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The cellular role of Atoh1 in development and regeneration in the mammalian cochlea

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By

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B.S., University of Rochester, 2005

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Abstract

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By Michael C. Kelly

Intricate neuroanatomical structures within our inner ears impart us with the ability to detect sound with incredible detail. This ability is largely due to the function of the auditory sensory end organ, known as the organ of Corti, which is made up of an exquisitely arranged mosaic pattern of specialized mechanosensitive hair cells that convert sound into a neuronal signal and non-sensory supporting cells. Over the course of development, multiple pathways converge to generate and pattern the cells of organ of Corti. Auditory hair cell damage can result from a variety of genetic and environmental factors, and their loss results in sensorineural hearing impairment. Since the mature mammalian ear does not spontaneously regenerate hair cells, these associated hearing deficits are permanent.

The transcription factor Atoh1, which has an essential role during development directing the differentiation of sensory hair cells within the inner ear, under certain conditions can promote the generation of new hair cells when delivered to cochlear epithelial cells. Understandably, Atoh1 has become an important target for promoting hair cells regeneration. However, the potential and limitations of Atoh1-mediated hair cell generation have not yet been systematically tested. In order to determine which cells within the cochlear epithelium are competent for Atoh1-mediated hair cell generation at various developmental timepoints, I generated inducible constructs and transgenic mouse models to direct the temporal and spatial activity of Atoh1.

Using these models, I show that Atoh1 can generate new hair cells within the cochlear epithelium, but that the competency for hair cell generation changes significantly depending on cell type and developmental stage. Interestingly, Atoh1-generated hair cells arise within patterned ectopic sensory regions that are reminiscent of endogenous sensory regions, and that this patterning is mediated through Notch signaling. Extended Atoh1 induction results in an expansion of regional competency for hair cell differentiation in non-sensory regions and a conversion of supporting cells. Intriguingly, Atoh1 induction can also cause the normally post-mitotic cochlear epithelium to re-enter cell cycle. These studies outline important parameters for Atoh1 hair cell generation and suggest that Atoh1 can help pattern the cochlear epithelium through cell fate decisions and proliferation control.

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Chapter I: General Introduction

This dissertation examines the effect of forced expression of the transcription factor Atoh1 within the mammalian cochlear epithelium in order to test the potential and limitations of Atoh1-mediated hair cell generation. Using various inducible Atoh1 expression constructs and transgenic mouse models, I tested the following central hypotheses: *(1) Forced Atoh1 expression can direct hair cell differentiation within cochlear epithelial cells, including the conversion of postnatal supporting cells into auditory hair cells. (2) The relative competency of cochlear epithelial cells to Atoh1-mediated hair cell generation depends both on cell type and developmental stage, with more mature cells exhibiting decreased competency. (3) Ectopic Atoh1 expression and resulting hair cell generation can elicit effects on surrounding non-sensory cells.* The studies described in this dissertation demonstrate important parameters for Atoh1 hair cell generation and illustrate various additional intriguing effects of Atoh1 on cell fate decisions and proliferation control within the mammalian cochlear epithelium.

In order to appreciate the motivation for these studies and to provide a contextual framework in which to interpret the various effects of forced Atoh1 expression, it is important to understand relevant background. In this chapter, I will first describe some of the anatomical and physiological properties of the auditory system contained within the mammalian inner ear. I will then summarize what is known about the developmental program that generates this precisely organized system, including the essential role that Atoh1 plays in hair cell differentiation. I will continue by describing the mechanisms and effects of auditory hair cell loss within mammals, by comparing the hair cell regenerative

capacity of other vertebrates to that of the non-regenerating mammalian inner ear, and by discussing possible strategies to promote regeneration in the mammalian system. Furthermore, I will comprehensively review what is already known about Atoh1's effects with the cochlea, including its potential use as a gene therapy tool to promote hair cell regeneration, and highlight how my central hypotheses aim to answer remaining questions important for our understanding of Atoh1's role in development and its potential therapeutic uses.

1.1 Basic Mammalian Inner Ear Anatomy and Auditory Function

The ability to detect sound is an important sensory modality for many organisms. Hearing provides a forewarning against danger, an ability to locate the unseen, a means through which to communicate, and is a source of environmental enrichment. Selective evolutionary pressure has presumably enhanced the sensitivity and extended the detectable frequency range of particular species based on specific fitness demands. Mammals have highly specialized structures that are optimized for detecting and distinguishing sounds across a broad range of intensity and frequency. This incredible degree of hearing sensitivity and resolution is largely due to unique cellular phenotypes and tissue architecture within the auditory sensory end organ of the mammalian inner ear that allows for efficient and accurate interpretation of mechanical sound vibrations.

A. Anatomy of the Inner Ear

The mammalian inner ears are paired structures that reside within the temporal bone on either side of the skull and contain structures necessary for both auditory and vestibular function (Fig. 1*A*). The cochlear duct on the more ventral portion of the inner ear is dedicated to audition, whereas structures important for vestibular function are on

the more dorsal half. The entire inner ear is encased within bone and it contains fluid-filled chambers organized into an intricate membrane-lined ductwork (Fig. 1*A)*.

The vestibular chambers include three semicircular canals and associated sensory regions, known as cristae, which allow detection of angular acceleration as it relates to head motion in the three-dimensional space. Two other vestibular sensory regions, known as maculae, sense the gravitational and linear acceleration that is critical for our sense of balance.

The spiral-shaped cochlear duct contains all the cellular components responsible for the conversion of sound, a mechanical force, into a neuronal signal that can be relayed centrally for processing. The interpretation of this information ultimately leads to our perception of hearing. Membrane divides the cochlea duct into three separate fluid-filled chambers, the scala vestubli, scala media, and the scala tympani (Fig. 1*B*). The auditory sensory end organ, known as the organ of Corti, is made up of a group of cells that reside on the basilar membrane within the scala media. The lateral wall of the cochlea contains the stria vascularis, which pumps K^+ ions into the scala media and generates the endocochlear potential necessary for auditory function. Many other specialized cell types and structures within the inner ear play important roles in auditory function, but the primary mechanosensitive activity of the cochlea happens within the specialized group of cells that form the organ of Corti.

The organ of Corti spans the entire length of the cochlea, with more basal regions having increased responsiveness to high-frequency sounds and apical regions more responsive to low-frequency. Both auditory and vestibular sensory regions are made up of two general classes of cells, the sensory hair cells and the non-sensory supporting

cells. Spiral ganglion neurons connect the sensory hair cells to more central brain structures that process the various aspects of the sound stimulus including frequency, intensity, temporal and location information.

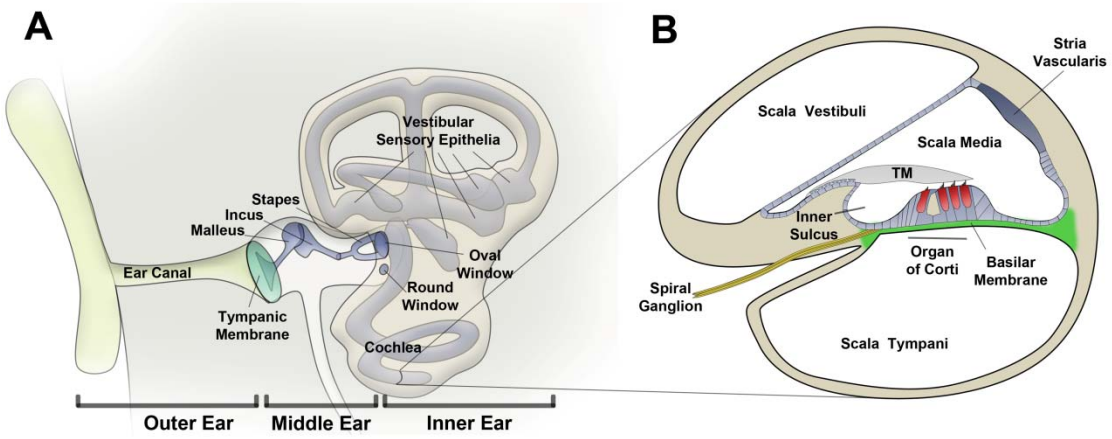

Fig. 1 - Gross Inner Ear Anatomy

(*A*) Diagram of the basic anatomical structures of the outer, middle, and inner ear. Sound travels through the ear canal until it reaches the tympanic membrane, which separates the outer ear from the middle ear space. Sound causes the tympanic membrane to vibrate and this motion is transmitted along a series of three ear ossicles, the malleus, incus and stapes within the middle ear. The foot of the stapes sits against a membranous opening in the bone-encased inner ear. Movement of the stapes causes oscillatory pressure changes within a continuous membranous duct within the cochlea that terminates at another elastic membranous opening called the round window. The vestibular structures and associated sensory epithelia reside in more dorsal portions of the inner ear. (*B*) A cross-section of the cochlea duct shows a division into three chambers, the scala tympani, scala vestibuli, and the scala media. The scala tympani and scala vestibuli represent the continuous duct that originates at the oval window and terminates at the round window, and they are connected at the apical tip of the spiraled cochlea. Within the scala media, the auditory sensory organ, the organ of Corti sits on the basilar membrane and is composed of

supporting cells (*dark grey*) and four rows of hair cells (*red*). Spiral ganglion neurons innervate hair cells and convey information to central targets for processing. The tectorial membrane (*TM*) is an acellular structure that spans the inner sulcus, sits on top of the organ of Corti and is connected to the outer hair cell stereocilia. The stria vascularis resides on the lateral wall and generates the distinct high [K^+] solution of the scala media called endolymph that bathes the apical surface of hair cells and results in an endocochlear potential that provides the ionic driving force necessary for mechanotransduction. Reissner's membrane separates the scala media from the scala vestibuli, which is filled with a solution similar to extracellular fluid called perilymph.

B. Sensory Region Architecture: Hair Cells and Supporting Cells

Both auditory and vestibular organs are organized in a mosaic pattern of hair cells surrounded by supporting cells (Fig. 2). The organ of Corti is composed of a highly ordered arrangement of cells with unique morphologies (Fig. 2*A*,*C*). Four rows of hair cells run the entire length of the cochlea and are surrounded by specific subclasses of supporting cells (Fig. 2*A*). These supporting cells are morphologically and molecularly highly differentiated and play important functions in the structural integrity and homeostasis of the tissue (Hudspeth 2000; Zhang *et al.* 2005; Ramirez-Camacho *et al.* 2006). Auditory hair cells are also divided into two subclasses. Inner hair cells form a single row on the more medial or neural side of the organ of Corti, and outer hair cells form three rows towards the more lateral or abneural side of the sensory region. In comparison, vestibular sensory regions display a relatively simple mosaic of hair cells and supporting cells (Fig. 2*B*,*D*), with some variations existing between and within individual vestibular sensory organs.

A defining feature of all sensory hair cells is their distinctive array of microvillus protrusions on their apical surfaces, known as stereocilia. Some clear differences exist in the morphology of stereocilia bundles from different classes of hair cells. Within the auditory sensory organ, outer hair cells have several rows that are organized from a clear chevron-shaped template (Fig. 2*E*) and inner hair cells have only a few rows of neatly arrayed stereocilia bundles that originate form from a crescent-shaped template (Fig. 2*F*). In comparison, vestibular hair cells have longer stereocilia originating from a circular template (Fig. 2*G*).

In both the vestibular and the auditory systems, stereocilia are organized in a staircase-like gradient from one side of the cell to the other, exhibiting an intrinsically asymmetric cell polarity across the plane of their apical surface (Fig. 2*E*-*G*). Moreover, throughout the entire sensory organ, all hair cells are coordinately oriented across a single axis, thereby exemplifying a cellular phenomenon known as planar cell polarity (Fig. 2*A*-*D*). Although supporting cells lack a polarized stereocilia bundle, supporting cells of the organ of Corti also have intrinsic and planar cell polarity phenotypes in that they have coordinately arranged asymmetric cell morphologies that help build the structure of the organ of Corti (Fig. 2*A*).

Within the sensory regions of the inner ear, function is integrally tied to the structure of the individual cell morphologies and the overall tissue architecture. This is especially apparent within the organ of Corti, where the highly organized tissue is able to accomplish an incredible feat of detecting sounds across a broad range of intensity and frequency.

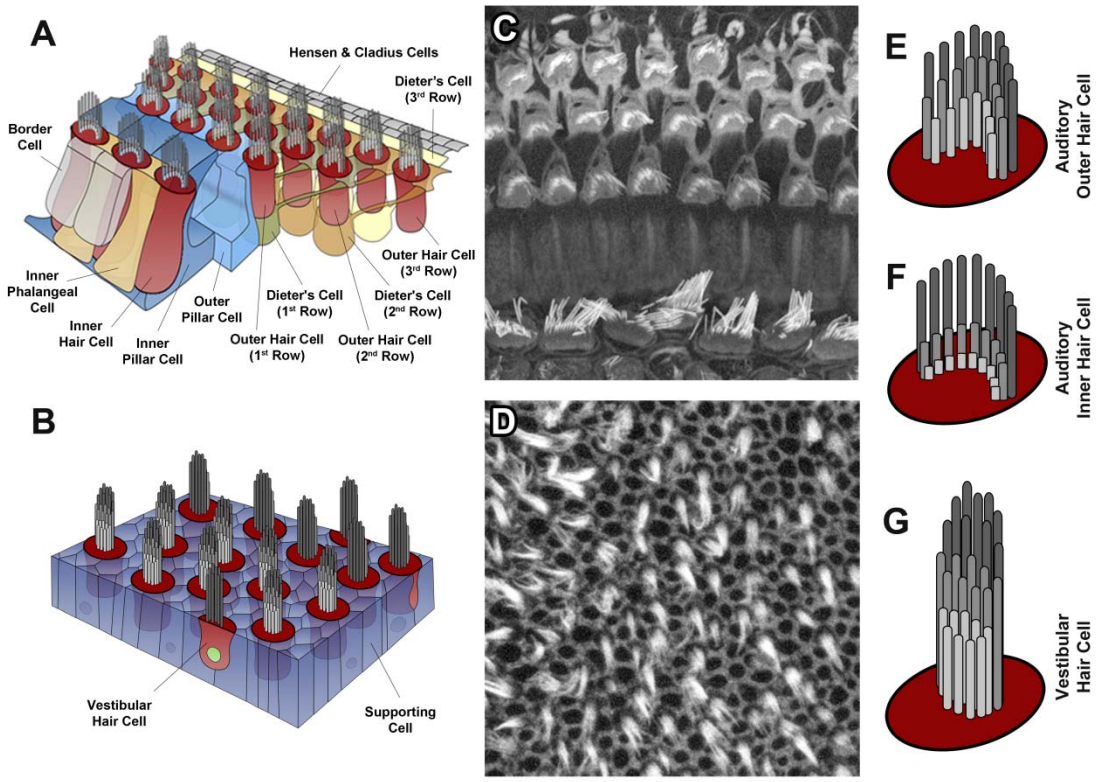

Fig. 2 - Inner Ear Sensory Organ Patterning

(*A*) Three-dimensional diagram of the auditory sensory epithelium, the organ of Corti, showing the variety of cell types and their distinct morphologies. Inner hair cells form a single row of cells and outer hair cells form three rows of cells that run the length of the cochlea, all of which display coordinately polarized stereocilia bundles on their apical surfaces. Inner and outer pillar cells separate the inner hair cell row from the outer hair cell region. Dieters' cells sit below outer hair cells and send highly-polarized phalangeal processes to form a lumenal surface that separates outer hairs of the next row, but at a location three positions away. (*B*) Three-dimensional diagram of a representative vestibular sensory epithelium showing mosaic patterning of hair cells and supporting cells, with hair cells displaying a coordinated polarization of their stereocilia bundles. (*C*) Example of a phalloidin-stained mature P14-stage mouse cochlea. (*D*) Example of a phalloidin-stained P6-stage mouse utricle. (*E-G*) Diagrams of the differing morphologies of inner

and outer auditory hair cells and vestibular hair cells. All classes of hair cells have intrinsic polarity in the shape and graded length of their stereocilia rows. Outer hair cell stereocilia (*E*) are formed from a chevron-shaped template, but inner hair cell stereocilia (*F*) originate from a crescent-shaped template. Vestibular stereocilia (*G*), which are typically much longer that those of auditory hair cells, are generated from a more circular template.

