-scale screens performed in the 1990s, many mutations have been found that affect the development of the ear, lateral line or the mechanotransduction of hair cells (Asai et al., 2006, Kappler et al., 2004, Malicki et al., 1996, Nicolson et al., 1998, Starr et al., 2004, Whitfield et al., 1996). Additionally, several labs have recently embarked on mutagenesis screens for genes required for hair cell regeneration (Piotrowski et al., unpublished, Raible et al., unpublished; (Behra et al., 2009)).

To date, only one mutant, *phoenix*, has been reported with defects in hair cell regeneration (Behra et al., 2009). *phoenix* mutants develop normally, but fail to completely regenerate neuromast hair cells. *phoenix* is expressed in neuromast support cells and possesses a predicted single membrane-spanning domain, however the function of *phoenix* is unknown (Behra et al., 2009). No other vertebrate homologs were found by sequence similarity. Hair cell regeneration likely fails in *phoenix* mutant larvae due to reduced support cell proliferation after induction of hair cell death (Behra et al., 2009). Importantly, this regeneration defect is specific to lateral line hair cells as tail fin regeneration occurs normally in *phoenix* mutant larvae.

The dearth of mutants published thus far is likely a reflection of the extensive follow up work required once a regeneration phenotype is observed. Any mutation that causes a defect in proliferation or compromised health of the larvae could cause a decrease in the number of regenerated hair cells. Additionally, if any genes are required for both hair cell development and regeneration, they will not be detected in this type of screen.

An alternative, and complement, to mutational screens are chemical screens. Compared to genetic screens, chemical screens are less labor intensive, however they also require a significant amount of follow up work to identify which pathways are targeted by the compound (Peterson and Fishman, 2011, Taylor et al., 2010). Chemical screens are relatively easy to perform in zebrafish because chemical compounds can be diluted in the

medium rather than injected. Drugs can be added to the water at any developmental stage, thereby circumventing any embryonic requirement for the pathway that is targeted by the compound. Chemical inhibitors often inhibit all or several homologs of a gene, which may overcome genetic redundancy. Additionally, chemical inhibitors of particular pathways are valuable as they allow one to quickly test the function of this pathway without knowing which pathway members are expressed in the studied organ system. Additionally, once other screens have identified genetic mutations that affect regeneration, chemical screens can be performed for compounds that rescue the regeneration phenotype and that could potentially be used as therapeutic targets to trigger regeneration in mammals in the future.

The rationale of chemical screens during hair cell regeneration in zebrafish are to identify compounds that either prevent or enhance regeneration (Esterberg et al., 2013a). Chemical enhancers of zebrafish hair cell regeneration are good candidate molecules to be tested in non-regenerative systems. Signaling pathways affected by any compounds found to change during regeneration could then be studied genetically to examine how they function during regeneration. One such screen identified both inhibitors and enhancers of hair cell regeneration (Namdaran et al., 2012). All the chemical inhibitors of regeneration inhibited support cell proliferation, again illustrating proliferation is vital for hair cell regeneration in zebrafish (Namdaran et al., 2012). Two chemical enhancers of regeneration are the glucocorticoids dexamethasone and prednisolone, which act in part by increasing proliferation of support cells (Namdaran et al., 2012). Additionally, both chemical and mutagenesis screens are being performed that have identified suppressors of aminoglycoside induced hair cell death, which could allow the continued use of these antibiotics without the loss of hair cells (Coffin et al., 2010, Ou et al., 2012, Ou et al., 2010, Ou et al., 2009, Owens et al., 2008).

TRANSCRIPTOMICS OF ZEBRAFISH HAIR CELL REGENERATION

The studies described above have contributed greatly to our understanding of the function of particular pathways and genes, especially during development. However, they do not reveal how a particular pathway integrates and interacts with all the other pathways that together orchestrate hair cell development and regeneration.

