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Hair Cell Fate Decisions in Cochlear Development and Regeneration

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Abstract

The discovery of avian cochlear hair cell regeneration in the late 1980's and the concurrent development of new techniques in molecular and developmental biology generated a renewed interest in understanding the genetic mechanisms that regulate hair cell development in the embryonic avian and mammalian cochlea and regeneration in the mature avian cochlea. Research from many labs has demonstrated that the development of the inner ear utilizes a complex series of genetic signals and pathways to generate the endorgans, specify cell identities, and establish innervation patterns found in the inner ear. Recent studies have shown that the Notch signaling pathway, the Atoh1/Hes signaling cascade, the stem cell marker Sox2, and some of the unconventional myosin motor proteins are utilized to regulate distinct steps in inner ear development. While many of the individual genes involved in these pathways have been identified from studies of mutant and knockout mouse cochleae, the interplay of all these signals into a single systemic program that directs this process needs to be explored. We need to know not only what genes are involved, but understand how their gene products interact with one another in a structural and temporal framework to guide hair cell and supporting cell differentiation and maturation.

Keywords

Cochlea; Hair Cell; Supporting Cell; Genetic Regulation; Notch Pathway; Development; Regeneration

Hearing impairment affects almost 49 million people in the US and over 249 million worldwide. Approximately 17% of children under age 18 have a hearing loss and the incidence increases with age: roughly 30% of people over 65 and over 90% of people over 80 have a substantial hearing loss. Although rarely life-threatening, hearing loss affects more people than epilepsy, multiple sclerosis, spinal injury, stroke, Huntington's and Parkinson's diseases combined (Hudspeth, 1997) and has a huge financial impact on our economy and lifestyle. Hearing impairment is mainly caused by damage to the hair cells, the sensory cells in the cochlea (Figure 1). In mammals, the absence of these cells results in permanent hearing loss

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because they are generated only during embryonic development (Ruben, 1967) and must last throughout a lifetime.

In birds, however, this is not an issue, as they have the ability to regenerate cochlear hair cells throughout their lifetime (Cotanche, 1987; Cruz et al., 1987; Corwin & Cotanche, 1988; Ryals & Rubel, 1988; Lippe et al., 1991). This is not merely a delayed developmental response, as six-year-old quails (which is three years beyond their average lifespan) can regenerate hair cells as readily as newborn chicks (Ryals & Rubel, 1988). If this regeneration could be induced in the mammalian cochlea, it would offer a potential therapeutic treatment for sensorineural hearing loss in humans. Based on the recent clinical successes of the electronic cochlear implant, the goal of current researchin this area is to utilize regeneration to develop a “biological cochlear implant” to restore hearing function in the human cochlea. Thus, understanding the mechanisms that regulate avian hair cell regeneration and how this correlates with mammalian hair cell development, will be important for pursuing strategies to induce mammalian hair cell regeneration. These strategies can be approached either through the manipulation of existing, intrinsic cochlear cells to become replacement hair cells or through the engineering of stem cells for transplantation and differentiation within a damaged mammalian cochlea to make new hair cells.

There is significant evidence from the developmental literature that the Notch signaling pathway plays two key roles in cochlear development. The first of these occurs early in otocyst development and involves Notch1 and Jagged1 in defining the presumptive sensory epithelium through a lateral induction process (Daudet & Lewis, 2005; Kiernan et al., 2006; Daudet et. al., 2007; Hayashi et al., 2008). The second mechanism occurs later in cochlear development and is involved in determining hair cell and supporting cell fates through a lateral inhibition receptor/ligand interaction (Adam et al., 1998; Lanford et al., 1999; Morrison et al., 1999; Kiernan et al., 2005b). It has been proposed by several groups that the Notch lateral inhibition pathway initiates hair cell differentiation. However, detailed analyses of the published data indicate that the initial differentiation of hair cells is independent of the Notch lateral inhibition pathway. However, once the initial four rows of cochlear hair cells differentiate, the Notch pathway is apparently utilized to keep other progenitor cells from differentiating as supernumerary hair cells. Subsequently, if some of those initial four rows of hair cells are damaged or lost during the late embryonic or early post-natal periods, the dynamics of the Notch pathway are altered so that the progenitor cells formerly prohibited from differentiating into hair cells can now do so. Thus, the Notch pathway does appear to play a critical role in limiting the hair cell population during development. During hair cell regeneration in the bird cochlea, the Notch pathway is recruited once again to limit hair cell fates in the re-establishment of the hair cell and supporting cell mosaic in the sensory epithelium (Stone & Rubel, 1999; Daudet et al.,2009). Moreover, if regeneration can be manipulated to help generate new hair cells in the mature mammalian cochlea, then these cells will also need to express the Notch pathway genes at the appropriate time and place.