C. Pathway of Hearing: From Sound to Hair Cell Response

Sound generated by a resonating source travels through air as oscillating changes in pressure. These sound waves propagate outward in all directions and continue until their energy dissipates or they encounter an obstacle. Organisms adapted to sense sounds within the frequency range of that particular wave of oscillating pressure might be able to perceive the sound, if it arrives at the organism's auditory sense organ with sufficient intensity, or amplitude. Most natural auditory environments are noisy and complex, with many simultaneous sources and many sounds that have elaborate frequency, intensity, and temporal profiles. It therefore becomes important not only for an organism to be able to detect a sound, but also be able to decipher multiple aspects about that sound.

In a mammal, a series of events occur to transmit the sound from the external environment to the specialized auditory structures within the inner ear. Sound waves reaching the external ear can enter through the ear canal and make their way to the tympanic membrane, commonly known as the eardrum (Fig. 1*A*). The tympanic membrane, which separates the external ear from the middle ear space, vibrates in response to the oscillating pressure changes. On the opposing side of the tympanic membrane, within the middle ear space, a series of three bones called the ear ossicles, are connected in series and articulate in response to tympanic membrane movement. The third bone in the series of ear ossicles, known as the stapes, contacts a membranous opening on the cochlea and pushes on the fluid-fluid scala vestibuli, thereby recreating an oscillating difference in pressure within the cochlea (Fig. 1*B*). These pressure disturbances cause the basilar membrane to move in an up and down motion, resulting in mechanical forces that act upon the cells within the organ of Corti (Fig. 3*A*).

The organization of and adhesion between cells within the organ of Corti creates a degree of rigidity that allows the motion of the basilar membrane to be transmitted to the mechanosensitive structures of hair cells without compromising cellular or tissue integrity. Hair cells are able to detect the motion of the basilar membrane through relative changes in the tilt of their stereocilia bundles on their apical surface (Fig. 3*B*). Even minute angular tilts of the stereocilia bundle can lead to a robust hair cells response (Hudspeth 1989); however, the angle of deflection must occur along the line of intrinsic polarization in order to elicit maximal response (Shotwell *et al.* 1981), making it clear why a coordinately polarized sensory epithelium is essential for inner ear function. The deflection of the stereocilia bundle towards the tallest row leads to the opening of mechanosensitive ion channels and a subsequent influx of potassium (K^+) and calcium (Ca^{2+}) ions into the hair cells, causing a rapid depolarization (Fig. 3*C*). Deflections in the opposite direction cause the closure of these ion channels, thereby allowing both a positive and negative change relative to baseline resting conductance and hair cell membrane potential. This process of mechanoeletrical transduction has been studied extensively and experimental data has been comprehensively reviewed (Hudspeth 1989; Grant and Fuchs 2007; Vollrath *et al.* 2007; Phillips *et al.* 2008; Gillespie and Muller 2009; Grillet *et al.* 2009).

Changes in hair cell membrane potential, as well as increased intracellular calcium, lead to events at the hair cell basolateral surface. This includes graded alterations in the release rate of neurotransmitter at synaptic active zones (Fig. 3*C*), which modify the firing rate of postsynaptic spiral ganglion neurons (Nouvian *et al.* 2006). Voltage and calcium-dependent channels on the basolateral surface also respond to quickly bring the

hair cell back to its resting state. A depolarization of the hair cell, for example, causes increased K^+ and $Ca2^+$ permeable channel opening, an outward current and a repolarization of the cell (Fig. 3*C*) (Marcotti *et al.* 2003). These deflections and resulting changes in conductance occur on a very small timescale and allow large degree of temporal resolution. The entire process of sensory transduction is nicely summarized and illustrated in expert reviews and book chapters (Hudspeth 2000; Corey 2003; Schwander *et al.* 2010).

Although the mechanotransduction ion channels within hair cells have not yet been identified, evidence suggests that they are located on stereocilia tips and attached to the extracellular linkages that connect individual stereocilia tips to each other (Fig. 3*D*). These so called "tip-links" are composed of extracellular dimerizations of proteins Cadherin23 and Protocadherin15 (Kazmierczak *et al.* 2007; Muller 2008; Sakaguchi *et al.* 2009; Alagramam *et al.* 2011). Advanced imaging techniques have recently localized the mechanotransduction channel to a location near the tip link insertion on the lower stereocilia (Beurg *et al.* 2009). Bundle deflection toward the tallest stereocilia results in increased tip-link tension and a synchronized opening of a large number of these mechanotransduction channels (Fig. 3*D*). Mechanical deflection of inner hair cell stereocilia therefore allows for a very efficient, accurate, and precise means of translating basilar membrane movements into ionic currents within individual hair cells.

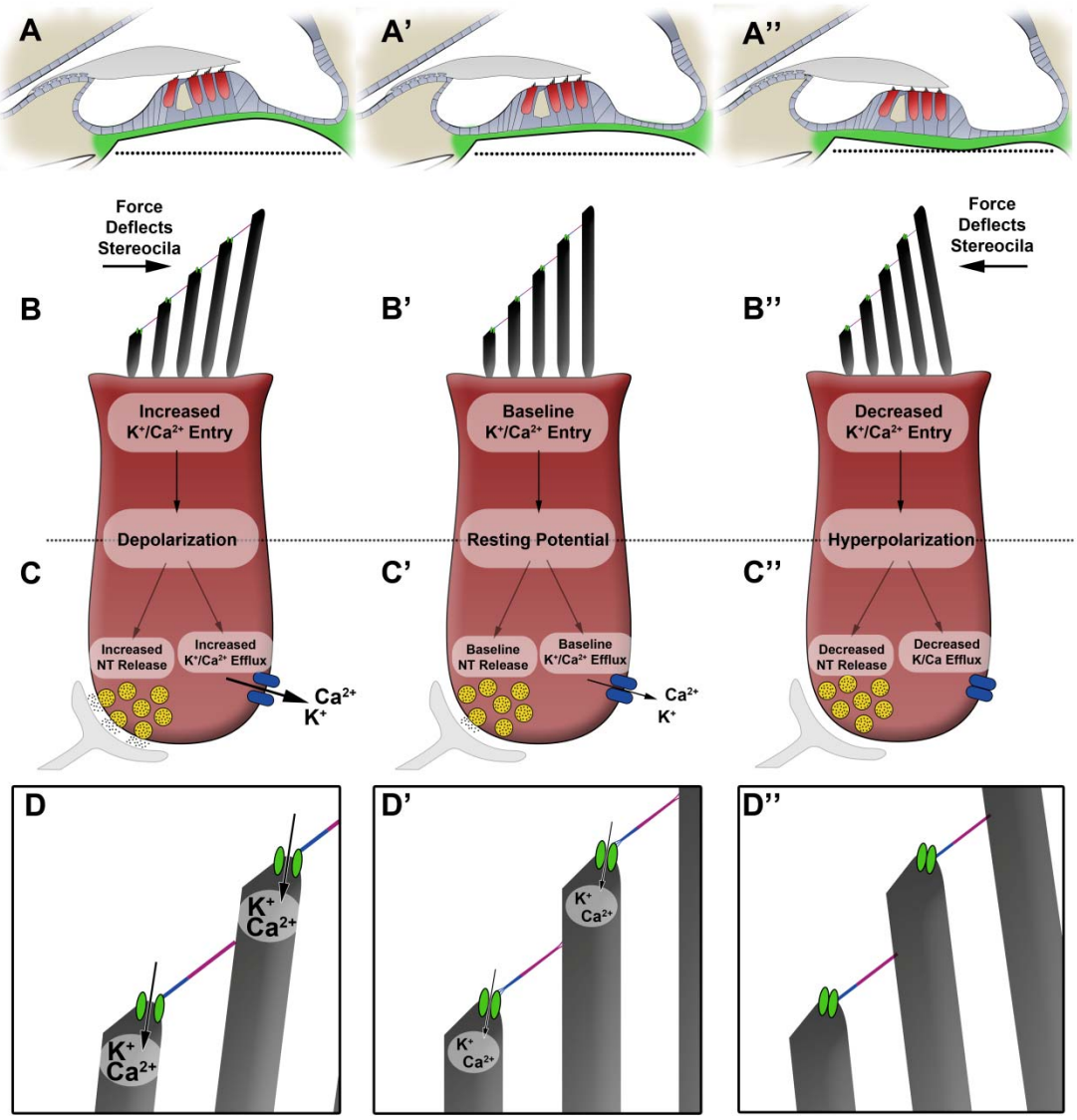

Fig. 3 - The Organ of Corti Converts Sound Into Current

(*A*) Diagram of the effects of oscillatory pressure changes, representing sound, within the cochlea. Increased pressure within the scala vestibuli and scala tympani causes the basilar membrane (*green*) to move in an upward manner compared to its location at rest (*A'*). The negative counterpart of the oscillating pressure change results in a downward movement of the basilar membrane (*A''*). (*B*) Movement of the basilar membrane generates force that causes stereocilia bundle deflection and changes in ion conductance. Upward movement of the basilar membrane results in a force that cause bundle deflection towards the tallest stereocilium and an increase in permeability to K+ and Ca^{2+} relative to the baseline conductance occurring in unperturbed

bundles (*B'*). The influx of K+ and Ca^{2+} leads to a depolarization of the hair cell from its resting potential. Conversely, downward basilar membrane movement is associated with a deflection of the bundle in the opposite direction, causing a decrease in K+ and Ca^{2+} permeability and resulting in hyperpolarization of the cell (*B''*). (*C*) The rate of synaptic neurotransmitter (NT) release and amplitude of basolateral outward rectifying current depends on the membrane potential of the hair cell. A more depolarized cell releases more NT and has a higher K+ and Ca^{2+} conductance than a cell at resting potential (*C'*). These graded changes occur in the opposite direction with hyperpolarization (*C''*). (D) A closer look at the tip-link and mechanotransduction channel function at stereocilia tips during deflections. Deflection towards the tallest stereocilium causes tension along all tip links, leading to a coordinated further opening and increased influx of K+ and Ca^{2+} from baseline amounts (*D'*). Deflection in the opposite direction causes a coordinated closer and decreases in ion flux.

Since many important aspects of our auditory environment, including verbal communication are not limited to sounds of a single frequency, the auditory system must have a way of reliably interpreting complex sounds. Within the mammalian auditory system, this is accomplished in part by graded biophysical properties along the length of the cochlea. Differences in cochlear structure, most importantly the variations in the thickness and width of the basilar membrane, cause individual frequency sounds to effect maximal displacements at a unique position along the length of the cochlea (von Bekesy 1970). This frequency tuning appears to be further refined by differences in the intrinsic properties of hair cells along the length of the cochlea, such as stereocilia length and electrophysiological properties (Lim 1980; Mammano and Ashmore 1996). Sensitivity and frequency selectively of the inner hair cells, which are the primary cells involved in auditory transduction, is likely further amplified by the electromotive activity of outer hair cells (Dallos *et al.* 2006; Dallos 2008). A combination of these factors causes inner hair cells towards the base of the cochlea, and closer to the oval window, to respond best to high-frequency sounds, and the inner hair cells towards the apex of the cochlea to respond best to low-frequency sound. This allows for an anatomical parsing of sounds of different frequencies that permit simpler central processing of a complex stimulus.

1.2 Development of the Auditory Inner Ear

The incredible cellular organization and associated function of the mammalian auditory system are the products of a complex developmental program. Inner ear formation begins early within the embryo as a small population of cells that expand and segregate to form all the sensory and non-sensory structures of the inner ear. Within auditory sensory regions, cells are patterned using multiple overlapping signaling

pathways to direct the growth and maturation of the organ of Corti. Using various experimental model systems, many aspects of the development of the inner ear have been elucidated, although important questions still remain.

A. Inner Ear Induction & Cochlear Duct Outgrowth

The inner ear begins as a patch of ectoderm immediately lateral to the developing hindbrain on either side of the head. This patch, known as the otic placode, is possibly derived from larger domain of thickened ectoderm that also gives rise to other cranial sensory placodes, including those that contribute to the eye and olfactory epithelium. The "pre-placodal" domain appears to form some time soon after the end of gastrulation as a band adjacent to the neural plate and has a distinct gene expression profile (Streit 2007). Signaling molecules, such as fibroblast growth factors (FGF's) and Wnt's (a class of proteins named after the *Drosophila* gene *wingless* and the vertebrate gene *Integration 1*), expressed by the underlying mesoderm and nearby developing hindbrain induce the formation of the otic placode (Wright and Mansour 2003; Urness *et al.* 2010). The size of the otic placode is restricted in part by signaling that restricts the effect of these factors on the ectodermal tissue (Mahoney Rogers *et al.* 2011). Comprehensive reviews detail the experimental evidence important for our current understanding of the specification and induction of the otic placode (Barald and Kelley 2004; Ohyama *et al.* 2007).

The otic placode invaginates and pinches off from the surface ectoderm to form what is known as the otic vesicle, or otocyst. The pathways involved in controlling this morphogenetic event are currently poorly understood. Soon after the otocyst is formed, regional specification and patterning of the otocyst begins. Neuronal precursors begin to delaminate and are driven by intrinsic and extrinsic factors to eventually form the ganglia

that innervate the auditory and vestibular sensory epithelia of the inner ear (Abello and Alsina 2007; Appler and Goodrich 2011; Yang *et al.* 2011). Cells remaining as part of the otocyst are patterned along three-dimensional coordinates to control the morphogenesis of the various structures and cell types of the inner ear (Bok *et al.* 2007a; Bok *et al.* 2011). Molecules expressed along regional domains of the hindbrain are likely candidates for directing these axes, although overlapping roles of multiple factors make interpretation of the experimental evidence difficult (Schneider-Maunoury and Pujades 2007).

The specification of the cochlea duct, which is derived from cells on the ventral portion of the otocyst, relies heavily upon direct and indirect effects of Hedgehog (Hh) signaling originating from the notochord or neural tube floor plate (Riccomagno *et al.* 2002; Brown and Epstein 2011) (Fig. 4*A*). Gradients of Hh activators and repressors further refine this axis, leading to differences between proximal and distal ends of the cochlear protrusion (Bok *et al.* 2007b). Within mouse, the cochlea begins as a small out-pocketing at embryonic day 10.5 (E10.5) and over the course of the next eight days grows significantly to reach its nearly mature length at E18.5 (Morsli *et al.* 1998). Evidence suggests that conserved pathways driving convergent extension activity within the epithelium may play an important role in driving extension of the cochlea duct during this time (Chen *et al.* 2002; Wang *et al.* 2005). Although the mechanism by which the outgrowth and subsequent spiraling of the cochlea is induced remains unclear, it appears that the presence of spiral ganglion neuron precursors, which will innervate the organ of Corti, and a presumptive sensory domain are important for developing a full-length cochlea (Bok *et al.* 2007a; Yang *et al.* 2011). This highlights the idea that the

morphogenesis of the inner ear structures is dependent on the proper development of surrounding tissues and inner ear sensory regions.