Several laboratories have undertaken gene discovery approaches, such as RNA-Seq, to uncover genes and pathways whose expression changes during the course of hair cell regeneration in zebrafish (Jiang et al., 2014, Liang et al., 2012, Schuck et al., 2011, Steiner et al., 2014). Such experiments generate large amounts of data, creating the challenge of deciding which genes to functionally test. Bioinformatics tools, such as Gene Ontology analysis (GO term) and pathway analysis software are used to group genes into signaling pathways that may be functionally important. These types of analyses have been performed on noise-damaged sensory epithelia in zebrafish ears (Liang et al., 2012, Schuck et al., 2011), and support cells from chemically damaged lateral line neuromasts (Jiang et al., 2014, Steiner et al., 2014).

In the zebrafish ear, *growth hormone 1* (GH) expression is greatly increased two days after noise-induced damage (Schuck et al., 2011). Functional tests revealed that addition of

recombinant GH immediately after noise-induced damage decreased apoptosis and increased both proliferation and hair cell regeneration in vivo, while blocking GH function decreased proliferation in zebrafish ears (Schuck et al., 2011, Sun et al., 2011). By RNA-seq analysis, *growth hormone 1* expression was not changed in neuromast support cells within five hours after hair cell loss (Jiang et al., 2014, Steiner et al., 2014), suggesting the role of GH might be specific to the ear.

A second study of regenerating ear sensory epithelia and lateral line hair cells described an inhibitory role for the Jak/Stat pathway (Liang et al., 2012). Immediately after hair cell death *jak1*, *stat3* and *socs3a* and *socs3b* are upregulated in the ear and lateral line neuromasts (Jiang et al., 2014, Liang et al., 2012, Steiner et al., 2014). Stat3 and Socs3 act in a negative feedback loop in which Stat3 induces *socs3*, which then inhibits Stat3 activation (Carow and Rottenberg, 2014, Liang et al., 2012). *Stat3/Socs3* is a commonly induced pathway after injury, as its induction has also been observed during early heart and retina regeneration in zebrafish (Elsaeidi et al., 2014, Fang et al., 2013). The generation of a transgenic line that allows inducible dominant-negative Stat3 (dnStat3) expression revealed that Stat3 activity is required for regeneration of the zebrafish heart (Fang et al., 2013). In contrast, pharmacological inhibition of Stat3 signaling leads to faster hair cell regeneration in the lateral line (Liang et al., 2012). Pharmacological inhibition of Stat3 also caused the downregulation of *socs3a* (Liang et al., 2012). As Socs3 is known to limit regeneration in some systems (Elsaeidi et al., 2014, Smith et al., 2009), the decrease in *socs3a* after Stat3 inhibition could be what is allowing for the faster hair cell regeneration (Liang et al., 2012). However, as pharmacological inhibitors could have as yet unknown functions, hair cell regeneration should be tested in the dnStat3 transgenic line.

Two groups performed transcriptomic analysis of support and mantle cells during lateral line regeneration (Jiang et al., 2014, Steiner et al., 2014). To specifically examine gene expression changes within the support cell populations both groups isolated GFP-positive lateral line support cells by fluorescent activated cell sorting (FACS) from transgenic lines. Jiang *et al.*, isolated both inner support cells and mantle cells from *Tg(SqET20:gfp)* larvae before and after neomycin treatment (Jiang et al., 2014). Steiner *et al.*, generated a transgenic line using the alkaline phosphatase promoter, *Tg(-4.7alpl:mCherry)*, which labels the mantle cell population only, and isolated expressing cells before and after copper treatment (Steiner et al., 2014). The mantle cell population is interesting because, as described above, they function as stem cells during sensory organ formation in regenerating tail in axolotl and likely zebrafish (Jones and Corwin, 1993, Dufourcq et al., 2006). However, in contrast to inner support cells, mantle cells do not proliferate significantly after hair cell death and thus do not give rise to hair cells (Andres Romero-Carvajal and Tatjana Piotrowski, unpublished observations). It is possible that mantle cells are only activated after depletion of most inner support cells or, alternatively, mantle cells play a different role during whole neuromast versus hair cell regeneration. Mantle cells might also signal to inner support cells, as changes in gene expression were observed within the mantle cell population during regeneration (Steiner et al., 2014).