Hair Cell Regeneration in the Avian Cochlea

Hair cell regeneration in the mature avian cochlea is a response to damage of the existing sensory cells caused by sound exposure, aminoglycoside treatment, laser ablation, or genetic mutation (Cotanche, 1987; Cruz et al., 1987; Corwin & Cotanche, 1988; Ryals & Rubel, 1988; Lippe et al., 1991; Warchol & Corwin, 1996; Gleich et al., 1997; reviewed in Stone & Cotanche, 2007; Cotanche, 2008). The loss of hair cells induces non-sensory supporting cells to undergo either direct transdifferentiation, where a supporting cell changes its gene expression to become a hair cell without dividing, or mitotic proliferation, where a supporting cell divides to produce two progenitor cells, in order to generate replacement hair cells. This results in a nearly complete structural and functional recovery of the cochlea. Yet, the

regenerative response is limited in both time and quantity, so that the number of new cells produced is just enough to replace the number of hair cells lost, as well as replacing the supporting cells utilized in direct transdifferentiation and mitotic proliferation (Stone & Cotanche, 1994; Bhave et al., 1995). Hair cell differentiation through supporting cell transdifferentiation and proliferation occurs either concurrent with or directly after hair cell death and ejection from the sensory epithelium (Corwin et al., 1991; Stone & Cotanche, 1994; Bhave et al., 1995; Stone et al., 1999; Mangiardi et al., 2004; Duncan et al., 2006). Our recent work has focused on demonstrating that the dying hair cells in the gentamicin-treated chick cochlea regulate the onset and progression of supporting cells through both direct transdifferentiation and mitotic proliferation. Evidence for this is seen in the regenerating chick utricle, where blocking hair cell death with apoptotic inhibitors rescues these cells, but also reduces the level of ongoing supporting cell mitoses needed to generate replacement cells (Matsui et al., 2002). Moreover, the number of dying hair cells will specifically regulate the choice of direct transdifferentiation or mitotic proliferation as a means for producing new hair cells (Stone & Cotanche, 1994; Cotanche, 2008). Identifying the genes and proteins involved in the regulation of regeneration will enable us to experimentally induce new hair cell production and potentially harness this process for therapeutic replacement of hair cells in mammals and ultimately in humans suffering from sensorineural hearing loss.

Dual mechanisms for regenerating hair cells

While supporting cell mitotic proliferation is the major source of regenerated hair cells, there is also clear evidence that up to a third of the new hair cells arise through direct transdifferentiation, where supporting cells change their gene expression and differentiate into hair cells without going through mitosis (Roberson et al., 1996; Adler et al., 1997; Baird et al., 2000; Roberson et al., 2004; Morest & Cotanche, 2004; Duncan et al., 2006;Cafaro et al., 2007). While direct transdifferentiation appears to be a rapid and early source of new hair cells (Roberson et al., 2004; Duncan et al., 2006), its overall effectiveness is limited because there is a loss of one supporting cell for each new hair cell made. Thus, there must be a point where supporting cell mitotic proliferation is activated not only to make new hair cells, but also to replace the supporting cells lost to direct transdifferentiation. Ultimately, the regenerating sensory epithelium must produce enough new hair cells and supporting cells to structurally and functionally repopulate the damaged region. Estimates from previous studies (Cotanche et al., 1987) indicate that there are approximately 2000 hair cells in the proximal 30% of the chick BP, the area damaged by a single gentamicin injection (Roberson et al., 2000). At a ratio of 3 to 4 supporting cells per hair cell in the proximal basilar papilla (Goodyear & Richardson, 1997), there should be 6000 to 8000 supporting cells in this region. All 2000 of the hair cells in the proximal 30% of the basilar papilla are lost during gentamicin damage in our model. About one-third (667) of these hair cells are replaced by direct transdifferentiation (Roberson et al., 1996;Roberson et al., 2004). The other two-thirds (1334) are replaced by mitosis. Thus, 667 supporting cells (~8% of the total) should be lost due to direct transdifferentiation, while 1334 supporting cells(~16%) should be involved in undergoing mitosis to produce one new hair cell and one new supporting cell. That means that 4000–6000 of the supporting cells (76%) within the region of hair cell loss should not be directly involved in regeneration. Yet, according to data from Bhave et al. (1995) all the supporting cells in the damaged region (proximal 30%) and beyond down-regulate statin and up-regulate PCNA in response togentamicin treatment. Moreover, our *in situ* hybridization data for α-tectorin indicate that all of the supporting cells in the basal 20–30% down-regulate expression of α-tectorin by 24h after gentamicin with a recovery seen by 48–72h (Figure 2). So even though only a third of the supporting cells are actively involved in regeneration, all of them within the region of hair cell loss seem to respond, at least initially, to the loss of the hair cells.

The signals that induce direct transdifferentiation in a subset of the supporting cells as an initial response to hair cell loss remain undefined, as are those that lead to the switch from direct transdifferentiation to mitotic proliferation. However, studies of both the developing mouse cochlea and the regenerating chick cochlea suggest that cell fate pathways, such as the Notch signaling pathway and the Atoh1 signaling pathway, regulate the precursor cells in the sensory epithelium to select either a hair cell or supporting cell fate (Fekete et al., 1998; Bermingham et al, 1999; Lanford et al., 1999; Stone & Rubel, 1999; Kelley, 2002; Woods et al., 2004; Kelley, 2007; Cafaro et al., 2007; Daudet et al., 2009). We hypothesize that very early on in the dying process, hair cells send signals to adjacent supporting cells to activate Atoh1 and initiate hair cell differentiation through direct transdifferentiation. Subsequently, there must be a threshold reached in the number of supporting cells available to commit to direct transdifferentiation that stops this pathway and leads to the activation of the mitotic proliferation pathway instead.