B. Auditory Sensory Domain Specification & Cell Cycle Exit

Specification of the sensory domains of the inner ear involves multiple signaling pathways that define the cell populations that will give rise to the hair cells and supporting cells. Although the six sensory domain of the inner develop as independent structures, they may arise from a common prosensory patch within the otocyst and later separate (Kelley 2006; Ohyama *et al.* 2007). Within these separated patches, *Eya1,* a homolog of *Drosophila* gene *eyes absent,* and *Six1,* a homolog of *Drosophila* gene *sine oculis,* encode for transcription factors that have been shown to have important roles in the regional specification of otocyst, including the specification and proper development of the prosensory patch (Zheng *et al.* 2003; Zou *et al.* 2008). This early prosensory patch is derived from the same regional domain of the otocyst as the delaminating neuronal precursors, and prosensory cells are inhibited from the neuronal fate in part by expression of the transcription factor T-box 1 (Tbx1) (Raft *et al.* 2004; Xu *et al.* 2007). LIM homeobox transcription factor 1 alpha (Lmx1a) also appears to be involved in these neuronal versus prosensory decisions, but is later expressed exclusively within non-sensory cells and is necessary for the proper separation of the various sensory organs within the inner ear (Huang *et al.* 2008; Nichols *et al.* 2008; Koo *et al.* 2009).

Within the developing cochlea, the prosensory region that eventually generates the organ of Corti, is defined by converging activity of fibroblast growth factor (FGF), bone morphogenetic protein (BMP), Hedgehog (Hh) and Notch signaling pathways (Fig. 4*B*). Although multiple FGF ligands and receptors play a role in multiple aspects of the

development of the cochlea, FGF20 signaling through FGF receptor 1 (Fgfr1) is clearly important in the formation the auditory prosensory domain (Pirvola *et al.* 2002; Hayashi *et al.* 2008). As for BMP signaling, a gradient originating from BMP4-expressing cells immediately lateral to the prosensory regions appears to be important for defining the sensory and non-sensory domains along the floor of the cochlea (Ohyama *et al.* 2010). Hh signaling, presumably from underlying spiral ganglia precursors, helps define the sensory region by inhibiting expansion towards the medial non-sensory domain (Driver *et al.* 2008). The role that canonical Notch signaling plays in prosensory region induction remains controversial due to confusing and somewhat conflicting results (Yamamoto *et al.* 2006; Basch *et al.* 2011). Despite these results however, Jagged1, which is a ligand for the Notch receptor, also appears to have a role in promoting prosensory formation and can initiate expression of the prosensory marker Sox2 (Kiernan *et al.* 2001; Brooker *et al.* 2006; Kiernan *et al.* 2006; Dabdoub *et al.* 2008). Similarly, ectopic delivery of the active portion of the Notch receptor can lead to the formation of Sox2-positive *de novo* sensory regions (Hartman *et al.* 2010; Pan *et al.* 2010), suggesting that Notch can have a potent effect of inducing sensory region formation.

The expression of several transcription factors marks the presumptive sensory domain, including Isl1 and Prox1, although their exact role in the prosensory region is unclear (Radde-Gallwitz *et al.* 2004; Bermingham-McDonogh *et al.* 2006). The transcription factor Sox2, which also marks the prosensory domain, is known to be required for the development of the inner ear sensory organs (Kiernan *et al.* 2005b). However, ectopic delivery and continual expression of Sox2 cannot form new sensory regions (Dabdoub *et al.* 2008).

Within the prosensory region the spatial and temporal regulation of cell cycle exit is tightly controlled. Interestingly, cells within the prosensory region become post-mitotic before the surrounding non-sensory epithelial cells (Ruben 1967; Chen and Segil 1999) (Fig. 4*C*). This appears to be associated with the expression of the cyclin dependent kinase inhibitor Cdkn1b, or p27/Kip1, which is expressed strongly within the prosensory domain (Chen and Segil 1999; Lowenheim *et al.* 1999). Expression of Cdkn1b begins in the apical turn of the mouse cochlea around E12.5 and progresses as a gradient towards the base, completing the coordinating of prosensory cell cycle exit around E14.5 (Lee *et al.* 2006). Interestingly, this cell cycle exit occurs before the cochlea has fully completed its elongation and before sensory cell fate decisions are made.

Various factors are involved in the coordination and maintenance of cell cycle exit within the developing and mature organ of Corti, and genetic deletions of *Cdkn1b*, as well as other cyclin dependent kinase genes *p19* and *p21*, lead to abnormal proliferation of these cells (Lowenheim *et al.* 1999; Chen *et al.* 2003; Laine *et al.* 2007; Oesterle *et al.* 2011). Similarly, inactivation of the *Retinoblastoma* (*Rb*) gene family members results in continued proliferation of cells within the sensory region (Mantela *et al.* 2005; Sage *et al.* 2005; Yu *et al.* 2010; Rocha-Sanchez *et al.* 2011). Although the exact mechanism is unknown, Notch signaling also appears to play a role in controlling cell cycle with the prosensory domain, as deletion of some components of this pathway lead to aberrant proliferation (Kiernan *et al.* 2005a). Notch signaling may represent a point of intersection between prosensory domain induction, cell cycle control, and later cell fate decisions, all of which have crucial importance in generating an organ of Corti with the correct relative location, number of cells, and overall cellular patterning.

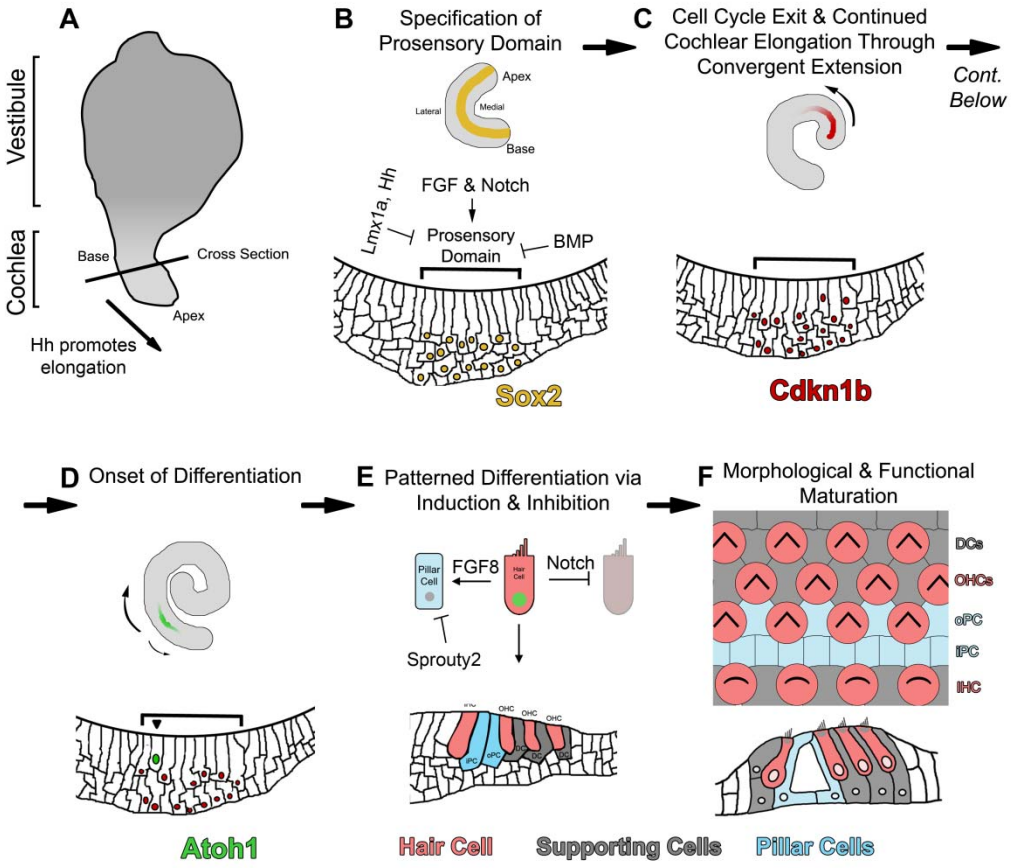

Fig. 4 - Multiple Pathways Control The Development of The Organ of Corti

(*A*) The cochlear duct begins as an expansion of the ventral-most portion of the otocyst. Initial specifications of prosensory domain most likely begin as soon as the cochlear duct is recognizable. (*B*) The prosensory domain is further restricted to a group of cells on the floor of the cochlea duct (*shown with bracket*) by the action of Sox2, Notch and Hedgehog (Hh) signaling, and Lmx1a. Diagrams for the whole-mount cochlea (*top*) and the cross-section of the cochlea at this stage highlight the expression of Sox2 (*yellow*) in the prosensory domain. (*C*) The sensory precursor cells become postmitotic under the control of several cyclin-dependent kinase inhibitors, including Cdkn1b. Cell cycle exit is initiated in the cells in the apical region and progresses toward the basal region, coinciding with the gradient of Cdkn1b onset in the cochlea.

The curved arrow indicates the direction of onset of Cdkn1b and the gradient of cell cycle withdrawal along the longitudinal axis of the cochlear duct. (*D*) The first sign of differentiation within the postmitotic prosensory domain is the expression of the transcription factor Atoh1, which starts in the mid-basal region and progresses toward both the apex and the base of the cochlea. There is also a second gradient along the medial-lateral axis of the cochlea from the inner to outer hair cells. The curved arrows indicate the longitudinal gradient of Atoh1 expression onset and hair cell differentiation. (*E*) Inductive and inhibitory signaling creates the correct cellular patterning of the organ of Corti. Much of this appears to be mediated by Notch signaling, which inhibits hair cell neighbors from adopting the same fate. Furthermore, the initial differentiation of the inner hair cells appears to direct the differentiation of other cells types, such as the Pillar cells (*blue*) through FGF8 and Hey2 in a Notch-independent manner. Sprouty2 further restricts the differentiation of pillar cells. (*F*) During terminal differentiation and maturation, all cells in the organ of Corti coordinate their cellular morphologies under the regulation of the planar cell polarity (PCP) signaling pathway. In mice, late embryonic and early postnatal hair cell and supporting cell types all undergo morphological and maturational changes that ultimately result in a highly sensitive sensory structure that is functional by two weeks after birth. Source: Modified from (Kelly and Chen 2009) – used with permission.

C. Cell Fate Decisions, Arrangement & Patterning in the Cochlea

In order for the prosensory region to be transformed from a population of post-mitotic sensory precursors into a fully developed organ of Corti, cell fate and patterning decisions must be carefully coordinated. The presumptive sensory region can be identified morphologically as a thickening within the floor of the cochlear epithelium. One of the earliest signs of cellular differentiation within the prosensory region is the expression of the proneural basic helix-loop-helix (bHLH) transcription factor Atonal homolog 1 (Atoh1), formerly Math1 (Fig. 4*D*). Early studies showed that Atoh1 was a hair cell differentiation factor that was necessary and sufficient for directing the sensory hair cell fate within the developing cochlear epithelium (Bermingham *et al.* 1999; Zheng and Gao 2000). Expression of the Atoh1 protein is first detected on the very medial edge of the prosensory domain beginning in the mid-base region at E13 in the mouse cochlea (Chen *et al.* 2002). Expression of Atoh1 progresses as a gradient along the length of the cochlea and from the medial to lateral side of the sensory region (Lanford *et al.* 2000; Chen *et al.* 2002) (Fig. 4*D*). The initiation of Atoh1's expression may be in part initiated by FGF20 signaling through Fgfr1, although the exact mechanism is still unclear (Hayashi *et al.* 2008). Atoh1's expression may be restricted within the early prosensory region as least in part by activity of inhibitor of differentiation and DNA binding proteins (Id's), which are expressed throughout the region and can block Atoh1's ability to promote its own expression through a positive auto-regulatory loop (Helms *et al.* 2000; Jones *et al.* 2006). Interestingly, differentiation of the organ of Corti follows a gradient

opposite to the apical to basal gradient of cell cycle exit, leaving the earliest born cells to be the last to differentiate in the apical turn of the cochlea.

The cascade of events that follow the onset of Atoh1 expression within developing hair cells is poorly understood. Despite its developmental importance, only a few direct binding targets of Atoh1 within hair cells have been indentified (Scheffer *et al.* 2007a; Scheffer *et al.* 2007b). A more complete targetome has been elucidated within Atoh1-positive cells of the cerebellum and intestinal epithelium, but it is clear that tissue-specific differences exist, suggesting that results may be non-transferrable (Bermingham *et al.* 2001; Klisch *et al.* 2011; Lai *et al.* 2011). Though there is a paucity of direct Atoh1 binding targets identified in hair cells, several factors are known to be downstream of Atoh1 and essential for proper hair cell development. The unconventional myosins, MyoVI and MyoVIIa, are observed very early in hair cell differentiation, provide a clear hair cell marker, and are essential for hair cell development (Hasson *et al.* 1997; Friedman *et al.* 1999). POU domain, class 4, transcription factor 3 (Pou4f3), also known as Brn-3c, is also expressed soon after Atoh1, but is only required for the later maturation and survival of hair cells (Xiang *et al.* 1997; Xiang *et al.* 1998; Keithley *et al.* 1999). Pou4f3's essential role in hair cell survival may be through its direct promoting of expression of growth factor independent I (GfiI), which is also necessary for hair cell viability (Wallis *et al.* 2003; Hertzano *et al.* 2004). Several other factors expressed early within developing hair cells are important for controlling the cell fate decisions of surrounding prosensory cells.

Notch signaling plays a crucial role in hair cell versus supporting cell decisions within the organ of Corti that aids in defining the mosaic pattern of these cell types (Fig. 4*E*).

Notch ligands, Delta1, Delta3, and Jagged2, expressed in developing hair cells activate Notch1 receptors in surrounding cells and prevent them from sharing the default proneural hair cell fate through a lateral inhibitory mechanism (Lanford *et al.* 1999; Zine and de Ribaupierre 2002; Kiernan *et al.* 2005a; Hartman *et al.* 2007). Binding of a Notch ligand to the receptor leads to γ-secretase-mediated proteolytic cleavage of the receptor and initiation of a signaling cascade that leads to the expression of Notch target genes (Andersson *et al.* 2011). Many Notch signaling target genes, including *Hes1*, *Hes5*, and *Hey2* are expressed within supporting cells and have been shown to inhibit hair cell differentiation (Lanford *et al.* 2000; Zheng *et al.* 2000; Zine *et al.* 2001; Li *et al.* 2008). Not surprisingly, genetic disruption of Notch signaling components at various point along the pathway or pharmacological inhibition of γ-secretase cleavage lead to supernumerary hair cells and disrupted sensory region patterning (Takebayashi *et al.* 2007; Driver and Kelley 2009; Yamamoto *et al.* 2011).

