INNER EAR SENSORY EPITHELIA DEVELOPMENT AND REGULATION IN ZEBRAFISH

A Dissertation

by

ELLY MAE SWEET

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2010

Major Subject: Biology

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ABSTRACT

Inner Ear Sensory Epithelia Development and Regulation in Zebrafish. (August 2010)

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The inner ear is a complex sensory organ of interconnected chambers, each with a sensory epithelium comprised of hair cells and support cells for detection of sound and motion. This dissertation focuses on the development and regulation of sensory epithelia in zebrafish and utilizes loss of function, gain of function and laser ablation techniques. Hair cells and support cells develop from an equivalence group specified by proneural genes encoding bHLH transcription factors. The vertebrate Atoh1 bHLH transciption factor is a potential candidate for this role. However, data in mouse has led some researchers to conclude it does not have a proneural activity, but, rather, is involved in later stages of hair cell differentiation. In addition, the factors regulating Atoh1 are mostly unknown. We address these issues in zebrafish and show that the zebrafish homologs *atoh1a* and *atoh1b* are required during two developmental phases, first in the preotic placode and later in the otic vesicle. They interact with the Notch pathway and are necessary and sufficient for specification of sensory epithelia. Our data confirm *atoh1* genes have proneural function. We also go on to show Atoh1 works in a complex network of factors, Pax2/5/8, Sox2, Fgf and Notch. Misexpression of *atoh1* alters axial patterning and leads to expanded sensory epithelia, which is enhanced by misexpression

of either *fgf8* or *sox2*. Lastly, we examine the role of *sox2* in sensory epithelia development and regeneration. Sox2 has been implicated in maintainence of pluripotent stem cells as well as cell differentiation. In the inner ear, *Sox2* is initially expressed in the prosensory domain and is required for its formation. Eventually, *Sox2* is downregulated in hair cells and maintained in support cells; however, its later role has not been determined. We show that in the zebrafish inner ear, *sox2* is expressed after sensory epithelium development has begun and, like in mouse, expression is down regulated in hair cells and maintained in support cells. Our data demonstrate a role for *sox2* in maintenance of hair cells and in transdifferentation of support cells into hair cells after laser ablation. Additionally, *sox2* is regulated by Aoth1a/1b, Fgf, and Notch.

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CHAPTER I

INTRODUCTION

RELEVANCE

The inner ear is a complex structure for hearing and balance. Millions of Americans suffer from hearing deficits as a result of environmental and genetic factors (Hudspeth, 2000). Hearing damage is primarily due to the permanent loss of sensory hair cells of the inner ear. Mammals are unable to regenerate lost hair cells; however, non-mammalian vertebrates are capable of regeneration after death or damage of hair cells. Restoration of hair cells and hearing in humans may be possible with therapeutic approaches; however, for this to be realized, there needs to be a better understanding about the molecules involved in hair cell development and regeneration. The use of model organisms with the ability to regenerate hair cells, such as zebrafish, may provide a useful approach to gathering this information.

INNER EAR DEVELOPMENT AND FUNCTION

The inner ear begins to develop during gastrulation as a thickening in the ectoderm that eventually becomes a highly complex organ for hearing and balance (Baker and Bronner-Fraser, 2001). This thickening in the ectoderm, the otic placode, converts into a simple epithelial vesicle by cavitation in zebrafish rather than invagination as it does in other vertebrates such as chick and mouse. The otic vesicle

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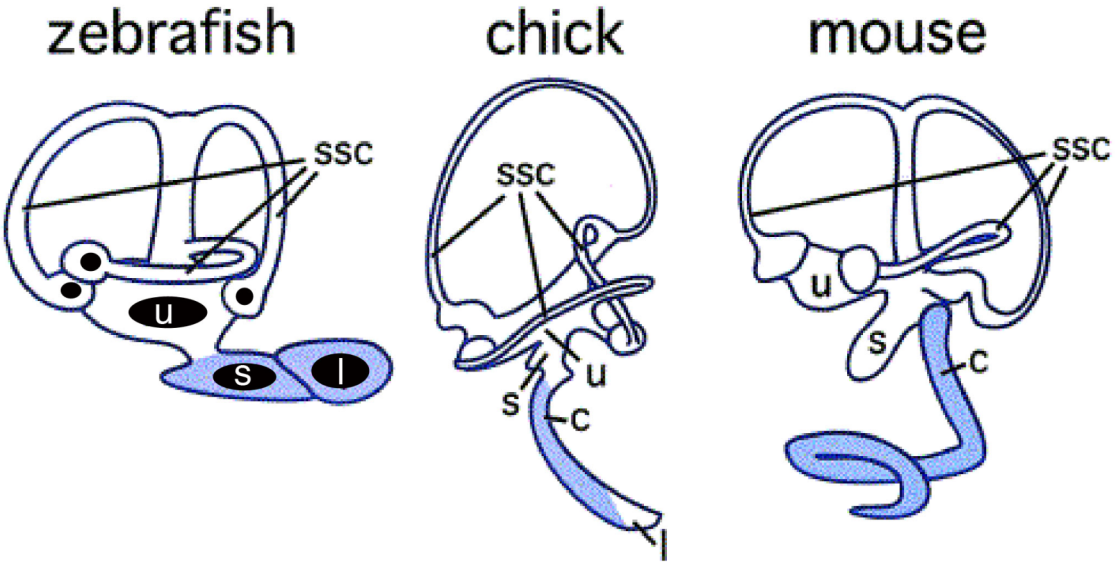

Figure 1.1. Structure of the vertebrate inner ear.
Representation of adult zebrafish, chick and mouse inner ear structures. Chambers colored in light blue are auditory chambers. All others are vestibular chambers. Black patches indicate sensory epithelia in zebrafish. Abbreviations: u: utricle, s: saccule; l: lagena, c: cochlea, ssc: semicircular canals. (Adapted from Riley and Phillips, 2003).

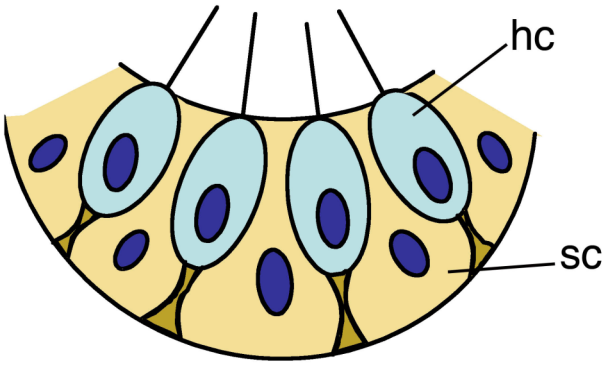

Figure 1.2. Inner ear sensory epithelium.
Representation of arrangement of hair cells (hc) and support cells (sc) within the sensory epithelium of zebrafish.

then undergoes rearrangements to form a complex structure of inter-connected chambers (Haddon and Lewis, 1996). Of these chambers, the utricle and semicircular canals make up the vestibular apparatus, which is highly conserved in structure and function among all vertebrates (Riley and Phillips, 2003). The auditory chambers, however, have undergone evolutionary modification. In mammals and birds, the primary auditory chamber is the cochlea, while in amphibians and fish there is no such counterpart. The saccule and lagena function as the primary auditory chambers in fish (Fig. 1.1).

Each chamber is associated with a sensory epithelium consisting of sensory hair cells and support cells (Fig 1.2). Hair cells are mechanosensory cells that are stimulated by lateral deflection of ciliary bundles and facilitate hearing and balance. Hair cells of the maculae, sensory patches in the utricle, saccule and lagena, are associated with otoliths, dense calcium carbonate crystals attached to hair cell bundles that help transmit accelerational forces and sound vibrations. Although the function of support cells is less understood, it is thought that they aid in the maintenance of hair cells and serve a stem cell like function in regeneration of hair cells (Stone and Cotanche, 2007). In zebrafish, sensory hair cells and support cells begin to develop before otic vesicle formation and prior to development of sensory neurons of the statoacoustic ganglion (SAG) that transduce signals from the hair cells to the brain. SAG neuroblasts delaminate from the anteroventral surface of the otic vesicle, differentiate, and innervate hair cells in various sensory patches of the inner ear (Haddon and Lewis, 1996). In mammals and chicks SAG development begins prior to hair cell and support cell development.

In addition to sensory hair cells of the inner ear, zebrafish utilize another mechanosenory organ, the lateral line, to detect changes in water flow. The lateral line consists of a series of neuromasts along the body of the animal. Each neuromast is made up of innervated hair cells surrounded by support cells. There are several structural and molecular similarities between hair cells of the lateral line and inner ear in zebrafish.

OTIC INDUCTION

The otic placode, which forms adjacent to the hindbrain, is induced by signals from surrounding mesoderm and neural tissue. In all vertebrates examined, Fibroblast Growth Factors (Fgfs) are expressed in tissues neighboring the otic placode and have been identified as the primary otic inducing factors. *fgf3* and *fgf8* have been shown in zebrafish to act redundantly to induce the otic placode (Phillips, et al., 2001; Leger and Brand, 2002; Maroon et al., 2002). *fgf3* mutants do not exhibit any morphological ear defects while *fgf8* mutants typically produce a small otic vesicle. Double knockdown of *fgf3* and *fgf8* results in complete loss of otic tissue. In mouse Fgf3, Fgf8, and Fgf10 all act to induce the otic placode (Wright and Mansour, 2003, Ladher et al., 2005). Knockouts of either *Fgf3* or *Fgf10* result in mild otic vesicle defects; however, double mutants produce microvesicles or complete loss of otic tissue. Otic induction is also impaired by loss of both *Fgf3* and *Fgf8*. Studies in chick indicate a role for Fgf3, Fgf8, and Fgf19 in otic induction. Knockdown of *Fgf8* impairs otic induction but it does so by also reducing *Fgf19* expression in mesoderm. Knockdown of *Fgf3* and *Fgf19* independently causes modest otic induction defects while loss of both severely impairs

otic tissue. These studies all support redundant roles for Fgf during otic placode induction. Fgfs continue to be expressed in the hindbrain after otic placode induction and may be important for maintaining otic tissue as well as for patterning the otic vesicle, as will be discussed later.

RESPONSE TO OTIC INDUCING FACTORS

In response to otic inducing factors, transcription factors are upregulated in the preotic region. The paired boxed transcription factor subfamily Pax2/5/8 are expressed during various stages of otic development (Pfeffer et al., 1998). One of the earliest known markers of the preotic development is *pax8*. In most vertebrates, otic expression of *pax8* begins by late gastrulation, although *Pax8* is not present in the chick genome. *pax2* expression begins during somitogenesis stages, after *pax8* has already turned on. In mouse, *Pax8* knockout does not exhibit an otic phenotype (Mansouri et al., 1998) while *Pax2-/-* mutants only show late otic vesicle defects (Torres et al., 1996). In zebrafish *pax8* knockdown, small otic placodes give rise to a small otic vesicle with fewer hair cells. There are two *Pax2* homologs in zebrafish *pax2a* and *pax2b*. Loss of both results only in reduced hair cell production; however, *pax2a-pax2b-pax8* deficient embryos initially form a small otic placode with complete loss of otic tissue by otic vesicle stages (Mackereth et al. 2005). These data indicate that *pax8* and *pax2* are partially functionally redundant and together are required to maintain otic fate.

PATTERNING OF THE EARLY INNER EAR

Many of the same signals involved in placode induction are also important for patterning of the early inner ear. The complex organization of the inner ear arise from otic placod regionalization caused by signaling interactions with neighboring tissues. This can be seen molecularly by asymmetric expression of specific genes. Signals from the hindbrain, for example, continue to regulate otic development even after otic placode induction and are mediated in part by *pax2/5* genes (Kwak et al., 2002).

Transplantation experiments and otic vesicle rotation experiments in chick have demonstrated the importance of signals from adjacent tissues. Expression of Pax2 in the chick ear is restricted to the dorsal medial wall, and rotation of the ear field by 180 degrees results in new expression of Pax2 adjacent to the hindbrain in the region that previously comprised the ventral lateral wall (Hutson et al., 1999). Additionally, loss of hindbrain signals near the otic regions by ablation of rombombere 5 (r5) and rombomere (r6) results in more uniform expression of Pax2 throughout the otic vesicle (Hutson et al., 1999). These data suggest that Pax2 is influenced by its environment and that hindbrain signals help to specify the medial-lateral axis in chick.

Fgf signaling in zebrafish may also regulate some aspects of medial-lateral axis formation. *fgf3* expression in the hindbrain starts prior to placodal formation and continues through the beginning of otic vesicle formation (Phillips et al., 2001; Kwak et al., 2002; Leger and Brand, 2002). The source of Fgf signaling then changes and *fgf3* and *fgf8* become expressed in the sensory epithelia of the otic vesicle (Leger and Brand, 2002). In zebrafish, *pax2a* is initially expressed in cells of otic placode and requires Fgf

signaling (Leger and Brand, 2002). We speculate that this expression is highest in placodal cells closest to the hindbrain. Expression of *pax2a* is progressively restricted to the medial wall of the otic vesicle. It eventually shows upregulation in developing hair cells (Riley et al., 1999). We hypothesize that Fgf signaling may be important for regulation of medial-lateral axis formation; however, further data are needed to support this.

In zebrafish, Fgf signaling from the hindbrain has been demonstrated to play important roles in regulation of the anterior-posterior axis. In zebrafish, *valentino* (*val*) encodes a bZip transcription factor that regulates *fgf3* in the hindbrain (Kwak et al., 2002). In *val* mutants, expression of *fgf3* is expanded from r4 into r5 /r6 region. Small ears with an expansion of anterior otic vesicle markers, *pax5* and *nkx5.1,* and loss of posterior makers can be seen in *val* mutants. Hair cells in *val* mutants are produced in excess and throughout the medial wall of the otic vesicle. All these phenotypes can be rescued by knocking down *fgf3* in *val* (Kwak et al., 2002). These data suggest a role for Fgf3 in specifying anterior fates and inducing utricular (anterior) macula development. In addition, *lia* (*fgf3*) mutants also show a reduction in hair cell number by 24 hpf, supporting a role for Fgf3 in development of the urtricular macula (Kwak et al., 2006). Hair cell formation is also potentially regulated by *fgf8*. Although *fgf8* is no longer expressed in the hindbrain after 14 hpf, it is expressed in sensory epithelia along with *fgf3* beginning at otic vesicle stages (Leger and Brand, 2002). Additionally, loss of *fgf8* does impair hair cell formation (Ledger and Brand, 2002). These data suggest a role for Fgf signaling in regulation of A-P axis and hair cell formation.

Similar to otic placode formation, Fgf signaling during otic vesicle stages also appears to be mediated in part by *pax2/5*. As mentioned earlier, *pax2a* is initially regulated by Fgf signaling until otic vesicle stages at which point it becomes upregulated in hair cells. *pax2a* is required for normal hair cell production, and loss of *pax2a* results in an overproduction of hair cells due to diminished Delta expression needed for lateral inhibition, discussed in the next section (Riley et al., 1999). *pax5* appears to work in conjunction with *fgf3* in the utricle. Embryos deficient for *fgf3* have a severe reduction in expression of *pax5* (Kwak et al., 2002; Kwak et al., 2006). Embryos knocked down for *pax5* have normal numbers of hair cells at 24 hpf. At later stages there is a 30% reduction in the number of hair cells in the utricular macula but not in the saccular macula. This deficiency of hair cells in the utricle arises from death of a subset of mature hair cells undergoing apoptosis (Kwak et al., 2006). These data further suggest a role for Fgf signaling in hair cell development through regulation of *pax2/5* genes. The involvement of Fgfs and *pax2/5* genes in sensory epithelia development will be examined in Chapters II and IV.

HAIR CELL DEVELOPMENT

Sensory epithelial patches located in the auditory and vestibular chambers of the inner ear mediate the perception of sound and balance. These patches comprise hair cells and support cells that arise from a common equivalence group in response to lateral inhibition through Delta-Notch signaling (Muller and Littlewood-Evans 2001; Fekete and Wu 2002; Whitfield et al., 2002). The process of lateral inhibition leads to a

precisely patterned spacing of alternating hair cell and support cell fates within the sensory epithelium. Initially, all cells of the equivalence group express low levels of Delta and Notch leading to mutual inhibition. Eventually some cells up-regulate Delta and become hair cells. Up-regulation of Delta in hair cells elevates Notch activity in neighboring cells. This prevents neighboring cells from becoming hair cells and forces them to adopt an alternate, support cell, fate (Fig 1.3).

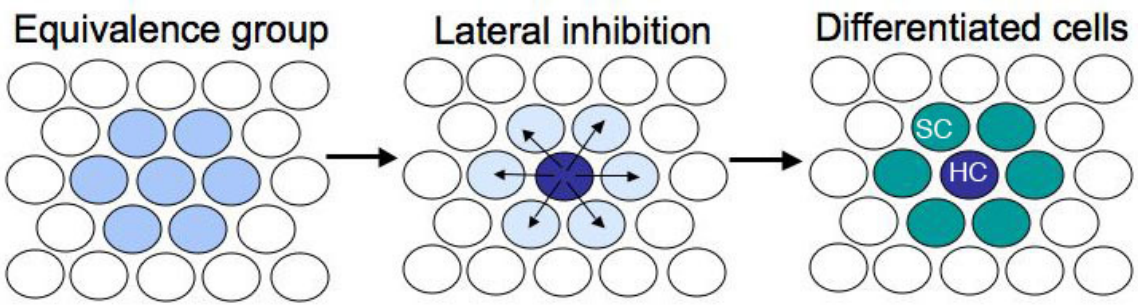

Figure 1.3. Model for lateral inhibition.
All cells of the equivalence group (blue) express low levels of Delta and Notch. Delta is upregulated in some cells (dark blue) and become hair cells (HC). Delta signals to neighboring cells upregulating Notch signaling and inhibiting hair cell fate in those cells forcing them to become support cells (SC).

There are several studies supporting a role for Notch in the mosaic pattern of hair cells and support cells. In zebrafish, blocking Notch signaling, via a mutation in *mind bomb* encoding an E3 ubiquitin ligase essential for Notch function, results in an ear containing an excess of hair cells and no support cells (Haddon et al., 1998; Riley et al., 1999). Examination of embryos with a dominant negative point mutation in *delta A* (*dlA*), a Notch ligand, shows an increase in hair cells and a decrease in support cells

(Riley et al., 1999). Loss of function studies in mouse involving *Notch1*, *Jagged 2*, *Delta-like 1 Hes1* or *Hes5* results a variable over-production of hair cells (Lanford et al., 1999; Zheng et al., 2000; Zine et al., 2001; Keirnan et al., 2006). Disrupting Notch signaling with a pharmacological inhibitor also leads to excess hair cells (Yamamoto et al., 2006; Takebayashi et al., 2007).

Equivalence groups are initially marked by expression of proneural genes. In *Drosophila,* proneural genes are required for specification of sensory organ precursors and formation of sensory organs (Brunet and Ghysen, 1999). Proneural genes encode basic Helix-Loop-Helix (bHLH) transcription factors that have DNA binding and dimerization abilities. In *Drosophila* these proteins are divided into families with the *achaete-scute* gene family specifying external sensory bristles while the *atonal* (*ato*) gene family specifies photoreceptors and chordotonal organs (Jarman et al., 1993; Jarman et al., 1995). In *Drosophila* photoreceptors and chordotonal organs arise from proneural clusters (equivalence groups) resembling the equivalence groups from which vertebrate sensory patches develop. Research on *Drosophila* sense organ specification may provide insight into sensory epithelia specification. During photoreceptor development, *ato* is expressed in a broad band of cells marking the equivalence group that is later restricted to discrete clusters expressing *ato* (Jarman et al., 1995). Equivalence group restriction occurs through activation of Delta-Notch signaling. Finally lateral inhibition further refines intermediate groups into alternating cell fates, *ato* expressing R8 precursor cells and non-*ato* expressing cells, through a balance of Ato and Notch activity (Baker et al., 1996; Baker and Yu, 1997). Notch has two roles during

photoreceptor development. During an early proneural phase expression of *ato* can be enhanced by forced Notch activation through expression of the Notch intracellular domain (NICD) (Baker and Yu, 1997). This early inductive phase is still poorly understood and involves a non-canonical Notch pathway not requiring Su(H) (Ligoxygakis et al., 1998). During later stages Notch signaling, through the canonical Notch pathway, represses *ato* expression (Baker and Yu, 1997). Loss of Notch results in an overproduction of R8 photoreceptor cells due to a failure to repress *ato* expression (Baker et al., 1996). Expression of *delta* requires *ato;* thus, in an *ato* mutant Notch mediated restriction fails and *ato* expression continues in a broad band in cells, though subsequent differentiation is blocked (Jarman et al., 1995; Baker and Yu, 1997).

In vertebrates, *Atoh1* is a candidate proneural gene for establishing sensory epithelia and hair cell specification. However, interpretation of the proneural role of *Atoh1* has been in dispute. Certain predictions can be made about the role of proneural genes based on *Drosophila* studies as mentioned in the previous section. In mouse there has been difficulty determining the pattern of early *Atoh1* expression in the cochlea. Depending on the method used some studies have come to the conclusion that *Atoh1* is expressed early in a broad domain consistent with a proneural role (Bermingham et al., 1999; Lanford et al., 2000; Wood et al., 2004). Others have concluded *Atoh1* expression is restricted to committed hair cells (Chen et al., 2002). Loss of *Atoh1* leads to a complete loss of hair cells; however, some cells remain and resemble support cells (Bermingham et al., 1999). Thus, it has been suggested that *Atoh1* does not act as a proneural gene required for specification of sensory equivalence group (Bermingham et

al., 1999; Chen et al., 2002; Fritzsch et al., 2005). Closer examination of these remaining cells with molecular markers does reveal support cell development is disrupted; however, expression of early markers preceding fate specification is initially present (Woods et al., 2004). Overexpression of *Atoh1* is sufficient to induce hair cells in the cochlea (Zheng and Gao, 2000; Kawamoto et al., 2003; Woods et al., 2004). However, this would be expected if *Atoh1* functioned as either a proneural gene or a later hair cell differentiation factor. Data are still needed to determine if *Atoh1* has a proneural role in sensory epithelia development.

In zebrafish there are two *ato* homologs, *atoh1a* and *atoh1b,* expressed in the developing otic vesicle (Adolf et al., 2004). Functional studies of *atoh1a/1b* will be described in Chapters II and IV and provide strong support for classic proneural function.

In mouse it has been suggested that another molecule, Sox2, a high-mobility-group (HMG)- box transcription factor, may play the role of a proneural gene in inner ear development (Kiernan et al., 2005b). Expression of *Sox2* in the cochlea begins earlier than *Atoh1* with expression in progenitors of both hair cells and support cells. Eventually, *Sox2* is lost from hair cells but remains in support cells (Kiernan et al., 2005b; Hume et al., 2007; Neves et al., 2007, Dabdoub et al., 2008). Additionally *Sox2* and *Atoh1* antagonize one another (Dabdoub et al., 2008). Mutants for *Sox2* have an absence of sensory epithelium or reduction in hair cells (Kiernan et al., 2005b). Overexpression of *Sox2* also inhibits formation of hair cells in the cochlea (Dabdoub et al., 2008). There is clearly an early role for *Sox2* in otic patterning and sensory epithelia

although the exact role and whether or not it has proneural function is yet to be determined.

An alternate role for *Sox2* may be in the regulation of hair cell maintenance and regeneration. In addition to its expression in the otic vesicle, it is expressed in the developing CNS as marker of neural stem cells (Ellis et al., 2004). It is also a key factor in the maintenance of mouse embryonic stem (ES) cell pluripotency. Loss of *Sox2* function results in a loss of pluripotency in mouse ES cells (Masui et al., 2007), while addition of *Sox2* aids in reprogramming of differentiated somatic cells into induced pluripotent stem cells (Takahahsi and Yamanaka, 2006). Since support cells are involved in hair cell regeneration as discussed in the next section, Sox2 may be involved in this process.

In zebrafish *sox2* is expressed in the otic placode after the onset of *atoh1* expression (Okuda et al., 2006). Additionally, it is expressed in support cells of the lateral line that proliferate during regeneration of hair cells (Hernandez et al., 2007). The role of *sox2* in zebrafish sensory epithelia development has not been determined and will be examined in Chapter IV of this dissertation.