In addition to isolating cells from different transgenic lines, the two groups analyzed the results differently. Steiner *et al.* focused on the most highly up- and downregulated genes to

identify novel pathways, whereas Jiang *et al.* focused on characterizing the behavior of all signaling pathways known to be crucial for hair cell development (Jiang et al., 2014, Steiner et al., 2014). The rationale behind this latter approach is the assumption that many of these developmental pathways will be re-employed during regeneration. For example, as described above, manipulation of the Notch or Wnt/ β -catenin pathways leads to extra hair cell formation in mouse, chicken and zebrafish. Unfortunately, even though these results are promising, these experiments have not lead to the restoration of a fully functional epithelium in mammals. One of the reasons for the limited success of these studies is that the precise timing of when these pathways are activated or inhibited and how these pathways interact in regenerating species is still unknown. In addition, Notch inhibition in the mouse produced hair cells that were generated via transdifferentiation of support cells, which may lead to their eventual depletion. Therefore, it is crucial to identify the factor(s) that trigger support cell proliferation directly after hair cell death.

Jiang *et al.* determined the activation status of signaling pathways at 1, 3 and 5 hours post hair cell death. Interestingly, the Notch pathway is downregulated 1 hour after hair cell death and re-activated at 3 hours (Jiang et al., 2014). A sampling of different signaling pathway members whose expression changes is shown in Figure 2G. Importantly, during chicken utricle hair cell regeneration, a transient decrease in Notch target genes *HES5* and *HES7* has also been observed (Ku et al., 2014). The function of the immediate but transient downregulation of Notch signaling in both chicken and zebrafish is unclear. Possibly, a transient down regulation of Notch signaling could contribute to the immediate increase in *atoh1a* expression after injury (Jiang et al., 2014, Ma et al., 2008, Wibowo et al., 2011). The reactivation of Notch signaling at 3 hours post injury likely functions to limit the number of hair cells regenerated and to set up proper hair cell polarities in the lateral line (Jiang et al., 2014, Ma et al., 2008, Mirkovic et al., 2012, Wibowo et al., 2011).

Notch signaling is not the only pathway that limits hair cell differentiation. Cyclin dependent kinase inhibitors block support and hair cell proliferation after hair cell differentiation in the mouse Organ of Corti (Kwan et al., 2009, Schimmang and Pirvola, 2013). Mouse mutants of the cyclin dependent kinase inhibitors p27^{Kip1}, retinoblastoma (pRb) or p19Ink4d show increased support cell proliferation leading to increased hair cell numbers, but the hair cells eventually die and do not contribute to a functional epithelium (Chen and Segil, 1999, Chen et al., 2003, Lowenheim et al., 1999, Sage et al., 2005). Characterizing the timing of cell cycle inhibitor expression during zebrafish regeneration is important, as it might provide clues to which signaling pathways or factors show similar temporal expression dynamics, and could therefore be involved in downregulating the cell cycle inhibitors and activating proliferation. In zebrafish, the cell cycle inhibitors *cdkn1b* (p27/kip1), *cdkn1c* (p57/kip2) and *rbl2* (retinoblastoma-like 2) are downregulated between one to three hours post hair cell death (Fig. 2G) (Jiang et al., 2014). The functions of these cell cycle inhibitors have not been studied in zebrafish hair cell development or regeneration, but they are likely downregulated to allow support cell proliferation to occur. The characterization of hair cell development and regeneration in the recently identified *rbl* (pRb) zebrafish mutant will reveal if cyclin dependent kinase inhibitors play a similar role in controlling proliferation in the zebrafish ear and lateral line as in the mammalian inner ear

(Gyda et al., 2012). Although proliferation occurs as part of normal regeneration in fish, avians and axolotls, as described above, an increase in proliferation is not observed after Notch inhibition in regenerating mouse or chicken sensory epithelia (Daudet et al., 2009, Mizutari et al., 2013), suggesting cell cycle inhibitors are still expressed. Possibly, temporarily blocking both cell cycle inhibitors and Notch signaling will enable enhanced regeneration in mammals.