While Notch signaling indirectly preserves the supporting cell population from the default hair cell fate, other factors actively induce the formation of certain classes of supporting cells. The asymmetric cellular patterning, including the stereotyped relative location of various subtypes of supporting cells across the mediolateral axis of the organ of Corti suggest that the induction of non-sensory cell phenotypes must be tightly controlled. Although much is still unknown about how this pattern is specified, important progress has been made recently. FGF8, which is expressed by early-forming row of inner hair cells, acts through Fgfr3 to direct and maintain the differentiation the inner pillar cell row (Colvin *et al.* 1996; Mueller *et al.* 2002; Hayashi *et al.* 2007; Jacques *et al.* 2007; Puligilla *et al.* 2007; Doetzlhofer *et al.* 2009) (Fig. 4*E*). This FGF signaling acts

through a Notch-independent mechanism to induce expression of Hey2, which prevents developing Pillar cells from converting to hair cells (Doetzlhofer *et al.* 2009). Sprouty2 antagonizes FGF signaling in more lateral sensory region cells, preventing the formation of supernumerary pillar cells (Shim *et al.* 2005). FGF signaling also appears to function with BMP signaling to regulate the number of Deiters' cells that are formed, possibly by defining the size of the outer hair cell region (Hayashi *et al.* 2007; Puligilla *et al.* 2007; Ohyama *et al.* 2010). In addition to FGF8 expression within the inner hair cell row and BMP4 expression from cells immediately lateral to the sensory region, other factors may also be important for the asymmetric cellular patterning within the organ of Corti. The mammalian homologue of the fly sine oculis-binding protein gene, *Sobp* or *Jxc1*, appears to be import for the asymmetry of the organ of Corti, and its genetic deletion sometimes leads to the formation of mirror symmetry of the sensory region (Chen *et al.* 2008).

At the time that cell fate decisions are being made within the prosensory region, the cochlea is still actively elongating. Within the differentiating sensory domain, an active process most likely representing convergent extension drives the tissue to thin, narrow and extend towards the apex (Chen *et al.* 2002; McKenzie *et al.* 2004; Wang *et al.* 2005). This process requires the proper functioning of a set of conserved proteins, known as the core planar cell polarity (PCP) proteins, and the associated experimental evidence has been comprehensively summarized in several reviews (Jones and Chen 2007; Kelly and Chen 2007; Chacon-Heszele and Chen 2009; Rida and Chen 2009). The directed forces needed to drive these movements appear to be generated at least in part by the asymmetric expression and activity of Myosin II within prosensory cells (Yamamoto *et al.* 2009). It is possible that other factors such as differential cell adhesion amongst

different sensory cell populations may also be involved. In addition to the long-range morphogenetic movements of cochlear extension, individual cell subtypes must refine their final relative location and contacts with neighboring cells to achieve the highly stereotyped cellular arrangement of the organ of Corti. Little is known about how this exact cellular pattern is defined. However, differential binding between the class of adherens junction-associated proteins known as Nectins have been recently shown to ensure that hair cells remain insulated by surrounding supporting cells (Togashi *et al.* 2011). Regardless of the exact mechanisms involved, these movements must be well regulated in order to distribute the correct number and type of cells along the length of the cochlea and to tightly control their relative location and cellular contacts within the organ of Corti.

As hair cells and supporting cells differentiate, they take on characteristic morphologies, many of which have distinctly asymmetric polarizations across the plane of the epithelium. Hair cells develop asymmetrically-polarized stereocilia bundles on their apical surfaces, all of which become coordinately aligned with vertices pointed towards the lateral side (Fig. 4*F)*. Although supporting cells are devoid of stereocilia bundles, many have extended asymmetric processes that are also coordinately polarized (Fig. 2*A*). Many of the core PCP proteins involved in the directed cellular movements necessary for cochlear extension are also functionally required in directing these processes. Because the organ of Corti represents such a striking example of planar polarized cells, it has become an active research model for studying vertebrate PCP and recent progress has been the subject of several reviews (Jones and Chen 2007; Kelly and Chen 2007; Chacon-Heszele and Chen 2009; Rida and Chen 2009).

D. Postnatal Cochlear Maturation and Hearing Onset

The functional maturation of the various components of cochlea must be coordinated to allow for all of the systems to cooperate in the onset of hearing. Final maturation of mechanotranduction and synaptic components of hair cells must occur and other parts of the cochlea must also undergo changes that are important for the proper functioning of the auditory system. Much of this process can be considered continuous with the development of the cochlea.

In mouse, the onset of hearing is around two weeks after birth and this coincides with the time that hair cells become functional mechanotranducers of sound (Geleoc and Holt 2003). This involves the development of mature stereocilia bundles in a polarized staircase-like pattern, assembly of mechanotransduction components and regulation extracellular linkages (Frolenkov *et al.* 2004; Nayak *et al.* 2007; Kelly and Chen 2009; Petit and Richardson 2009; Schwander *et al.* 2010). Relative stereocilia height is regulated in cells along the length of the cochlea and may be one of the intrinsic properties of individual hair cells that impart preferential sensitivity to certain frequencies along the tonotopic gradient (Lim 1980; Raphael and Altschuler 2003; Manor and Kachar 2008). Interestingly, hair cells appear to gain their ability to transduce sound along the same basal to apical gradient in which they differentiate (Lelli *et al.* 2009). Refinement of the distinct differences between inner and outer hair morphology and function, including the distinct stereocilia shapes, outer hair cell electromotility, and synaptic maturation, are coordinated in part via thyroid hormone signaling and dependent upon miR-96 activity (Rusch *et al.* 2001; Weber *et al.* 2002; Sendin *et al.* 2007; Kuhn *et al.* 2011).

Little is known about the pathways involved in the maturation of individual supporting cell subtypes, which have highly differentiated morphologies necessary of auditory function within the mature organ of Corti (Fig. 4*F*). Early supporting cell fate decisions appear to be more of a passive consequence of inhibition of hair cell fate, as experimental manipulations where these inhibitory signals are removed in embryonic or neonatal cochlea lead to their conversion to hair cell (Kelley *et al.* 1995; White *et al.* 2006; Doetzlhofer *et al.* 2009). Consistent with this, several proteins, including Cdkn1b, Sox2, and Prox1, which are expressed within the entire prosensory domain and later downregulated in differentiated hair cells, are maintained within the supporting cells population (Chen and Segil 1999; Kiernan *et al.* 2005b; Bermingham-McDonogh *et al.* 2006; Dabdoub *et al.* 2008). However, at least two of these factors, Prox1 and Sox2, can also actively repress hair cell differentiation, raising the possibility of a role in non-sensory cell identity (Dabdoub *et al.* 2008; Kirjavainen *et al.* 2008). The supporting cell inducing effects of FGF8 signaling through Fgfr3, particularly in defining the inner pillar cell subtypes that help form the tunnel of Corti, are one case where a positive factor is known (Mueller *et al.* 2002; Hayashi *et al.* 2007; Jacques *et al.* 2007; Puligilla *et al.* 2007; Doetzlhofer *et al.* 2009). However, the exact mechanism leading to the mature pillar cell phenotype is unknown and disruption of this signaling pathway still leads to a conversion to the default hair cell fate. Other fairly early markers of supporting cells exist, but their exact role within supporting cell identity is unknown and some lack a very specific supporting cell specific expression (von Bartheld *et al.* 1991; Gestwa *et al.* 1999; Knipper *et al.* 1999; El-Amraoui *et al.* 2001; Rio *et al.* 2002; Sato *et al.* 2006; Brors *et al.* 2008; Smeti *et al.* 2011). Amongst these, glial fibrilary acidic protein (GFAP) appears to

have relatively well defined expression within differentiated supporting cells of the organ of Corti (Rio *et al.* 2002; Smeti *et al.* 2011). Overall, more work is needed to define the pathways involved in the differentiation and maturation of these crucial cell types within the maturing organ of Corti.

Many other portions of the cochlear epithelium undergo significant changes leading up to onset of hearing. The stria vascularis on the lateral wall of the cochlea matures and begins generating the endocochlear potential within the scala media (Schmidt and Fernandez 1963; Sagara *et al.* 1995; Wangemann 2006). The acellular tectorial membrane extends fully over the organ of Corti and forms contacts with the outer hair cell stereocilia (Rueda *et al.* 1996). Kölliker's organ cells, underneath the tectorial membrane, regress in a basal to apical gradient to form the inner sulcus (Hinojosa 1977). Non-sensory cells within and surrounding the organ of Corti form a gap-junction mediated network, allowing biochemical coupling necessary for cochlear function, including the recycling of potassium to the stria vascularis (Kikuchi *et al.* 2000; Zhang *et al.* 2005; Kelly *et al.* 2011). Multiple aspects of the maturation of the cochlear epithelium, in addition those mentioned within the organ of Corti, appear to be dependent upon thyroid hormone (TH) signaling (Knipper *et al.* 1999; Rusch *et al.* 2001; Weber *et al.* 2002; Sendin *et al.* 2007). Although many pathways are likely involved, the coordination of the various final developmental steps allows for all components to begin functioning together for the onset of hearing.

1.3 Sensorineural Hearing Loss & Regeneration

Our sense of hearing depends on the proper development and continued functioning of the many complex components that make up our auditory system. This includes the

survival of the hair cells, which are responsible for the conversion of sound into neural signals. In normal situations, the auditory system functions throughout a lifetime. However, damage to the auditory sensory structures within the inner ear, most commonly the loss of auditory hair cells, can have devastating effects and lead to impairment known as sensorineural hearing loss. Whereas other species retain the ability to regenerate hair cells and recover hearing, the mature mammalian auditory system lacks a spontaneous hair cell regeneration response and hearing impairments due to hair cell loss are permanent. Since hearing is important for quality of life and sensorineural hearing loss affects a significant portion of the human population (NIDCD, *http://www.nidcd.nih.gov/health/statistics/hearing.html*), permanent hair cell loss represents a serious public health concern. Several active avenues of research explore ways to promote regeneration in the mammalian inner ear.

A. Hair Cell Death

The essential function of hair cells in the conversion of mechanical sound forces into neural signals, as well as the tonotopic specificity of individual hair cells along the length of the cochlea, make the role of each hair cell important to hearing sensitivity to the full spectrum of sound frequencies. Hair cell damage can be due to a variety of genetic and environmental causes, with deficits manifesting at the time of birth, progressively over the course of ageing, or suddenly at any time in between. While some post-traumatic recovery of damaged hair cells has been observed in culture sensory epithelia (Sobkowicz *et al.* 1996), the extent to which this occurs *in vivo* is unknown and is clearly insufficient for the maintenance of functional hair cells throughout life.

Genetic causes of hearing loss, which represent a large percentage of cases of impairment in the human population, often act directly or indirectly through damage to hair cells (Lenz and Avraham 2011). For example, mutations in the gene encoding the gap-junction protein Connexin26, which accounts for up to half of all cases of hereditary prelingual deafness, causes the disruption of cochlear non-sensory cell biochemical coupling and leads to the progressive degeneration of hair cells (Cohen-Salmon *et al.* 2002). In addition to those already identified, new genetic mutations associated with hearing impairment continue to be identified, and the study of how they relate to auditory function remain an active area of research (Steel and Brown 1996; Petit *et al.* 2001; Raviv *et al.* 2010; Shearer *et al.* 2011). The results of these studies will likely provide a better understanding of multiple factors that contribute to the survival and functioning of hair cells and for auditory function. However, any potential therapy developed to treat hearing loss due to a genetic mutation, including one that promotes the replacement of hair cells, will also have to consider the possible continued deleterious effects of the mutation. Although mutations in key genes can alone cause hair cell death and hearing loss, other mutations may cause an increase in sensitivity to environmental factors.

A number of factors within the environment can have detrimental effects on the survival of hair cells, including mechanical overstimulation, toxic chemicals, and infection. This may be in addition or in combination with the loss associated with what might be considered the "natural" aging process. Recent work has uncovered some of the underlying mechanisms by which these factors cause the death of hair cells and identified possible targets of therapeutic interventions for the prevention of hair cell loss (Raphael 2002; Cheng *et al.* 2005; Warchol 2010). While these therapies may help prevent new

hair loss and further hearing impairment, they will likely need to be administered as a prophylactic or soon after injury. Furthermore, they will be ineffective at reversing hearing loss associated with previous hair cell death.

Regardless of the underlying cause, hair cell death leads to changes in the organization of the mammalian sensory epithelium. This includes the sealing of apical surface of the organ of Corti by supporting cells in the region where hair cell are lost, likely to prevent the K^+-rich endolymph from having toxic effects on the basolateral surfaces of remaining hair cells and spiral ganglion neurons (Raphael 2002). A similar active involvement of supporting cells in the restructuring of the sensory epithelium following hair cell death is also seen within avian sensory epithelia (Bird *et al.* 2010). However, supporting cells within the organ of Corti do not share all the same cellular responses to hair cell loss with the avian system, and mammalian supporting cells often lose their highly-differentiated morphologies and eventually regress to form a flat epithelium (Kim and Raphael 2007). Loss of hair cells also leads to a progressive degeneration of spiral ganglion innervation (Spoendlin 1984), likely due to a loss of neurotrophic support from hair cells (Fritzsch *et al.* 2004).

Unfortunately, following the death of existing hair cells, mammalian sensory epithelia appear to have an extremely limited ability to spontaneously generate replacement cells. Although some regeneration has been reported in some mammalian vestibular sensory epithelial, even in mature organs (Forge *et al.* 1993; Warchol *et al.* 1993), the organ of Corti loses its regenerative response during early development. Embryonic cochlear supporting cells have a certain degree of plasticity and convert to hair cells following the ablation of the original hair cells (Kelley *et al.* 1995), but hair cells lost from the mature

mammalian cochlea do not appear to be replaced (Forge *et al.* 1998). This makes the changes associated with the loss of auditory hair cells, including hearing impairment, permanent.

B. Hair Cell Regeneration in Non-Mammalian Species

It seems that the survival advantages granted by the ability to detect and process stimuli across multiple modalities, including sound, would make it advantageous for an organism to be able to maintain hearing sensitivity throughout its lifetime. Many primary sensory cells, including retinal photoreceptors, olfactory neurons, and hair cells, are subject to many environmental factors and some of the most likely components of our sensory organs to require replacement after damage. In fact, it appears that many species utilize a fairly conserved process for regenerating these sensory cells and the similarities these processes have been recently reviewed (Bermingham-McDonogh and Reh 2011). Despite this, the ability to replace hair cells differs greatly between species, with mammals unable to accomplish the same regeneration as that of other vertebrate classes (Warchol 2011).

The phenomenon of hair cell regeneration in non-mammalian species has been known for quite some time (Stone 1937), but more extensive characterization began in the 1980's when it was observed that birds had the ability to regenerate hair cells following specific hair cell ablation (Corwin and Cotanche 1988; Ryals and Rubel 1988). Since then, much work has been done to characterize the process of functional hair cell regeneration observed in multiple species, and several expert reviews have summarized these results and made comparisons to mammalian inner ear sensory organs (Stone and

Cotanche 2007; Brignull *et al.* 2009; Cotanche and Kaiser 2010; Bermingham-McDonogh and Reh 2011; Warchol 2011).

Whereas some non-mammalian organs continually replace or add hair cells, the avian auditory sensory organ, known as the basilar papilla, only generates new hair cells following damage, making it one of the better models for understanding the regenerative response (Stone and Cotanche 2007). Initial studies with tritiated thymidine labeling showed that following damage, supporting cells divided and that some of these labeled cells became new hair cells (Corwin and Cotanche 1988; Ryals and Rubel 1988). During these regeneration events, supporting cell nuclei moved apically away from the basilar membrane before undergoing mitosis (Raphael 1992; Tsue *et al.* 1994). It was later determined that the generation of hair cells from the supporting cell population did not require proliferation, at least within the basilar papilla, suggesting that hair cell regeneration could occur through a process of phenotypic conversion called direct transdifferentiation (Adler and Raphael 1996). Soon after the ablation of existing hair cells, cells with morphologies consistent with a supporting cell to hair cell transition can be observed, including cells with a lumenal migration of the their nucleus and basolateral surface, as well as the formation of immature stereocilia bundles (Adler and Raphael 1996). In fact, the earliest regenerated hair cells appear to arise through direct transdifferentiation, and it has been hypothesized that supporting cell division may only be a secondary response to replace the converted cells (Roberson *et al.* 2004). If this is actually the case, then processes of mitotic regeneration versus direct transdifferentiation might be a continuation of the same event, with both undifferentiated and differentiated supporting cells being potential hair cell progenitors.