HAIR CELL REGENERATION

Hearing deficits can arise from death or damage to hair cells and potential therapies for such hearing loss have focused on regeneration of hair cells in the mammalian cochlea. Of vertebrates only mammals lack the ability to regenerate hair cells. Studies in

vertebrates that can regenerate hair cells have provided a basis for studying regeneration in mammals.

Birds have the ability to regenerate hair cells in both the auditory and vestibular regions of the inner ear after damage (Corwin and Cotanche, 1988; Ryal and Rubels, 1988, Roberson et al., 1992). After loss of hair cells the surrounding support cells replace the damaged hair cells. This regenerative response occurs through two mechanisms. One mechanism is transdifferentiation, the change of a support cell into a hair cell. The second mechanism involves support cells re-entering the cell cycle and undergo asymmetric cell division to give rise to both a support cell and a new hair cell (Stone and Cotanche, 1994; Alder and Raphael, 1996; Roberson et al., 1996). During regeneration markers of hair cell development are reinitiated, for example *Atoh1a* is up-regulated in transdifferentiating and mitotically active support cells (Cafaro et al., 2007). The potential for inducing hair cell formation through forced activation of genes involved in hair cell development has received much attention.

Regeneration studies in mammals have attempted to induce hair cell regeneration by overexpression of *Atoh1* in both normal and deafened ears. Overexpression of *Atoh1* in cochlear cultures induces extra hair cells (Zheng and Gao, 2000). Studies in mature deafened guinea pigs using an adenovirus to overexpress *Atoh1* showed the presence of both hair cells and support cells with some improvement of auditory thresholds. However, the results were variable from animal to animal and cell morphologies appeared abnormal (Izumikawa et al., 2005). Additionally, the presence of both hair cells and support cells could mean either Atoh1 acts as a proneural gene or the observed

pattern reflects residual cells resulting from incomplete ablation. It is not clear to what extent overexpression of *Atoh1* can repair a damaged epithelium. It is likely that competence of cochlear cells to respond to Atoh1 may require additional factors to fully reprogram non-sensory cells. Additionally, the competence of cochlear cells may change as the organ of Corti matures. These issues of competence of otic tissue to respond to Atoh1 and the addition of other factors for enhanced response will be addressed in zebrafish in Chapter IV of this dissertation.

Studies in hair cell regeneration have been initiated in zebrafish. Chemically damaged hair cells of the lateral line regenerate from both transdifferentiation and asymmetric cell division of support cells (Hernandez et al., 2007). This is consistent with data from chick regeneration studies (Stone and Cotanche, 1994; Alder and Raphael, 1996; Roberson et al., 1996). Studies in zebrafish lateral line have reported *Sox2* expression in dividing support cells suggesting a role in regenerative cell division, but functional studies have not been reported (Hernandez et al, 2007). Very little has been studied on regeneration in zebrafish otic sensory epithelium. The role of Sox2 in maintenance and regeneration of otic hair cells will be examined in Chapter III.

DISSERTATION OBJECTIVES

The objective of this dissertation is to address the regulation and development of sensory epithelia by *atoh1*, *sox2*, Fgf and Notch using zebrafish as a vertebrate model system.

Atoh1 has been shown to be required for hair cell formation in mammals. However, its role as a proneural gene in specification of the sensory equivalence group

has been in question, and the factors regulating *Atoh1* are mostly unknown. To investigate the role of *atoh1* and its upstream activators we performed loss of function studies using mutants, morpholino knockdown technology, and chemical inhibitors. Chapter II, a collaboration with my colleague Bonny Millimaki, shows zebrafish *atoh1a* and *atoh1b* genes function as proneural genes to specify the sensory equivalence group, which gives rise to both hair cells and supports. Additionally, induction and maintenance of *atoh1* genes require Fgf and members of the Pax2/5/8 family of transcription factors.

Although Atoh1 is both necessary and sufficient for hair cell formation, few studies have examined the ability for Atoh1 to produce the entire sensory equivalence group, including hair cells and support cells. Additionally, competence to form sensory epithelia may change with maturity and additional factor could enhance the competence of tissue to respond to Atoh1. Chapter IV characterizes the effects of *atoh1* misexpression in zebrafish. It shows Atoh1 is sufficient to induce sensory epithelia containing both hair cells and support cells consistent with its proneural function. Additionally, sensory competence is greater during placodal stages although hair cells are not produced on the lateral or dorsal walls of the otic vesicle. The zone of competence can be expanded to include the lateral and dorsal walls when *atoh1a* is misexpressed along with *fgf8* or *sox2.*

Chapter III, a collaboration with the first author, my colleague Bonny Millimaki, addresses the role of Sox2 in sensory epithelia maintenance and regeneration. I contributed to portions of Figures 3.1, 3.3, 3.4 and 3.5, and thus I include it here as a

record of my work. We show that contrary to the role for *Sox2* in sensory epithelium development in mouse, zebrafish *sox2* is expressed after sensory epithelium development begins and is regulated by Atoh1a/1b, Fgf and Notch. It is also required for maintenance and regeneration of hair cells in the inner ear. This provides the first test of such function in any vertebrate.

CHAPTER II

ZEBRAFISH *atoh1* GENES: CLASSIC PRONEURAL ACTIVITY IN THE INNER EAR AND REGULATION BY FGF AND NOTCH*

INTRODUCTION

Sensory epithelia of the vertebrate inner ear consist of two cell types, hair cells and support cells. Both are produced from a prosensory equivalence group initially marked by expression of *Atoh1*, a homolog of the *Drosophila* proneural gene *atonal* (*ato*) (Bermingham et al., 1999). As the equivalence group develops, a few cells upregulate *atoh1* expression and complete differentiation as hair cells. The rest lose expression of *atoh1* and become support cells. As the principal regulator of hair cell differentiation, *Atoh1* has received great attention in recent years in both basic and applied research (Shailam et al., 1999; Lanford et al., 2000; Zheng and Gao, 2000; Itoh and Chitnis, 2001; Chen et al., 2002; Wang et al., 2002; Woods et al., 2004; Fritzsch et al., 2005; Izumikawa et al., 2005; Kelley, 2006). However, despite extensive analysis of *Atoh1*, a number of fundamental issues still need to be resolved. Most notably, there are conflicting reports as to the precise role(s) of *Atoh1* in otic development. Although *Atoh1* is maintained only in hair cells, it may function earlier to specify the equivalence group itself – a definitive proneural function. Accordingly, disruption of mouse *Atoh1*

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(*Math1*) ablates all hair cells and support cells in the cochlea (Woods et al., 2004). However, the persistence of cells expressing some early markers of sensory epithelia has been interpreted to mean that mouse *Atoh1* is not required for specifying the equivalence group per se, but instead only promotes the final stages of hair cell development (Bermingham et al., 1999; Chen et al., 2002; Fritzsch et al., 2005). Additionally, a key aspect of prosensory development does not require *Atoh1*: Prospective sensory cells begin to express $p27^{kip1}$ and exit the cell cycle prior to expression of *Atoh1*, and this process still occurs in *Atoh1* mutants. On the other hand, $p27^{kip1}$ expression and cell cycle withdrawal could be regulated independently from equivalence group specification. Indeed, sensory epithelia still form in $p27^{kip1}$ mutants despite the failure of cells to properly exit the cell cycle (Chen and Segil, 1999). This leaves open the question of when the equivalence group forms and whether *Atoh1* acts early or late in the process.

Work on *Drosophila ato* provides a useful paradigm for testing vertebrate *Atoh1* function (Fig. 2.1). *ato* is initially expressed in a broad pattern (the equivalence group) well before cell fate specification (Jarman et al., 1995). The equivalence group then restricts its own size through activation of Delta-Notch (Dl-N) signaling (Baker et al., 1996; Baker and Yu, 1997). In this process, N-dependent downregulation of *ato* breaks the equivalence group into discrete "intermediate groups" of *ato*-expressing cells separated by non-expressing cells that are excluded from the sensory structure. Subsequently, the balance of ato and N activity selects between alternate fates within intermediate groups (lateral inhibition). Because *ato* is required for *Dl* expression, *ato*

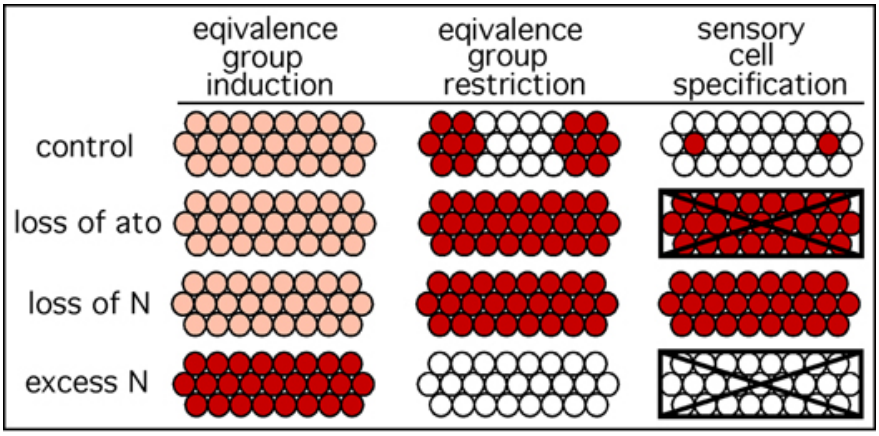

Figure 2.1. *Drosophila ato* as a paradigm for proneural regulation and function. Red circles represent *ato*-expressing cells. Stage-dependent refinement of the expression pattern is altered in distinctive ways by perturbing *ato* or *N* function. In addition, loss of *ato* or excess *N* blocks specification of sensory cells (crossed-out fields of cells).

mutants fail to activate N-mediated restriction of *ato*, resulting in retention of a broad field of *ato*-expressing cells that are otherwise blocked from further development (Jarman et al., 1995; Baker and Yu, 1997). Similarly, *N* mutants also fail to restrict *ato* expression, but in this case all cells differentiate as sensory cells (Baker et al., 1996). Paradoxically, during the prosensory phase of development elevating N activity by expressing N intracellular domain (NICD) enhances *ato* expression (Baker and Yu, 1997). This involves a poorly characterized branch of the N pathway not requiring Su(H) (Ligoxygakis et al., 1998). During subsequent phases of development, NICD activates the canonical N pathway and abolishes *ato* expression. This work provides clear predictions for how vertebrate *Atoh1* might function assuming it acts as a classic proneural gene. In contrast, terminal differentiation factors like NeuroD are insensitive

to N activity and are not required for cell fate specification (Chitnis and Kintner, 1996; Reviewed by Brunet and Ghysen, 1999; Hassan and Bellen, 2000).

A crucial determinant of proneural gene function is the regulatory context in which it operates (Niwa et al, 2004). Activation of *ato* requires combinatorial signaling and specific regional identity genes like *eyeless* (*Pax6*), which also modify the sensory fate specified by *ato* (Niwa et al., 2004). The factors that induce *Atoh1* in the ear and cooperate in its function are largely unknown. *Sox2* is expressed broadly in the early otic vesicle in mouse and is required for induction of *Atoh1* several days later (Kiernan et al., 2005b). The lag in *Atoh1* expression suggests that Sox2 works combinatorially with other factors to initiate prosensory development. A number of signaling molecules have also been implicated in sensory epithelium development (Pirvola et al., 2002; Stevens et al., 2003; Daudet and Lewis, 2004; Brooker et al., 2006; Kiernan et al., 2006; Pujades et al., 2006), but their relationships to *Atoh1* expression remain unknown. Identifying the upstream activators of *Atoh1* is essential for understanding the regulatory network leading to formation and maintenance of hair cells.

Here we investigate the role of zebrafish *atoh1* genes, *atoh1a* and *atoh1b*, in hair cell development. Gene knockdown shows these genes play essential roles during successive stages of hair cell development, beginning in the preotic placode. Interactions with the Delta-Notch pathway strongly support a classic proneural role for *atoh1*. We also show that Fgf and members of the Pax2-5-8 family of transcription factors are required for induction or maintenance of *atoh1* expression. These data reveal

a complex gene network in which *atoh1* genes play vital roles at multiple stages of sensory epithelium development.

MATERIALS AND METHODS

Strains and developmental conditions

The wild-type strain was derived from the AB line (Eugene, OR). The *mib*^{ta52b} and *noi*^{tu29a} mutations are likely null alleles (Lun and Brand, 1998; Itoh et al., 2003). *b380* is a deletion of *dlx3b* and *dlx4b* and mutants are easily identified after 1l hpf by lack of somitic segmentation (Fritz et al, 1996). The *hsp70-dnSu(H)* line was developed by Latimer et al. (2005), and the *hsp70-Gal4* and *UAS-NICD* lines were developed by Scheer and Campos-Ortega (1999). About 25% of embryos were affected by dnSu(H) and NICD in these lines, respectively. Embryos were developed in fish water containing methylene blue at 28.5° and staged according to standard protocols (Kimmel et al., 1995). At least 30 embryos were observed for each time-point except where noted.

In situ hybridization

In situ hybridization was performed at 67°C as described (Jowett and Yan, 1996; Phillips et al., 2001).

Immunofluoresence

Antibody staining was performed as described by Riley et al., (1999). Primary antibodies: Pax2 (Covance, diluted 1:100), acetylated tubulin (Sigma T-6793, diluted

1:100). Secondary antibodies: Alexa 546-conjugated goat anti-rabbit IgG (Molecular Probes A-11010, diluted 1:50) or Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes A-11001, diluted 1:50).

Misexpression

The *atoh1a* plasmid was obtained from Reinhard Köster. To misexpress *atoh1a* under the control of the CMV promoter, 30-90 pg of plasmid was injected into 1-cell embryos. For RNA misexpression, wild-type mRNA was synthesized in vitro using mMessage mMachine kit (Ambion). A total of 60-80pg of mRNA was injected into 1-cell embryos, or was coinjected with *atoh1a/atoh1b* double MO.

Morpholinos

Morpholinos were obtained from Gene Tools, Inc. For most experiments, 5 ng of morpholino was injected into 1-cell embryos. Morpholinos for *dlx3b*, *dlx4b*, *fgf3, foxi1, pax2b* and *pax8* were described previously (Solomon and Fritz, 2002; Mackereth et al. 2005). Additional morpholino sequences are as follows: *atoh1b*-MO 5' TCATTGCTTGTGTAGAAATGCATA T 3'; *atoh1a*-MO1 5' TCTGTTGGTTTGTGCTTTTGGGAGG 3'; *atoh1a*-MO2 5'AAAGTTTGTGGCTAT GGATACAGGG 3'; *atoh1a*-MO3 5' ATCCATTCTGTTGGTTTGTGCTTT T 3'. *atoh1a*-MO3 was used for most experiments. The phenotypes caused by injection of *atoh1a* and/or *atoh1b*-MOs affected 90-100% of embryos, except where noted.

SU5402 inhibitor treatment

SU5402 was dissolved in DMSO to prepare a 40 mM stock solution. Embryos were treated in their chorions with 50 µM SU5402 (10-14 hpf), 80 µM (12-18 hpf), or 100 µM (18-24 hpf). Controls were incubated in an equal concentration of DMSO as that of treated embryos. To terminate treatment, embryos were washed several times and either allowed to develop further or fixed and processed immediately.

RESULTS

Requirement of *atoh1* genes for hair cell development

It was shown previously that zebrafish *atoh1a* (formerly *zath1*) is expressed in hair cells in the inner ear and lateral line (Itoh and Chitnis, 2001; Whitfield et al., 2002). We designed three different morpholino oligomers (MOs) to block translation of *atoh1a*, all of which affected hair cell development. While two of these MOs caused varying degrees of non-specific cell death in the neural tube, the third was effective at a dose that had no discernable toxicity and was therefore used for the remainder of this study. Injection of *atoh1a*-MO strongly impairs formation of hair cells in the inner ear (Fig. 2.2 U). Tether cells, an early-forming hair cell required for otolith localization (Riley et al., 1997), are not affected in *atoh1a* morphants and otoliths form normally (Fig. 2.2 G). Tether cells, named for their precocious kinocilia, initially form in pairs at both ends of the nascent otic vesicle and later adopt the morphology of fully developed hair cells by 22 hpf. Normally, later-forming hair cells begin to accumulate soon after 24 hpf.

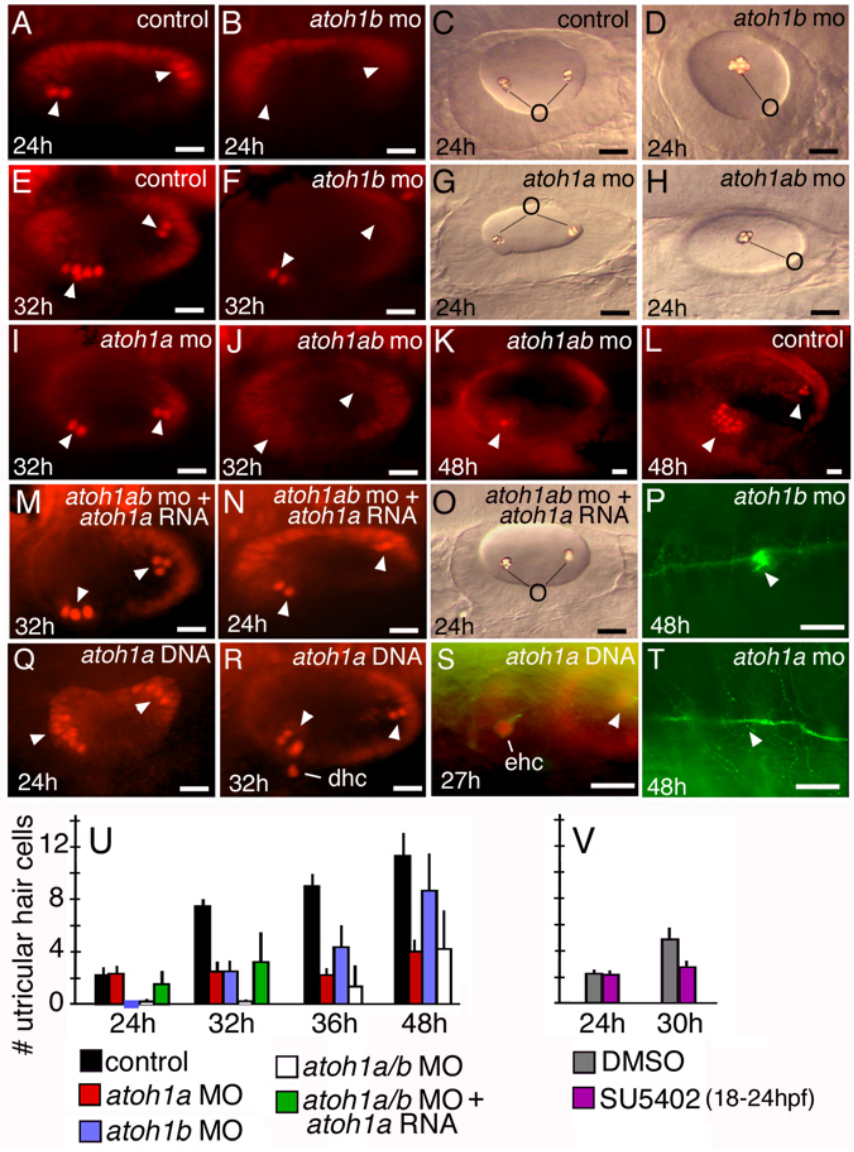

Figure 2.2. Requirement for *atoh1* in hair cells in the ear and lateral line. All panels show dorsolateral views with anterior to the left and dorsal up. (A, B, E, F, I-N, Q-S) Pax2 antibody staining of otic hair cells (arrowheads) at the indicated times in control embryos (A, E, L), *atoh1a* morphant (I), *atoh1b* morphants (B, F), *atoh1ab* double morphants (J, K), *atoh1ab* double morphant coinjected with *atoh1a* mRNA (M, N), and embryos injected with *atoh1a*-plasmid (Q-S). *atoh1a*-plasmid stimulates production of supernumerary hair cells at 24 hpf (Q), but these are not maintained at 32 hpf (R), and instead displaced hair cells (dhc) appear ventrally within subjacent mesenchyme, leaving gaps in the hair cell layer. An ectopic hair cell (ehc) is revealed anterior to the otic vesicle by co-staining with Pax2a (red) and acetylated-tubulin (green) (S). (C, D, G, H, O) Otoliths (o) produced in control (C), *atoh1a* morphant (G), *atoh1b* morphant (D) *atoh1ab* double morphant (H) and *atoh1ab* double morphant coinjected with *atoh1a* RNA (O). (P,T) Acetylated-tubulin staining of the lateral line and neuromasts (arrowheads) in *atoh1b* morphant (P) and *atoh1a* morphant (T) at 48hpf. (U, V) The mean (± standard deviation) of Pax2-postive hair cells present in the utricle at the indicated times and under the indicated conditions. Sample sizes ranged from 15-35 embryos per time point. Scale bar, 15 µm.

However, later-forming hair cells are profoundly impaired in all *atoh1a* morphants as additional hair cells are not evident until 48 hpf (Fig. 2.2 I,U, and data not shown).

Adolf et al. (2004) recently described a second zebrafish *atonal* homolog, *atoh1b*, that we hypothesized might also play a role in hair cell development. In contrast to *atoh1a*-MO, injection of *atoh1b*-MO ablates tether cells in both the utricle and saccule (Fig. 2.2 B) in all specimens. Later-forming hair cells are still produced, albeit more slowly than normal (Fig. 2.2 F,U). A single otolith is produced but initially forms as an untethered mass due to the absence of tether cells (Fig. 2.2 D). Otoliths eventually bind to utricular hair cell cilia after 30 hpf (not shown).

Coinjection of *atoh1a*-MO and *atoh1b*-MO ablates all hair cells in the inner ear in > 90% of specimens (Fig. 2.2 J,U). This was confirmed using phalloidin to mark stereocilia and antiactetylated tubulin staining of kinocilia (not shown). A single untethered otolith is produced (Fig. 2.2 H) reflecting loss of tether cells. Hair cells do begin to form by 48 hpf in *atoh1ab* double morphants (Fig. 2.2 K,U), probably reflecting diminishing capacity of the MOs to knock down *atoh1* function at later stages. Thus, *atoh1* function is essential for hair cell formation in zebrafish as in mouse. Moreover, the data support a model in which *atoh1b* preferentially regulates development of tether cells while *atoh1a* regulates later forming hair cells.

Neuromasts of the lateral line are also ablated by knocking down *atoh1a* (Fig. 2.2 T). However, knocking down *atoh1b* has no effect on neuromasts (Fig. 2.2 P). These data are consistent with findings that neuromasts express and require *atoh1a* but not *atoh1b* (Itoh and Chitnis, 2001; Sarrazin et al., 2006; and our unpublished observations).

Misexpression of *atoh1a*

To test whether the effects of *atoh1*-MOs on hair cell development could be rescued, *atoh1ab* double morphants were coinjected with 80 pg of *atoh1a* mRNA. More than half of these coinjected embryos produce tether cells, tethered otoliths, and later-forming hair cells (Fig. 2.2 M-O), indicating substantial rescue from the effects of the MOs. These data show that loss of hair cells in *atoh1*-morphants is a specific consequence of disrupting *atoh1* function.