Regeneration as a recapitulation of development

One of the initial hypotheses proposed in our early regeneration studies was that the new hair cells were arising by processes similar to those which occurred during embryonic development in the cochlea (Cotanche, 1987; Corwin & Cotanche, 1988). Indeed, morphological and ultrastructural studies of regeneration and chick cochlear development showed many common events between the two. Moreover, a number of structural similarities were seen between development and regeneration in the chick cochlea and embryonic development in the mouse organ of Corti. These include an initial period of cell proliferation followed by a differentiation of the post-mitotic progenitor cells into hair cells and supporting cells, culminating in a maturation of the stereociliary bundles and establishment of neural connectivity. As tools developed in mouse genomics and data from the human genome project became available during the 1990's, researchers were able to begin assessing the genetic mechanisms that regulate the development of the mouse cochlear sensory epithelium and the cell fate choices made during the differentiation of hair cells and supporting cells within the organ of Corti (Barald & Kelley, 2004; Kelley, 2007). Again, as with structural development, many common genetic pathways were found between the developing mouse cochlea and the developing and regenerating chick cochlea (Lewis, 1991; Lee & Cotanche, 1995, 1996; Adam et al, 1998; Fekete & Wu, 2002; Daudet & Lewis, 2005, Stone & Rubel, 1999).

However, one major difference identified between mammals and birds was the expression of proliferation inhibitors during organ of Corti development (Chen & Segil, 1999; Lowenheim et al., 1999; Chen et al, 2003; Mantela et al., 2005; Sage et al., 2005, 2006), and this was thought to be responsible for the mature mammalian cochlea's inability to regenerate. While p27 is expressed in hair cells and supporting cells of the mature avian basilar papilla (Torchinsky et al., 1999), its presence does not seem to be altered in supporting cells during regeneration. Furthermore, the continued presence of p27 does not appear to inhibit the initiation of regeneration. The presence of other proliferation inhibitors in the chick basilar papilla, such as Rb and p19, has not been defined. Genetic manipulation of the mammalian cochlea has shown that when these proliferation inhibitors are knocked down the supporting cells can continue to proliferate beyond their normal embryonic time window, which can lead to the production of excess supporting cells and supernumerary hair cells (Chen & Segil, 1999; Lowenheim et al., 1999; Chen et al., 2003; Mantela et al., 2005; Sage et al., 2005, 2006; Weber et al., 2008). However, this abnormal proliferation of the supporting cells induces a massive wave of apoptosis in both hair cells and supporting cells that leads to a complete disruption of the sensory epithelium and a subsequent profound hearing loss. Thus, future attempts to induce mammalian cochlear regeneration will need to find a way to temporarily turn off the cell proliferation inhibitors and block the signals that result in widespread cell death in the sensory epithelium. Moreover, once supporting cell proliferation is reinitiated, it will be important to

induce the new cells to assume a cochlear progenitor fate and differentiate into new hair cells and supporting cells. Evidence from *in vitro* studies of the embryonic mouse cochlear cells expressing Atoh1 suggest that these cells can also regulate the differentiation of their neighboring cells to produce both new hair cells and supporting cells in a pattern reminiscent of the normal organ of Corti (Woods et al., 2004). Whether this can occur in a mature, damaged sensory epithelium is not yet clear.

Genetic specification of the cochlear sensory epithelium

The Notch signaling pathway comprises a complex of genes that have been found to control cell fate determination in developing tissues of all metazoans (Doe & Goodman, 1985; Lewis, 1991; Artavanis-Tsakonas et al., 1999; Louvi & Artavanis-Tsakonas, 2006). Early in otocyst development, the Notch pathway is thought to define the presumptive sensory epithelium through a lateral induction process (Daudet & Lewis, 2005; Kiernan et al., 2006; Daudet et. al., 2007; Hayashi et al., 2008). Once the sensory epithelium is established, the cells undergo a series of Notch-directed lateral inhibition choices to determine which cells will differentiate into hair cells and which will become supporting cells (Figure 3). These decisions are made by the segregating of Delta1, Jagged2/Serrate2, and Atoh1 into presumptive hair cells, while Notch1, Jagged1/Serrate1, and the Hes family of genes are restricted to supporting cells (Kelley, 2006, 2007). At embryonic day 12.5 (E12.5) in the mouse, Notch1, Jagged1, and Sox2 are expressed in all cells within the presumptive cochlear sensory epithelium (Lanford et al., 1999; Woods et al., 2004; Kiernan et al., 2005b; Dabdoub et al., 2008). Most of these signals are initially found in the base of the cochlea and spread to the apex with time. However, p27, an inhibitor of cell proliferation, is expressed in the opposite pattern. It initially appears in the cochlear apex beginning at E12.5 and reaches the base by E14 (Chen & Segil, 1999; Lee et al., 2006). This pattern matches the wave of terminal mitoses in the mouse cochlea originally described by Ruben (1967) and defines a region in the presumptive sensory epithelium termed the Zone of Nonproliferating Cells, or ZNPC (Chen & Segil, 1999). The ZNPC is thought to represent the boundaries of the region specified to become the developing organ of Corti.