Fgf signaling is also required for multiple aspects of ear development (Wright and Mansour, 2003, Munnamalai and Fekete, 2013). During neuromast hair cell regeneration, Fgf pathway members show a similar expression pattern to that of Notch pathway members with transient downregulation followed by a more prolonged recovery starting at 5 hours post injury (Jiang et al., 2014). The regulation and function of the Fgf pathway is relatively well understood in the lateral line primordium, however its role in mature, deposited neuromasts has not been examined. In the lateral line primordium, Fgf signaling is required for Notch signaling activation, and Notch signaling feeds back to enhance Fgf signaling (Matsuda and Chitnis, 2010, Nechiporuk and Raible, 2008). Whether Fgf and Notch signaling also interact during hair cell regeneration has not been tested. Fgf signaling is also required for proper development of support cell types in the mouse cochlea and atypical Fgf ligand and receptor interaction can change support cell fate in the mouse cochlea (Mansour et al., 2013, Mueller et al., 2002). Additionally, during zebrafish primordium migration, Fgf signaling is required for proliferation (Aman and Piotrowski, 2011). Therefore Fgf signaling could be functioning to maintain support cell types within neuromasts and/or regulate their proliferation.

Interestingly, in the chicken, *FGF20* is downregulated immediately after hair cell death and when added exogenously inhibits support cell proliferation during hair cell regeneration, however regeneration of hair cells was not examined (Ku et al., 2014). Likewise, addition of Fgf2, but not Fgf1, inhibited proliferation in young chicken cochlea and vestibular cultures (Oesterle et al., 2000). The Fgf receptor *Fgfr3* is expressed in support cells of the chicken cochlea, and is down regulated after hair cell death (Bermingham-McDonogh et al., 2001, Ku et al., 2014). Pharmacological inhibition of Fgfr activity during development of cultured chicken cochlea leads to increased hair cell numbers, however, this activity was not due to increased proliferation, but transdifferentiation of support cells into hair cells (Jacques et al., 2012a). These results in chicken suggest that Fgf signaling is actively inhibiting proliferation or differentiation during homeostasis and that it needs to be downregulated for hair cell regeneration to occur. As proliferation rates strongly increase in zebrafish after hair cell death, Fgf signaling, separately or in collaboration with Notch, could also function to inhibit proliferation during neuromast homeostasis. Thus, downregulation of these two pathways in neuromasts could be responsible for triggering proliferation.

Transcriptional profiling of regenerating chicken cochlear sensory epithelia revealed that multiple Fgf ligands, in addition to *FGF20*, are expressed (Ku et al., 2014). As different Fgf ligands and receptors can have different activities (Belov and Mohammadi, 2013, Mason, 2007, Turner and Grose, 2010), global loss of Fgf signaling through pharmacological inhibition may not reveal the complete picture of Fgf signaling during hair cell regeneration. Loss of function studies of individual Fgf ligands or receptors would therefore be more physiologically relevant. For example, blocking all Fgfr signaling pharmacologically does

not increase support cell proliferation during chicken sensory regeneration, even though addition of Fgf20 inhibits proliferation (Ku et al., 2014).