Injecting 80 pg of *atoh1a* mRNA (with or without MOs) did not lead to formation of excess or ectopic hair cells. This is in contrast to mouse in which misexpression of *atoh1* promotes formation of ectopic hair cells in tissues immediately surrounding endogenous sensory epithelia (Zheng and Gao, 2000; Woods et al., 2004; Izumikawa et al., 2005). Because injected mRNA may not be stable enough to strongly affect later stages of otic development, we injected zebrafish embryos with plasmid DNA to misexpress *atoh1a* under the control of the powerful and ubiquitously expressed CMV promoter. Injection of 90 pg of *atoh1a* plasmid caused axial truncation in up to 30% of embryos whereas injection of 30 pg or 60 pg did not alter overall embryonic morphology (not shown). Embryos injected with 60 pg or 90 pg of *atoh1a* plasmid often showed expanded sensory patches at 24 hpf (Fig. 2.2 Q). By 30 hpf, however, many supernumerary hair cells are lost while isolated Pax2-positive cells appear sporadically in the subjacent mesenchyme (Fig. 2.2 R). The latter are likely to be dying hair cells as suggested by general elevation of acridine orange staining (not shown). We showed in another study that dying hair cells are often extruded from the otic vesicle to the

underlying mesenchyme (Kwak et al., 2006). This also occurs in *mind bomb* (*mib*) mutants, which form supernumerary hair cells that are later extruded as they undergo apoptosis (Haddon et al., 1999). It is possible that excess hair cells die because forced expression of *atoh1a* bypasses vital processes required for hair cell maintenance. We also cannot exclude the possibility of non-specific toxicity associated with concentrated plasmid-injection. In addition to changes in the otic vesicle, about 1/3 of embryos injected with *atoh1a* plasmid also formed ectopic Pax2a-positive cells in the surface ectoderm just anterior or posterior to the otic vesicle. Double labeling with acetylated tubulin antibody confirms that some of these cells are hair cells (Fig. 2.2 S). Although ectopic hair cells formed at the level of the lateral line, *pax2a* expression indicates these are not lateral line neuromasts. These data show that in zebrafish as in mouse, *atoh1* misexpression can induce excess and ectopic hair cells, but only in regions close to the endogenous hair cell domains. This is consistent with findings that bHLH proteins work combinatorially with other transcription factors, such as Hox and Pax proteins, whose regional expression establishes restricted zones of competence (Niwa et al., 2004; reviewed by Westerman et al., 2003).

Expression of *atoh1a* and *atoh1b* during normal development

Otic expression of *atoh1a* begins at 14 hpf in two domains in the otic placode, marking the primordia of the utricular and saccular sensory epithelia (Fig. 2.3 A). As hair cells begin to differentiate, *atoh1a* expression upregulates in the hair cell layer but weak expression is also detected in the basal cell layer. The latter may represent nascent hair

cells in the earliest stages of differentiation (Fig. 2.3 C). Expression continues in the sensory maculae through at least 48 hpf. Expression is also seen in the sensory cristae by 48 hpf (not shown).

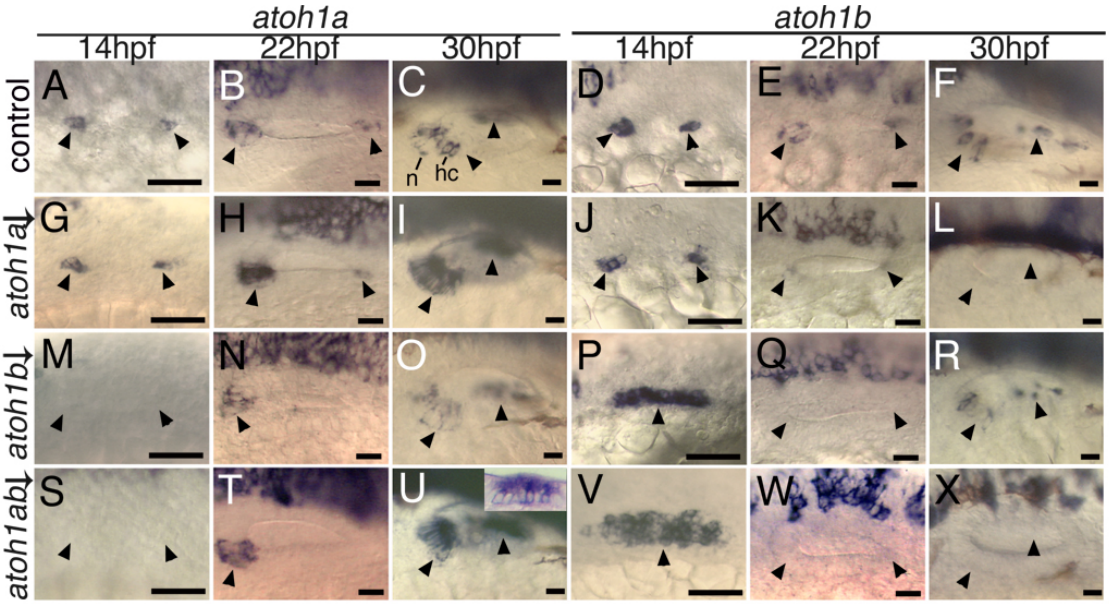

Figure 2.3. Atoh1-dependent and independent expression of *atoh1* genes. Dorsolateral views (anterior to left) showing expression of *atoh1a* (A-C, G-I, M-O, S-U) and *atoh1b* (D-F, J-L, P-R, V-X) in control (A-F) *atoh1a* morphant (G-L), *atoh1b* morphant (M-R) and *atoh1ab* double morphant (S-X) embryos at the indicated times. Expression of *atoh1a* at 32 hpf in mature hair cells (hc) and putative nascent hair cells (n) is indicated in (C). Arrowheads indicate observed or expected domains of otic expression. Inset in U shows a parasagittal section through the anterior *atoh1a* expression domain. Scale bar, 15 µm.

Expression of *atoh1b* begins much earlier, marking the medial edge of the preotic placode by 10.5 hpf (Fig. 2.3 A,B). This pattern resolves into two discrete patches by 14 hpf, encompassing the future sensory epithelia (Fig. 2.3 D). At this stage, expression of *atoh1b* overlaps with *atoh1a*, but *atoh1b* is expressed at a higher level (compare Fig. 2.3

A,D). By 22 hpf, *atoh1b* expression diminishes and marks only a subset of the *atoh1a* domain (Fig. 2.3 E,F). These differences in temporal expression are consistent with the notion that *atoh1b* acts early in otic development while *atoh1a* predominates during later development of sensory epithelia.

Auto- and crossregulation of *atoh1* gene expression

Because proneural genes often regulate their own expression, we examined expression of *atoh1a* and *atoh1b* in embryos knocked down for either or both functions. In *atoh1b* morphants, preplacodal expression of *atoh1b* is not altered (not shown). However, *atoh1b* expression fails to become restricted to two sensory primordia in the otic placode at 14 hpf (compare Fig. 2.3 D,P). Expression of *atoh1b* ceases by 16 hpf in *atoh1b* morphants (Fig. 2.3 Q and data not shown), indicating that *atoh1b* is required to maintain its own transcription. Interestingly, macular expression of *atoh1b* returns after 24 hpf (Fig. 2.3 R).

atoh1a is not expressed in *atoh1b* morphants until around 20 hpf and is limited to the utricular (anterior) macula (Fig. 2.3 M,N). By 30 hpf, *atoh1b* morphants show *atoh1a* expression in both utricular and saccular maculae, although the level of expression is lower than normal (Fig. 2.3 O). These data show that *atoh1a* requires *atoh1b* for expression in the otic placode but not in the otic vesicle after 20 hpf. Once activated, *atoh1a* could be responsible for reactivation of *atoh1b* expression after 24 hpf (Fig. 2.3 R).

In *atoh1a* morphants, *atoh1a* and *atoh1b* are expressed normally through 20 hpf (Fig. 2.3 G,J, and data not shown). By 22 hpf, *atoh1a* morphants begin to express *atoh1a* at higher than normal levels (Fig. 2.3 H,I). Conversely, *atoh1b* expression is nearly extinguished by 22 hpf and cannot be detected after 24 hpf (Fig. 2.3 K,L). These data show that *atoh1a* is necessary to maintain *atoh1b* expression after 22 hpf and that *atoh1a* limits its own expression.

In *atoh1ab* double morphants, *atoh1b* is expressed in an expanded domain at 14 hpf but is not maintained in the ear after 16 hpf (Fig. 2.3 V-X and data not shown). Expression of *atoh1a* cannot be detected until 22 hpf, after which it is expressed at higher than normal levels (Fig. 2.3 S-U). Sections show that the epithelium has only a single layer of columnar cells that express high levels of *atoh1a* (Fig. 2.3 U inset).

Taken together, these data show that *atoh1b* acts early to establish and refine the sensory equivalence group and to induce early expression of *atoh1a*, while *atoh1a* is required later to maintain expression of *atoh1b* and to limit its own expression. The requirement for *atoh1b* to restrict its own expression domain at such an early stage is consistent with the possibility that it acts as a classic proneural gene (Fig. 2.1). The data also confirm that *atoh1b* is required for differentiation of tether cells whereas *atoh1a* is required for later forming hair cells.

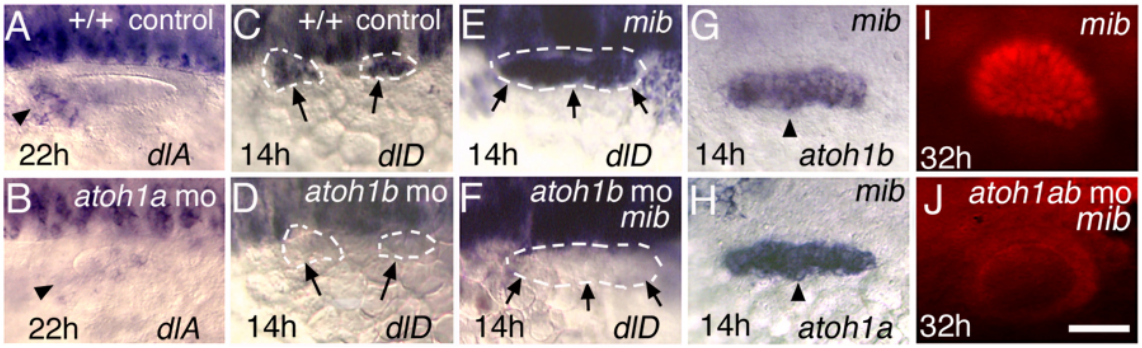

Figure 2.4. Interactions between *atoh1* and the Delta-Notch pathway. (A, B) Expression of *dlA* at 22 hpf in a control embryo (A) and *atoh1a* morphant (B). (C-F) Expression of *dlD* at 14 hpf in a control embryo (C), *atoh1b* morphant (D), *mib* mutant (E), and *mib* mutant-*atoh1b* morphant (F). (G, H) *mib* mutants show expanded otic domains of *atoh1b* (G) and *atoh1a* (H) at 14 hpf. (I, J) Pax2 antibody staining at 32 hpf reveals supernumerary hair cells in a *mib* mutant (I) but no hair cells in a *mib* mutant coinjected with *atoh1a*-MO and *atoh1b*-MO (J). Arrowheads and arrows indicate otic regions. All images are dorsolateral views with anterior to the left. Scale bar, 30 μm (A, E, I-P) or 15 μm (B-D, F-H).

Involvement of *atoh1* genes in Delta-Notch signaling

Proneural genes often limit their own expression by transcriptional activation of Delta (Dl), which in turn stimulates Notch (N) and thereby inhibits subsequent proneural gene expression (Baker and Yu, 1997; Parks et al., 1997). In support, knocking down *atoh1b* strongly inhibits expression of *dlA* and *dlD* in the ear at 14 hpf (Fig 2.4 C,D, and data not shown). Similarly, knocking down *atoh1a* diminishes *dlA* and *dlD* expression at 22hpf (Fig 2.4 A,B, and data not shown). Thus, *atoh1* genes are required for normal activation of *delta* gene expression.

To further investigate the role of Dl-N feedback, we examined *atoh1* function in *mind bomb* (*mib*) mutants. The *mib* gene encodes an E3 ubiquitin ligase essential for Dl-N signaling (Itoh et al., 2003). *mib* mutants produce an enlarged domain of both *atoh1a*

and *atoh1b* at 14 hpf, mimicking the failure to restrict expression seen in *atoh1b* morphants (Fig. 2.4 G,H). Because both *atoh1* genes remain fully active in *mib* mutants, *delta* gene expression is also greatly expanded and all cells in the equivalence group complete differentiation as hair cells (Haddon et al., 1999; Riley et al., 1999; Fig 2.4 E,I). However, injection of *atoh1a*-MO and *atoh1b*-MO into *mib* mutants fully suppresses these latter defects, blocking *delta* gene expression and ablating all hair cells in all specimens (Fig. 2.4 F,J). These data further support a role for *atoh1* genes as upstream activators of Dl-N signaling that normally acts to limit and refine *atoh1* expression and function.

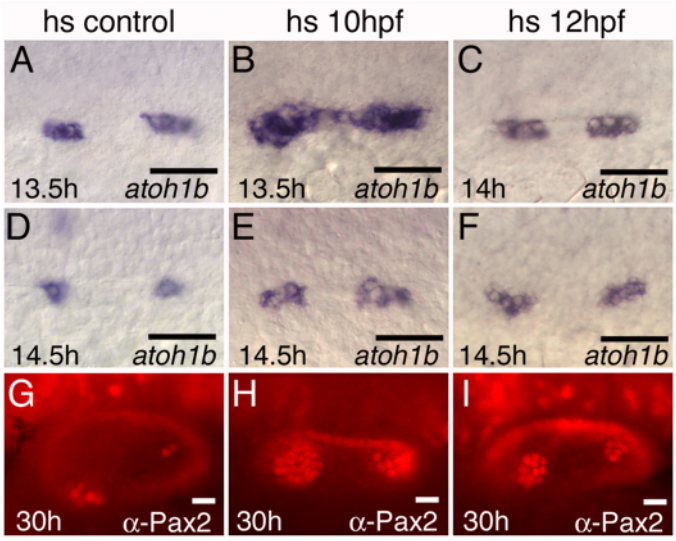

Figure 2.5. Heat shock induction of dnSu(H). Expression of *atoh1b* at 13.5 or 14 hpf (A-C), *atoh1b* at 14.5 hpf (D-F) and Pax2 at 30 hpf (G-I) as seen in control embryos heat shocked at 10 hpf (A, D, G) or *hsp70-dnSu(H)* transgenic embryos heat shocked at 10 hpf (B, E, H) or 12 h pf (C, F, I). Images show lateral views with anterior to the left. Scale bar, 15 µm.

To test the temporal requirements for the canonical N pathway, we used a transgenic line to express a dominant-negative form of Su(H) (dnSu(H)) under the control of *hsp70* promoter (Wettstein et al., 1997; Shoji et al., 1998; Latimer et al., 2005). This promoter induces high-level transcription within 15 minutes following heat shock, providing a pulse of protein accumulation lasting several hours (Scheer et al., 2002). Heat shock induction of dnSu(H) at 8 hpf does not alter *atoh1b* expression or hair cell development (not shown). However, heat shock at 10 hpf causes the initially broad domain of *atoh1b* to be maintained through at least13.5 hpf, about 2 hours longer than normal (Fig. 2.5 B). By 14.5 hpf, expression becomes restricted to two discrete domains that are larger than normal (Fig. 2.5 E). This domain restriction presumably reflects resumption of Dl-N signaling as the pulse of dnSu(H) subsides. However, the enlarged domains show no further reduction after 14.5 hpf and go on to form supernumerary hair cells (Fig. 2.5 H). Heat shock at 12 hpf (after equivalence-group restriction has already begun) also results in maintenance of two large domains and production of excess hair cells (Fig. 2.5 C,F,I). Heat shock at 14 hpf has little effect on *atoh1b* expression or hair cell formation (not shown). These data show that equivalence group restriction can still occur after 13.5 hpf but then *atoh1b* expression stabilizes by 14.5 hpf regardless of domain-size, defining an interval during which cell fates are specified.

To test how N gain-of-function affects *atoh1* gene expression (as in Fig. 2.1), we used a heat shock-inducible Gal4-UAS system to drive expression of N intracellular domain (NICD) (Scheer and Campos-Ortega, 1999). In this system, heat shock induces

sustained NICD expression for at least 17 hours (Scheer et al., 2002). Heat shock induction of NICD at 9 hpf or 10 hpf does not prevent induction of *atoh1b* in the preotic placode (Fig. 2.6 B). However, *atoh1b* expression is lost by 12 hpf (Fig. 2.6 D). In addition, *atoh1a* is never activated and no hair cells are produced (not shown). Heat shock induction of NICD at 18 hpf also rapidly extinguishes *atoh1* expression and blocks hair cell formation (not shown). We also examined the effects of NICD in *atoh1b* morphants, which usually have no functional equivalence group until 20 hpf when *atoh1a* is first expressed. In *atoh1b* morphants, activation of NICD at 18 hpf induces *atoh1a* by 19 hpf, one hour earlier than without NICD (Fig. 2.6 E-G). Expression then subsides by 20 hpf and no hair cells are produced (Fig. 2.6 H, and data not shown). Thus, NICD initially stimulates, or at least does not block, upregulation of *atoh1* genes as the equivalence group forms but then rapidly extinguishes *atoh1* expression at all later stages.

In summary, the relationship between *atoh1* function and the Dl-N pathway is consistent with all predictions of the fly *ato* paradigm (Fig. 2.1). Moreover, *atoh1*-dependent restriction of the equivalence group precedes fate specification by several hours. These findings strongly support a classic proneural mechanism of action for zebrafish *atoh1* genes.

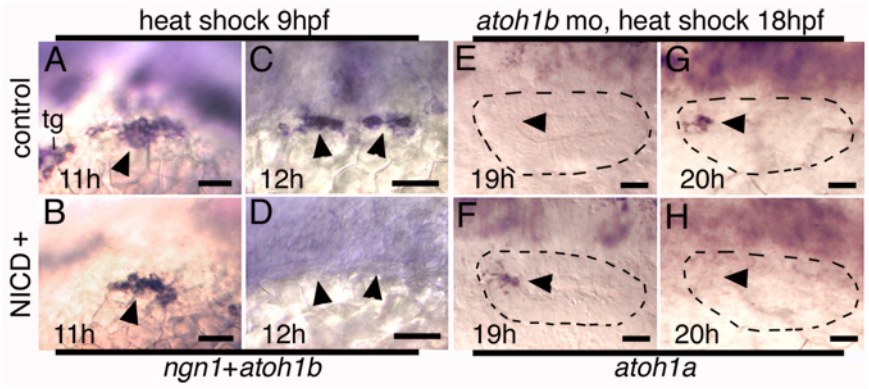

Figure 2.6. Heat shock induction of NICD. (A-D) Expression of *atoh1b* and *ngn1* at 11 hpf (A, B) and 12 hpf (C, D) in control embryos (A, C) or NICD-positive embryos (B, D) heat shocked at 9 hpf. Loss of *ngn1* expression, which is non-overlapping with *atoh1b*, confirms effective NICD-induction. (E-H) Expression of *atoh1a* at 19 hpf (E, F) and 20 hpf (G, H) in *atoh1b* morphants without NICD (E, G) or with NICD (F, H) heat shocked at 18 hpf. Otic vesicles are outlined. Arrowheads mark otic expression domains. tg, trigeminal ganglion. All are lateral views with anterior to the left. scale bar, 15 μm.

Regulation of *atoh1b* in preotic cells

Expression of *pax8* is the earliest known marker of otic placode induction (Pfeffer et al., 1998). *atoh1b* is expressed in a subset of *pax8*-expressing cells in the preotic placode (Fig. 2.7 A,B), raising the possibility that *pax8* is required for early activation of *atoh1b*. Knocking down *pax8* reduces the size of the preotic domain of *atoh1b* (Fig. 2.7 F), but the level of expression appears normal. We next asked whether factors that act upstream of or parallel to *pax8* might also regulate *atoh1b*. Induction of *pax8* requires Foxi1 autonomously within the preplacodal ectoderm, as well as stimulation by Fgf3 and Fgf8 secreted from adjacent hindbrain tissue (Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002; Liu et al. 2003; Solomon et al., 2003; Hans et al., 2004). Knocking down *foxi1* causes severe reduction of *atoh1b* expression (Fig. 2.7 G). To test the role of

Fgf, embryos were treated with the Fgf signaling inhibitor SU5402. Induction of *atoh1b* is blocked in embryos treated from 10-14 hpf (not shown). When SU5402 is added beginning at 10.5 hpf, after the onset of *atoh1b* expression, expression of *atoh1b* is lost in all specimens by 12.5 hpf (Fig. 2.7 D). Expression of *atoh1a* is also blocked (Fig. 2.7 I), consistent with a requirement for *atoh1b* in *atoh1a* induction. Embryos coinjected with *fgf3*-MO and *fgf8*-MO also do not express *atoh1* genes (not shown). Thus, Foxi1 and Fgf signaling are required to initiate and maintain expression of *atoh1b* in the preotic placode, and Pax8 is needed to produce a normal sized domain.

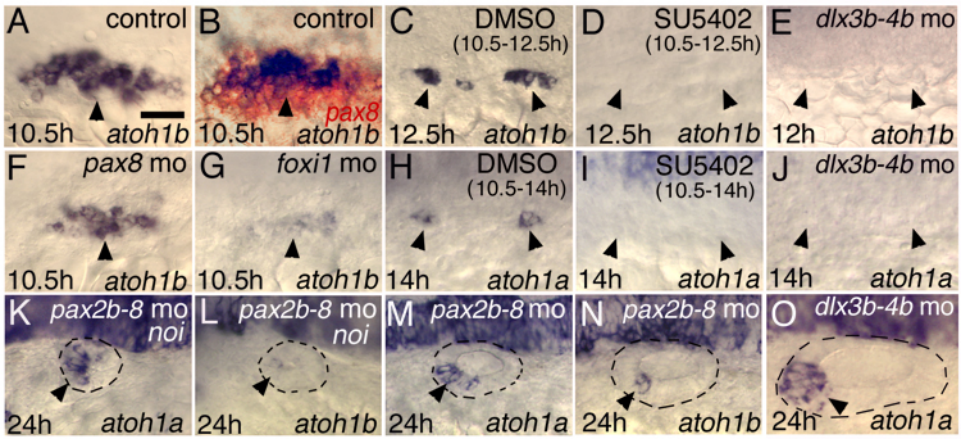

Figure 2.7. Inducers of early *atoh1* expression. (A, B, F, G) Expression of *atoh1b* at 10.5 hpf in a control embryo (A, B), *pax8* morphant (F) and *foxi1* morphant (G). The specimen in (B) was double stained to reveal *pax8* expression (red). (C, D) Expression of *atoh1b* at 12.5 hpf in embryos treated from 10.5-12.5 hpf with DMSO alone (C) or SU5402 in DMSO (D). (H, I) Expression of *atoh1a* at 14 hpf in embryos treated from 10.5-14 hpf with DMSO alone (H) or SU5402 in DMSO (I). (E, J, O) *dlx3b-dlx4b* morphants showing expression of *atoh1b* at 12 hpf (E) or *atoh1a* at 14 hpf (J) or 24h hpf (O). (K-N) Expression at 24 hpf of *atoh1a* (K,M) and *atoh1b* (L,N) in *noi* mutants injected with *pax2b-pax8*-MO (K,L), and in wild-type embryos injected with *pax2b-pax8*-MO (M,N). All are dorsolateral views with anterior to the left. Arrowheads indicate observed or expected domains of otic expression. Scale bar, 30 µm (A, B, F, G, K-O) or 10 µm (C-E, H-J).

Distal-less genes *dlx3b* and *dlx4b* also regulate early otic development but in a distinct pathway acting parallel to *foxi1-fgf-pax8*. Loss of *dlx3b* and *dlx4b* does not block induction of *pax8* but subsequent steps in otic development fail (Solomon and Fritz, 2002; Liu et al., 2003; Hans et al., 2004). Accordingly, neither *atoh1a* nor *atoh1b* are expressed in *dlx3b-dlx4b* morphants during placodal development (Fig. 2.7 E,J). Similarly, *b380* mutants, which are deleted for *dlx3b* and *dlx4b* (Fritz et al., 1996) also fail to express *atoh1* genes in the otic placode (not shown). Later in development, *dlx3b-dlx4b* morphants produce small otic vesicles containing only anterior (utricular) sensory patches. Tether cells do not form, consistent with loss of early *atoh1b*, but later hair cells begin to form after 24 hpf (not shown) in association with belated expression of *atoh1a* (Fig. 2.7 O). Dlx proteins could act directly on *atoh1b* transcription or indirectly by regulating competence to respond properly to Fgf after initial otic induction, as suggested by recent studies (Hans et al., 2004; Solomon et al., 2004).