Genetic expression patterns during mammalian embryonic hair cell differentiation

Atoh1 (the gene formerly known as Math1) has been identified as the earliest hair-cell-specific gene required for definitive hair cell development, because the loss of Atoh1 results in the failure of any hair cell differentiation in the mouse cochlea (Bermingham et al., 1999). Although the deletion of Sox2 or Eya1 also leads to a lack of hair cell differentiation, this is thought to involve a loss of the progenitor cell population in general and not a specific block in hair cell differentiation (Kiernan et al., 2005a; Kalatzis et al., 1998; Zou et al., 2008). At E13.5, Atoh1 mRNA is broadly expressed in cells throughout the presumptive sensory epithelium, as determined by *in situ* hybridization (Lanford et al., 2000; Woods et al., 2004; Matei et al., 2005). These Atoh1-positive cells are the progenitor cells that will subsequently differentiate further into presumptive hair cells and supporting cells. Interestingly, a single row of cells within the Atoh1-positive progenitor cells labels with antibodies to myosin VI at this time (Montcouquiol & Kelley, 2003). These myosin VI-positive cells will give rise to the single row of inner hair cells in the organ of Corti. One day later, at E14.5, the presumptive inner hair cells begin to express higher levels of Atoh1 than their neighbors, as determined by Atoh1-GFP reporter expression(Bermingham-McDonogh et al., 2006). In addition, these same cells begin to expressDelta1 and Jagged2 mRNA (Morrison et al., 1999; Lanford et al., 1999; Hartman et al., 2007). AtE15.5the three rows of OHC can be identified with the Atoh1-GFP reporter mouse(Bermingham-McDonogh et al., 2006)and by E17 only the four rows of hair cells express Atoh1 mRNA (Lanford et al., 2000). AtE16.5-E18Delta1 and Jagged2 mRNA

can be localized withinthe three rows of OHC, as well (Morrison et al., 1999; Lanford et al., 1999; Murata et al., 2006; Kelley, 2007).

There appears to be a temporal discepancy in the localization of Atoh1 mRNA between the *in situ* hybridization experiments and those using the Atoh1-GFP reporter mouse. It has been suggested that the promoter used in the Atoh1-GFP mouse enables the Atoh1 protein to act on its own promoter, therefore enhancing the GFP signals in cells where it is most active (Kelley, personal communication). Therefore, the signal would be stronger in the presumptive hair cells at a time when both the presumptive hair cells and presumptive supporting cells still express the Atoh1 mRNA. This is suggested in the results presented above, but a careful comparative study of mRNA expression and Atoh1-GFP expression during the key E13.5 to E17 time window is lacking.

Currently, it is not known if myosin VI labels the differentiating outer hair cells before they can be identified by Atoh1-GFP atE15.5. A second hair cell-specific protein, myosin VIIa, appears in the presumptive inner hair cells of the cochlea by E15.5, coincident with the localization of Delta1 mRNA to these cells (Chen et al., 2002). Myosin VIIa protein is localized to the three rows of outer hair cells by E16, 12 hours after Atoh1-GFP and 12 hours before Delta1 mRNA are localized to these same cells (Chen et al., 2002;Bermingham-McDonogh et al., 2006; Morrison et al., 1999).

Thus, myosin VI appears to be the earliest overt marker to appear in the subset of cells that will become hair cells within the broaderband of Atoh1 mRNA-positive cells of the developing organ of Corti. Delta1 and Jagged2 mRNA, and myosin VIIa protein exhibit a similarlyrestricted expression in hair cellstwo to three days later. Interestingly, while both myosin VI and myosin VIIa are expressed very early in hair cell specification, neither are required for hair cell differentiation, as mutant mice lacking either of the two genes develop normally throughout embryogenesis and the hair cells only begin to fall apart during postnatal maturation (Self et al., 1998, 1999). However, a double knockout for both myosin VI and VIIa may show that hair cell differentiation cannot occur when both are missing. Thus, even though myosin VI and myosin VIIa expression do not appear to be critical to early hair cell differentiation, they do specifically identify those cells that have already committed to a hair cell fate even earlier than can be detected with Atoh1 mRNA or Atoh1-GFP expression. Either we have not yet been able to detect the changes in Atoh1 mRNA or protein levels that signal the discrete beginning of hair cell differentiation in these cells, or there is someas-yet-unidentified gene that isactivated in the presumptive hair cells before Atoh1 levels are increasedwhich also induces myosin VI expression. This protein could then play a role in localizing and reinforcing the expression of Atoh1 within these presumptive hair cells. This would then be coordinated with a turning off of Atoh1 expression in adjacent progenitor cells and an activation of signals specific for determining supporting cell fate. There is some evidence that Eya1 may play such a role (Zou et al., 2008).