One recurring theme in nearly all systems that regenerate hair cells is that the processes involved reconstitute many aspects of the same developmental program that generated the original organ (Cotanche and Kaiser 2010; Bermingham-McDonogh and Reh 2011). Following damage to the avian basilar papilla, Atoh1 is upregulated in a large population of supporting cells following damage, but then only persists in the cells that differentiate into replacement hair cells (Cafaro *et al.* 2007). Notch signaling components are also upregulated in the damaged epithelium and Notch activity is required to specify the correct ratio of hair cells and supporting cells for the regenerating organ (Stone *et al.* 1999; Daudet *et al.* 2009). As has been shown previously within a regenerating zebrafish hair cell-based sensory organ, regenerated hair cells within the basilar papilla regain the proper coordinated orientation of their stereocilia bundles utilizing what appears to be the same conserved set of PCP proteins required during development (Lopez-Schier and Hudspeth 2006; Warchol and Montcouquiol 2010).

Why does the mammalian auditory system seem incapable of hair cell regeneration, while all other vertebrate classes show some degree of recovery? Despite the progress made in understanding the hair cell regeneration process that can occur in various non-mammalian sensory organs, many questions about how the process is regulated and why it is not initiated in the mammalian cochlea still remain unanswered. It is unclear how the knowledge of the regeneration process will translate to the mammalian system; however, there is hope that a better understanding of the process of hair cell regeneration of non-mammalian species may help identify key differences from non-regenerative mammalian species.

C. Strategies to Promote Hearing Recovery in Mammals

The prevalence of hearing impairment in the human population and the lack of a natural regenerative process makes the improvement of hearing a significant public health concern. Although prostheses are available, they have limitations and are bounded by other biological considerations. Guided by a continually expanding body of work dissecting pathways involved in the normal development of the auditory system and fueled by knowledge of non-mammalian functional hair cell regeneration, researchers continue look for ways to promote functional repair in mammals. There are theoretically multiple ways to accomplish this, including releasing endogenous progenitors from an inhibitory signal that is preventing regeneration, delivering replacement progenitor cells to produce new hair cells, or by inducing regeneration via gene therapy or drug. While research along all of these avenues has shown promise, no clinical therapeutic has yet been developed and more work has yet to be done (Oshima *et al.* 2010b; Shibata and Raphael 2010).

Auditory prostheses are currently the only treatment option for those with sensorineural hearing loss. Hearing aids, which work simply as sound amplifiers and still rely upon existing hair cells, are only helpful in turning up the signal gain in individuals with residual auditory function. Cochlear implants, on the other hand, convert environmental sounds into electrical stimulation that bypasses auditory hair cells and directly activate spiral ganglion neurons at different points along the length of the cochlea, utilizing principles of tonotopic innervation to encode frequency information (Rubinstein and Miller 1999). Although the cochlear implant electrode bypasses a non-functional organ of Corti, it still requires spiral ganglion neurons and requires a relatively

invasive procedure to implant the electrode. This strategy is most appropriate for children, who may have relatively healthy cochlear innervation and a higher capacity to learn how to interpret the simple parsed signals created by the prostheses as representations of complex sounds, including language. While cochlear implants are an important medical advancement, their significant overall limitations warrant further study into other possible means of promoting hearing recovery.

The regenerative response in other vertebrate classes and a certain degree of plasticity within the developing embryonic and neonatal inner ear, suggest that the adult mammalian ear may be actively inhibiting a conserved repair mechanism (Kelley *et al.* 1995; White *et al.* 2006; Doetzlhofer *et al.* 2009; Warchol 2011). An inability to replace lost hair cells may be the result of an evolutionary trade-off for high-frequency hearing, which is achieved through the highly stereotyped cellular architecture of the organ of Corti and may be disrupted by the replacement of cells. By blocking the inhibitory signals that might be preventing cellular conversion or cell cycle re-entry, a functional organ of Corti may be regenerated, even if it doesn't have the same degree of sensitivity (Brigande and Heller 2009; Kwan *et al.* 2009). Although a blockade of Notch signaling may seem like a reasonable strategy based on its role during development, Notch signaling components appear to be down-regulated well before hearing onset and blocking Notch signaling does not cause supporting cell conversion after P3 in mouse (Yamamoto *et al.* 2006; Hartman *et al.* 2009). The lack of conversion following hair cell loss or Notch signaling blockade may also be due to the relatively high morphological and molecular differentiation that mammalian supporting cells undergo. A correlation between regenerative processes and some aspects supporting cell differentiation has been

made within vestibular sensory epithelia, but the causal role and possible manipulations towards regeneration have not been directly tested (Meyers and Corwin 2007; Burns *et al.* 2008; Collado *et al.* 2011).

Using knowledge of the pathways involved in the coordination and maintenance of the post-mitotic state of cells within the organ of Corti, one might also attempt to create new sensory progenitors through mitotic events by disrupting of cell cycle inhibition (Lowenheim *et al.* 1999; Mantela *et al.* 2005; Sage *et al.* 2005; Laine *et al.* 2007). However, *in vivo* disruption of the involved components appear to have a detrimental effects on the long-term survival of these sensory cell and many of the cycling cells never differentiate into mature hair cells (Chen *et al.* 2003; Sage *et al.* 2006; Yu *et al.* 2010). Furthermore, there is evidence to suggest that supporting cells would require expression of cyclin D1, which is downregulated before the time of hearing onset (Laine *et al.* 2010). More recently, it has been demonstrated that a temporal inactivation of cell cycle inhibitors in the adult can lead to proliferation and survival of cochlear epithelial cells, although it is unknown if new hair cells are produced as a result (Oesterle *et al.* 2011). Like Notch signaling, the task of inducing proliferation may not be as simple as removing the inhibitory factors.

The progress made in efficient methods in the culture and *in vitro* differentiation of embryonic-derived and induced pluripotent stem cells has been informative and promising for many biomedical fields, and the inner ear biology field is no exception (Li *et al.* 2004). It was discovered that the mammalian inner ear harbored true stem cell populations that could be isolated from vestibular sensory epithelial of embryonic and adult mice (Li *et al.* 2003a). However, the ability to isolate and culture a stem cell

population from the cochlear epithelium was significantly less than that of the vestibular regions, and this small population decreased progressively with age (Oshima *et al.* 2007). Although this suggests that few endogenous cochlear epithelia stem cells are present in adult mammals, a relatively efficient *in vitro* differentiation protocol has been reported for the creation of highly differentiated and "functional" hair cells (Li *et al.* 2003b; Oshima *et al.* 2010a). Despite these exciting results, attempts at injecting various populations of cultured stems cells into the mammalian have so far been unsuccessful at generating new auditory hair cells (Tateya *et al.* 2003). Though a clinical application for stem cell generated replacement hair cells may have many difficulties to overcome, it has become a useful tool for inner ear biology, particularly in understanding the factors that influence hair cell differentiation (Beisel *et al.* 2008; Oshima *et al.* 2010b).

One of the most promising strategies to promote hearing recovery is the induction of developmental genes that will lead to a functional regenerated cochlear epithelium. The induction of ectopic sensory regions has been demonstrated in the mouse inner ear by activation of Notch signaling at various embryonic stages within non-sensory regions (Hartman *et al.* 2010; Pan *et al.* 2010). However, it is unclear if how long this non-sensory plasticity for prosensory formation persists, particularly in cells immediately adjacent to a damaged organ of Corti where Notch signaling's lateral inhibitory effects might compete. Two other genes thought to play an important role in prosensory domain formation, *Prox1* and *Sox2*, may have similar caveats as activated Notch in that their later expression has the effect of inhibiting hair cell differentiation (Dabdoub *et al.* 2008; Kirjavainen *et al.* 2008). Atoh1, which is required for the differentiation of sensory hair cells from the sensory precursor domains during development, has been a particularly

appealing target for promoting hair cell regeneration. Early work has shown that ectopic delivery of Atoh1 to embryonic and neonatal cochlear epithelia can generate new sensory hair cells (Zheng and Gao 2000; Shou *et al.* 2003; Woods *et al.* 2004; Gubbels *et al.* 2008). Furthermore, evidence from viral transfection suggests that Atoh1 can direct the formation of new hair cells in the adult mammalian cochlea (Kawamoto *et al.* 2003; Shou *et al.* 2003; Izumikawa *et al.* 2005). Consequently, Atoh1 has been an important target for hair cell regeneration strategies and represents one of the only strategies demonstrated so far to be capable of functional hair cell regeneration *in vivo* (Edge and Chen 2008; Brigande and Heller 2009; Shibata and Raphael 2010).

1.4 Atoh1: An Incomplete Story in Development and Regeneration

Atoh1 is a member of the basic helix-loop-helix (bHLH) transcription factor family that is known to have important roles during development of many tissues. In mammals, expression of Atoh1 was first discovered within the nervous system and was determined to be a closely related homologue of the fly gene *atonal* (Akazawa *et al.* 1995). In fact, *atonal* can serve as a functional replacement for a null allele of mouse *Atoh1* (Wang *et al.* 2002), illustrating not only Atoh1's conservation, but also its diversity of function depending on cellular context. Atoh1 has been shown to direct the differentiation of numerous unique cells types throughout the mammalian body, including cerebellar granule cells, cerebellar rhombic lip interneurons, dorsal commissural interneurons, goblet cells within the intestine, Merkel touch-sensitive cells with the skin, and of course the sensory hair cells of the inner ear (Ben-Arie *et al.* 1997; Helms and Johnson 1998; Bermingham *et al.* 1999; Bermingham *et al.* 2001; Yang *et al.* 2001; Machold and Fishell 2005; Maricich *et al.* 2009). Atoh1 appears to be essential for the differentiation of many

of these cell types, as mice lacking the Atoh1 gene do not generate those cells (Ben-Arie *et al.* 1997; Bermingham *et al.* 1999; Bermingham *et al.* 2001; Yang *et al.* 2001; Machold and Fishell 2005; Maricich *et al.* 2009). Although Atoh1 is generally thought to promote the terminal differentiation of particular cell types, it can also be expressed in certain progenitor cell populations (Machold and Fishell 2005; Miesegaes *et al.* 2009). Furthermore, the expression of Atoh1 has even been associated with the formation of medulloblastomas (Zhao *et al.* 2008; Flora *et al.* 2009; Ayrault *et al.* 2010), showing that in some situations it may work to promote an undifferentiated and proliferating state.

In addition to the variety of Atoh1's context-dependent effects, classical work with homologues of mouse *Atoh1* suggest that there may be two phases to its activity, with its strong cell-specific expression being proceeded by broader expression within a group of precursors. Within *Drosophila* eye development, Atonal is initially expressed throughout the proneural field and then later resolved through Notch signaling lateral inhibition to individual cells that differentiate into a particular type of photoreceptor (Jarman *et al.* 1994; Hsiung and Moses 2002). Similarly, in the regenerating avian auditory epithelium, Atoh1 expression is initially in a broad field before being upregulated specifically in cells that will become the regenerated sensory hair cells (Cafaro *et al.* 2007). Zebrafish appear to have two homologues of *Atoh1*, *atoh1a* and *atoh1b*, and while there appears to be similar roles in defining both the proneural equivalence domain and in sensory cell differentiation, these two functions seem to be divided between *atoh1a* and *atoh1b* (Millimaki *et al.* 2007). In these systems, the Atoh1 homologue is first involved in the formation of the precursor domain; its expression is then refined and is later required for the differentiation of an individual cell type.

Atoh1's function within the normal development of the sensory structures of the mouse inner ear appear to be limited to the differentiation of hair cells. Although *Atoh1* promoter activity and expression of *Atoh1* transcript has been reported earlier within the prosensory domain (Bermingham *et al.* 1999; Lanford *et al.* 2000), Atoh1 protein has only been detected within the subset of postmitotic precursor cells that will differentiate as hair cells (Chen *et al.* 2002). Furthermore, though *Atoh1* is required for the differentiation of hair cells, it does not appear to be required for the formation of the prosensory domain or the coordination of cell cycle exit within this domain (Bermingham *et al.* 1999; Chen *et al.* 2002; Woods *et al.* 2004).

Despite this apparent disconnection from specification of the inner ear sensory regions, expression of Atoh1 within this prosensory is refined through Notch signaling in a manner similar to *Atoh1*'s homologues in other systems. Disruption of Notch signaling at early timepoints leads to an overproduction of hair cells at the expense of the supporting cell population (Zine *et al.* 2001; Kiernan *et al.* 2005a; Doetzlhofer *et al.* 2009). Furthermore, the removal of existing hair cells from the embryonic and neonatal organ of Corti allows supporting cells to upregulate Atoh1 and spontaneously converts to hair cells (Kelley *et al.* 1995; White *et al.* 2006). These results show that most of the precursor cells in inner ear sensory regions express Atoh1 when inhibitory cues are removed, suggesting that Atoh1 expression and hair cell differentiation is the default and that developing sensory regions actively repress Atoh1 expression to generate the mosaic pattern of hair cells and supporting cells.

Atoh1's ability to generate hair cells appears to not be limited to the predefined sensory precursor domain, at least within the cochlear epithelium. Previous work has

shown that ectopic delivery of Atoh1 to embryonic (Woods *et al.* 2004; Gubbels *et al.* 2008) and neonatal (Zheng and Gao 2000; Shou *et al.* 2003; Qian *et al.* 2006) cochlear epithelia can generate new sensory hair cells within non-sensory regions surrounding the organ of Corti. Intriguingly, *Atoh1*-transfected cells appear to inhibit adjacent transfected cells from hair cell differentiation, induce the formation of surrounding supporting cells, and recruit innervation similar to hair cells within the developing sensory region (Kawamoto *et al.* 2003; Woods *et al.* 2004; Qian *et al.* 2006; Gubbels *et al.* 2008). In these situations, it is unclear whether Atoh1 promotes the induction of new prosensory domains in these ectopic regions or if these regions represent repressed sensory domains that Atoh1 expression reactivates.

While Atoh1 has potent effects within relatively undifferentiated non-sensory cells of the developing cochlea, the conversion of more highly differentiated cell types may represent a different scenario. Various non-sensory cochlear epithelial become highly specialized during development and maturation of the inner ear (Kelly and Chen 2009) (Fig. 2*A*). Since it is these mature cell populations, including the supporting cells within a damaged organ of Corti, that are the likely targets for any Atoh1-mediated hair cell regeneration strategy, it is important to compare Atoh1's effect within various cell types in the developing and mature inner ear. Although evidence from viral transfection suggests that Atoh1 can direct the formation of new hair cells in the adult mammalian cochlea (Kawamoto *et al.* 2003; Shou *et al.* 2003; Izumikawa *et al.* 2005), the extent to which this possible and the source of these new hair cells is uncertain.

While Atoh1's importance during normal inner ear sensory region development is clear, and promising reports suggest that Atoh1 can induce the generation of functional

new hair cells in the mammalian cochlea, several important questions about Atoh1's effect within the inner ear still remain:

- Which cell types of the cochlear epithelium are competent for Atoh1-mediated hair cell generation?
- Does this competency change as the cochlear epithelium matures and cells become more highly differentiated?
- Can supporting cells be converted new hair cells by forced Atoh1 expression?
- What effects does ectopic Atoh1 expression and hair cell generation have on surrounding non-sensory cell populations?

Here, I report a series of experiments utilizing Atoh1 expression vectors and transgenic mouse models to spatially and temporally control Atoh1 activity within the cochlear epithelium. Using both *in vitro* and *in vivo* mouse models, I attempt to address these remaining questions and add to our current understanding of Atoh1's role within the development of the inner ear and its potential uses and limitations for hair cell regeneration. This study delineates the potent effects of Atoh1 within the young mammalian cochlea and provides valuable models for the further exploration of Atoh1's effects within the inner ear as well as other tissues.