As mentioned above, Wnt/ β -catenin signaling is crucial for the regeneration of many organ systems and controls proliferation during regeneration of lateral line neuromasts and the vertebrate ear (Head et al., 2013, Jacques et al., 2013). Jiang *et al.* reported that Wnt/ β -catenin signaling is absent from homeostatic neuromasts and is not activated during the first 10 hours of regeneration, even though support cell proliferation already happens during that time and the first hair cells have begun to differentiate (Fig. 2G) (Jiang et al., 2014). This result demonstrates that Wnt/ β -catenin signaling cannot be involved in triggering the initial phase of proliferation. The inhibition of neuromast proliferation seen after over expression of the Wnt/ β -catenin inhibitor *dkk1b* (Head et al., 2013), may be due to an effect on a later phase of proliferation. Inhibiting Wnt/ β -catenin signaling at different time points after hair cell death should reveal when it is required for proliferation.

Additional signaling pathways that are modulated after hair cell death include BMP, Fat protocadherin family proteins, insulin, TNF- α , apoptosis pathways, nitric oxide and reactive oxygen species signaling (Jiang et al., 2014, Steiner et al., 2014). Downregulation of *fat2* in mantle cells is interesting as the Fat family of proteins are involved in planar cell polarity signaling and negative regulation of cell proliferation, both of which are important for development and regeneration (Reddy and Irvine, 2008, Steiner et al., 2014, Thomas and Strutt, 2012). However, as yet, functional data is lacking for most of the genes identified in these transcriptomic analyses.

One caveat of these gene expression comparisons is that the analyses only focused on support cell populations. Therefore, these studies do not reveal possible signals from hair to support cells or other factors that influence support cells non-cell-autonomously. For instance, there is robust, but transient, immune cell migration towards the lateral line after copper or neomycin induced hair cell death (d'Alencon et al., 2010). Generally, inflammation induced by the immune response is detrimental to homeostatic tissue and regeneration (David and Kroner, 2011). However, in the adult zebrafish brain the immune response is necessary for regeneration (Kyritsis et al., 2012). During tail regeneration in axolotl increased immune cell migration towards neuromasts near the wound has also been observed (Jones and Corwin, 1993). Increased macrophage migration into chicken cochlea after antibiotic induced damage has also been described, but no effect on hair cell regeneration or clearance of dead hair cells was seen in vitro after depletion of macrophages (Warchol et al., 2012). Likewise, glucocorticoids, such as dexamethasone and prednisolone inhibit the immune cell migration to the injured lateral line but increase neuromast hair cell regeneration (d'Alencon et al., 2010, Namdaran et al., 2012). Therefore, one could speculate that the immune response is not important for hair cell regeneration and may, in fact, be detrimental.

The comparative temporal analysis of the activation statuses of several signaling pathways by Jiang et al. provides an important framework for when to manipulate each pathway in mammals that will hopefully aid in the restoration of a functional sensory epithelium (Jiang et al., 2014). Based on this analysis and on published results in various model organisms,

one may want to transiently inhibit Notch signaling to induce transdifferentiation of support cells into hair cells, followed by a transient Wnt/ β -catenin activation, or inhibition of cyclin dependent kinase inhibitors, to replenish support cells. The zebrafish is a great model system to perform and test such combinatorial pathway manipulations. Results from these experiments will then aid in determining which combinations of pathways to manipulate in the mouse where such experiments are more labor-intensive and more costly.

FUTURE STUDIES

As mentioned above, transcriptome analyses are powerful tools to identify gene expression changes after hair cell death. However, the amount of data that is generated is daunting and genes need to be prioritized for further analysis, as functional studies are time consuming. One strategy to identify candidate genes would be to compare gene lists generated in the zebrafish to those recently generated in chicken and mouse models (Hawkins et al., 2003, Hawkins et al., 2007, Ku et al., 2014, Sinkkonen et al., 2011). Genes or pathways that are similarly regulated during hair cell regeneration in these different model organisms likely play important roles and should be functionally analyzed. Likewise, comparative in situ hybridization analyses between the different species with these candidate genes might help to homologize support cell types between zebrafish, chicken and mice. Results from such studies will hopefully provide clues into which mammalian support cells might be the most amenable to regenerate hair cells after experimental manipulation.