Pax2 and Pax8 proteins maintain *atoh1b*

Pax8 normally cooperates with closely related proteins Pax2a and Pax2b to maintain the otic placode (Hans et al., 2004; Mackereth et al., 2005). Knockdown of *pax8* and *pax2b* in embryos homozygous for a null mutation in *pax2a* (*noi* mutants, Lun and Brand, 1998) causes progressive loss of otic tissue and no vesicles are produced. Accordingly such embryos do not express *atoh1a* or *atoh1b* in the otic region (not shown). Reducing the MO concentration by half allows the majority of *pax2a-pax2b-pax8*-deficient embryos to produce small otic vesicles. In 100% of these specimens, *atoh1a* is

expressed at a high level in a nearly normal number of cells at the anterior end of the otic vesicle whereas *atoh1b* expression is barely detectable in any specimen (Fig. 2.7 K,L). Partial knockdown of *pax8* and *pax2b* in wild-type embryos results in a moderately diminished otic vesicle expressing normal levels of both *atoh1a* and *atoh1b* (Fig. 2.7 M,N), although *atoh1b* is typically expressed in only one or two cells. These data show that full expression of *atoh1b* requires Pax8 and Pax2 functions. In contrast, *atoh1a* expression is not strictly dependent on Pax2/8 function.

Continuing requirements for Fgf

As the otic vesicle forms, *fgf3* and *fgf8* begin to be expressed in domains encompassing the sensory epithelia (Leger and Brand, 2002). To test whether Fgf signaling regulates *atoh1* expression after placode formation, embryos were treated with SU5402 for various intervals at successively later stages of development. Treatment from 12-18 hpf did not affect *atoh1b* but reduced expression of *atoh1a* (Fig. 2.8 A-D). When embryos were treated at 18 hpf for one, two, four or six-hour intervals, expression of both *atoh1a* and *atoh1b* were strongly reduced but not eliminated (Fig. 2.8 E-H). We hypothesized that the period of SU5402-insensitivity of *atoh1b* from 12 to 18 hpf reflects maintenance of *atoh1b* by autoregulation. Furthermore, since *atoh1a* and *atoh1b* help maintain each other at later stages, cross-regulation could account for residual expression seen in SU5402-treated embryos. In support, *atoh1b* morphants fail to express either *atoh1a* or

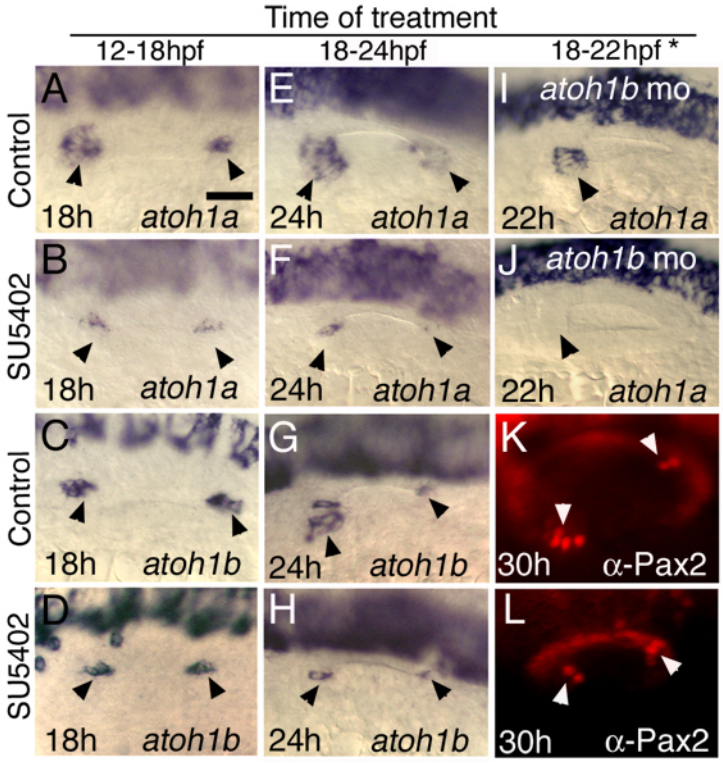

Figure 2.8. Stage-dependent requirements for Fgf. Embryos were treated with DMSO (control) or DMSO plus SU5402 for the indicated time intervals. (A-H) Expression of *atoh1a* in control and SU5402 treated embryos at 18hpf (A, B) and 24hpf (E, F), and expression of *atoh1b* in control and SU5402 treated embryos at 18hpf (C, D) and 24hpf (G, H). (I, J) Expression of *atoh1a* at 22 hpf in *atoh1b* morphants treated with DMSO (I) and DMSO and SU5402 (J). (K, L, *treatment from 18-24 hpf) Pax2 staining of hair cells at 30hpf in embryos treated with DMSO (K) or DMSO and SU5402 (L). All images are dorsolateral views with anterior to the left. Black arrowheads indicate otic expression. White arrowheads indicate sensory epithelia. Scale bar, 30 µm.

atoh1b when treated with SU5402 from 18-22 hpf (Fig. 2.8 J). We next tested the effects of SU5402 on hair cell formation. In embryos treated from 18-24 hpf, tether cells were produced normally (not shown). This was not unexpected because tether cells are already present in the otic vesicle at 18 hpf and hence their specification cannot be blocked by this treatment. However, production of later forming hair cells was strongly impaired during the 6-hour period following removal of the inhibitor (Fig. 2.8 L, Fig. 2.2 V). Presumably the severe reduction in *atoh1* expression seen at 24 hpf delays resumption of macular development. These data show that *atoh1* expression and hair cell development require ongoing Fgf signaling. This marks the first identification of a signaling molecule required to both induce and maintain *atoh1* expression in the vertebrate inner ear.

atoh1-dependent and -independent expression of macular genes

We next tested whether *atoh1* function affects *fgf* or *pax* gene expression. Otic expression of *fgf3* and *fgf8* is normal in *atoh1ab* double morphants (Fig. 2.9 A-D). Likewise, expression of *pax5* in the utricle, which is regulated by Fgf signaling (Kwak et al., 2002; Kwak et al., 2006), is also unaltered in *atoh1ab* double morphants (Fig. 2.9 F). In contrast, knockdown of both *atoh1a* and *atoh1b* strongly reduces the level of *pax2b* expression (Fig. 2.9 H). *pax5* and *pax2b* are both required for normal development and maintenance of hair cells (Whitfield et al., 2002; and our unpublished observations) but only the latter is affected by *atoh1* function. Thus, expression of *fgf* genes and some

downstream targets (*pax5*, *atoh1a*) continue in the macular region despite disruption of *atoh1* function and the absence of a sensory epithelium.

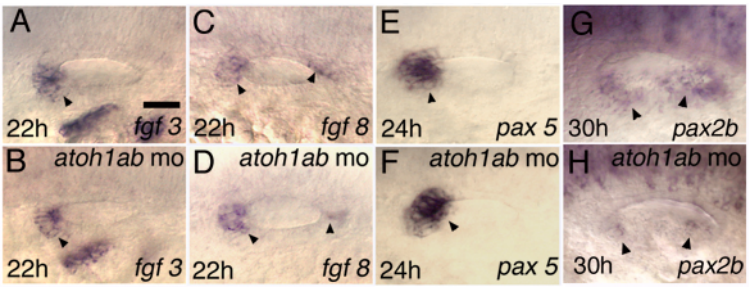

Figure 2.9. Expression of macular genes. Expression of *fgf3* (A, B) and *fgf8* at 22 hpf (C, D), *pax5* at 24 hpf (E, F) and *pax2b* at 30 hpf (G, H) in control embryos (A, C. E. G) and atoh1ab double morphants (B, D, F, H). All panels show dorsolateral views with anterior to the left and dorsal up. Arrowheads indicate expression in sensory epithelia. Scale bar, 30 µm.

DISCUSSION

Our data support a model in which *atoh1a* and *atoh1b* act in a complex network leading to the establishment of a sensory equivalence group and subsequent differentiation of hair cells (Fig. 2.10). There are two distinct phases of *atoh1* function. In the first phase, *atoh1b* establishes a single prosensory domain during preplacodal development and subsequently activates Delta-Notch feedback to split the domain into separate utricular and saccular primordia in the nascent otic placode by 12 hpf. Lateral inhibition and specification of tether cells occurs by 14 hpf, when Atoh1b also activates expression of *atoh1a*. In the second phase, beginning soon after formation of the otic vesicle, *atoh1a* expression predominates in the maculae and maintains *atoh1b* in a subset of cells.

Moreover, *atoh1a* is primarily responsible for specifying later forming hair cells and activating Delta-Notch mediated lateral inhibition.

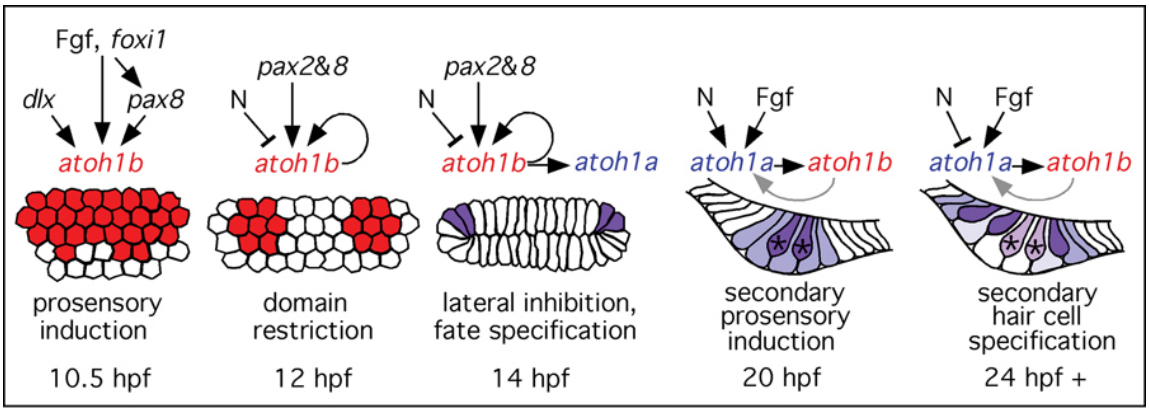

Figure 2.10. Summary of *atoh1* regulation and function. *fgf-foxi1-pax8* and *dlx* pathways induce expression of *atoh1b* (red) in medial preotic cells, specifying the prosensory equivalence group. By 12 hpf, the domain is restricted into two intermediate groups by Dl-N activity, which is activated by *atoh1b* function. Tether cells are specified around 14 hpf as *atoh1a* is activated (blue, coexpression with *atoh1b*, purple). At 20 hpf, N and Fgf activate a wider domain of *atoh1a* associated with later forming hair cells. Tether cells (asterisks) terminally differentiate. *atoh1a* is required to maintain or activate *atoh1b* in differentiating cells, and *atoh1b* helps maintain high levels of *atoh1a*. At 24 hpf and thereafter, later forming hair cells begin to differentiate and coexpress *atoh1* genes and N activity limits *atoh1* expression. Mature tether cells and hair cells downregulate *atoh1* expression.

Fgf signaling is an essential upstream activator of *atoh1* expression during both phases, though *atoh1b* becomes independent of Fgf after 12 hpf. This could reflect the onset of *atoh1b*-autoregulation. A similar transition occurs with *Drosophila ato*, which becomes autoregulatory as it initiates domain-restriction and lateral inhibition (Sun et al., 1998). Fgf may facilitate *atoh1b*'s transition to autoregulation, similar to the role of EGFR and MAP Kinase activity in promoting autoregulation of *Drosophila ato* during

sensory organ development (zur Lage et al., 2004). Unlike *atoh1b*, maintenance of *atoh1a* remains heavily dependent on Fgf but is not dependent on *atoh1* function after 20 hpf. Indeed, *atoh1ab* morphants maintain higher than normal expression of *atoh1a*. This is probably because *fgf* genes continue to be expressed (Fig. 2.9) and promote *atoh1a* expression in the absence of N-mediated feedback inhibition.

The overlapping yet distinct functions of zebrafish *atoh1* genes likely reflects evolutionary "subfunctionalization" (Force et al., 1999). Following a genome duplication thought to have occurred early in the teleosts lineage, duplicate copies of genes often diverge in regulation to subdivide the ancestral function. Only *atoh1b* is required for development of tether cells, which are analogous to primary neurons. Because such precocious cell types are typical of anamniote embryos, this probably reflects an ancestral *atoh1* function. *atoh1a* has apparently lost regulatory elements required to respond to the *fgf-foxi1-pax* and *dlx* pathways involved in *atoh1b* induction. However only *atoh1a* is essential for later hair cells, which continue to form well beyond embryonic development. This too is probably an ancestral *atoh1* function. Sensory epithelia continue to expand throughout life in teleosts, suggesting ongoing recruitment of new cells into the equivalence group. Fgf-dependent induction of *atoh1a* in adjacent cells might account for such recruitment, a function similar to the role of EGFR and *ato* in recruiting new sensory organ precursors in the *Drosophila* chordotonal organs (zur Lage et al., 1997). The two Atoh1 proteins probably retain similar DNA-binding properties, however, as misexpression of *atoh1a* can restore tether cell formation in *atoh1ab* double morphants (Fig. 2.2 M-O).

Zebrafish *atoh1* genes have proneural function

There have been differing opinions as to whether vertebrate *Atoh1* genes act as classic proneural genes or only as terminal differentiation factors (Reviewed by Kelley, 2006). Specific comparisons between zebrafish *atoh1* genes and *Drosophila ato* (Fig. 2.1) reveal striking parallels. More generally, various authors have used four criteria to define proneural function (Brunet and Ghysen, 1999; Hassan and Bellen, 2000; Westerman et al., 2003) that can be applied to zebrafish *atoh1* genes. First, proneural genes are expressed prior to sensory fate specification. *atoh1b* is induced broadly in the preotic placode at 10.5 hpf whereas specification of tether cells (stabilization of *atoh1* expression) does not occur until 14 hpf. Second, proneural genes are subject to lateral inhibition (and the related process of domain-restriction) via N-mediated repression. Zebrafish *atoh1* genes, once induced, are readily repressed by N activity. Moreover, both *atoh1* genes facilitate their own repression by autonomously activating *delta* expression. Third, proneural function is necessary for producing the equivalence group for the entire sensory structure. *atoh1ab* morphants produce only a simple epithelium lacking hair cells; and while support cell markers are not known in zebrafish, it is important to note that the epithelium continues to express *atoh1a*. Since loss of *atoh1* expression marks the first step in support cell specification, these cannot be support cells. Fourth, proneural function is sufficient to induce ectopic sensory development. Misexpression of *atoh1a* induces ectopic hair cells, though only in limited regions near the otic vesicle or endogenous sensory epithelia, as has been shown for *Atoh1* in mammals (Zheng and Gao, 2000; Woods et al., 2004; Izumikawa et al., 2005).

Competence to respond appropriately to Atoh1 may require a unique combination of additional factors. The zone of competence could be influenced by *pax2-5-8* genes, which are coregulated with *atoh1* genes by Fgf signaling. Other signaling pathways have also been implicated in this process. Misexpressing components of the Notch or Wnt pathways in chick can also induce ectopic sensory patches, but only in restricted regions near endogenous sensory patches (Steven et al., 2003; Daudet and Lewis, 2004). Combinatorial signaling and restricted zones of competence also influence the functions of proneural genes in *Drosophila* (Westerman et al., 2003; Niwa et al., 2004). Thus, while many additional details need to be resolved, zebrafish *atoh1* genes meet all four criteria used to define proneural function.

Conserved mechanisms

While mammals show no early phase of specification analogous to tether cell development, and sensory epithelia develop only during a limited stage of embryogenesis, some aspects of sensory development have been conserved. The clearest example is the role of N signaling. *Dll1* and *Jag2* encode N ligands that regulate the balance of hair cells and support cells in the mouse cochlea. Loss of *Jag2* causes a modest increase in hair cells (Lanford et al., 1999; Kiernan et al., 2005a), as does anti-sense knockdown of *N1* in cochlear cultures (Zine et al., 2000). Loss of *Dll1* causes a larger increase in hair cells (Brooker et al., 2006), and disrupting both *Dll1* and *Jag2* causes a dramatic increase in hair cells and a modest decrease in support cells (Kiernan et al., 2005a). The number of support cells is greater than expected because support

cells continue to divide longer than normal, partially offsetting earlier deficiencies. Although no phenotype comparable to zebrafish *mib* has been described in mouse, the mouse data nevertheless support the lateral inhibition model well. Residual support cell development likely reflects the activity of another N ligand, Jag1. *Jag1* is initially expressed throughout the prospective sensory region and later becomes restricted to support cells during differentiation. It has been proposed that Jag1 signaling between support cells augments lateral inhibitory signals from hair cells (Eddison et al., 2000). Indeed, partial loss of *Jag1* also leads to excess hair cell production (Zine et al., 2000; Kiernan et al, 2001). However, conditional knockouts of *Jag1* ablate much, though not all, of the sensory epithelia (Brooker et al., 2006; Kiernan et al., 2006). This supports a model in which Jag1's function changes with time, initially promoting the early inductive phase of N signaling and later augmenting lateral inhibition. While the mechanistic basis for the shift from inductive to repressive N signaling remains unknown, similar transitions occur in the regulation of *Drosophila ato* and zebrafish *atoh1a* (Baker and Yu, 1997; Fig. 2.6 F,H). It is not known whether mouse also shows N-dependent restriction of the initial equivalence group.

Fgf signaling may also play a conserved role in mammals. A number of Fgfs are expressed in the otic vesicle and developing sensory epithelia in mouse, but in most cases their role in hair cell formation is obscured by severe morphogenetic defects caused by specific gene knockouts. However, hypomorphic alleles of *Fgfr1* severely reduce hair cell production in the cochlea without blocking morphogenesis (Pirvola et al., 2002). Furthermore, Pirvola et al. (2002) have proposed that Fgfs produced by inner

hair cells in the Organ of Corti stimulate differentiation of later forming outer hair cells through activation of Fgfr1.

A potential difference between mouse and zebrafish is the question of whether mouse *Atoh1* has proneural activity (reviewed by Kelley, 2006). This is especially evident when considering the mammalian cochlea, which is a highly derived structure that differs in important ways from the more primitive maculae and cristae. However, as summarized below, available data are complex and can be considered inconclusive. *Atoh1* is necessary for hair cell differentiation and is sufficient for inducing ectopic hair cells (Bermingham et al., 1999; Zheng and Gao, 2000; Woods et al., 2004; Izumikawa et al., 2005). *Atoh1* is also subject to autoregulation (Helms et al., 2000), which in other species facilitates pattern refinement during lateral inhibition. Unfortunately a direct link between lateral inhibition and *Atoh1* has not been shown in mouse. *Atoh1* is initially expressed in a broad domain that spans the full depth of the epithelium, approximately 4-5 cells thick (Bermingham et al., 1999; Lanford et al., 2000; Chen et al., 2002; Woods et al., 2004), but expression is not uniform and some cells appear to express little or no *Atoh1*. These data do not distinguish whether there is an earlier stage of low uniform *Atoh1* expression followed by rapid upregulation and pattern-refinement or, alternatively, whether *Atoh1* marks only differentiating hair cells after fate-specification. Several groups have concluded that mouse *Atoh1* lacks proneural activity based in part on the observation that sensory regions in *Atoh1* knockout mice contain a single layer of cells that morphologically resemble support cells (Bermingham et al., 1999). However, these cells express no definitive markers of mature support cells

(Woods et al., 2005). Early non-restricted expression of *Jag1* occurs normally, but later expression normally associated with support cells is lost. Thus support cell differentiation is disrupted, though it is not clear whether the defect lies in specification or maintenance. Another early marker of the sensory epithelium, $p27^{kip1}$, normally precedes *Atoh1* in expression and continues to be expressed in the prosensory region in *Atoh1* mutants (Chen et al., 2002). This has been interpreted to mean that cells of the equivalence group are specified but fail to differentiate. However, $p27^{kip1}$ plays no role in fate-specification and there are no independent indicators of when the equivalence group forms in mouse. While expression $p27^{kip1}$ is regulated partly by the same inductive signals that specify the equivalence group (Kiernan et al., 2006), upregulation of fate-specifying gene(s) need not follow precisely the same timecourse. Moreover, even if *Atoh1* were necessary for prosensory induction, loss of *Atoh1* would not be expected to block any of the initial transcriptional responses to inductive signals. Thus expression of $p27^{kip1}$ and *Atoh1* in the absence of *Atoh1* function (Bermingham et al., 1999; Chen et al., 2002; Fritzsch et al., 2005) could simply reflect ongoing parallel responses to common upstream activators in cells that are otherwise blocked at an early stage. Similarly, we have shown that several early markers of sensory epithelia in zebrafish (*atoh1a*, *pax5*) are coregulated by Fgfs and continue to be expressed in *atoh1ab* morphants (Fig. 2.9). A similar situation has been documented in *Drosophila ato* mutants, which produce no photoreceptors in the eye but continue to coexpress genes normally preceding formation of the prosensory equivalence group, including *ato* and the N target gene *hairy* (Jarman et al., 1995). In summary, gene expression and genetic

studies in mouse do not necessarily contradict the notion that *Atoh1* might have proneural activity, but key supportive data are also lacking. Resolving this issue will require assessment of precisely when fate specification occurs relative to expression of *Atoh1* and $p27^{kip1}$, how these genes are coregulated, and the epistatic relationships between the various upstream factors including Sox2, Jag1 and Fgf.

CHAPTER III

SOX2 IS REQUIRED FOR MAINTENANCE AND REGENERATION, BUT NOT INITIAL DEVELOPMENT, OF HAIR CELLS IN THE ZEBRAFISH INNER EAR*

OVERVIEW

This is a published account (Millimaki et al. 2010) of the role of *sox2* in maintencance and regeneration of inner ear hair cells. It is primarily the work of my colleague, B.B. Millimaki. However, since I contributed to portions of Figures 3.1, 3.3, 3.4 and a majority of Figure 3.5, I include it here as a record of my work.

INTRODUCTION

The capacity for maintenance and regeneration are fundamental properties of many mature tissues and organ systems. Regeneration often involves reactivation of developmental regulatory factors that coordinate growth and differentiation of pluripotent progenitor cells or stem cells. In the inner ear, sensory epithelia comprise interspersed patterns of sensory hair cells and support cells that in most vertebrates are capable of self-renewal (Corwin and Oberholtzer, 1997; Ozeki et al., 2007; Edge and Chen, 2008).

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* Reprinted with permission from "Sox2 is required fir maintenance and regeneration, but not initial development, of hair cells in the zebrafish inner ear"; by **Millimaki, B. B., Sweet, E. M., Riley, B. B.** *Dev. Bio.* **338**, 262-269.

Hair cells are highly susceptible to a number of environmental insults that can trigger apoptosis. Lost hair cells can be regenerated from support cells through either of two processes: Support cells may directly transdifferentiate into hair cells or, alternatively, undergo asymmetric division to yield a hair cell and another support cell (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Adler and Raphael, 1996). Unfortunately, the capacity for regeneration has been lost in the mammalian cochlea (Ozeki et al., 2007; Corwin and Oberholtzer, 1997; Edge and Chen, 2008), accounting for progressive irreversible hearing loss in humans as we age. To some extent this may be due to elevated expression levels of the mitotic inhibitors p27(Kip1) and Ink4d in support cells (Chen and Segil, 1999; Lowenheim et al., 1999; Chen et al., 2003), thereby preventing regeneration through asymmetric cell division. However, it is not clear why cochlear support cells cannot undergo transdifferentiation.

A candidate for a regulator of maintenance and regeneration of hair cells is *Sox2*. *Sox2* encodes a transcription factor well known for its role in maintaining pluripotent stem cell populations, as well as differentiation during early development. For example, *Sox2* is required to maintain pluripotency in mouse embryonic stem cells (Avilion et al., 2003; Masui et al., 2007) whereas misexpression of *Sox2* facilitates conversion of adult differentiated cell types into pluripotent stem cells (Takahahsi and Yamanaka, 2006; Yu et al., 2007). *Sox2* is also one of the first regulators of early specification of neurectoderm during vertebrate gastrulation (Kishi et al., 2000; Graham et al., 2003). How *Sox2* orchestrates the mutually exclusive activities of maintaining pluripotency vs.

stimulating differentiation is not fully understood. In sensory epithelia of the inner ear, *Sox2* is initially expressed in progenitors of both hair cells and support cells (Kiernan et al., 2005; Hume et al., 2007; Neves et al., 2007). It is eventually lost from hair cells after differentiation but is maintained in support cells. The role of *Sox2* in support cells is unknown. In mouse, disruption of *Sox2* blocks initial formation of the entire sensory epithelium, thereby obscuring its subsequent role in support cells, as well as its possible involvement in hair cell maintenance (Kiernan et al., 2005).