Chapter II: Potency of Atoh1 Delivery to Cochlear Explants

2.1 Introduction

In order to determine Atoh1's effect within various cochlear epithelial cells, I first needed a method of reliably and efficiently expressing Atoh1 within various cells types. To accomplish this, I began with an *in vitro* model of the cochlear epithelium and generated expression vectors for delivering Atoh1 to various cell types and

While other groups had successfully transiently transfected *Atoh1* to the inner ear *in vivo* (Kawamoto *et al.* 2003; Izumikawa *et al.* 2005; Gubbels *et al.* 2008; Schlecker *et al.* 2011), the associated technical difficulties and limited transfection efficiency with these *in vivo* delivery methods made it incompatible with the variety of experimental goals I looked to accomplish. In contrast, the cochlear epithelium can be relatively easily extracted from embryonic and early postnatal mice with minimal damage to the tissue. The explanted cochlea can then be cultured for several days *in vitro*, and used as an *ex vivo* model of the cochlear environment (Sobkowicz *et al.* 1975; Qian *et al.* 2007).

Despite the ease in extracting and culturing embryonic and early postnatal cochlea, the delivery of recombinant expression constructs is relatively difficult and posed a potential technical hurdle. Though commercial transfection reagents typically do not work, other methods such as electroporation have been improved and used with moderate success (Driver and Kelley 2010). In fact, several groups have used electroporation for delivering Atoh1 to cultured cochlear epithelium and shown that new hair cells can be produced (Zheng and Gao 2000; Woods *et al.* 2004). Combinations of constructs can also be electroporated together to examine the interaction of different genes (Zheng *et al.* 2000; Jones *et al.* 2006; Qian *et al.* 2006; Dabdoub *et al.* 2008), further extending the utility of

this method. However, because of the mechanical and physiological strain put upon the tissue by the required manipulations and electric field, there is an inherent trade-off between the overall health of the culture and the number of transfected cells.

In addition to electroporation, viral-mediated transfection has also been used to deliver genes to cultured inner ear sensory epithelium (Luebke *et al.* 2001; Holt 2002; Di Pasquale *et al.* 2005; Stone *et al.* 2005). These methods use modified, replication-incompetent virus containing the gene of interest to transfect the cochlear epithelium without subjecting the tissue to the stresses of electroporation. Several different classes of virus have been used for these purposes, and individual serotypes and promoters appear to target specific populations of cells (Holt 2002; Stone *et al.* 2005; Luebke *et al.* 2009; Sheffield *et al.* 2011). Lentivirus, in particular, has the advantage of being able to transfect dividing and postmitotic tissue without eliciting an immune response (Dissen *et al.* 2009). Viral delivery can also be used to deliver a gene to early postnatal mice (Sheffield *et al.* 2011), making it potentially feasible to move to *in vivo* transfections following confirmation of a construct's efficacy *in vitro*.

With both electroporation and viral transfection of cochlear explant, I anticipated that some, but not all of our questions would be answered. Our goal, therefore, was to utilize these *in vitro* transfection methods as a way to develop and test an inducible Atoh1 design that could be used in the generation of a transgenic mouse model. These *in vitro* models could tell us, at least, if I could successfully regulate Atoh1's function as a transcription factor temporally and if transient activity of Atoh1 would be sufficient to generate new hair cells.

Here I optimized conditions for the culturing postnatal mouse cochlear explants for use as an *ex vivo* model. I also developed tools and constructs to deliver an inducible version of Atoh1 to cochlear epithelial cells and showed that I could temporally regulate its activity by controlling its subcellular localization. Furthermore, I tested the potency of an inducible Atoh1 and demonstrated that an inducible form of Atoh1 could generate new hair cells in the postnatal cochlear epithelium.

2.2 Methods

Care and use of animals

Wildtype mice from the FVB or C57BL/6 background were bred to produce offspring used in these *in vitro* studies. Postnatal day 0 (P0) was determined as the day pups were born. Animal care and use was in accordance with NIH guidelines and was approved by the Animal Care and Use Committee of Emory University.

Culturing of postnatal cochlear explants

Before attempting to ectopically deliver Atoh1 to cochlear epithelial cells to study its effects, I first had to establish the conditions for effective culturing of the tissue. Using published methods (Sobkowicz *et al.* 1975) and previously established protocols in the lab (Wang *et al.* 2005; Qian *et al.* 2007) as a starting point, various parameters were tested for the optimal culturing of early postnatal cochleae.

Cochleae were removed from the inner ear of P0-2 mice, cut into three separate pieces (Fig. 5*A*), and placed on a poly-D-lysine (Sigma, Cat: P6407) coated glass bottom dishes (MatTek Corp, Cat: P35G-0-14-C) with the lumenal surface of the organ of Corti facing upwards (Fig. 5*B*). The three pieces were roughly equal lengths and corresponded to the

apical, middle, and basal turns of the spiral-shaped cochlea. The media formulation used by Qian et al (2007) (Fig. 5*C*, *CM1*), which works well for embryonic tissue, unfortunately led to the death of middle and basal turn hair cells in our postnatal cultures. I therefore tried variations of this culturing media and analyzed the survival of hair cells throughout the three turns (Fig. 5*C*). Media without Streptomycin, which is an antibiotic that belongs to the aminoglyside class known to negatively affect the survival of hair cells, was determined to be responsible for the death of hair cells in the more basal half of the cochlea. The media formulation used for all subsequent experiment was *CM4*: DMEM/F12 (Gibco, Cat: 11039) with 10% FBS (Atlanta Biologicals, Cat: S11150H), 2% B-27 supplement (Gibco, Cat: 17504-044) and 1% 104U/mL Penicillin G (Sigma, Cat: P3032).

Non-sensory tissue surrounds the organ of Corti, and when culturing the cochlea, some membranes sit above the sensory region (Fig. 5*D*). Depending on the application, this can make transfection and subsequent imaging of the cochlear epithelium surrounding the organ of Corti difficult. Therefore, two other techniques of culturing cochlear explants with varying amounts of surrounding non-sensory tissue removed were established. Cochlear ducts could be cut on their medial edge (Fig. 5*A*) and the roof and lateral wall could either be laid flat on the lateral side of the culture (Fig. 5*E*) or removed entirely (Fig. 5*F*) for better access to the sensory region. These are the methods of culturing that will be used for all subsequent experiments.

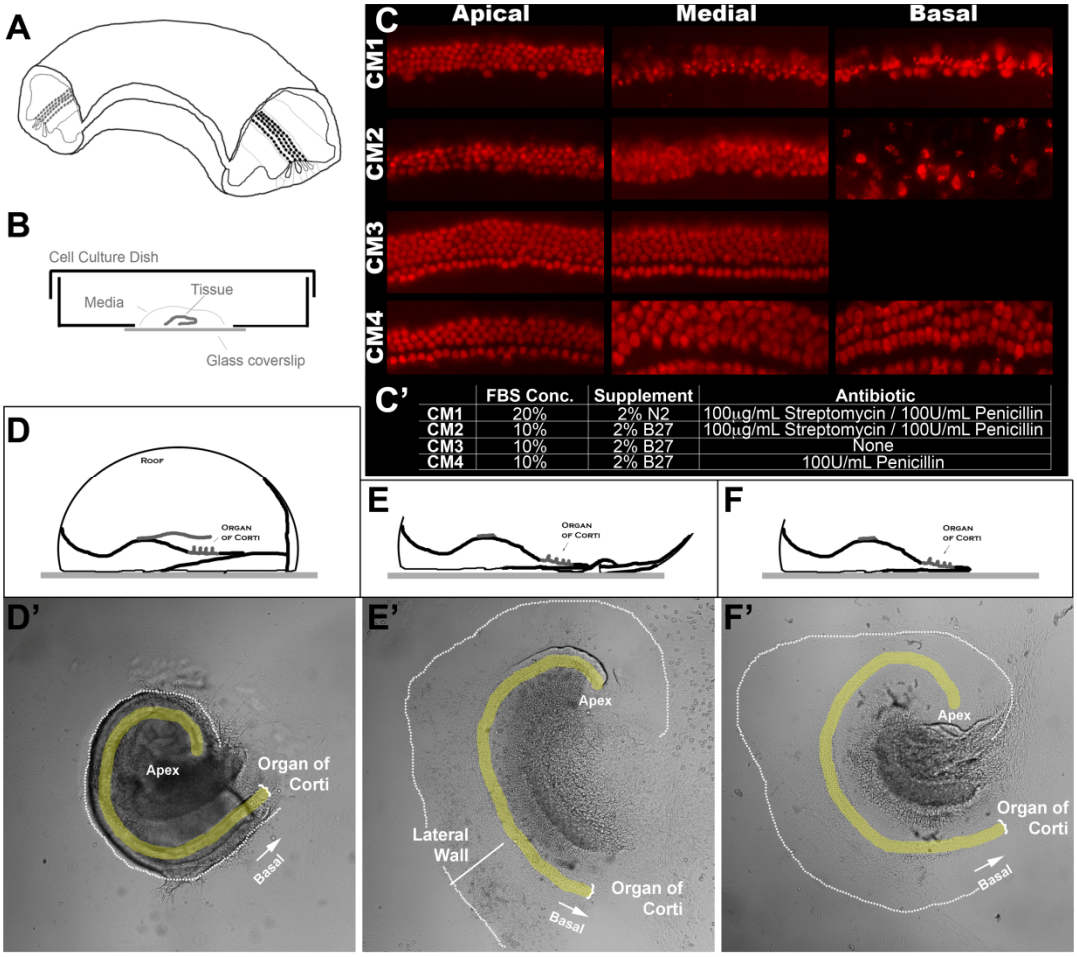

Fig. 5 - Postnatal Cochlear Explant Culture Provides *In Vitro* Model

(*A*) Diagram of a cut section of mouse cochlea duct showing the relative location of the sensory region and surrounding non-sensory tissue. (*B*) Diagram of the *in vitro* culturing setup used. The tissue sits on a glass-coverslip and is immersed in media. (*C*) Comparison of the effect on hair cell survival, as shown by MyoVI immunostaining, of various complete media (CM) formulations (*C'*) used to culture postnatal cochlea regions from the apical, middle, and basal turn. (*D-F*) Apical turns of cochleae cultured with varying amounts of surrounding tissue removed. Cochlea with entire roof and lateral wall and tectorial membrane intact (*D*), with the lateral wall laid flat and tectorial membrane removed (*E*), and with lateral wall and tectorial membrane completely removed (*F*). (*D"-F'*) Example images illustrating actual cochlear explant cultures with different methods.

Design and construction of Atoh1-HA-ER ieGFP expression vector

The entire *Atoh1* open reading frame was PCR amplified from mouse genomic DNA using a forward primer that contained SalI and a reverse primer that encoded the HA tag. The modified tamoxifen-sensitive *estrogen receptor ligand binding domain (ER^t(LBD))* was PCR amplified from genomic DNA from the CreESR1 mouse line (B6;129-Gt(ROSA)26Sor$^{tm1(cre/Esr1)Nat}$/J JAX Stock: 004847) using a forward primer that encoded the HA tag and a reverse primer with BamHI. These fragments were confirmed by sequencing and then cloned into a TA vector (Invitrogen, Cat: 45-0640). *Atoh1* was excised with SalI and AatII, and *ER^t(LBD)* was excised with AatII and BamHI. These fragments were then subcloned into a pIRES2-EGFP expression vector (BD Biosciences Cat: 6029-1) at the SalI and BamHI sites.

Cell culture transfection and electroporation of cochlear explants

Atoh1-HA-ER ieGFP vector DNA was amplified and purified by Qiagen midiprep (Cat: 12143). To confirm proper expression and function of the Atoh1-HA-ER fusions protein, human embryonic kidney (HEK) cells were plated on glass coverslips and allowed to grow to a medium (~50%) confluency before transfection using Fugene6 transfection reagent (Roche Cat:11814443001). For use in electroporations, *Atoh1-HA-ER ieGFP* plasmid DNA was diluted to a concentration between 1.0-2.0 mg/mL. Electroporations of cochlear explant cultures were performed as previously described (Qian *et al.* 2006), although the number of pulses, pulse length, and voltage was varied slightly between experiments to optimize transfection efficiency and health of the tissue. Electroporated cochleae were cultured with the roof and lateral wall removed (Fig. 5*F*).

Design and subcloning of Atoh1-HA-ER ieGFP lentivirus

The *Atoh1-HA-ER ieGFP* sequence was subcloned from the pIRES2-eGFP expression vector into a pFUGW replication-incompetent lentiviral vector. First, the BamHI site between *Atoh1-HA-ER* and *ieGFP* sequence was cut, Klenow filled-in and blunt-end ligated to ablate this site. Next, an adapter fragment, containing a BamHI site was inserted upstream of *Atoh1-HA-ER* by subcloning into the *Atoh1-HA-ER pIRES2-eGFP* vector at the AseI-SalI region. A BamHI-MfeI fragment containing the *Atoh1-HA-ER ieGFP* sequence was then excised and subcloned into a BamHI/EcoRI-digested pFUGW vector, with EcoRI being compatible with the MfeI overhang. The Emory Viral Core transfected HEK cells and packaged the virus, then purified the lentivirus to a titer of ~$1*10^7$. Aliquots were stored at -80C and thawed for individual experiments.

Cochlear explant transfection with lentivirus

Postnatal cochlear explant cultures were established with the roof, lateral wall and tectorial membrane removed (Fig. 5*F*). Cochlear explants were allowed to attach to the coverslip for about two hours before addition of lentivirus. pFUGW *Atoh1-HA-ER ieGFP* lentivirus was thawed on ice, then 0.5-2uL of purified lentivirus (titer of ~$1*10^7$) was added directly to the media. Cultures were incubated overnight with lentivirus, before the media was removed and replaced by fresh complete media. Tips, media and other possibly contaminated reagents and consumables were treated with a bleach solution overnight before being autoclaved and discarded in biohazardous waste.

Sample treatment, fixation, immunostaining, and imaging

Transfected HEK cell cultures and organ cultures were allowed to grow overnight before treatment with the activated form of Tamoxifen, 4-Hydroxytamoxifen (Sigma Cat: H7904). Cultures were fixed the following day for analysis of translocation of the Atoh1-HA-ER fusion protein, or changed to fresh media and allowed to incubate for two more days for analysis of ectopic hair cell production. Cultures were fixed in 4% paraformaldehyde solution in PBS for 10mins, before immunostaining. Immunostaining was performed as previously described (Qian *et al.* 2006; Li *et al.* 2008). Primary antibodies used were: MyoVI antibody (Proteus Biosciences Cat: 25-6791) and HA antibody (Cell Signaling Cat: 2367S). Hoechst stain (Molecular Probes) was used to mark cell nuclei. Samples were mounted on glass slides in Fluoromont-G (SouthernBiotech Cat: 0100-01) and sealed for long-term storage. Mounted samples were imaged using either an Olympus IX71 inverted microscope with a Zeiss high-resolution monochrome AxioCam HRm camera or scanned using a Zeiss LSM510 confocal. Images were processed and compiled with Photoshop CS3 software (Adobe).