At E16-E17, activated Notch1, Jagged1, p27, Prox1, and Hes5 expression are localized to supporting cells, and Sox2 expression is much stronger in supporting cells than in hair cells (Lanford et al., 2000; Kiernan et al., 2005a; Murata et al., 2006; Bermingham-McDonogh et al., 2006; Kelley, 2007; Dabdoub et al., 2008). Sox2 disappears completely from hair cells by P0, but is retained in supporting cells throughout maturity (Oesterle et al., 2008; Dabdoub et al., 2008). The Notch pathway genes are thought to function through lateral inhibition to prevent adjacent progenitor cells from differentiating as hair cells during cochlear development. These Notch interactions do not appear to have any role in stimulating the differentiation of hair cells, because selective knockouts of Delta1 and Jagged2 result in excessive hair cell differentiation at the expense of supporting cells, rather than eliminating the differentiation of hair cells (Kiernan et al., 2005b; Brooker et al., 2006). This second wave

of Notch pathway genes appears to be involved in preventing neighboring cells from acquiring a hair cell fate once the original four rows of hair cells have differentiated. Moreover, altering the Notch pathway does not seem to be able to induce hair cell specification in undamaged mature avian basilar papillae, or in damaged mammalian organs of Corti (Daudet et al., 2009; Batts et al., 2009).

Between postnatal day 0 (P0) and P3 the hair cell fate genes Atoh1, Delta1, and Jagged2 are selectively down-regulated in the developing hair cells and by P7 are no longer detected in the organ of Corti (Lanford et al., 2000; Murata et al., 2006). Meanwhile, activated Notch1 and Hes5 are down-regulated in the differentiated supporting cells, while expression of Sox2, p27, and Jagged1 are retained through maturity (Chen & Segil, 1999; Lanford et al., 2000; Oesterle et al., 2008). Once the key Notch pathway genes have performed their developmental roles in establishing the sensory epithelium and defining the correct mosaic of hair cells and supporting cells, these genes are no longer required to maintain cell identity in the mature organ of Corti and are down-regulated. Therefore, these developmentalcell fate genes are only needed during the initial stages of development in the cochlear sensory epithelium and appear to function as genetic “training wheels”. Once the cells have established their identities as hair cells or supporting cells, they can maintain these cell fates throughout the remainder of their lives without the active functioning of the Notch lateral inhibition pathway genes or the Atoh1/Hes family of genes.

The genes that continue to be expressed in mature supporting cells only, i.e., Sox2, p27, Notch1, and Jagged1, are initially found in all cells of the prosensory epithelium and become restricted to supporting cells only late in development, well after the hair cells have differentiated. This corroborates the hypothesis that supporting cells are the direct descendants of the multipotent progenitor cells in the sensory epithelium and, therefore, they may be good targets for initiating a regenerative capacity in the mature mammalian organ of Corti. In addition, since Notch1, Sox2, p27, and Jagged1 are retained in the presumptive hair cells as they undergo differentiation and are only lost from the sensory cells later in development, it suggests that continued expression of these particulargenes at the levels present in hair cells from E13.5 through P0 does not actively interfere with hair cell differentiation during development. It is possible that the activity of the proteins for these genes is diminished or inactivated during this period, even though their mRNAs are still present. However, overexpression of these genes in experimental situations may lead to levels of their respective proteins high enough to disrupt hair cell differentiation (Dabdoub et al., 2008).

Gene expression patterns during avian embryonic hair cell differentiation

While most of the research on the Notch signaling pathway has been carried out in the embryonic mouse organ of Corti, a few parallel studies have been performed in the developing and regenerating avian cochlea (Figure 4). The benefit of the avian system in these experiments is that we can explore how closely the developmental pathways match those in the mouse and, in addition, how they are recapitulated during regeneration in the mature cochlea. In development, Notch1 is expressed as early as E1.5-E2 in the otic placode and is present in all otocyst cells by E5 (Adam et al., 1998). Jagged1/Serrate1 is localized to the ventral surface of the otocyst by E3.5 and in the prosensory epithelium by E5. Notch1 and Jagged1/Serrate1 expression at these early times is thought to function in the lateral induction of the sensory epithelia (Daudet & Lewis, 2005;Daudet et al., 2007). Sox2 is localized to the prosensory epithelium by E3-E4 and is found in all cells of the developing basilar papilla by E5, but by E8 it is already restricted to the supporting cells (Neves et al., 2007). Atoh1 mRNA appears in the anterior and posterior semicircular canals by E3 and is strongly expressed in a subset of these cells by E4 (Pujades et al., 2006). In our preliminary studies, we see strong expression of Atoh1 protein in the lagena and saccule as early as E6 and faint label in the cells of the

basilar papilla by E9. Since hair cells begin differentiating in the distal basilar papilla by E6 and reach the proximal region by E9, Atoh1 expression in the presumprive hair cells would be expected to precede this time window. Currently, we are working on refining the expression patterns of Atoh1 in the developing chick basilar papilla between E5 and E9. Delta1 appears in the prosensory epithelium by E5, is seen throughout the differentiating hair cells in the basilar papilla by E6-E8, but is only transiently expressed and is gone from the developing sensory epithelia by E9 (Adam et al., 1998). In our preliminary studies, we see clear labeling for myosin VI protein in the distal basilar papilla at E6 and throughout the entire sensory epithelium by E9. This early evidence parallels our previous SEM studies which show that the first hair cells are identifiable by E6 in the distal end and throughout the basilar papilla by E9 (Cotanche & Sulik, 1984). Espin, a critical component of the developing stereociliary bundle, is seen in the distal hair cells by E8 and throughout all the hair cells by E12 (Li et al., 2004;Sekerková et al., 2006). Unfortunately, there is not yet enough data for the embryonic chick basilar papilla on the expression of many of the genes or proteins that have been defined in detail for the embryonic mouse cochlea. In general, the data that have been collected so far suggest that the genetic program forgenerating hair cells and supporting cells in the avian inner ear are similar to those utilized in the developing mouse cochlea. However, further detailed studies are needed to make more accurate comparisons between the two systems.