We have investigated the role of *sox2* in zebrafish, taking advantage of the fact that it is not required for establishment of the sensory epithelium during early otic development. We find that knockdown of *sox2* does not prevent the emergence of hair cells and support cells but does lead to subsequent sporadic cell death of hair cells, and possibly support cells as well. We further show that, in wild-type embryos, regeneration of hair cells following laser-ablation involves transdifferentiation of support cells but not cell division, and that knockdown of *sox2* totally blocks the regeneration process. These findings suggest that *sox2* is required to maintain support cells in a pluripotent state or, alternatively, *sox2* facilitates a discrete aspect of support cell differentiation that provides the facultative ability to transdifferentiate under appropriate conditions. The data further indicate that *sox2* is required for survival of at least some hair cells, either directly by regulating early stages of hair cell differentiation or indirectly by regulating essential non-autonomous functions of support cells.

MATERIALS AND METHODS

Strains and analysis of gene expression

The wild-type strain was derived from the AB line (Eugene, OR). *hsp70:Gal4, UAS:NICD* and *brn3c:gfp* lines were previously described (Scheer and Campos-Ortega, 1999; Xiao et al., 2005). In situ hybridization was performed at 67°C as described (Millimaki et al., 2007). Where indicated in the text, statistical significance was assessed using t-tests.

Misexpression

To generate heat shock vectors for misexpression, full length cDNAs of *fgf8, atoh1a,* or *sox2* (Pujic et al., 2006) were ligated to *hsp70* heat shock promoter (Shoji et al., 1998) with flanking *I-SceI* meganuclease sites (Thermes, 2002; Rembold et al., 2006). Recombinant plasmid (10-40 pg/nl) was coinjected with *I-SceI* meganuclease (NEB, 0.5 U/µl) into 1-cell stage embryos. Stable transgenic lines *Tg(hsp70:fgf8a)*^{x17}, *Tg(hsp70:atoh1a)*^{x20} and *Tg(hsp70:sox2)*^{x21} were generated by raising injected embryos to adulthood and screening by in situ hybridization for overexpression of the transgene or PCR for germline transmission.

Morpholinos

Translation-blocking morpholino oligomers (MOs) were obtained from Gene Tools, Inc. Embryos were injected at the one-cell stage with MOs as follows: 5 ng *sox2*-MO, 5'-AACCGATTTTCTGAAAGTCTACCC-3' (Pujic et al., 2006); 2.5 ng *atoh1a*-MO, 5'-

ATCCATTCTGTTGGTTTGTGCTTTT-3'; 7.5 ng *atoh1b*-MO, 5'-TCATTGCTTGTGTAGAAATGCATAT-3' (Millimaki et al., 2007). In all knockdown experiments, embryos were coinjected with 7.5 ng of *p53*-MO (Robu et al., 2007) to inhibit non-specific cell death sometimes caused by off-target effects of MOs. Under the conditions used here, co-injection of *atoh1a*-MO, *atoh1b*-MO and *p53*-MO (2.5, 7.5 and 7.5 ng, respectively) resulted in complete absence of hair cells through at least 48 hpf in more than 90% of morphants. Efficacy of *sox2*-MO was confirmed by showing that staining with Sox2 polyclonal antibody (Millipore, 1:100 dilution) was undetectable in the otic vesicles of *sox2*-morphants at 36 hpf, and staining in the brain was strongly reduced (data not shown). Uninjected embryos of comparable stage and genetic background were used as controls for knockdown experiments.

SU5402 and DAPT inhibitor treatment

SU5402 was dissolved in DMSO to prepare a 20 mM stock solution. DAPT was dissolved in DMSO to prepare a 10mM stock solution and was diluted 100x for incubations. Embryos were treated in their chorions with 110 μM SU5402 and/or 100 μM DAPT beginning at 26 hpf, and then fixed at 30 hpf to examine changes in *sox2* expression.

Cell transplantation and laser-ablation

Ablations were performed using a MicroPoint laser system with either a 40x or 100x objective. Multi-cell ablations required sequential targeting of individual cells. For

lineage-tracing experiments, donor embryos were injected with lineage tracer (lysine fixable rhodamine 10,000 MW dextran, mixed 1:4 with biotinylated dextran in 0.2 M KCl) at the one-cell stage. Labeled donor cells were transplanted to unlabeled host embryos at the blastula stage. After allowing chimeras to develop to the indicated stages, hair cells in close proximity to lineage-labeled support cells were laser ablated. During ablations, we frequently observed temporary photo-bleaching of GFP in non-targeted hair cells. GFP fluorescence typically recovered within two hours. Laser irradiation also caused varying degrees of photo-bleaching of rhodamine-dextran in nearby support cells. Although rhodamine-fluorescence was still readily detectable several hours later, fluorescence often continued to diminish with time as lineage label accumulated in vesicles and appeared to be secreted into the lumen of the otic vesicle. In some cases rhodamine fluorescence could no longer be detected by 24 hours post-ablation. In such cases, staining for biotinylated dextran usually permitted detection of lineage-labeled cells. In other experiments, embryos were examined for evidence of regeneration 17 hours post-ablation, prior to complete loss of rhodamine fluorescence. Loss of lineage-label was never observed in non-laser irradiated embryos.

BrdU incorporation

BrdU pulse labeling was performed as described by Gray et al. (2001). Dechorionated embryos were incubated in fish water containing 10 mM BrdU and 10% DMSO for 30 min at 33°C. Embryos were rinsed and incubated twice in fish water for 15 min at 33°C. Embryos were then fixed in MEMFA (see in situ hybridization), briefly rinsed,

and incubated in 2N HCl for 1 h at 37°C. Embryos were washed and stained with anti-BrdU (Beckton-Dickinson, 1:250).

Cell death assay

For acridine orange staining, dechorionated embryos were incubated in 7 ml of 1μg/ml acridine orange solution in fish water for 30 minutes. Embryos were then washed with fish water 3 times, 10 minutes each wash. Analysis was completed immediately.

RESULTS

Expression of *sox2*

Otic expression of *sox2* begins at around 14 hpf in the nascent otic placode (Fig. 3.1 A). This is 4 hours after the onset of *atoh1b*, the main gene responsible for specifying the prosensory equivalence group (Millimaki et al., 2007). Expression of *sox2* is contiguous along the medial edge of the otic placode with elevated expression in two domains marking the future utricular and saccular maculae. Expression is eventually restricted to the macular domains, which increase in size as the maculae expand within the otic vesicle (Fig. 3.1 B). Sectioning reveals that nascent hair cells at the periphery of the maculae still express *sox2* but expression is lost as hair cells mature (Fig. 3.1 C). Support cells maintain *sox2* expression, as has been seen in mouse and chick (Fig. 3.1 C) (Hume et al., 2007; Neves et al., 2007). By 48 hpf, primordia of the cristae also begin to express *sox2* (data not shown). Otic expression of *sox2* continues through at least 72 hpf, the latest stage examined (data not shown).

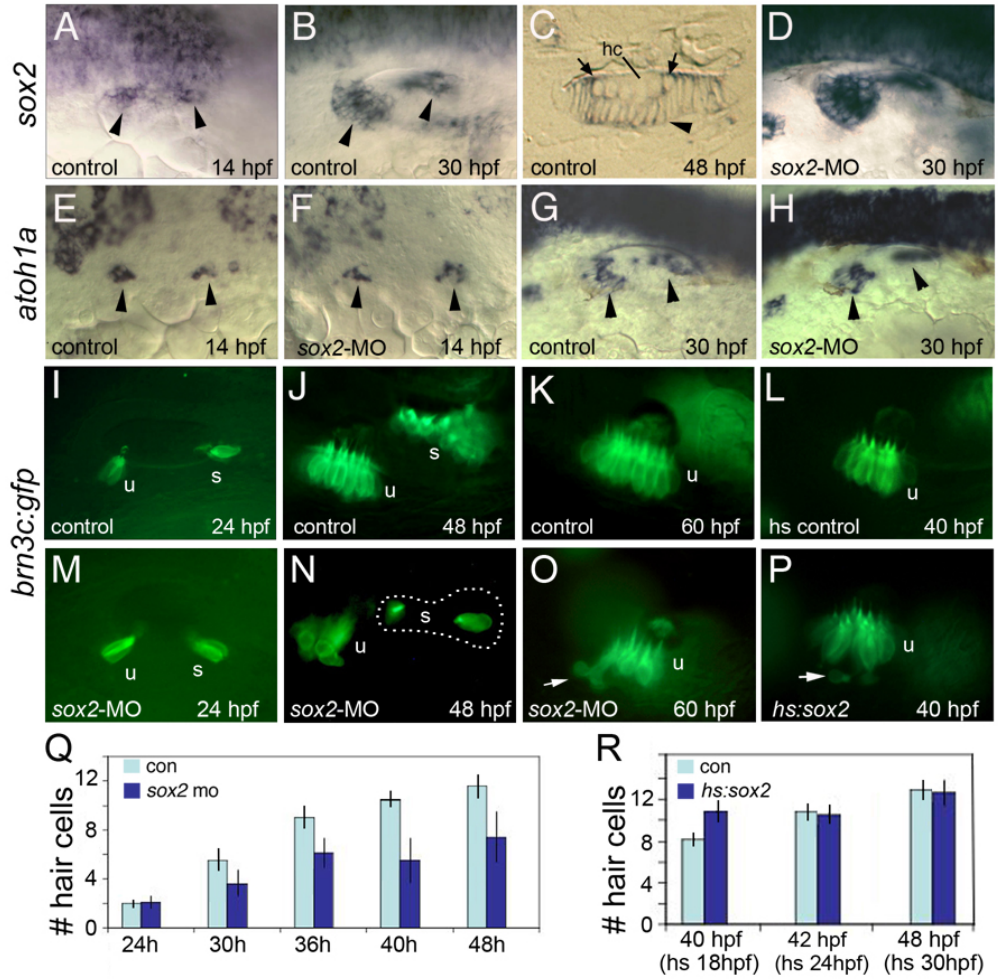

Figure 3.1. Sox2 is not required for hair cell development. (A-C) *sox2* expression in control embryos at 14 hpf (A), 30 hpf (B) and in a cross section of the utricular macula at 48 hpf (C). *sox2* expression is lost from mature hair cells (hc) but is still detected in recently formed hair cells (arrows) and all surrounding support cells (arrowhead). (D) *sox2* expression at 30 hpf in a *sox2* morphant. (E-H) Expression of *atoh1a* in control embryos (E, G) and *sox2* morphants (F, H) at the indicated times. Arrowheads mark macular expression domains. (I-P) *brn3c:gfp* expression in control embryos at 24 hpf (I), 48 hpf (J) and 60 hpf (K); expression in a control embryo heat shocked at 24 hpf and photographed at 40 hpf (L); expression in *sox2* morphants at 24 hpf (M), 48 hpf (N) and 60 hpf (O); and expression in a *hs:sox2* transgenic embryo heat shocked at 24 hpf and photographed at 40 hpf (P). Positions of the utricular (u) and saccular (s) maculae are indicated. Note the absence of hair cells in the middle of the saccular macula in the *sox2* morphant (N). Arrows in (O, P) show hair cells being extruded from the utricular macula. All images show lateral views with anterior to the left and dorsal to the top. (Q) A time course showing the mean number of utricular hair cells in control embryos (con) and *sox2* morphants (*sox2* mo). Sox2 morphants exhibited a normal number of hair cells at 24 hpf ($p = 0.88$) but showed significantly fewer hair cells at later time points ($p < 0.0001$ for each time point). (R) Number of utricular hair cells in control embryos and *hs:sox2*/+ embryos subjected to heat shock at 18, 24 or 30 hpf, and counted at 40, 42 or 48 hpf, respectively. Transgenic embryos heat shocked at 18 hpf produced significantly more hair cells than normal ($p < 0.0004$), whereas the number of hair cells was not altered by heat shocking at 24 or 30 hpf ($p = 0.78$ or 0.73, respectively). Error bars in (Q, R) represent standard deviations, with $n \geq 15$ for each time point.

Effects of knocking down *sox2*

We next assessed the consequences of knocking down *sox2*. Injection of translation-blocking morpholino oligomer (MO) to knockdown *sox2* in zebrafish did not block early expression of *atoh1a* or *atoh1b* in the otic placode (Fig. 3.1F and data not shown). At later stages, the macular domains of *atoh1a* expression were nearly normal or slightly reduced in size (Fig 3.1H). The macular domain of *sox2* expression appeared relatively normal in *sox2* morphants, though the level of transcript was higher than normal (Fig. 3.1 D). To determine whether knockdown of *sox2* perturbs hair cell formation, we injected *sox2*-MO into transgenic embryos expressing *brn3c:gfp*, a marker of differentiated hair cells (Xiao et al., 2005). Tether cells, the first hair cells to differentiate during otic development (Riley et al., 1997), formed on time and appeared normal in *sox2*-depleted embryos (*sox2* morphants) (Fig. 3.1 M). At later stages, additional hair cells continued to form but accumulated significantly more slowly than normal ($p < 0.0001$) (Fig. 3.1 N, Q). Additionally, the saccule of *sox2* morphants usually showed a notable gap between newly forming hair cells (anterior) and the initial tether cells (posterior) (Fig. 3.1N). Finally, hair cells appeared disorganized in *sox2* morphants, and some hair cells appeared to be extruded into the underlying mesenchyme (Fig. 3.1 O). Such displacement has been previously associated with loss of cells undergoing apoptosis (Kwak et al., 2006). Thus, hair cell production is not blocked in *sox2* morphants, but nevertheless occurs slowly and shows signs of irregular patterning. Such deficiencies could indicate faulty hair cell maturation or an increase in hair cell death or both.

To test whether *sox2*-deficiency causes increased cell death, we stained *sox2* morphants and control embryos with the vital dye acridine orange (AO) at 48 hpf. In *sox2* morphants, AO- positive cells were observed in the otic vesicle in 31 of 33 specimens examined and, on average, 2.6 positive cells were seen per ear (Fig. 3.2 B). The majority (66%) of AO-positive cells were seen within the developing maculae of *sox2* morphants and marked both the apical and basal layers of the sensory epithelium, indicating the presence of dying hair cells and possibly support cells as well (Fig. 3.2 C, D, F). In control embryos, only 20 of the 33 specimens exhibited AO-positive cells with an average of only 1 positive cell per ear examined. Moreover, only a single control specimen showed any AO-positive cells within the maculae (Fig 3.2 A, E), a far lower incidence than was seen in *sox2* morphants ($p < 0.0001$). Thus, cell death is normally quite rare in sensory epithelia but is common in *sox2* morphants, confirming that *sox2* directly or indirectly influences hair cell survival.

Effects of *sox2* misexpression

Injection of *sox2* mRNA caused severe patterning defects throughout the embryo, confounding interpretation of its effects in the inner ear (data not shown). We therefore generated a transgenic line to misexpress *sox2* under the control of the heat shock-inducible promoter *hsp70* (Shoji et al., 1998). Activation of *hs:sox2* at 18hpf caused a 20-30% increase in the number of hair cells produced by 40 hpf (Fig. 3.1 R). The resulting maculae appeared somewhat disorganized and occasionally (≤ 10% of embryos) exhibited hair cells being ejected from the macula (Fig. 3.1 P). In contrast,

activation of *hs:sox2* at 24 hpf or later had no discernable effect (Fig. 3.1 R). At no time did activation of *hs:sox2* result in production of ectopic hair cells beyond the endogenous macular domains, indicating that, unlike *atoh1a/b* (Millimaki et al., 2007), *sox2* is not sufficient to establish a prosensory equivalence group.

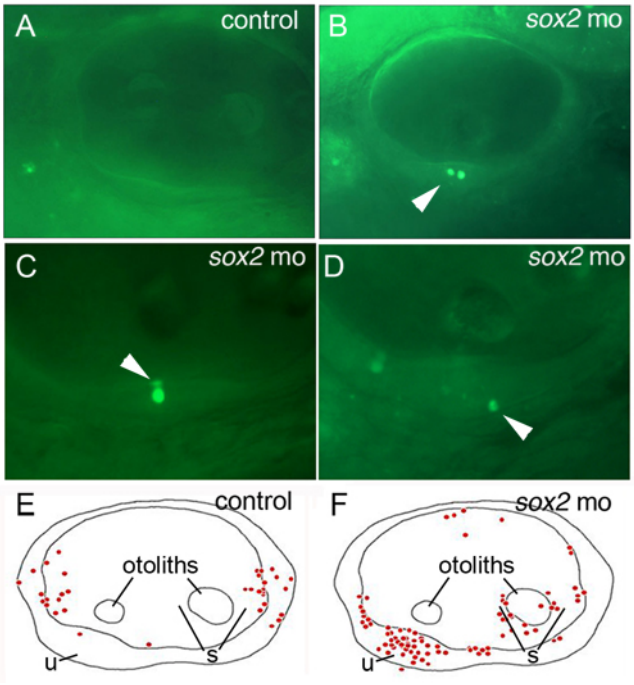

Figure 3.2. Loss of Sox2 results in macular death. (A-D) AO-labeling of dying cells in a control embryo (A) and *sox2* morphants (B-D). Morphants often contained multiple dying cells within sensory epithelia (B), and were observed in apical (C) or basal (D) regions of the maculae (arrowheads). (E, F) Schematic maps depicting the distribution of all AO-positive cells seen in otic vesicles of 33 control embryos (E) or 33 *sox2* morphants (F) at 48 hpf. Positions of the utricular macula (u), saccular macula (s) and otoliths are indicated. No AO-positive cells were detected in the lateral wall of the otic vesicle. All images show lateral views with anterior to the left and dorsal to the top.

Co-misexpression of Sox2 and Atoh1a

Misexpression studies in mouse suggest that Sox2 and Atoh1 are mutually antagonistic with respect to cell fate specification in the cochlea (Dabdoub et al., 2008). We therefore tested whether *hs:sox2* could block the ability of *hs:atoh1a* to stimulate hair cell production. Activation of *hs:atoh1a* at 24 hpf resulted in production of excess and ectopic hair cells throughout the ventromedial wall of the otic vesicle by 33-34 hpf (Fig. 3.3 A). Co-activation of *hs:atoh1a* and *hs:sox2* also led to formation of ectopic hair cells (Fig. 3.3 4B), similar to activation of *hs:atoh1a* alone. Thus, misexpression of *sox2* does not antagonize *atoh1a* function sufficiently to block hair cell differentiation in zebrafish. However, the pattern of ectopic hair cells was less orderly following co-activation of *hs:sox2* and *hs:atoh1a* (note the absence of straight rows of hair cells in Fig. 3.3 B), suggesting that excess Sox2 weakly impairs the ability of Atoh1a to pattern the macula.

Regulation of *sox2* by Atoh1, Fgf and Notch

To better understand the role of sox2 in macular development, we examined its functional relationship to other genes known to regulate early steps in the process, Atoh1a/b, Notch, and Fgf (Millimaki et al., 2007). In *atoh1a/b* double morphants, which lack hair cells and support cells, *sox2* expression was not detectable until 20 hpf, a delay of six hours (Fig. 3.3 E and data not shown). At 30 hpf, *atoh1a/b* double morphants continue to express *sox2* in two macular domains, though both domains are smaller than normal (compare Figs. 3.3 F and 3.1 B). These data show that Atoh1a/b activity is

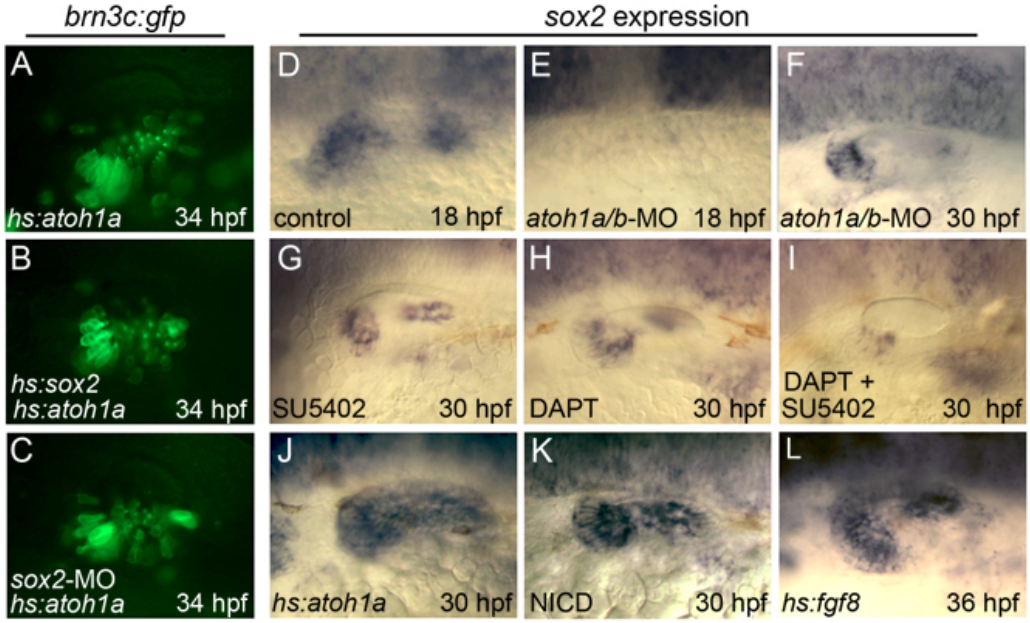

Figure 3.3. Relationship between Sox2 and upstream regulators of hair cell development. (A-C) Expression of *brn3c:gfp* in *hs:atoh1a*/+ transgenic embryos (A, C) and a *hs:atoh1a*/+;*hs:sox2*/+ double transgenic embryo (B) heat shocked at 24 hpf and photographed at 34 hpf. The specimen in (C) was also injected with *sox2*-MO. The inset in (A) shows a heat-shocked *brn3c:gfp*/+ control embryo at 34 hpf. (D-L) *sox2* expression in a control embryo (D), *atoh1a/b* morphants (E, F), wild-type embryos exposed to SU5402 (G), DAPT (H), or both DAPT and SU5402 (I) beginning at 26 hpf, a *hs:atoh1a*/+ embryo heat shocked at 24 hpf (J), a *hs:gal4*/+;*UAS-NICD*/+ embryo heat shocked at 24 hpf (K), and a *hs:fgf8*/+ embryo heat shocked at 30 hpf (L). *sox2* expression is shown at 30 hpf, except (D, E, 18 hpf) and (L, 36 hpf). Expression in control embryos does not change appreciably between 30 and 36 hpf. All images show lateral views with anterior to the left and dorsal to the top.

required for initiation of *sox2* expression at the correct time. To block Fgf signaling we incubated embryos with the pharmacological inhibitor SU5402. This does not block *sox2* expression but reduces its level of expression (compare Figs. 3.3 G and 3.1 B). To block Notch signaling embryos were treated with DAPT, which blocks proteolytic processing necessary to activate Notch. This also reduced the level of *sox2* expression (Fig. 3.3 H). Treatment with both SU5402 and DAPT nearly eliminated *sox2* expression (Fig. 3.3 I), suggesting that these signals act in parallel to regulate *sox2*.