2.3 Results

A. Tamoxifen inducible Atoh1-ER generates ectopic hair cells

In order to temporally control Atoh1's activity, I first designed and tested a construct that would allow the regulation of Atoh1's function as a hair cell differentiation factor. Previous work has shown that the fusion of the estrogen receptor with another protein causes the chimeric protein to be retained within the cytoplasm until ligand binding, which results in its nuclear translocation (Picard 1994). The particular ligand-binding domain of the estrogen receptor used, ER^{t}(LBD), contains a mutation that makes it

specifically sensitive to 4-hydroxytamoxifen (Tam) (Littlewood *et al.* 1995). This strategy has been used to temporally regulate the activity of the Cre-recombinase protein for genetic manipulation studies in a wide array of tissues within mouse (Hayashi and McMahon 2002). Since Atoh1 activity requires dimerization with E-proteins and binding to genomic targets (Cabrera and Alonso 1991; Bertrand *et al.* 2002), I generated a expression vector construct that expressed an Atoh1 protein fused to a modified tamoxifen-sensitive estrogen receptor ligand-binding domain (ER^t(LBD)) (Fig. 6*A*).

It was important that Atoh1's activity could be tightly controlled within *in vitro* studies before I moved forward with the production of a transgenic mouse line based upon this design. Although another group had previously reported that a similar *Atoh1-ER* construct could be successfully electroporated into the cochlear epithelium and led to generate ectopic hair cells (Woods *et al.* 2004), I wanted to ensure that its function was tightly controlled by Tam-induction. For this reason, I included an HA tag within our fusion protein, which allowed us to immunolocalize our fusion protein in Tamoxifen and untreated conditions (Fig. 6*B*).

I initially confirmed the proper regulation of the fusion by transfecting cultured HEK cells, treating with Tam, and then immunostaining for the HA tag in the Atoh1-HA-ER fusion protein. Whereas, untreated control cultures transfected with the Atoh1-HA-ER pIRES2-eGFP vector showed the fusion protein retained within the cytoplasm (Fig. 6*C*), transfected cultures treated with 25nM Tam showed that the Atoh1-HA-ER fusion protein was efficiently translocated to the nucleus (Fig. 6*D*). This showed that the spatial localization of the Atoh1-HA-ER fusion protein was highly dependent on the presence of Tam and suggested that Atoh1's nuclear access may be regulated by Tam-induction.

To test whether our Atoh1-HA-ER fusion protein could generate ectopic hair cells following induction, I electroporated our Atoh1-HA-ER pIRES2-eGFP expression vector into P0-2 cochleae, treated with Tam and then immunostained for the hair cell marker MyoVI (Fig. 6*E*,*F*). Unfortunately, many of electroporated samples had poor tissue morphology or did not appear to contain any transfected cochlear epithelial cells, making these cochlear explant cultures unacceptable for further analysis. In the small number of satisfactory samples, I observed at least one culture where new ectopic hair cells appeared to be generated following Tam-induction in a region separate from the endogenous organ of Corti (Fig. 6*E*). Upon closer examination, these hair cells expressed eGFP, which suggested that they contained the *Atoh1-HA-ER ieGFP* expression vector and that I was truly observing *Atoh1-HA-ER* generated hair cells (Fig. 6*F*). Most of the MyoVI and eGFP-positive hair cells seemed to have the characteristic flask-shaped hair cell morphology. Interestingly, not all the eGFP-positive cells within this region expressed the same level of MyoVI or hair cell shape, with some transfected cells in clusters having little detectable MyoVI (Fig. 6*F*).

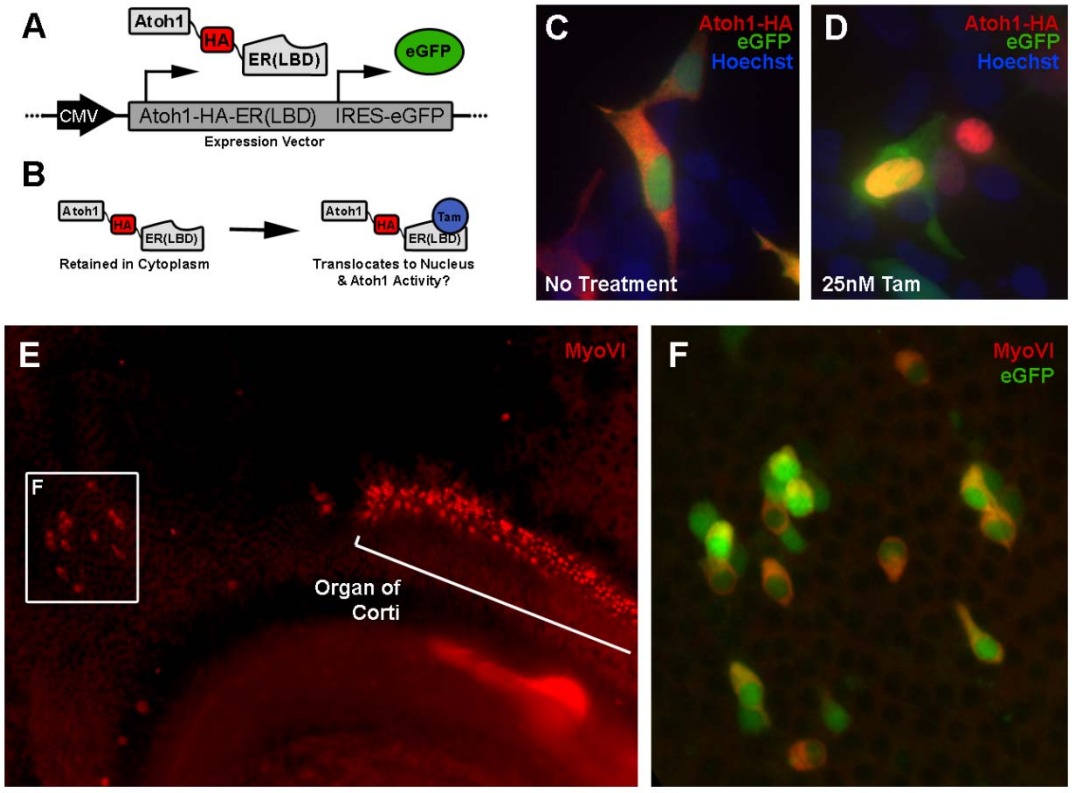

Fig. 6 - Tamoxifen-Inducible Atoh1-HA-ER Generates Hair Cells

(*A*) Diagram of the *Atoh1-HA-ER pIRES2-eGFP* expression vector. A cytomegalovirus (CMV) promoter drives expression of the Atoh1-HA-ER fusion protein and an independently translated eGFP reporter. (*B*) Diagram of Atoh1-HA-ER 4-hydroxytamoxifen (Tam)-inducible function. Tam binding to the tamoxifen-sensitive estrogen receptor ligand-binding domain (ER^t(LBD)) allows the fusion protein to be translocated to the nucleus, where Atoh1 can act. (*C-D*) HEK cells transfected with the *Atoh1-HA-ER ieGFP* expression vector expressing eGFP. Immunostaining for HA shows that the fusion protein is retained within the cytoplasm under normal conditions (*C*), but is efficiently translocated to the nucleus when transfected cells are incubated in media containing as low as 25nM Tam (*D*). (*E-F*) Electroporated P0 cochlear epithelium with ectopic hair cells generated in a region separate from the organ of Corti (*E*), with the ectopic hair cells and some surrounding cells clearly expressing the eGFP reporter.

B. Lentiviral Transfection of Cochlear Explants with *Atoh1*

To explore other methods of delivering Atoh1 with better efficiency to the cultured cochlear epithelium, I tested the feasibility of viral transfections within our explant model. At the time, there was limited work characterizing the efficiency of various classes of viral vectors and specific promoters within the inner ear (Stone *et al.* 2005). I therefore opted to test the transfection efficiency of the pFUGW lentiviral vector, which was recommended by the Emory Viral Core. Using a *GFP* control version of the pFUGW lentivirus (*a gift from the Emory Viral Core*) (Fig. 7*A*), I transfected P1-stage cochlear explant cultures and looked for transfection of the epithelium. Mesenchymal, neuronal and epithelial cells towards the edge of the explants appeared be transfected with high efficiency, and a few isolated cells near the sensory regions were sometimes observed (Fig. 7*B*). A high magnification view of the transfected sensory region cells showed that some resided between the inner and outer hair cells rows and had a distinctive Pillar cell morphology that suggested that this particular supporting cell type was successfully transfected (Fig. 7*C*). Other cultures showed transfected cells within the outer hair cell region, with GFP-positive cells residing between MyoVI-positive hair cells (Fig. 7*D*), which suggested that Deiters' cells could also be transfected. No hair cells in any of the *GFP* pFUGW lentivirus-treated cultures appeared to be GFP-positive.

Based on the preliminary success of transfecting cochlear explants with control GFP lentivirus, I designed and produced an *Atoh1-HA-ER ieGFP* pFUGW lentivirus for use in our studies. Using the *Atoh1-HA-ER ieGFP* cassette from our previously constructed and tested *Atoh1-HA-ER* pIRES2-eGFP expression vector (Fig. 6), I replaced the GFP sequence within the pFUGW backbone vector. The completed *Atoh1-HA-ER ieGFP*

pFUGW vector was transfected into HEK cells and confirmed to have similar inducible nuclear translocation function (Fig. 7*F,G*). Unfortunately, since the *Atoh1-HA-ER ieGFP* lentivirus was packaged and subsequently tested on cochlear explant cultures, no transfected cochlear epithelial cells have so far been observed. This is possibly due to the relatively low viral titer ($\sim 1*10^7$) of the *Atoh1-HA-ER ieGFP* lentivirus. However, transfected cells at the periphery of these cultures also appear to translocate the expressed Atoh1-HA-ER fusion protein following Tam-induction (*Data not shown*).

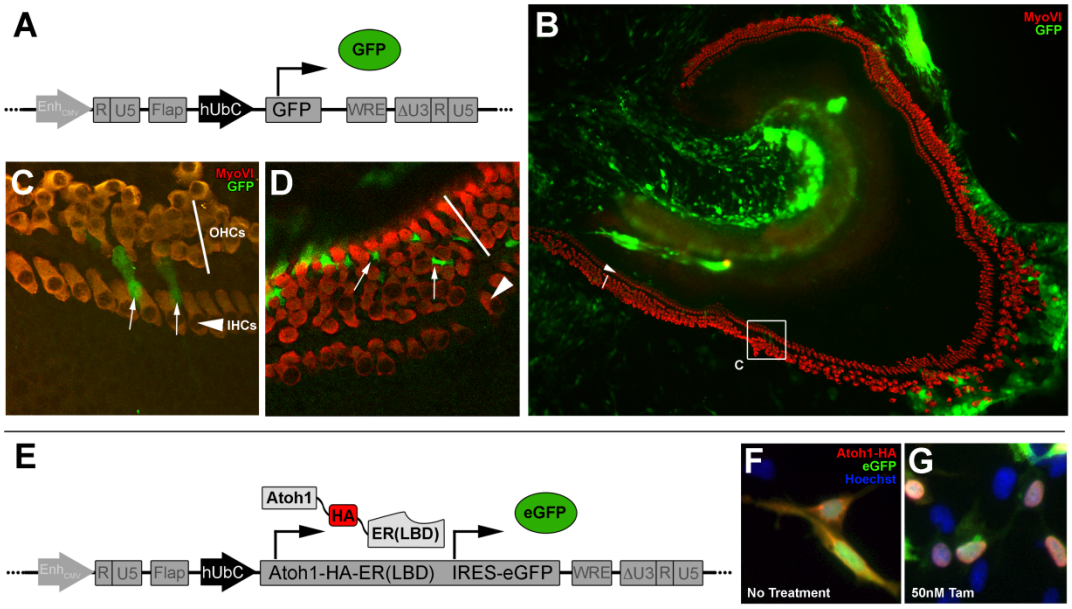

Fig. 7 - Lentiviral Transfections of Cochlear Explants

(A) Diagram of control *GFP* pFUGW lentiviral vector used to test for transfection efficiency of cochlear explant cultures. (B) A cochlear explant culture transfected with *GFP* pFUGW lentivirus, showing individual supporting cells within the sensory region expressing GFP. (C) A confocal image of region of culture shown in (B), showing GFP-positive supporting cells (arrows), likely Pillar cells between the inner hair and outer hair cell region. (D) Individual supporting cells (arrows) surrounding MyoVI-positive outer hair cells expressing GFP, likely representing lentiviral-transfected Dieters' cells. (E) Diagram of *Atoh1-HA-ER IRES-eGFP* pFUGW lentiviral vector for ectopic expression of inducible Atoh1-HA-ER fusion protein, as well as an independently translated eGFP reporter. (F) HEK cells transfected with *Atoh1-HA-ER IRES-eGFP* lentivirus shows the fusion protein is retained within the cytoplasm, but is efficiently translocated to the nucleus when treated with low dose Tamoxifen (Tam).

2.4 Discussion

The use of cochlear explants has become an important model for studies of the inner ear because of the relative ease of culturing, the ability to manipulate and monitor live cultures and the degree of which it represents the *in vivo* tissue (Sobkowicz *et al.* 1975; Driver and Kelley 2010). Here, I optimized culturing conditions for the study of early postnatal cochleae. I discovered that the conditions commonly used for embryonic cultures (Qian *et al.* 2007) led to a basal to apical gradient hair cell loss due to the presence of the ototoxic antibiotic Streptomycin (Fig. 5*C*). Ototoxic aminoglycosidic antibiotics are thought to gain access and exert their toxic effects on intracellular processes of hair cells via open mechanotransduction channels at stereocilia tips (Marcotti *et al.* 2005). Since hair cells begin mechanotransduction postnatally along the basal-to-apical gradient (Geleoc and Holt 2003), the gradient of sensitivity likely represents differences in hair cell maturity along the length of the cochlea. With optimized media conditions for the survival of cultures up to seven days, I compared three different techniques for dissection and culturing of the postnatal cochlea (Fig. 5*D-F*). Culturing methods with the roof and tectorial membrane removed appeared to be significantly better for the visualization of cells within the sensory region (Fig. 5*E',F'*) and did not appear to significantly affect tissue morphology.

Using this established *ex vivo* model, I was able to test our inducible Atoh1 constructs. Cell culture work showed that our novel Atoh1-HA-ER fusion protein could be efficiently regulated by treatment with low concentration Tamoxifen (Fig. 6*C,D,* Fig. 7*F,G*). Furthermore, when our *Atoh1-HA-ER ieGFP* expression vector was electroporated into a postnatal cochlear epithelium and induced with Tamoxifen, ectopic hair cells could

be observed, suggesting that our fusion protein also worked successfully as a hair cell differentiation factor even with a relatively short induction time period (Fig. 6*E,F*). Previous work with the electroporation of *Atoh1* into cochlear non-sensory regions suggested that Atoh1-generated hair cells exerted non-cell autonomous effects on neighboring cells, including inhibition of adjacent transfected cells from hair cell differentiation and inducing supporting cell markers (Woods *et al.* 2004). Interestingly, not all the transfected cochlear epithelial cells in our cultures expressed hair cell markers (Fig. 6*F*), which may represent a non-cell autonomous effect of lateral inhibition. However, the limited numbers of cells transfected prevented a closer examination of this process and a more definitive conclusion.

Overall, I encountered significant difficulty in transfecting epithelial cells and maintaining healthy cultures following electroporation. Furthermore, despite the promise of preliminary results using a control lentivirus (Fig. 7*A-D*), our *Atoh1-HA-ER ieGFP* pFUGW lentivirus failed to provide the additional efficiently and reproducibility that I desired. Because other groups have used these methods successfully, it is likely that further *in vitro* work optimizing conditions could lead to more consistent positive results; however, it is unlikely that these results would further greatly add novel findings to what has been reported previously using similar methods (Woods *et al.* 2004). However, recent work has also shown that expression constructs can be delivered to the inner ears of live embryos by electroporation (Gubbels *et al.* 2008) and by viral transfection (Sheffield *et al.* 2011). This introduces the possibility of early *in vivo* delivery of *Atoh1-HA-ER* constructs, when transfection efficiency may be increased, and later induction and study of Atoh1's function either *in vivo* or *in vitro*. In these experiments, the specificity of

promoters and viral serotypes may be important for the proper targeting of individual cell types (Stone *et al.* 2005; Sheffield *et al.* 2011).