Genetic regulation of avian cochlear regeneration

The mature chick cochlea exhibits continued expression of Notch1 and Jagged1/Serrate1 in the supporting cells of the basilar papilla (Stone & Rubel, 1999; Daudet et al., 2009). During regeneration induced by gentamicin, Atoh1 protein appears in the supporting cells very early, within 9–15h after the gentamicin injection (Cafaro et al., 2007; Chapman et al., 2008). By 3–4 days, Delta1 mRNA is upregulated in the region of damage (Stone & Rubel, 1999; Daudet et al., 2009). Myosin VI first appears in newhair cells generated by direct transdifferentiation at 78h and myosin VIIa appears at 90h (Duncan et al., 2006). By 10D, both Atoh1 and Delta1 expression has been down-regulated in the new hair cells (Stone & Rubel, 1999; Chapman et al., 2009). Thus, at least at the level of gene expression, the signaling pathways utilized in embryonic development are re-employed during the differentiation of hair cells and supporting cells during regeneration.

Summary and Conclusions

Studies of the gene pathways and expression patterns in the development and regeneration of hair cells and supporting cells in the mammalian and avian cochlea have demonstrated a number of proteins involved in the Notch lateral inhibition pathway and the Atoh1/Hes family of genes. It will be important to understand the normal cascade of these signals in the developing cochlea that give rise to cochlear progenitor cells and then guide their subsequent decisions to become either hair cells or supporting cells. While many of the individual genes involved in these decisions have been identified from studies of mutant and knockout mouse cochleae, the interplay of all these signals into a single systemic program that directs this process needs to be explored more fully. We need to know not only what genes are involved, but understand how their gene products interact with one another in a structural and temporal framework to guide hair cell and supporting cell differentiation and maturation.

Currently, the major obstacle to inducing regeneration in the mammalian cochlea seems to be the expression of several proliferation inhibitors during the later stages of cochlear development. Initial genetic experiments leading to the elimination or conditional knockout of these inhibitors have demonstrated renewed or continued proliferation within the sensory epithelium. However, this has not yet led to the production of new hair cells and often causes rampant apoptosis throughout the sensory epitheilum and the death of all the hair cells and

supporting cells. A greater understanding of the genetic components in progenitor cell proliferation and hair cell and supporting cell differentiation will be critical to our ability to regulate the regeneration induced by controlled down-regulation of specific proliferation inhibition signals in the mammalian cochlea.

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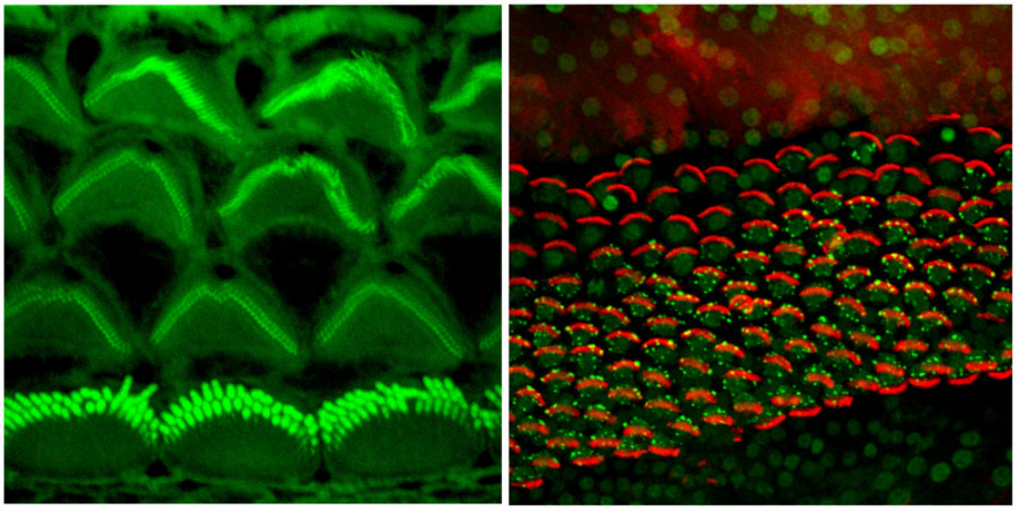

Figure 1.
Confocal micrographs of the luminal surface of the postnatal day 3 mouse organ of Cortiwhere actin is labeled with phalloidin (green) and of the regenerating mature chick basilar papilla where actin is labeled with phalloidin (red) and the early apoptosis marker TIAR is labeled with a specific antibody (green).