To further test their roles in *sox2* regulation, we used heat shock lines to misexpress Atoh1a, Fgf8 or an activated intracellular domain of Notch (NICD) (Scheer and Campos-Ortega, 1999). Activation of *hs:atoh1a* at 24 hpf led to a dramatic expansion of the *sox2* domain to cover the entire ventromedial wall of the otic vesicle by 30 hpf (Fig. 3.3 J). This correlated with production of ectopic hair cells in the same domain several hours later (Fig. 3.3 A). However, expansion of the domain of *sox2* expression is not required for ectopic hair cell production, since activation of *hs:atoh1* in *sox2* morphants also led to overproduction of hair cells (Fig. 3.3 C). Heat shock activation of NICD led to nearly as great an expansion in *sox2* expression (Fig. 3.3 K). Activation of *hs:fgf8* caused a modest expansion of the macular domains of *sox2*, as well as a low level of ectopic expression in intervening tissue (compare Figs. 3.3 L and 3.1 B). Under the conditions used here, neither NICD nor Fgf8 were sufficient to stimulate ectopic hair cell formation. Thus, Atoh1a, Notch and Fgf activity are all able to activate ectopic expression of *sox2*, but this response is neither necessary nor sufficient for ectopic hair cell production.

Analysis of hair cell regeneration and the role of *sox2*

Regeneration of hair cells in the inner ear has not been previously examined in zebrafish embryos. To do so, we used a laser to ablate GFP-positive hair cells in *brn3c:gfp/+* embryos and established a timeline for hair cell regeneration. We initially targeted only hair cells at the macular center to distinguish subsequent regeneration from normal developmental accumulation of hair cells along the periphery. When ablation was initiated at 48 hpf, the resulting gap in the macula was still easily discernable 12 hours later (Fig. 3.4 A). By 24 hours post-ablation most gaps had been largely filled with new hair cells (Fig. 3.4 B). Thus, substantial hair cell regeneration takes place between 12 and 24 hours post-ablation. Next, to assess the capacity for wholesale regeneration, we ablated all visible hair cells in the utricular macula at 30 hpf, taking care to examine embryos at 34 hpf to confirm that all hair cells had been killed. We then counted the number of hair cells present at 38 hpf (before there is discernable regeneration) and again at 50 hpf (after regeneration has occurred). In unablated controls, the number of hair cells increased by an average of 3.6 ± 0.9, representing normal hair cell production as the macula grows (Fig. 3.4 E, F). In ablated ears, 6.4 ± 0.5 hair cells were produced in this time, representing both normal and regenerative hair cell production (Fig. 3.4 E, F). We infer that the difference between control and laser-irradiated groups (2.8 hair cells/16 hours, $p < 0.005$) represents the number of hair cells produced through regeneration.

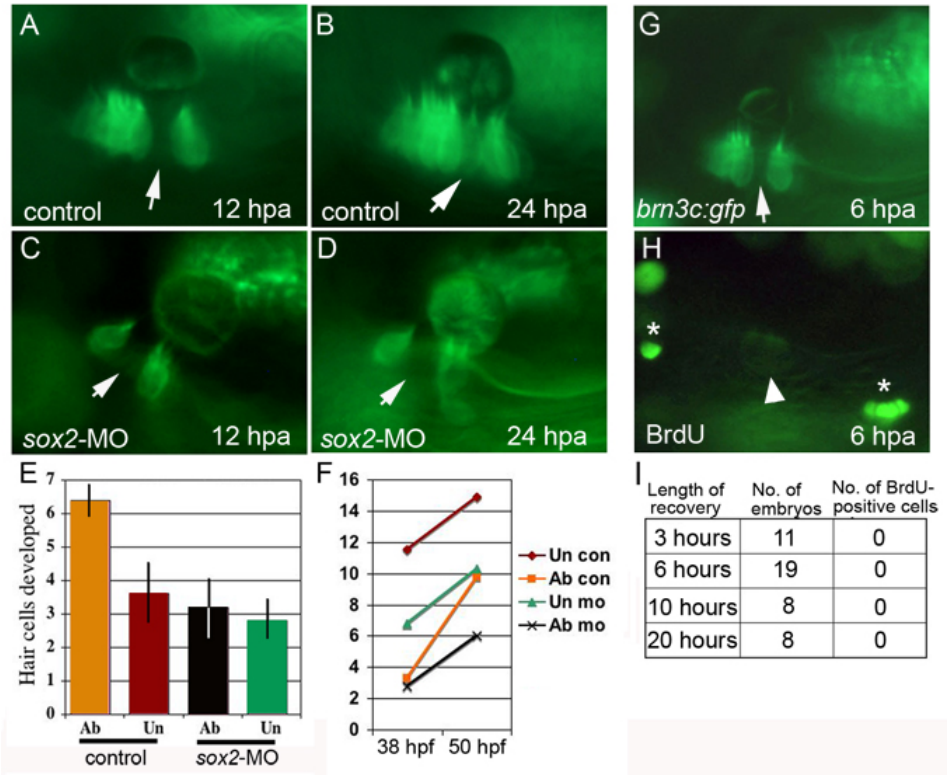

Figure 3.4. Hair cell regeneration requires *sox2* but does not involve cell division. (A-D) *brn3c:gfp* following ablation in a control embryo (A, B) and a *sox2* morphant (C, D). Hair cells were ablated at 48 hpf, and ablated regions (arrows) were still evident at 12 hours post-ablation (hpa) (A, C) and 24 hpa (B, D). By 24 hpa, the gap filled in with newly formed hair cells in the control (B) but not in the *sox2* morphant (D). (E, F) The number of hair cells produced following wholesale ablation of utricular hair cells. Ablation was conducted at 30 hpf, embryos were allowed to recover, and hair cells were counted at 38 hpf and again at 50 hpf. Typically 2 hair cells were produced during the recovery period. The number of hair cells produced between 38 and 50 hpf (E), and the total number of hair cells (F) are indicated for ablated (ab) and unablated (un) control embryos and *sox2*-morphants. Each time point shows the mean ± standard error of 3 or 4 experiments, with sample sizes of 19 to 23 embryos. (G-I) BrdU incorporation at various times following ablation initiated at 48 hpf. After 3, 6, 10 or 20 hours of recovery, embryos were incubated with BrdU for 3 hours and then fixed for processing. A specimen just before fixation at 6 hours post ablation (G) shows that the hair cell gap is still evident (arrow). After processing with anti-BrdU (H), dim GFP fluorescence is still detectable (arrowhead) and shows that no brightly labeled BrdU-positive cells (asterisks) are evident within the macula.

We next examined whether regeneration involves transdifferentiation or asymmetric cell division. To examine whether regeneration involves cell division, hair cells were ablated in the center of the utricular macula at 48 hpf, embryos were allowed to recover for 3, 6, 10 or 20 hours post-ablation, and then BrdU was added for a 3 hour pulse-label. We examined a total of 46 embryos, with at least 8 specimens per time point. Although BrdU-positive cells were detected in many regions of the embryo at each time point, no BrdU incorporation was detected in the macula in any specimen (Fig 3.4 G-I). This indicates that regeneration seen within 24 hours post-ablation does not involve asymmetric cell division. To test whether regeneration involves transdifferentiation, we performed a lineage analysis in laser-irradiated *brn3c:gfp* embryos. Rhodamine-labeled cells were transplanted into unlabeled host embryos at the mid-blastula stage, and host embryos were screened at 36 hpf (n = 310 embryos) or 48 hpf (n = 280 embryos) to identify rare cases in which lineage-label was detected in support cells but few or no hair cells (Fig. 3.5 A-C). Of 590 embryos (1180 ears) screened, 38 showed appropriate labeling patterns. In these specimens, hair cells near the lineage-labeled support cells were laser-ablated. Because laser-targeting sometimes causes photo-bleaching without killing hair cells, specimens were examined again 3 hours post-ablation to confirm that targeted hair cells had indeed been killed (Fig. 3.5 D-F). By 17-24 hours post-ablation, 16 out of 38 specimens showed rhodamine-positive hair cells, with a corresponding disappearance of rhodamine-positive support cells (Fig. 3.5 G-I). The remaining 22 specimens gave inconclusive results due to variable loss of

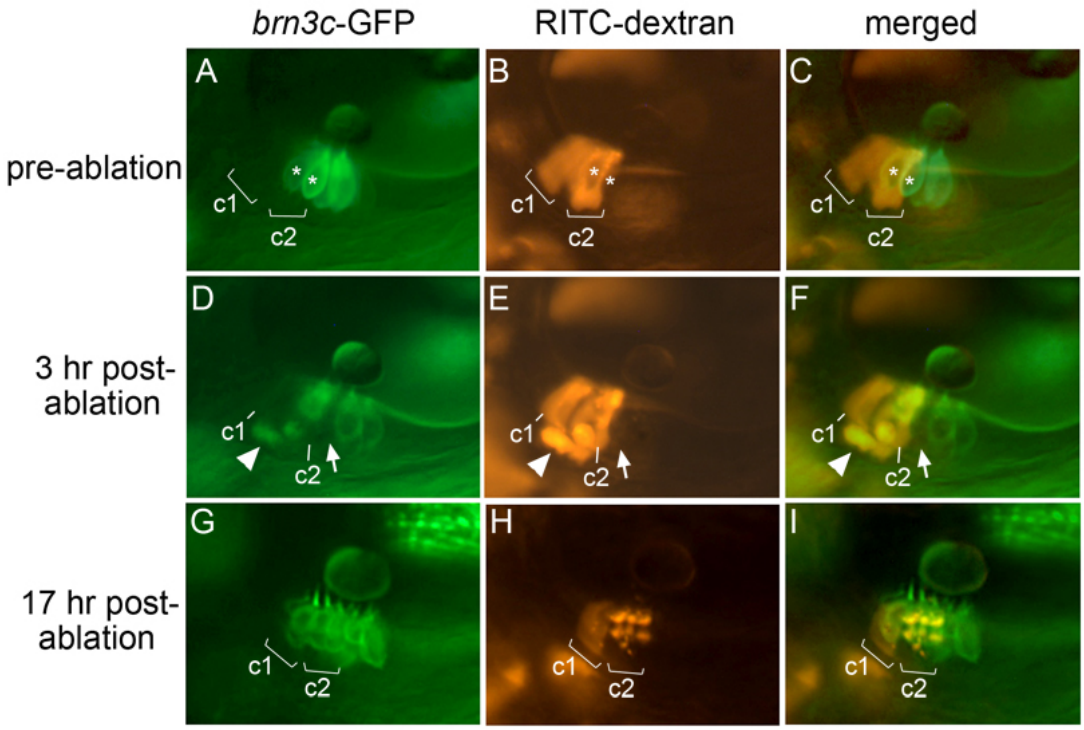

Figure 3.5. Regeneration occurs through transdifferentiation. (A-C) Lineage-labeled embryo at 48 hpf, just before laser-ablation, showing *brn3c:gfp* labeled hair cells in the utricular macula (A), two clusters (c1 and c2) of lineage-labeled cells (B) and an overlay showing both labels (C). Most lineage-labeled cells are support cells. Asterisks mark hair cells that were subsequently targeted for ablation. (D-F) The same specimen 3 hours post-ablation. A notable gap in the hair cell layer (arrow) marks the position previously occupied by one of the targeted hair cells. Accumulation of lineage-label plus GFP beneath the macula appears to show a fragmenting apoptotic hair cell being ejected from the macula (arrowhead). Labeled support cells are still evident in clusters c1 and c2. (G-I) The same specimen 17 hours post-ablation. Support cells in cluster c1 are still evident, though fluorescence intensity has decreased as described in Materials and Methods. In contrast, lineage-label is no longer visible in the support cell layer in cluster c2. Instead, lineage-labeled cells now occupy the hair cell layer and express *brn3c:gfp*. Much of the lineage label is concentrated in vesicles, as is typical at this stage following laser irradiation (see Materials and Methods). All images show lateral views with anterior to the left and dorsal to the top.

lineage label (see Materials and Methods). These data show that support cells can transdifferentiate into hair cells within 17-24 hours post-ablation, thereby facilitating regeneration in zebrafish embryos.

We next examined whether hair cell regeneration occurs in *sox2* morphants. Ablation of hair cells in the macular center in *sox2*-morphants at 48 hpf produced gaps that remained unfilled at 72 hpf, 24 hours after ablation (Fig. 3.4 C, D, n = 9). Similar results were obtained following wholesale ablation: In *sox2*-morphants in which all hair cells were ablated at 30 hpf, an average of 3.2+/- 0.9 hair cells were produced between 38 hpf and 50 hpf. In unablated *sox2*-morphants an average of 2.8 +/- 0.6 hair cells were produced (Fig 3.4 E, F). Because there was no difference in the number of hair cells produced in ablated and unablated embryos ($p = 0.75$), we infer that no regeneration occurred by 50 hpf. Together these data suggest that *sox2* is required for hair cell regeneration in zebrafish embryos.

DISCUSSION

We have shown a requirement for *sox2* in maintenance and regeneration of hair cells in the zebrafish inner ear. It is possible that both functions are co-regulated in support cells or, alternatively, they could reflect independent functions in hair cells and support cells, respectively. Although *sox2* is not required for overt hair cell formation, the sporadic cell death seen later could reflect faulty regulation of early hair cell differentiation. Alternatively, the requirement for hair cell survival could indicate that *sox2* regulates an essential non-autonomous function in support cells. Analysis of *mib* mutants in

zebrafish suggests that support cells are required for hair cell survival. In this background, the entire sensory equivalence group differentiates precociously as hair cells, all of which subsequently die by 36 hpf (Haddon et al., 1998). Deficiencies in support cell functions are clearly subtler in *sox2* morphants, and hair cell death occurs only sporadically over a protracted period. Additionally, it is possible that support cells themselves die in *sox2* morphants, though this is difficult to resolve without reliable support cell-specific markers.

The requirement for *sox2* in regeneration clearly points to an essential function in support cells. We find that support cells directly transdifferentiate into hair cells following laser ablation in zebrafish, as has been observed in neonatal mice (Kelley et al., 1995). Maintenance of *sox2* expression might allow support cells to retain developmental plasticity even as they differentiate enough to execute their essential functions. Alternatively, *sox2* might regulate a discrete aspect of support cell differentiation that enables them to respond to macular damage by transdifferentiation into hair cells. The mechanism governing transdifferentiation is not well understood, but studies in chick suggest that *Atoh1* is involved (Cafaro et al., 2007). In this case, downregulation of *sox2* might be required for upregulation of *Atoh1*. It is also known that *Atoh1*-null cells can sometimes become hair cells when surrounded by wild-type cells, indicating the existence of an alternate hair cell pathway (Du et al., 2007). The status of *sox2* in this pathway is unknown. It will be interesting to explore whether the loss of regenerative processes in the mammalian cochlea involve changes in *Sox2* regulation. Support cells in mouse might lack the ability to reduce expression of *Sox2*

enough to allow *Atoh1* activation. Alternatively, expression levels may be too low to maintain pluripotency. Cochlear support cells are highly specialized and differentiated, which could indicate a more stable commitment to these specific fates (Corwin and Oberholtzer, 1997). Expression of *sox2* in the lateral line in zebrafish is also consistent with a role in regeneration, though this can apparently occur by transdifferentiation or asymmetric cell division (Woods et al., 2004; Hernandez et al., 2007; Ma et al., 2008).

The role of *sox2* in patterning of the inner ear and sensory epithelium shows some interesting parallels between zebrafish and mouse, though there are clearly also some important differences. We have shown that zebrafish *sox2* expression begins within the maculae downstream of *atoh1a/b*, and knockdown of *sox2* does not block *atoh1a/b* expression. In contrast, mouse *Sox2* is initially expressed throughout the ventral half of the otic vesicle well before formation of the sensory primordia (Kiernan et al., 2005). Moreover, *Sox2* mutant mice produce no sensory cells and fail to express *Atoh1*. These observations have led to the suggestion that mouse *Sox2* acts as a proneural gene to establish the prosensory equivalence group (Kiernan et al., 2005; Dabdoub et al., 2009). As a potential correlate, we detected a 20-30% increase in hair cell production following activation of *hs:sox2* at18 hpf. This corresponds to a brief period in zebrafish when Notch activity stimulates *atoh1a* expression (Millimaki et al., 2007), suggesting that the pulse of *sox2* misexpression may help mediate this effect. However, in contrast to *Atoh1* (Woods et al., 2004; Millimaki et al., 2007), misexpression of *Sox2* is not sufficient to activate formation of ectopic sensory epithelia in mouse or zebrafish, arguing against a simple prosensory role. An alternative

explanation for the early requirement in mouse is that *Sox2* initially acts as a regional specifier for the floor of the otic vesicle without which all ventral fates are lost. This would explain why the prosensory inductive signal Jag1 is not expressed in *Sox2* mutants (Kiernan et al., 2005).

In a second phase of *Sox2* function, zebrafish and mouse appear much more alike in their expression and regulation of *Sox2*. In both species, *Sox2* is induced by Notch activity, and possibly Fgf signaling as well (Pirvola et al., 2002; Brooker et al., 2006; Kiernan et al., 2006; Hayashi et al., 2008) (Fig. 3.3). Interestingly, early expression of *Atoh1* is co-induced by these same signals (Pirvola et al., 2002; Woods et al., 2004; Brooker et al., 2006; Kiernan et al., 2006; Millimaki et al., 2007; Hayashi et al., 2008). Subsequent mutual antagonism between *Atoh1* and *Sox2* (Dabdoub et al., 2009) could then reinforce cell fate diversification mediated by Notch-dependent lateral inhibition (Haddon et al., 1998; Riley et al., 1999; Brooker et al., 2006; Kiernan et al., 2006; Millimaki et al., 2007). Perturbing the balance of these activities might explain why in our studies misexpression of *sox2* led to more chaotic arrangements of hair cells. However, unlike misexpression experiments in mouse (Dabdoub et al., 2009), we did not see a reduction in hair cell production following misexpression of *sox2*, arguing that Sox2 does not directly antagonize Atoh1 activity. It is possible that variation in the relative abundance or perdurance of misexpressed proteins influences how cells respond in different settings (Boer et al., 2007; Kopp et al., 2008).

CHAPTER IV

PATTERNING OF ECTOPIC SENSORY EPITHELIA INDUCED BY ATOH1A IN ZEBRAFISH AND ENHANCEMENT OF SENSORY COMPETENCE BY FGF

INTRODUCTION

The inner ear is an important structure for hearing and balance. Sensory epithelia of the inner ear, comprising hair cells and support cells, mediate the perception of sound and balance. Research has elucidated many of the factors involved in sensory epithelia development and regulation. In vertebrates, one of these key factors is a basic helix-loop-helix transcription factor, Atoh1. As a principle regulator of hair cell development, Atoh1 has received much attention in basic and applied research. Atoh1 is both necessary and sufficient for hair cell development (Chen et al., 2002, Woods et al., 2004, Izumikawa et al., 2005). Overexpression of *Atoh1* leads to production of hair cells both within the organ of Corti, the sensory organ of the cochlea, and in ectopic locations outside the organ of Corti (Zheng and Gao, 2000; Woods et al., 2004; Jones et al., 2006). However, the full extent of sensory competence has not been determined. Overexpression of *Atoh1* has been shown to induce hair cells surrounded by support cells (Woods et al., 2004; Izumikawa et al., 2005). This is consistent with a pronueral function in specifying a prosensory domain from which hair cells and support cells arise. *Atoh1* null cells can be induced to form support cells by overexpression of *Atoh1* in neighboring cells (Woods et al., 2004). Additionally, overexpression of *Atoh1* in deafened guinea pigs has revealed recovery of sensory epithelia with both hair cells and

support cells, although cell morphology was abnormal (Izumikawa et al., 2005). It is not clear whether this pattern of hair cells and support cells is a result of proneural function of *Atoh1* or of incomplete ablation of sensory epithelium. In addition, whether overexpression of *Atoh1* can induce expression of all factors involved in sensory epithelia development has not been examined. The ability of *Atoh1* to pattern the sensory epithelia needs further investigation.

In zebrafish, there are two *atoh1* genes, *atoh1a* and *atoh1b*, that have been shown to act as proneural genes during sensory epithelia development. Global misexpression of *atoh1a* induces formation of ectopic hair cells and likely support cells, although only hair cell formation was directly examined (Millimaki et al., 2010). However, misexpression of *atoh1a* results in production of hair cells only in limited regions of the otic vesicle. Most hair cells can be seen within the ventromedial wall of the otic vesicle while none were observed on the lateral wall (Millimaki et al., 2010). This raises questions as to the full effects of *atoh1* misexpression, the extent of sensory competence in the ear and its ability to pattern sensory epithelia.

Here we investigate the effects of *atoh1a* misexpression in zebrafish by examining temporal and spatial parameters that influence Atoh1 function. We demonstrate that misexpression of *atoh1a* can induce expanded sensory epithelia composed of both hair cells and support cells as is consistent with its proneural function. These expanded sensory epithelia express factors involved in sensory epithelia development including *deltaA* (*dlA*), *fgf3/8*, *sox2* and *pax2/5*. The effects of *atoh1a* misexpression are temporally and spatially restricted with increased competence to

respond at early placodal stages to *atoh1a* and with the addition of other factors mainly *fgf8*.

MATERIALS AND METHODS

Strains

The wild-type strain was derived from the AB line (Eugene, OR). *hsp70:Gal4, UAS:NICD* was developed by Scheer and Campos-Ortega (Scheer and Campos-Ortega, 1999) and the *brn3c:gfp* was developed by Xiao et al. (Xiao et al., 2005). *hsp70:atoh1a, hsp7:fgf8* and *hsp70:sox2* lines were previously described (Millimaki et al., 2010). At least 30 embryos were observed for each time-point.

Misexpression

Misexpression experiments using heat shock inducible transgenic lines were carried out in a water bath at 39°C for 30 minutes at time points described in the results.

In situ hybridization

In situ hybridization was performed at as described previously (Jowett and Yan, 1996; Phillips et al., 2001).

Sections

For cryosectioning of *brn3c:gfp*, embryos were fixed overnight in MEMFA (0.1 M

Mops at pH7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde). Embryos were then washed twice for 5 minutes in 1x PBS followed by two one hour long washes in PBT with 0.5% Triton-X and finally washed twice for 5 minutes in 1x PBS and transferred into a 30% sucrose solution made in PBS. Embryos were embedded in tissue freezing medium (Triangle Biomedical Sciences, H-TFM) and cut into 10 μm sections using a cryostat. Slides were dried overnight and subsequently washed in PBS. Cover slips were mounted and sections were observed. For sections of *sox2* and *brn3c:gpf,* embryos were stained whole mount for *sox2* and GFP and then embedded in Immunobed resin (Poly- sciences No. 17324) and cut into 7 μm sections.

Immunoflourescence

Antibody staining was performed as described by Riley et al. (Riley et al., 1999). Primary antibodies: anti-Pax2 (Covance diluted at 1:100) or anti-GFP (Santa Cruz Biotechnology diluted 1:200). Secondary antibodies: Alexa 546-conjugated goat anti-rabbit IgG (Molecular Probe diluted 1:50) or HRP-conjugated goat anti-rabbit IgG (Vector laboratories PI-2000 diluted 1:200).

RESULTS

Effects of *atoh1a* misexpression

It was shown previously that zebrafish *atoh1a* is necessary and sufficient for hair cell development (Millimaki et al., 2007). To further investigate the effects of *atoh1a* misexpression and determine the temporal requirements for *atoh1a,* we utilized a heat

shock inducible line to misexpress *atoh1a* (Millimaki et al., 2010). Induction of the *hsp70* heat shock promoter typically results in elevated transcript levels of the transgene for 90 minutes that gradually decay over the next few hours (Hans et al., 2007, and unpublished observations). However, we have found that the *hs:atoh1a* transgenic line leads to long lasting upregulation of *atoh1a* for 6 hours followed by low levels for an additional 3 hours (data not shown). This extended upregulation is likely to occur through the auto-regulatory activation of endogenous *atoh1a.* Activation of *hs:atoh1a* at 18 hpf in embryos expressing *brn3c:gfp,* a marker of differentialted hair cells, led to production of hair cells throughout the ventromedial quadrant of the ear at 30 hpf (Fig. 4.1 A, B). This domain of hair cells continued to span a domain that included the normal developing utricle and saccule and intervening epithelium at 42 hpf (Fig. 4.1 C, D). Under these conditions misexpression of *atoh1a* was able to expand hair cell production into the medial wall of the otic vesicle; however, no hair cells were seen in the lateral wall.