The fact that prenatal mice still possess the ability to regenerate a small number of hair cells, but lose this ability as they mature can be exploited to identify genes that change between these two time points. If these genes are similarly regulated during regeneration in the chicken or zebrafish, they could play an important role in hair cell regeneration and are prime candidates for functional analyses.

Even though transcriptome studies of support cells are powerful tools to identify genetic pathways in support cells that respond to hair cell death, these studies are not suitable for identifying the extrinsic signals that activate support cell proliferation. Uninjured hair cells could provide a signal that inhibits support cell proliferation or dying hair cells could release a signal that induces support cell proliferation. Therefore, comparisons of transcriptomes between hair and support cells during homeostasis or regeneration will be informative. Candidate proteins are those secreted by hair cells or are membrane-bound and whose target genes demonstrate expression changes within support cells after hair cell death.

Non-coding microRNAs (miRNA) are also candidates for triggering hair cell regeneration. As single miRNAs can regulate multiple genes in different signaling pathways, their activation or inhibition could quickly affect cell behavior, and therefore could play an important role during hair cell regeneration. Indeed, miRNAs are required for ear development in mouse and zebrafish and mutations in miRNAs have been associated with hearing loss in humans (Groves et al., 2013, Ushakov et al., 2013, Li et al., 2010, Li and Fekete, 2010, Mencia et al., 2009, Lewis et al., 2009). However, as yet, none of the gene expression analyses in zebrafish has searched for changes in miRNA expression after hair

cell death, as different methods are needed to detect transcriptional changes of these short RNAs.

Testing gene function in juvenile or adult zebrafish is not trivial, but important technical advances have been recently made. Because hair cell regeneration is studied in larval zebrafish, some of the gene knockdown techniques used to interrogate developmental processes in the early embryo are not suitable. For example, injected anti-sense morpholinos that inhibit translation or splicing are degraded over time and are no longer effective in 5–7 day old larvae, the time points typically used in neuromast regeneration studies. Electroporation of morpholinos is a possibility that has been successfully used during adult fin regeneration (Hyde et al., 2012, Thummel et al., 2006). However, an important caveat of morpholinos is they can have off target effects that induce p53-mediated cell death (Gerety and Wilkinson, 2011, Robu et al., 2007). For example, *tcf7*-morpholino toxicity causes increased cell death within the migrating lateral line primordium leading to decreased neuromast deposition that could be partially rescued by co-injection with a *p53* morpholino (Aman et al., 2011). Importantly, zebrafish mutant for *tcf7* do not show a lateral line phenotype, indicating the *tcf7*-morpholino phenotype was non-specific (Aman et al., 2011). Likewise, *epcam/tacstd*-morpholino injected embryos showed reduced neuromast deposition that was not recapitulated in the genetic mutant (Slanchev et al., 2009, Villablanca et al., 2006). Morpholino experiments therefore have to be properly controlled (Bill et al., 2009, Eisen and Smith, 2008), and should ideally be followed or substituted by the generation and analysis of mutants in the gene of interest. The use of new genome editing techniques, such as TALENs and CRISPRs, will catapult the zebrafish hair cell regeneration field forward as they can be used to produce genomic insertions, allowing the generation of conditional mutations that use the Cre/lox system to bypass any developmental requirements (Blackburn et al., 2013, Jungke et al., 2013, Bedell et al., 2012).