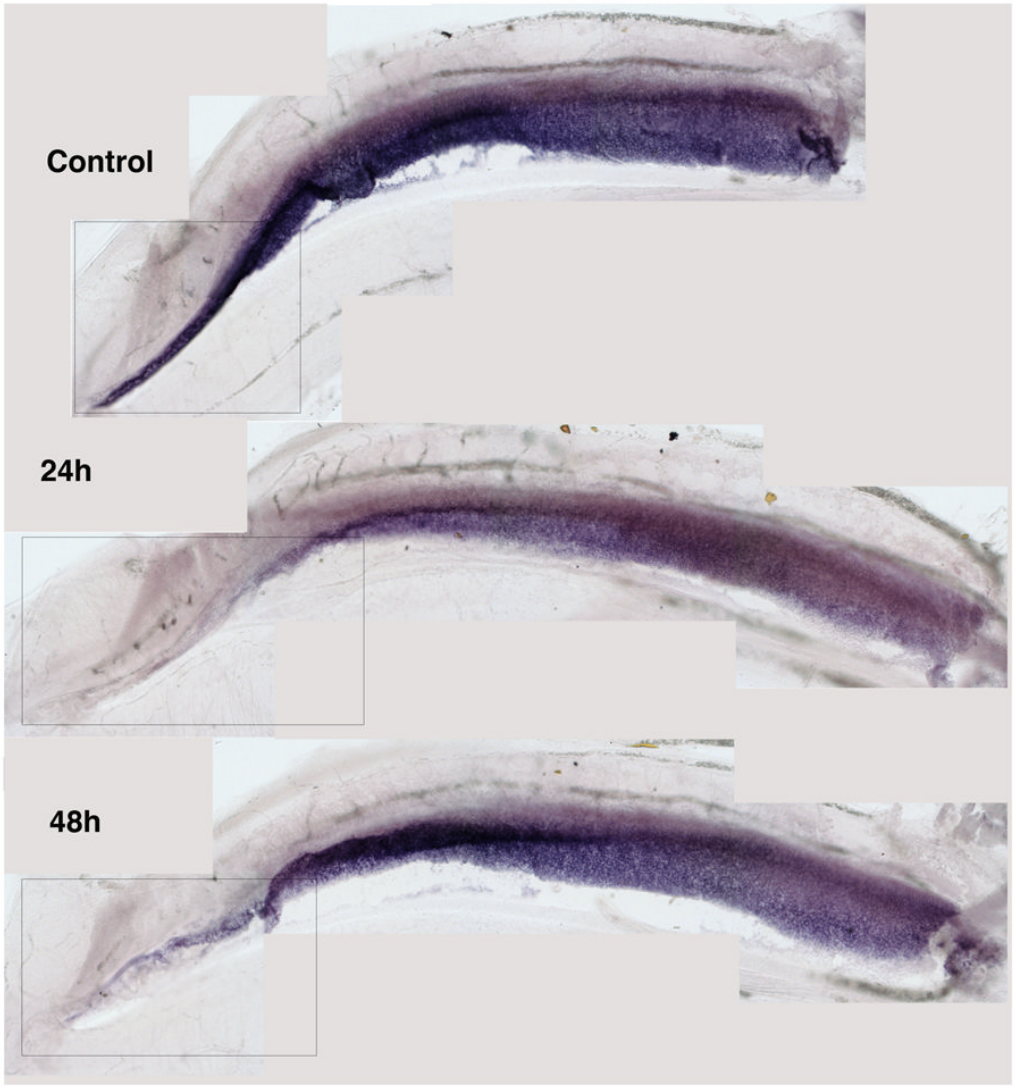

Figure 2.
Whole mount *in situ* hybridization of the chick cochlea forα-Tectorin, a gene expressed in supporting cells for synthesizing proteins needed to maintain the extracellular matrix of the tectorial membrane. This gene is down-regulated in all supporting cells within 24h of a single gentamicin injection, but begins to be re-expressed in some supporting cells by 48h after the gentamicin.

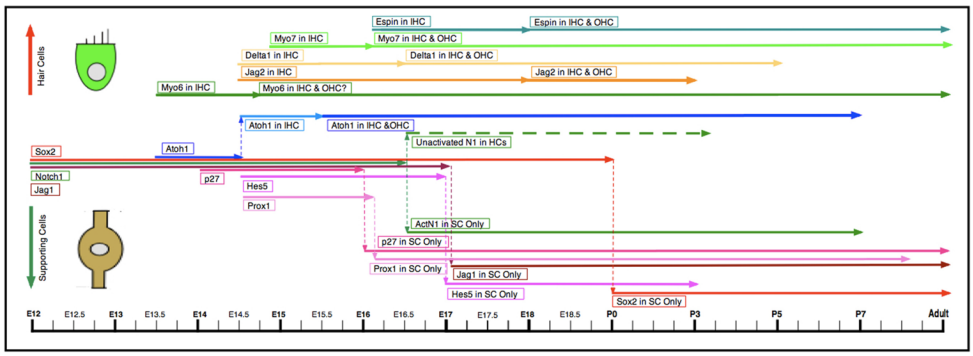

Figure 3.

Timeline of gene expression in mammalian cochlear hair cell/supporting cell differentiation. In the timeline, expression of the genes/proteins in all progenitor cells is represented by the lines in the center on the left side of the timeline. The line shifts up when one of these components becomes restricted to hair cells; while for those that become restricted to supporting cells, the line shifts down. The left end of each line represents the time when these genes/proteins are first expressed, while the right end of the line represents the time they stop being expressed. The lines that begin within the hair cell field (top) are for genes/proteins that are only ever expressed in hair cells, and not in progenitor cells. Lines that reach the left and right edges of the timeline represent genes/proteins that have begun their expression before E12, or continue to be expressed in the adult cochlea, respectively.