To further examine hair cell differentiation under these conditions, we examined Pax2 expression, which upregulates during development of all hair cells in the utricle, as well as 2-3 cells in the saccule (Riley et al., 1999). Nearly all cells within the otic vesicle that expressed *brn3c:gfp* were also positive for Pax2 within 15 hours of *atoh1a* activation (Fig. 4.1 E, F). Expanded hair cell domains additionally expressed macula markers *fgf8, fgf3* and *pax5* (Fig. 4.1 G-L). These data suggest that misexpression of *atoh1a* induces primarily utricular hair cells and expansion of genes involved in the hair cell developmental pathway, although only within a restricted region of the otic vesicle.

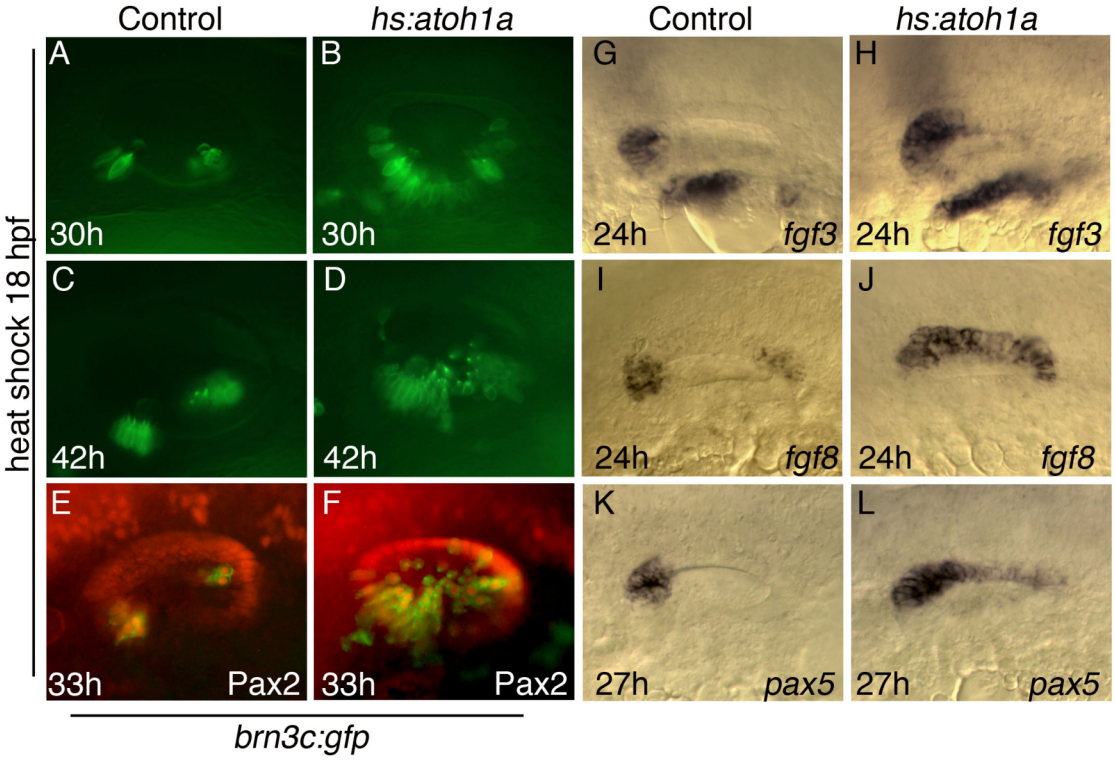

Figure 4.1 Effects of *atoh1a* misexpression at 18 hpf.
(A-L) Embryos heat shocked at 18 hpf. (A-F) Expression of *brn3c:gfp* (green) in the utricle and saccule of controls (A, C, E) and in *hs:atoh1a* embryos (B, D, F) at indicated times. (E, F) Co-staining with anti-Pax2 in red. (G-L) Otic expression of *fgf3, fgf8* and *pax5* in control embryos (G, I, K) and expanded expression in *hs:atoh1a* embryos (H, J, L) respectively. Arrowheads mark domains of otic expression. All images show anterior to the left and dorsal up. (A-H) Images show dorsolateral view and (I-L) show dorsal view.

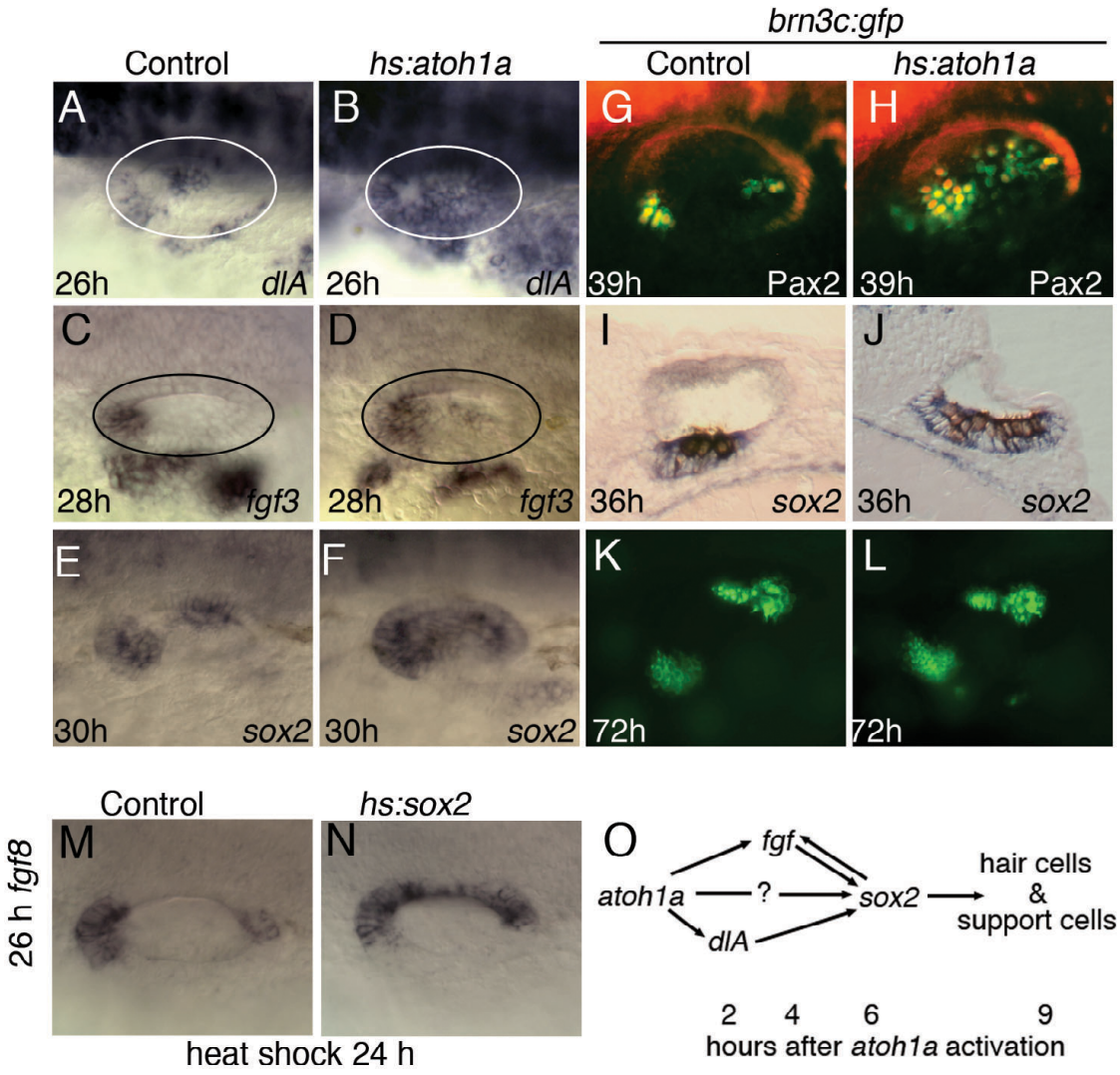

Figure 4.2. Temporal response of macular development to misexpression of *atoh1a*. (A-J) Embryos heat shocked at 24 hpf. (A-F) Otic expression at the indiacted times of *dlA, fgf3* and *sox2* in control embryos (A, C, E) and *hs:atoh1a* embryos respectively. (G-L) Expression of *brn3c:gfp* (green) and Pax2 (red) in otic hair cells at 39 hpf in control (G) and *hs:atoh1a* (H) embryos. (I, J) Expression of *sox2* (blue) and anti-GFP (brown) in control (I) and *hs:atoh1a* (J) embryo at 36 hpf. (K, L) Expression of *brn3c:gfp* in control (K) and *hs:atoh1a* (L) heat shocked at 48 hpf and photographed at 72 hpf. (M, N) Otic expression of *fgf8* control (M) and *hs:sox2* (N) embryos heat shocked at 24 hpf and photographed at 26 hpf. (O) Summary of response to *atoh1a* misexpression. All images show anterior to the left and dorsal up. (A-K) Images show dorsolateral view and (M, N) show dorsal views.

Temporal response of macular development to *atoh1a* misexpression

Previous results have shown that misexpression of *atoh1a* at 24 hpf leads to overproduciton of *brn3c:gfp* positive hair cells by 9 hours after activation of *hs:atoh1a* (Fig. 4.2 G, H; Millimaki et al., 2010). Additionally, a molecular response to *atoh1a* was observed many hours prior to hair cell differentiation, with expression of *sox2* seen throughout the ventromedial wall of the otic vesicle 6 hours after activation of *hs:atoh1a* (Fig. 4.2 E, F; Millimaki et al., 2010). The process of macular development is regulated by a network of factors in addition to *sox2* (Milimaki et al., 2007; Millimaki et al., 2010). Thus, we next examined the response of these factors to *atoh1a* misexpression. *atoh1a* genes are required for normal activation of *delta* genes (Millimaki et al., 2007). Induction of *hs:atoh1a* at 24 hpf led to a dramatic expansion of *deltaA* covering the entire ventromedial wall of the otic vesicle by 26 hpf (Fig. 4.2 A, B). This was followed by expansion of *fgf3* into the medial wall at 28 hpf (Fig. 4.2 C, D). In contrast to the 18 hpf heat shock, only minimal expansion of *fgf8* and *pax5* was observed (data not shown). Expansion of *sox2* was seen throughout the ventromedial wall at 30 hpf (Fig. 4.2 E, F, Millimaki et al., 2010). It has previously been shown that Notch and Fgf activity independently induce *sox2* expression, which likely contributes to the expansion of *sox2* domain after activation of *hs:atoh1a* (Millimaki et al., 2010). Hair cells, marked with *brn3c:gfp,* were observed 9 h after activation of *hs:atoh1a* and became Pax2 positive beginning 3 h later (Fig. 4.2 G, H; Millimaki et al., 2010). Response to *atoh1a* misexpression not only resulted in expansion of hair cells but also expansion of support cells as seen in transverse sections with *sox2* expression in support cells and *brn3c:gfp* in

hair cells (Fig. 4.2 I, J). These data show *hs:atoh1a* activates a full series of genetic responses leading to production of a singular large sensory epithelium spanning the region normally occupied by the utricular and saccular maculae plus intervening epithelium.

This ability to induce sensory epithelia diminished after 24 hpf. For example, activation at 48 hpf led only to an increase in hair cell production with few hair cells outside the endogenous macular domains (compare Fig. 4.2 K, L to Fig. 4.1 C, D). These data indicate a decrease in sensory competence of the otic vesicle to respond to *atoh1a* at later developmental stages. Some responses differ between induction of *hs:atoh1a* at 18 hpf and 24 hpf activation. Activation at later stages does not expand the domain of *fgf8* and *pax5* expression as it does with earlier *hs:atoh1a* induction although the reason for this is unclear. Pax2 is expressed in nearly all *brn3c:gfp* positive hair cells after an 18 hpf heat shock while a 24 hpf heat shock produces some hair cells that do not appear to be Pax2 positive. This may reflect expansion of a more anterior identity after a heat shock at 18 hpf. Additionally at stages later than 24 hpf, *hs:atoh1a* produces many fewer extra hair cells, with little or no ectopic hair cells beyond the utricular and saccular maculae.

Enhanced sensory competence by co-misexpression of *atoh1a* and other factors

To test the zone of sensory competence, we examined misexpression of *atoh1a* during late gastrulation when the ear may be more competent to respond to Atoh1a. Maximal effects of *atoh1a* misexpression could be seen after heat shocks at 14 hpf when the

placode is first visible. Earlier heat shocks resulted in expanded hair cells. However, these cells appear to be subjected to normal domain restriction and thus, result in less expansion when compared to that of misexpression at 14 hpf (data not shown). Subsequent experiments were carried out in serial heat shocks at 14 and 16 hpf. Serial activation of *hs:atoh1a* did not greatly enhance hair cell production compared to that of a single heat shock at 14 hpf (data not shown). Serial heat shocks, however, were necessary for phenotypes seen with co-misexpression of *atoh1a* and other factors in the next section. Misexpression of *atoh1a* after a serial heat shock at 14 hpf and 16 hpf leads to an expansion of hair cells throughout the ventral floor of the ear at 38 hpf (Fig. 4.3 A, B). Transverse sections reveal few if any cells on the dorsal, medial, or lateral walls of the otic vesicle (Figs. 4.3 I). Early misexpression of *atoh1a* did not reveal a broader zone of competence; however, this may be due to required interactions with other locally expressed genes.

We next examined co-misexpression of *atoh1a* and other factors involved in hair cell development and otic patterning to see if we could expand the zone of sensory competence into locations where hair cells are not otherwise produced. We first examined Fgf. Fgf from the hindbrain is required for medial expression of *atoh1a/1b.* By extension, Fgf may be important for inducing medial character and competence to respond to Atoh1a. Serial co-activation of hs:*atoh1a* and *hs:fgf8* at 14 hpf and 16 hpf led to an expansion of hair cells observed on all walls of the otic vesicle with a small region of the medial wall lacking hair cells (Fig. 4.3 C). In transverse sections of these embryos, a thicker epithelium was observed throughout the vesicle expect for a small

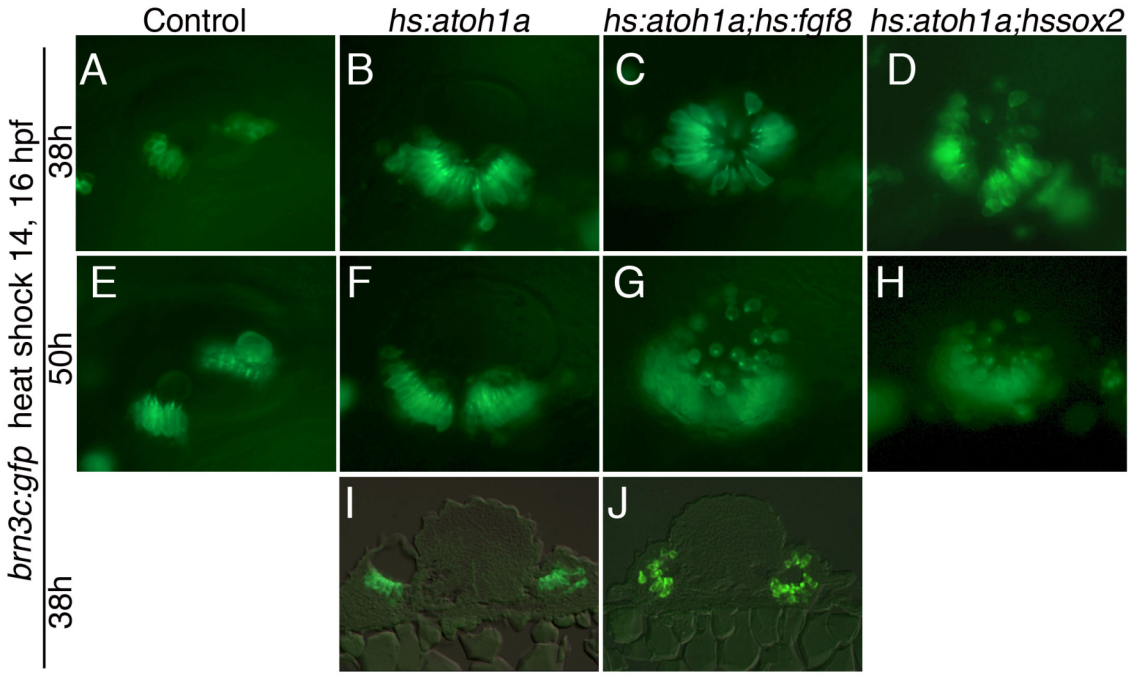

Figure 4.3. Co-misexpression of *atoh1a* and other factors.
(A-J) Expression of *brn3c:gpf* after serial heat shock at 14 and 16 hpf in control (A, E), *hs:atoh1a* (B, F, I), *hs:atoh1a;hs:fgf8* (C, G, J) and *hs:atoh1a;hs:sox2* (D, H) embryos photographed at the indicated times. Images in (I) and (J) are transverse sections. In many sections examined the otic vesicle was collapsed as seen in (I). This is an artifact of the sectioning technique and is probably due to the thin nature of the dorsal otic tissue. All other images are dorsolateral views with anterior to the left and dorsal to the top.

portion on the medial wall lacking hair cells (Fig. 4.3 J). Absence of hair cells in this region is likely due to cell death judging by distribution of macular markers. Serial heat shocks enhanced the effects seen with misexpression of *atoh1a* and *fgf8* as a single heat shock produced fewer ectopic hair cells (data not shown). This is probably due to the transient nature of *hs:fgf8* activity (data not shown). We next examined the ability of *sox2* to enhance hair cells production seen in *hs:atoh1a*. *sox2* is required for

maintenance of hair cells and misexpression of *sox2* at 18 hpf has been shown to cause a slight increase in hair cell production (Millimaki et al., 2010). Similar to co-misexpression of *atoh1a* and *fgf8,* activation of *hs:atoh1a* and *hs:sox2* produced hair cells located on lateral wall (Fig. 4.3 D). Hair cells produced after misexpression of *atoh1a* with either *fgf8* or *sox2* were still present at 50 hpf; however, cells on the lateral wall appeared more widely separated than normal (Figs. 4.3 E,F). This appears to result from expansion of intervening non-sensory cell populations rather than death of hair cells based on continued presence of these cells 12 hours after they were first observed. We also examined the ability of *pax* genes known to be co-factors for atonal in the fly eye (Zhang et al., 2006). Under all the conditions examined, activation of *hs:atoh1a* with either *hs:pax2a* or *hs:pax8* did not alter the production of hair cells seen in *hs:atoh1a* alone (data not shown).

Co-activation of *hs:atoh1a* together with other transgenes at 18 hpf or later did not enhance the effects seen with *hs:atoh1a* alone further suggesting diminished sensory competence over time (data not shown). Taken together these data suggest co-activation with *fgf8,* which could mimic hindbrain signals that specify medial fates, is sufficient to produce hair cells in locations not otherwise seen with misexpression of *atoh1a* alone. However, this effect was resticted to activation during placodal stages when the otic cells may be more plastic.

Misexpression of *sox2* induces *fgf* expression

To determine why co-misexpression of *sox2* and *atoh1a* resembles that of *atoh1a* and *fgf8,* we examined *fgf* expression after activation of *hs:sox2* at 24 hpf. Expression of *fgf8* was expanded into the medial wall of the otic vesicle at 26 hpf (Fig. 4.2 M,N). Rapid upregulation of *fgfs* in *hs:sox2* may explain why co-activation with *atoh1a* has such strong similarity to that of *atoh1* and *fgf8*. This supports a model where *atoh1a* can activate *delta* and *fgf* genes both of which in parallel induce *sox2*. *sox2* can then feedback to give more *fgf*. Finally, this leads to production of hair cells and support cells with alternating fates determined through lateral inhibition (Fig. 4.2 O).

Otic vesicle patterning is altered by misexpression of *atoh1a* and *fgf8*

Co-misexpression of *atoh1a* and *fgf8* stimulated more widespread hair cell production in the otic vesicle including the lateral wall suggesting altered patterning of the otic vesicle, especially of the lateral and dorsal cells, which normally never produce sensory epithelia. To test how axial pattering was altered under these conditions, we examined several axial markers after serial activation of *hs:atoh1a* alone or in combination with *hs:fgf8*. Several anterior markers, *fgf8*, *fgf3* and *pax5* were all expanded posteriorly in *hs:atoh1a* and more strongly expressed in combination with *hsfgf8* (Fig. 4.4 A-C''). Consistent with anteriorization of the otic vesicle, the posterior marker *pou2f3b* (previously zp23) was reduced by activation of *hs:atoh1a* and nearly absent following activation of *hs:atoh1a* and *hs:fgf8*, while the posterior marker *fsta* was completely absent after misexpression of *atoh1a* or *atoh1a* and *fgf8* (Fig. 4.4 D-E''). In addition to

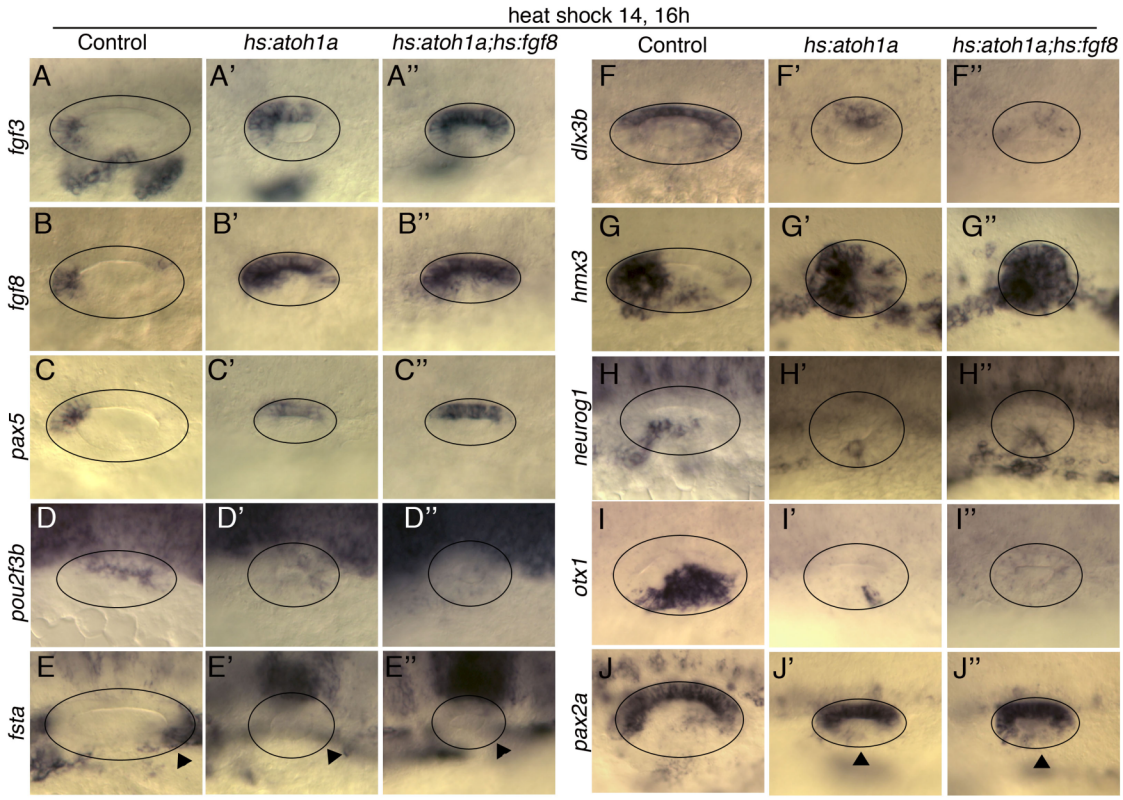

Figure 4.4. Axial patterning after co-misexpression of *atoh1a* and *fgf8*.(A-J'') Embryos heat shocked at 14 and 16 hpf and photographed at 26 hpf. Expression of various markers as indicated in control (A-J) *hs:atoh1a* (A'-J') and *hs:atoh1a;hs:fgf8* (A"-J"). Images (A-C") show dosal views and (D-J") are dosolateral views. Arrowheads in E-E" mark expected location of *fsta* in the posterior otic vesicle. Arrowheads in J-J" indicate expanded domain of *pax2a* in the lateral wall of the otic vesicle. All images show anterior to the left and dorsal to the top.