The ability to image regeneration in vivo is one of the most important strengths of zebrafish. Fluorescent reporters for various signaling pathways reveal in real time how gene expression changes occur in relation to cellular behaviors such as proliferation or differentiation. Transgenic reporters for Wnt/ β -catenin, Notch, Bmp, Fgf and the cell cycle have already been generated (Sugiyama et al., 2009, Laux et al., 2011, Molina et al., 2007, Dorsky et al., 2002, Moro et al., 2012, Parsons et al., 2009, Shimizu et al., 2012). However not all reporters express well in the lateral line and should be remade. The analysis of cell behavior is facilitated by fusion of fluorescent proteins to various targeting domains that either target fluorescent proteins to nuclei allowing for easier visualization of mitotic cells or to the membrane to label cellular protrusions. Transgenic lines that label the post-synaptic side of hair cell synapses can also be used to study synapse formation (Sheets et al., 2011). These new reporters will not only be useful for time-lapse analysis but also for cell-sorting and gene expression analysis of subpopulations of cells within neuromasts.

More detailed lineage analyses will be important to determine the relative contributions of the different support cell populations to hair cell regeneration. Generation of the Cre/lox system and Cre reporter lines that drive expression in the ear and lateral line will be useful for these lineage tracing experiments. Additionally, clonal analysis using the Zebrafish (Zebrafish Brainbow) technology could be used to investigate lineage relationships within

neuromasts (Pan et al., 2013b). We have recently tested a Zebrafish transgenic system and have been able to identify individual clones within mature neuromasts (Fig. 2D, Masataka Nikaido and Tatjana Piotrowski unpublished observation).

CONCLUSIONS

Zebrafish maintain a long-lived pool of stem cells capable of producing new support cells and hair cells during homeostasis and after trauma. Hair cell regeneration is robust and occurs in both the ear and the mechanosensory lateral line, both of which are experimentally accessible and genetically tractable. Combined, the knowledge we are gaining from regeneration studies in the zebrafish and other regenerating species, such as chickens, will identify common mechanisms of hair cell regeneration that can be exploited to induce regeneration in mammalian inner ears and ultimately improve hearing in people.

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Figure 1. Anatomy of the lateral line

(A) 5 day old zebrafish larvae stained with alkaline phosphatase to label the anterior and posterior lateral line system. (B) Higher magnification view of alkaline phosphatase positive posterior lateral line neuromasts and interneuromast cells (arrows). (C) Scanning electron micrograph of a posterior lateral line neuromast with stereocilia and longer kinocilia pseudocolored in yellow. (D) Transmission electron micrograph of a posterior lateral line neuromast with mantle cells pseudocolored in blue and hair cells pseudocolored in yellow. Inner support cells are unlabeled. (E–G) Visualization of hair cell regeneration in zebrafish.

(E) 5 day old zebrafish with neuromasts labeled by DASPEI (yellow) uptake by hair cells.
(F) Two hours after neomycin treatment no hair cells can be labeled with DASPEI. (G) Two days after neomycin treatment hair cells have regenerated.

Figure 2. Transgenic reporters and gene expression changes after hair cell death

(A–E) Various transgenic reporters used in lateral line research. (A) *Tg(cldnb:lyngfp)* labels all cells of the neuromast. (B) *Tg(SqET20:gfp)* labels mantle cells and some inner support cells. (C) *Tg(SqET4:gfp)* is expressed in immature and mature hair cells. (D) HGn39D labels pIIg axons that innervate neuromasts. (E) Zebrawow expression in mature neuromasts. (F) Still images of hair cell regeneration demonstrated using double *Tg(SqET20:gfp)* and *Tg(SqET4:gfp)* expressing fish. *Tg(SqET4:gfp)* positive hair cells are surrounded by a ring of *Tg(SqET20:gfp)* positive mantle cells. The majority of hair cells are gone 1 hour after

neomycin treatment. Hair cells remaining at 1 hour (arrows) were likely too immature to respond to neomycin. Newly regenerated hair cells can be seen starting at 5 hours post neomycin treatment (arrowheads). (G) Gene expression analysis after neomycin treatment illustrates changes in expression of various signaling pathways. Images from panels F and G are reproduced with permission from Jiang, *et al.* (Jiang et al., 2014).