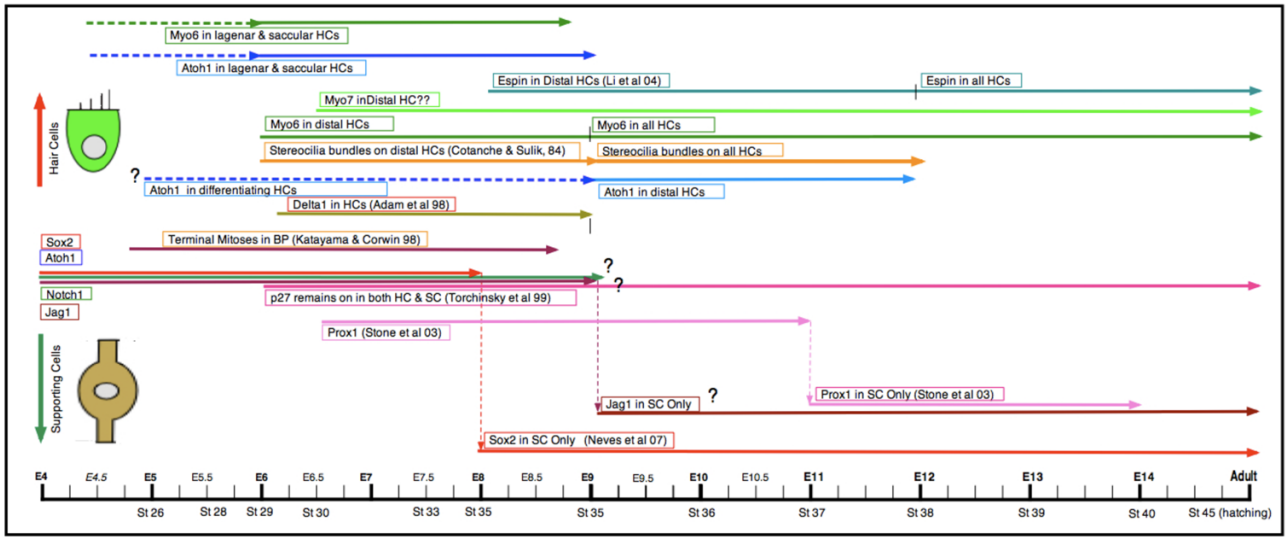

Figure 4.

Timeline of gene expression in avian cochlear hair cell/supporting cell development. In the timeline, expression of the genes/proteins in differentiating hair cells is represented by the lines beginning on the left side of the timeline. Data based on published results include the reference in the line descriptor. Data without references are based on preliminary studies in our lab.

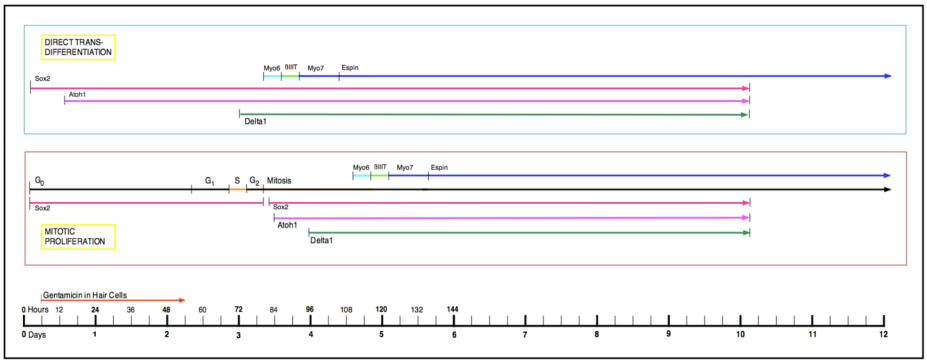

Figure 5.
Timeline of gene expression in avian cochlear hair cell/supporting cell regeneration. In the timeline, expression of the genes/proteins in regenerating hair cells produced by both direct transdifferentiation and mitotic proliferation are represented by the lines beginning on the left side of the timeline.

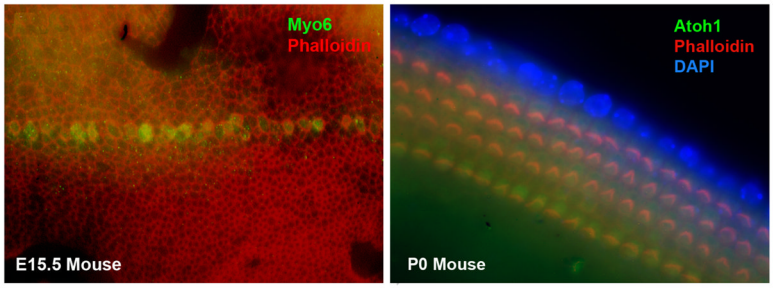

Figure 6.
Expression of myosin VI protein in early differentiation of inner hair cells in the mouse embryonic cochlea at E15 and of Atoh1 expression in the differentiating hair cells in the mammal cochlea at postnatal day 0.