AP markers we examined a dorsal marker *dlx3b*, which was also reduced in *hs:atoh1a* and nearly absent after co-activation of *hs:atoh1a* and *hs:fgf8* (Fig. 4.4 F-F''). An anterior and ventral marker *hmx3* was expanded to more posterior regions in *hs:atoh1a* alone and more strongly in double *hs:atoh1a; hs:fgf8* transgenic embryos (Fig. 4.4 G-G''). The expression of *neurog1* was diminished in level in *hs:atoh1a.* This is consistent

with data from mouse showing Neurog1 and Atoh1 antagonize one another (Raft etl al., 2007). In the *hs:atoh1a; hs:fgf8* embryos the level was not reduced but the lateral domain was shifted posteriorly (Fig. 4.4 H-H''). This compensation in expression level is possibly due to regulation of *neurog1* by Fgf. A lateral marker *otx1* was severely diminished in the otic vesicles of *hs:atoh1a* and completely gone in double transgenic animals (Fig. 4.4 I-I''). Consistent with loss of lateral markers an expansion of the medial marker *pax2a* into more lateral regions was observed in *hs:atoh1a* and more strongly altered in *hs:atoh1a*; *hs:fgf8* doubles (Fig. 4.4 J-J''). These data suggest *atoh1a* is able to alter patterning of the otic vesicle forcing more anterior, ventral and medial identity but only to a certain extent on its own. Co-activation of *hs:fgf8* and *hs:atoh1a* enhanced this activity.

DISCUSSION

We have shown sufficiency of *atoh1a* to induce full sensory epithelia development including the production of both hair cells and support cells. This is consistent with a proneural function for *atoh1* in sensory epithelia development. In mouse, overexpression of *Atoh1* has also been shown to induce hair cells and support cells. *Atoh1* null cells can be induced to form support cells by overexpression of *Atoh1* in neighboring cells (Woods et al., 2004). This is consistent with the role of *ato* in the fly eye where photoreceptor formation requires the function of the proneural gene *ato.* *ato* is specifically required for photoreceptor R8 selection and is not directly required for the other photoreceptors (R1-R7). R8 selection by *ato* is critical in recruiting neighboring

cells to become R1-R7 (Jarman et al., 1994). Atoh1 does not specify all fates but triggers signaling interactions that guarantee production of alternating pattern of hair cells and support cells.

Our misexpression approach produces a pulse of *atoh1a* from a transgene that results in an auto regulatory activation of the endogenous gene, which then interacts normally with other genes to generate a relatively normal pattern, albeit in an expanded spatial domain. Genes normally involved in sensory epithelia development are turned on sequentially after misexpression of *atoh1a. dlA* expression was initiated within 2 hrs followed shortly by *Fgf* expression 2 hrs after *dlA* expression. Fgf signaling has been shown to be an essential upstream regulator of sensory epithelium and *atoh1* expression (Millimaki et al., 2007, Hayashi et al., 2008). Our data support a feedback loop between Atoh1 and Fgf, which may aid in recruitment of prosensory domains as the sensory epithelia in fish continue to expand throughout the life of the animal. In addition to facilitating recruitment, Fgf signaling may also promote autoregulation of *atoh1* similar to the role of Egfr in *Drosophila* sensory organ development (zur Lage et al., 1997; zur Lage et al., 2004). Subsequently *sox2* expression is initiated 6h after induction of *hs:atoh1a*. It was previously shown that both Notch and Fgf signaling are necessary and sufficient for *sox2* expression in the otic vesicle. Thus the 6 hr delay in *sox2* expression may be due in part to the requirements for Notch and Fgf signaling. Finally, hair cells can be visualized with *brn3c:gpf* 9h after *atoh1a* misexpression followed by an upregulation of Pax2 in hair cells 3 hours after expression of *brn3c:gfp* (Fig.4.2 O). The kinetics of response to misexpression of *atoh1a* may depend on developmental stage.

Fgf3 and Fgf8 appear differentially activated at different times. The reasons for this are unclear and require further study.

Competence of the otic vesicle to respond to atoh1a is restricted both temporally and spatially. There was a diminished response to *atoh1a* misexpression over time. Activation of *hs:atoh1a* early lead to a great expansion of sensory epithelia whereas later activation at 48 hpf resulted in mild expansion of endogenous sensory domain with few hair cells seen outside these domains. Restriction of competence to respond to *atoh1a* over time may reflect commitment of the otic tissue to particular non-sensory fates making the tissue unresponsive to Atoh1a. Additionally, the competence to respond to *atoh1a* may require a particular set of regional factors as is seen with the function of proneural genes in *Drosophila* (Westerman et al., 2003; Niwa et al., 2004). Responsiveness possibly reflects axial patterning, partially established even before otic placode formation. The normal domain of *atoh1a/1b* is seen in medial cells. This requires Fgf signaling from the hindbrain, and thus, Fgf is able to induce medial character required for Atoh1 responsiveness. Hence, co-misexpression of *fgf* and *atoh1a* expands the domain of ventral-medial identity into the lateral-dorsal wall of the otic vesicle.

As a principle regulator of sensory epithelia development Atoh1 has received much attention as a potential candidate for gene therapy to aid in regeneration after hair cell death. Studies from mammals have shown overexpression of *Atoh1a* induces hair cells (Zheng and Gao, 2000; Kawamoto et al., 2003, Woods et al., 2004; Izumikawa et al., 2005). One such study by Izumikawa et al. was also able to demonstrate some

limited functional recovery of hearing in chemically deafened animals treated with Atoh1. However, hearing was not restored to normal levels and outer hair cells and support cells exhibited abnormal morphologies (Izumikawa et al., 2005). This could possibly be due to secondary death of hair cells as a result of limited competence. Our analysis of *atoh1a* misexpression may bring light to the possibility of enhancing sensory competence by the addition of other factors involved in sensory epithelia development. In both fish and mammals, Fgf signaling appears to play essential roles in sensory epithelia development (Millimaki et al., 2007, Privola et al., 2002; Hayashi et al., 2008). Co-misexpression of *Atoh1* and its upstream activators may be required for activation of important cofactors and could provide a potential way to improve hair cell regeneration after death or damage of hair cells in mammals.

CHAPTER V

SUMMARY AND CONCLUSIONS

This dissertation focused on the development and regulation of sensory epithelia in zebrafish inner ear. Sensory epithelia are comprised of hair cells and support cells, which arise from a common equivalence group and are essential for the perception of sound and balance. In Chapter II we show that *atoh1* genes, regulated by Fgf beginning during preotic stages and continuing throughout development of the otic vesicle, function as proneural genes to regulate sensory epithelia development. While our initial analysis indicated Atoh1 was sufficient to specify sensory epithelia, it was only shown to lead to a production of hair cells with no direct evidence for support cell induction. We further characterized the effects of *atoh1a* misexpression in Chapter III and show *atoh1* is sufficient to induce sensory epithelia with both hair cells and support cells. The gain-of-function experiments used in this study demonstrated Atoh1 was able to induce hair cells but with certain temporal and spatial limitations. Competence to respond to Atoh1 is greatest during placodal stages when addition of Fgf can further expand the domain of sensory competence leading to hair cell production in locations not otherwise seen. Misexpression of *atoh1a* activates either directly or indirectly a full program of genes involved in sensory epithelia development including *dlA*, *sox2*, *fgf* and *pax2/5*. In mouse as an alternative to *Atoh1*, *Sox2* has been proposed to play a proneural role in regulation of prosenory domain. However, its role in zebrafish had not been examined. The known role of Sox2 in stem cell maintenance along with its expression in support cells

suggested a later role in hair cell maintenance and regeneration. We investigated the role of zebrafish *sox2* in hair cell maintenance and regeneration in Chapter IV. We show that *sox2* is not required for hair cell formation but rather is necessary for survival of hair cells. Whether or not it is directly required for regulation of hair cell differentiation or indirectly by regulation of support cells is yet to be determined. In summary this study has expanded our understanding of the molecular regulation and requirements for sensory epithelia development and provides a framework for future studies on sensory epithelia development.

ZEBRAFISH *atoh1* GENES FUNCTION AS PRONEURAL GENES

The loss of function studies described in Chapter II along with the gain of function data in Chapter III demonstrate that Atoh1a and Atoh1b act as proneural genes to specify the prosensory equivalence group. It had long been hypothesized that proneural genes encoding bHLH transcription factors specify the prosensory equivalence group from which hair cells and support cells arise. There are conflicting reports on whether vertebrate *Atoh1* genes function as proneural genes or terminal differentiation factors. The data on *atoh1* genes in zebrafish are consistent with the paradigm set by studies on the role of proneural genes in *Drosophila* sensory organ development. Various authors have defined proneural genes using four criteria (Brunet and Ghysen, 1999; Hassan and Bellen, 2000; Westerman et al., 2003). First, proneural genes are expressed prior to specification of cell fate. Expression of *atoh1b* can be seen in the preotic region prior to specification of the first forming hair cells, tether cells. Secondly, proneural genes are

necessary for equivalence group specification. Morpholino knockdown of both *atoh1a* and *atoh1b* leads to complete loss of hair cells. There is no known marker of support cells in zebrafish, but in *atoh1a/1b* double morphants a simple epithelium expressing *atoh1* is produced. During support cell specification, expression of *atoh1* is lost suggesting that all remaining cells in the epithelium of *atoh1a/1b* double morphants expressing *atoh1* are not support cells. Third, proneural function is sufficient to induce ectopic sensory patches. Gain-of-function data using heat shock inducible *atoh1a* revealed induction of sensory epithelia containing both hair cells and support cells. Fourth, proneural genes are responsive to lateral inhibition via Notch signaling. After initial proneural gene expression within the equivalence group, the proneural domain becomes restricted via Delta-Notch (Dl-N) signaling. Initially, *atoh1b* is expressed in a broad domain, but later otic expression becomes restricted to two domains. In *mib* mutants, which lack Dl-N signaling, expression of *atoh1* genes fails to be restricted. Finally, misexpression of an active form of Notch, NICD leads to loss of *atoh1b* expression and loss of hair cells. These data indicate that *atoh1* is repressed by Notch activity. Our studies indicate zebrafish *atoh1* genes do have proneural function during sensory epithelia specification and development. This knowledge of *atoh1* genes provides a way to understand how they may function in recovery of damaged hair cells.

These data reveal some strong similarities to that of Atoh1 function in mouse as well as some potential differences. Most data on mouse *Atoh1* has examined its role in the cochlea. The mammalian cochlea is a much more highly derived structure than that of the vestibular maculae and cristae. Thus differences in the role of *Atoh1* in the

cochlea versus the maculae of zebrafish may explain some of the differences seen in the role of vertebrate *Atoh1*. In mouse, *Atoh1* is both necessary and sufficient for hair cell formation consistent with a role for *Atoh1* in prosensory specification. Although there is no direct evidence for a responsiveness of *Atoh1* to lateral inhibition, disruption of Notch signaling leads to increased hair cell numbers. Expression of *Atoh1* would be expected in a broad domain before fate specification; however, determination of the early expression of *Atoh1* in the cochlea has been inconclusive. Some studies, depending on the method used have shown *Atoh1* expression in a broad domain or more restricted expression to committed hair cells. These data do not address whether there is an initial broad domain that is later upregulated in some cells and further restricted with pattern refinement. Additionally, RT-PCR data reveal Atoh1 transcription begins 12 hours before mRNA or protein can be detected by in situ hybridization or immunolocalization (Matei et al., 2005). Therefore, current methods of visualizing Atoh1 expression are not sufficient to settle this issue in mouse.

Although Atoh1 is both necessary and sufficient for hair cell formation there are discrepancies about its role in specifying the entire equivalence group from which both hair cells and support cells arise. Examination of support cells in *Atoh1* null animals has shown loss of mature support cell markers in the organ of Corti (Woods et al., 2004). Markers, such as *Jag1,* expressed prior to *Atoh1* but ultimately restricted to support cells are, however, initially expressed early in both normal and *Atoh1* null mice. Although, later expression of *Jag1,* associated with support cells is lost in *Atoh1* null mice (Woods et al., 2004). This is consistent with a disruption of support cells. Additionally, *Atoh1*

null cells can be induced to form support cells by overexpression of *Atoh1* in neighboring cells (Woods et al., 2004). This does not differ from the situation in the fly eye where the formation of photoreceptors depends on the function of the proneural gene *ato*. *ato* is specifically required for photoreceptor R8 selection and is not directly required for the other photoreceptors (R1-R7). R8 selection by *ato* is critical in recruiting neighboring cells to become R1-R7 (Jarman et al., 1994). Examination of $p27^{kip1}$, a marker of cell cycle exit, has led some groups to conclude Atoh1 is not required for prosensory domain specification. Expression of $p27^{kip1}$ precedes that of Atoh1 and continued prosensory domain expression of $p27^{kip1}$ in *Atoh1* null mice has been interpreted to mean that *Atoh1* is not required for cell fate specification but rather for later stages of hair cell differentiation (Chen et al., 2002). However, it is also possible that cell cycle exit does not require Atoh1 function but is regulated by other factors similar to the continued expression of macular markers *pax5*, *fgf3* and *fgf8* in zebrafish *atoh1a/1b* morphants. A similar situation is also evident in *Drosophila* where *ato* mutants lacking photoreceptor cells continue to express genes that are normally expressed prior to prosensory equivalence group formation (Jarman et al., 1995). Although data from mouse varies from that of zebrafish it does not necessarily contradict a proneural role for *Atoh1*. Further analysis is needed to fully determine the role of Atoh1 in mouse sensory patch development. The ability to induce sensory epithelia containing both hair cells and support cells may be of importance during regeneration. Recovery of hearing is likely to require the correct number of both hair cells and support

cells. Regeneration of hair cells without support cells might not lead to a fully functional recovery.

THE ROLE OF FGF DURING SENSORY EPITHELIA DEVELOPMENT

Fgf signaling plays a role in several stages of otic development from otic placode induction to patterning of the otic vesicle. We show a role for Fgf during sensory epithelia development through regulation of *atoh1* and *sox2* genes in zebrafish.

Fgf signaling in mouse has also been implicated in sensory patch formation. Conditional deletion *FgfR1* results in a cochlear sensory epithelia lacking mostly outer hair cells. Additionally *Atoh1* is decreased in these animals (Privola et al 2002). A more recent examination of the role of Fgf in sensory patch formation suggests Fgf20 as a likely ligand for Fgfr1 and blocking Fgf signaling with a receptor inhibitor or blocking Fgf20 with antibodies results in decreased *Atoh1* expression and a reduction of hair cells and support cells (Hayashi et al., 2008). Both these studies are consistent with a role for Fgf signaling in sensory patch formation and regulation of Atoh1 as seen in zebrafish.

We have additionally shown that Fgf has the ability to expand the region responsive to misexpression of *atoh1a*. This may be through the regulation of other factors that influence the zone of sensory competence. Fgfs are also known to regulate Pax2/5/8 family of transcription factors. We speculate these transcription factors may be involved in sensory competence. They are regionally expressed and involved in different aspects of hair cell development. Moreover, they may function in a similar manner as is seen in *Drosophila* with Pax6 modifying *atol* function to activate eye-

specific genes rather than chordotonal genes (Zhang et al., 2006). Additionally, in mouse Pax6 enhances Atoh1 in the rhombic lip (Landsberg et al., 2005). Other factors are probably also involved; however, these details need to be resolved. Fgf does not only regulate *atoh1* and *sox2*; it is also induced by *atoh1* and *sox2* suggesting that a positive feedback mechanism is involved in sensory epithelia development. *fgf* expression may also be auto-inductive as laser induction of *hs:fgf8* in the hindbrain or expansion of *fgf3* in the hindbrain of *val* mutants expands expression of *fgf8* and *fgf3* throughout the medial wall of the otic vesicle (Kwak et al. 2002 and unpublished observations). These data suggest a model for how the sensory epithelia might continue to expand throughout the life of fish. In this model, Fgf in the maculae recruits new cells in the peripheral tissue to expand the sensory epithelia and a fine balance of factors involved in macular development may be required for this outward expansion.

Fgfs roles in patterning of the ear and axis formation are an important part of sensory epithelia formation. The prosensory domain formation is first evident by asymmetric expression of *atoh1b* in the medial portion of the otic placode. This requires Fgf signaling likely from the hindbrain as well as placodally expressed transcription factors of the Pax2/5/8 family. Later Fgf3 from the hindbrain is required for regulating expression of regional A-P markers of the otic vesicle and regulation of *pax5* a regional utricular hair cell maintenance factor (Kwak et al., 2002; Kwak et al., 2006). This is consistent with a role in specifying anterior fates. Although *fgf8* has not been directly shown to play a role in A-P patterning, it is possible that misexpression of *fgf8* enhances the overall levels of Fgf signaling and thus can alter A-P patterning. We show that co-

misexpressing both *atoh1a* and *fgf8* during late placodal stages expands the zone of sensory competence resulting in production of hair cells throughout much of the otic vesicle. This suggests Fgf may regulate other factors involved in sensory competence. Likely factors include *pax2/5/8* as well as unidentified factors.

MAINTENANCE FACTORS

Fgf signaling may also be important for macular maintenance through regulation of *pax2/5*. In the urtricle Fgf3 is both necessary and sufficient to activate *pax5* expression (Kwak et al., 2002). It has been shown that *pax5* regulates maintenance and function of the utricular macula. In embryos knocked down for *pax5*, hair cells in the utricle initially form but later begin to die (Kwak et al., 2006). Survival of only utricular hair cells requires pax5 and could possibly reflect regional identity induced by Fgf3. Whether defects seen in *pax5* morphants are due to support cells is unclear and requires further studies. A similar hair cell death phenotype is also seen in *pax2a* mutants (Kwak et al., 2006). Both *pax2a* and *pax5* may have roles in hair cell survival or cell death may be a result of loss of *pax5* seen in *pax2a* mutants. Additionally, we show Fgf signaling regulates *sox2* expression, which is necessary for hair cell maintenance. How these genes function is not clear. Expression patterns of *pax5* and *sox2* differ with *sox2* expression eventually restricted to support cells, while *pax5* expression is seen in both hair cells and support cells primarily of the utricle. They may function in different aspects of hair cell maintenance and survival. Fgf signaling appears to be able to control

aspects of hair cell specification through regulation of *atoh1* genes and survival/maintenance of hair cells through regulation of *pax5* and *sox2*.

DIFFERENTIAL ROLES OF FGF3 AND FGF8

Fgf signaling plays roles in patterning and specification in the otic vesicle; however, there may be some differences in the roles of Fgf3 and Fgf8. Interestingly although both *fgf3* and *fgf8* are initially expressed in the hindbrain followed by expression in the developing maculae, their expression patterns are not exactly the same. Expression of *fgf8* in the hindbrain is downregulated shortly after otic placode formation while *fgf3* continues to be expressed in the hindbrain until the otic vesicle forms. Both *fgf3* and *fgf8* are then expressed in the developing otic vesicle primarily in the anterior macula. However, expression of *fgf8* can also be seen in the posterior portion of the saccule. Eventually, we have found that *fgf3* expression can be seen in the anterior portion of the saccule (unpublished observations). Loss of either *fgf3* or *fgf8* disrupts hair cell production; however, loss of *fgf8* appears more severe and can often lead to entire loss of the saccule (Kwak et al., 2005, Leger and Brand, 2002). Additionally, *fgf3* and *fgf8* also appear to respond differently to misexpression of *atoh1a.* Both *fgf8* and *fgf3* are expanded after misexpression of *atoh1a* at early stages; however, at later stages only *fgf3* expression is strongly responsive to *atoh1a* misexpression. Although both Fgfs activate similar RTK pathways, these differences in function may arise in part from their differential expression in the hindbrain and or the additional function of Fgf3 to enter the nucleus and interact with proteins in the nucleolus to modify the response to RTK

activity (Kiefer and Dickerson, 1995). How and if these differences influence different aspects sensory epithelia development is unclear. One possibility might be specification of regional identity of sensory epithelia cells. It would be interesting to compare hair cell development after misexpression of *fgf3* and *fgf8*.

sox2 IN HAIR CELL MAINTENANCE

The studies in Chapter IV demonstrate a role for Sox2 in maintenance of hair cells in zebrafish inner ear. How exactly Sox2 regulates maintenance and regeneration is not known. Sox2 may regulate hair cell maintenance by providing some essential step necessary to maintain hair cell survival and/or by a function in support cells. However, due to a lack of support cell markers in zebrafish, it is difficult to resolve whether support cells also die in *sox2* morphants, and if this is also partially the cause for hair cell death. Hair cell survival appears to require support cells as indicated by death of hair cells seen in *mib* mutants lacking support cells (Haddon et al., 1998). A role for *sox2* in support cells is supported by its expression pattern in the otic vesicle. Expression of *sox2* is initially seen in both hair cells and support cells. Eventually it is lost from mature hair cells while remaining in supports cells. This is also the case in both chick and mouse (Hume et al., 2007; Neves et al., 2007). Expression of *sox2* is also seen in the support cells of the lateral line in zebrafish (Hernandez et al., 2007).

HAIR CELL REGENERATION

We also show a role for Sox2 in hair cell regeneration. Although this has not been directly examined in other vertebrates, it may function similarly. Hair cell regeneration in chick has been shown to occur through both transdifferentation as well as asymmetric proliferation of support cells (Stone and Cotanche, 1994; Alder and Raphael, 1996; Roberson et al., 1996). Similar regeneration is also seen in the lateral line of zebrafish; however, most regeneration occurs through proliferation of support cells expressing Sox2 (Hernandez et al., 2007). We show *sox2* is required for hair cell regeneration in the zebrafish ear. This regeneration occurs through transdiferentiation; however, our data do not rule out the possibility of a later proliferative response to hair cell damage. Transdifferentation may be a fast response to hair cell damage that can repair minimal damage quickly while more extensive damage may also require a long-term response such as asymmetric division of support cells. In chick, regeneration of hair cells through transdifferentiation occurs first followed by proliferative regeneration (Stone and Cotanche, 1994; Alder and Raphael, 1996; Roberson et al., 1996). A related response is seen in zebrafish lateral line hair cell regeneration such that mild hair cell damage leads to regeneration by transdifferentiation while more severe damage results in regeneration from asymmetric division of support cells (Hernandez et al., 2007). How transdifferentiation occurs is not well understood; however, it is likely to involve Atoh1. Upregulation of *Atoh1* is seen during regeneration in chick (Cafaro et al., 2007). Downregulation of *Sox2* may be necessary for this process and may be a reason for the lack of regeneration seen in the mouse cochlea. Consistent with this idea, expression of

Sox2 continues in support cells of mouse long deafened cochleas (Oesterle and Campbell, 2009). In mouse Atoh1 and Sox2 have been shown to antagonize each other such that overexpression of *Sox2* inhibits hair cell formation while reduction in *Sox2* function leads to increases in hair cells number. Moreover, overexpression of both *Atoh1* and *Sox2* together result in fewer hair cells than that of *Atoh1* alone. There are however caveats to these experiments. The level of Sox2 may be important in determining how Sox2 functions given its mutually exclusive activities in maintaining pluripotency and stimulating differentiation. The role of Sox2 in hair cell regeneration in chick, mouse and zebrafish lateral line has not been fully examine, but potentially, it functions similar to that of Sox2 in the zebrafish inner ear. It will be interesting to explore whether loss of regeneration in mammals involves changes in *Sox2* regulation.

CONCLUSION

Our studies have provided information on the regulation and development of sensory epithelia in zebrafish. Identifying the regulatory pathways that are lost and conserved among vertebrates may help to reveal molecular strategies for reactivating hair cell production in mammals. Our data have pointed at several key features that could be of potential interest in understanding how regeneration may be activated in mammals. Reinitation of developmental processes is important for hair cell regeneration. Our data using zebrafish have identified the proneural genes, *atoh1a* and *atoh1b,* required for sensory epithelia development and of equal importance upstream regulatory factors Fgf, Pax2/5/8, and Notch. Additionally, *sox2* is required for hair cell regeneration and

maintenance and is also regulated by both Fgf and Notch. How expression of these factors changes in response to hair cell damage has not been examined in zebrafish but may provide useful information on how non-mammals activate regeneration.

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