The initial positive results of our postnatal cochlear explant cultures and our inducible *Atoh1* constructs gave us the confidence to move forward with the generation of transgenic mouse models of Atoh1 expression. Furthermore, the limitations of our *in vitro* delivery methods made it clear that a stable genetic model was necessary to address our central questions. However, given that new *in vivo* delivery methods are being developed and that future gene therapies may rely upon targeted delivery methods, these inducible Atoh1-HA-ER tools may continue to be useful for other studies as well. Furthermore, the establishment of the cochlear explant model will enable *in vitro* manipulations of transgenic tissue that would otherwise be infeasible *in vivo*.

Chapter III: Potency of Atoh1 in the Entire Cochlea *In Vivo*

3. 1 Introduction

The potency of Atoh1 as a hair cell differentiation factor is supported by our generation of ectopic hair cells following *in vitro* delivery of Atoh1 to the postnatal cochlear epithelium (Chapter I: Fig. 6). These findings are consistent with a number of previously published reports that use electroporation or viral transfection methods to deliver Atoh1 either *in vitro* or *in vivo* (Kawamoto *et al.* 2003; Shou *et al.* 2003; Woods *et al.* 2004; Izumikawa *et al.* 2005; Qian *et al.* 2006; Gubbels *et al.* 2008). Intriguingly, within our study (Fig. 6*F*) and within a previous report (Woods *et al.* 2004), there is evidence to suggest both that Atoh1 not only initiates a hair cell differentiation program, but that it may also have non-cell autonomous effects on cells surrounding Atoh1-generated hair cells. Ectopic hair cells also appear to recruit innervation (Gubbels *et al.* 2008), even when induced in the postnatal cochlea (Kawamoto *et al.* 2003; Qian *et al.* 2006). These studies suggest that Atoh1 is not only capable of generating hair cells when expressed outside its normal developmental expression domain and timeframe, but that other aspects of sensory region development are also induced to pattern surrounding cells. These results point to a complex role of Atoh1 during development and hint at the possibility of Atoh1-mediated hair cell regeneration that includes appropriate cellular patterning and innervation.

Despite these interesting findings, the conclusions drawn from these studies are limited for several reasons. The delivery methods used thus far have relied upon random transfection of various types of cochlear epithelial cells. This lack of targeting together

with the small number of cells transfected overall, makes comparisons of Atoh1's effects within individual cell types difficult. An exploration of Atoh1's potential non-cell autonomous effects, such as cross-regulation across multiple cells within a tissue, is also limited to small clusters of transfected cells (Woods *et al.* 2004) (Fig. 6*F*). Furthermore, the difficulty associated with these delivery methods, particularly *in vivo*, preclude more a more comprehensive evaluation of Atoh1's effect across different developmental timepoints and along with other manipulations.

Because of the limitations imposed by other gene delivery methods, it was important to develop a stable model of Atoh1 expression for me to further explore Atoh1's effect within the cochlea. In order to critically evaluate the effect of Atoh1 within various cell types at different postnatal timepoints, I looked to force Atoh1 expression within a broad population of the cochlear epithelium in an inducible manner. Since Atoh1 is normally downregulated in mature hair cells during postnatal maturation (Zheng *et al.* 2000), and continued forced expression of Atoh1 may have unintended detrimental effects on the cells in which it is expressed, I thought it was also important that our forced expression of Atoh1 be reversible. Although many questions could be potentially answered using our cochlear explant model, an *in vivo* model of Atoh1 expression would presumably provide results with fewer experimental caveats associated with an *in vitro* system. Since the mouse has become an important model of the study of the mammalian inner ear and a diverse set of advanced genetic tools allow for efficient manipulations using transgenic mice (Tian *et al.* 2006), I decided to design and generate an inducible Atoh1 transgenic mouse model.

Here, I report the generation of a transgenic mouse line that allows for temporal and cell-specific targeting of Atoh1 in a stable and reproducible manner. I show that at postnatal stages, the cells of the cochlear lining that are competent to become hair cells are clustered in distinct regions in neonatal animals and that the competency becomes progressively more restricted up to the age of hearing onset, correlating with the expression of the prosensory marker Sox2. Atoh1 initiates a differentiation program that leads to the development of characteristic hair bundles, targeted innervation, and the physiological properties resembling that of endogenous hair cells. Furthermore, the induction of Atoh1 resulted in the up-regulation of Notch signaling and the generation of non-sensory supporting cells to encircle the ectopic hair cells in the ectopic sensory regions. Surprisingly, I also observed induced cell proliferation in the normally quiescent cochlear epithelium. These data together suggest that Atoh1 not only acts as a potent hair cell differentiation factor, but also activates pathways important for patterning the sensory epithelium through cell fate decisions and cell cycle control.

3. 2 Methods

Generation of Inducible Transgenic Atoh1 Mouse Line

The coding sequence for *Atoh1* was cloned into the SalI/EcoRV site of the pTET-Splice vector (Invitrogen). This vector was linearized by SapI/NotI digest and founder animals were generated by pronuclear injection. Positive founders were identified by PCR genotyping, confirmed by Southern blot using a probe that recognizes both wildtype and transgenic loci, and then bred onto an rtTA background (JAX Stock#005670 (Belteki *et al.* 2005)). Mice were crossed to one of the tissue-specific Cre lines (Foxg1^{Cre}, JAX

Stock#004337 (Hebert and McConnell 2000) or GFAP^{Cre}, a gift from Ken McCarthy (Casper and McCarthy 2006)) to generate samples to be used in this study.

Doxycycline Administration and Brdu Injections

Doxycycline Hyclate (Sigma, Cat: D9891) was administered to nursing female mice and young pups via restricted diet in a manner similar to Pan et al (2010). Food was prepared by dissolving 400mg of Dox and 5g of sucrose in 50mL of drinking water and mixing with 100g of crushed food pellets. Water was prepared by dissolving 200mg of Dox in 100mL of drinking water with 5g of sucrose. For longer induction studies, Dox dose was cut in half from those listed above. Mice administered Dox for longer than 2-6 days began to lose weight and died within a week, limiting studies to the reported timeframe.

For proliferation studies, neonatal mice were injected subcutaneously with a BrdU solution (5mg/mL in PBS pH7.0) once daily during the period of Dox-induction. Animals were euthanized and inner ear tissue was removed and fixed in 4% PFA in PBS.

Cochlear cDNA Synthesis and RT-PCR Analysis

Cochleae were isolated from transgenic and littermate control mice following doxycycline administration, snap-frozen in liquid nitrogen, then cells were lysed with QIAshredder columns (Qiagen, Cat 79654). Total RNA was isolated and purified using an RNeasy Micro-elute kit (Qiagen, Cat 74004). A cDNA library was synthesized using previously described methods (Chen and Segil 1999). Relative template representation was then determined following normalization of total cDNA template using specific

primers for Atoh1 fwd: TCTGCTGCATTCTCCCGAGC rev: CTCTGGGGGTTACTCGGTGC, Hes1 fwd: AGCGGAATCCCCTGTCTAC rev: CAACTGCATGACCCAGATCA, Hes5 fwd: TCCTCTGGATGTGGGAAGAC rev: CTTTGTATGGGTGGGTGCAT, or β-actin control fwd: GGGACCTGACAGACTACCTC rev: AGCCTTCCTTCTTGGGTATGG.

Cochlear Explant Organ Culture & Pharmacological Manipulations

P0-P4 stage pups were euthanized and cochlear tissue extracted. The lateral wall, Reissner's membrane, and tectorial membrane were removed before plating on poly-D-lysine (Sigma, Cat: P6407) coated glass bottom dishes (MatTek Corp, Cat: P35G-0-14-C) as reported by Qian et al (2007). Tissue was incubated in DMEM/F12 (Gibco, Cat: 11039) with 10% FBS (Atlanta Biologicals, Cat: S11150H), 2% B-27 supplement (Gibco, Cat: 17504-044) and 1% 104U/mL Penicillin G (Sigma, Cat: P3032). DAPT experiments were performed in the absence of FBS. Dox was added to complete culture media for a final concentration of 1ug/mL. For proliferation analysis, BrdU was added to media for a final concentration of 5ug/mL.

Immunohistochemistry and Tissue Staining

Tissue preparation, immunostaining and imaging were performed as previously described previously (Li *et al.* 2008). Primary antibodies used were: MyoVI (Proteus Biosciences Cat: 25-6791), Sox2 (Santa Cruz Biotech, Cat: sc-17320), Tuj1 (Covance, Cat: MMS-435P), BrdU (Roche, Cat: 1170376), GFP (Millipore, Cat MAB3580), Cdkn1b (BD Transduction Laboratories, Cat: K25020), and phospho-histone H3 (Millipore, Cat: 06-570). Hoechst stain (Molecular Probes) was used to mark cell nuclei.

BrdU and Cdkn1b staining required a 10mM citrate buffer steaming antigen retrieval step (Chen and Segil 1999; Tang *et al.* 2007). Fluorophore-conjugated Phal stains used were: AF488-Phal (Molecular Probes, Cat: A12379), Rhod-Phal (Molecular Probes, Cat: R415), AF635-Phal (Invitrogen, Cat: A34054). Secondary antibodies used were: AF488-conjugated Goat-anti-Mouse (Molecular Probes, Cat: A11001), Rhodamine-conjugated Goat-anti-Rabbit (Jackson IR, Cat: 111-295-144), Cy5-conjugated Donkey-anti-Goat (Jackson IR, Cat: 705-175-147), Cy2-conjugated Donkey-anti-Mouse (Jackson IR, Cat: 715-225-150). All samples were mounted in Fluoromont-G (SouthernBiotech Cat: 0100-01) and sealed for long-term storage.

Whole Cell Current Patch-clamp Recording

The flattened cochlear whole mount preparation was used to record the hair cells whole cell currents (Chang *et al.* 2008). These sets of experiments were performed in collaboration with Qing Chang from Xi Lin's laboratory, Emory University. Briefly, cochleae were isolated from either P5 or P6 pups inner ears and the lateral wall, Reissner's and the tectorial membrane were removed from the duct. The organ of Corti and ectopic sensory region of Tg^{Atoh1} samples were mounted on a glass coverslip. Ectopic and endogenous hair cells were identified by their specific location, cell shape and presence of characteristic stereocilia as seen under DIC optics on a Zeiss AxioSkope 2. The external bathing solution is made from standard HBSS (H4891, Sigma) and internal solution components are (mM): KCl 120, CaCl2 1.2 MgCl2 1 EGTA 10. The pH of the external and internal solutions was adjusted to 7.4 and 7.2 respectively, and the osmolarity was adjusted to 300 mOsm for both. The traditional techniques for patch-

clamping hair cells were used as previously described (Housley and Ashmore 1992). The electrode has an access resistance of about 3MΩ. The whole cell currents were recorded through an Axon 200B amplifier (Axon Ins, CA) and Digidata 1322A, and only hair cells with a rupture resistance ≥500MΩ were included for analysis. The current traces were analyzed using Clampfit 9.2 (Axon Ins, CA) and the associated I-V curve was plotted using Origin 7.0 (OriginLab Cor, MA).

Imaging, Cell Counts, and Statistical Analysis

Mounted samples were imaged using either an Olympus IX71 inverted microscope with a Zeiss high-resolution monochrome AxioCam HRm camera or scanned using a Zeiss LSM510 confocal. Images were processed and compiled with Photoshop CS3 software (Adobe). For DAPT/DMSO-treat Tg^{Atoh1} organ cultures, the percentage of hair cells was determined by calculating the number of MyoVI-positive cells over the total number of cells in the ectopic sensory (Fig. 13*D*,*F*). Comparisons were made using a two-tail unpaired type-2 Student's t-test.

Care and Maintenance of Animals

Animal care and use was in accordance with NIH guidelines and was approved by the Animal Care and Use Committee of Emory University. Tg^{Atoh1} mice were PCR genotyped for the presence of the pTET-Splice(Atoh1) transgene with fwd-primer: CGCGCAATTAACCCTCACTA and rev-primer: CGGGAGAATGCAGCAGATAC. rtTA mice were genotyped with fwd-primer: GAGTTCTCTGCTGCCTCCTG and rev-primer: AAGACCGCGAAGCGTTTGTC. $Foxg1^{Cre}$ mice were genotyped with fwd-

primer: AGTATTGTTTTGCCAAGTTCTAAT and rev-primer: TCCTATAAGTTGAATGGTATTTTG.

3. 3 Results

A. Generation of an Inducible Transgenic Atoh1 Mouse

To test the competency of postnatal cochlear epithelial cells to Atoh1-mediated hair cell differentiation, I generated inducible transgenic mice, Tg^{Atoh1}, in which the Atoh1 coding sequence is under the control of a tetracycline-response element (TRE). Atoh1 expression is activated by the reverse tetracycline trans-activator (rtTA), which requires doxycycline (Dox) to bind to the TRE and activate *Atoh1* transcription (Fig. 8*A*). I verified the presence of the transgene in four founder lines via Southern blot using a probe that recognized both the endogenous *Atoh1* and the Tg^{Atoh1} loci (Fig. 8*B*), allowing a relative comparison of transgene copy number between founder lines and a estimation of total transgene copies relative to the two-copies at the endogenous locus. To direct the expression of Tg^{Atoh1} following administration of Dox, I crossed Tg^{Atoh1} mice with a Cre-activated rtTA line (B6.Cg-Gt(ROSA)26Sortm1(rtTA,EGFP)Nagy/J) and the $Foxg1^{Cre}$ line (129(Cg)-Foxg1tm1(cre)Skm/J). I verified that rtTA expression was directed to the $Foxg1^{Cre}$-expessing cell population using GFP as an indirect marker (Fig. 8*C*).

Neonatal mice from each of the four founder lines carrying all three transgenic alleles fed Dox for two days had a strong up-regulation of *Atoh1* transcript within the cochlear epithelium compared to the normal level of *Atoh1* expression in Dox-treated littermate controls (Fig. 8*D*). Although all four lines had strong up-regulation of *Atoh1*, it was clear that some lines had a stronger response than others and that the transcript levels did not correlate with transgene copy number (Fig. 8*B,D*). I selected the line (Founder #03) that

carries an estimated 2-3 copies of the transgene (Fig. 8*B*), but still had a strong up-regulation of *Atoh1* transcript following two-days of induction (Fig. 8*D*) for use in all subsequent experiments. Additional experiments were performed using Tg^{Atoh1} mice from other founder lines and similar results were observed (*Data not shown*). I confirmed that *Atoh1* was specifically up-regulated in the cochlear epithelium of mice carrying both Tg^{Atoh1} and $Foxg1^{Cre}$ following two-days of Dox-induction, compared to normal levels expressed by littermate controls at postnatal day 2 (P0-2:P2) (Fig. 8*E*). This Atoh1 up-regulation was dependent upon Dox administration, as cochleae from triallelic Tg^{Atoh1} animals had similar levels of Atoh1 transcript as littermate controls at the P2-stage without Dox-induction (Fig. 8*F*). These triallelic transgenic mice, $Foxg1^{Cre}$;rtTA;Tg^{Atoh1} allowed targeted Atoh1 expression throughout the cochlear epithelium in a conditional and reversible manner upon administration of Dox.

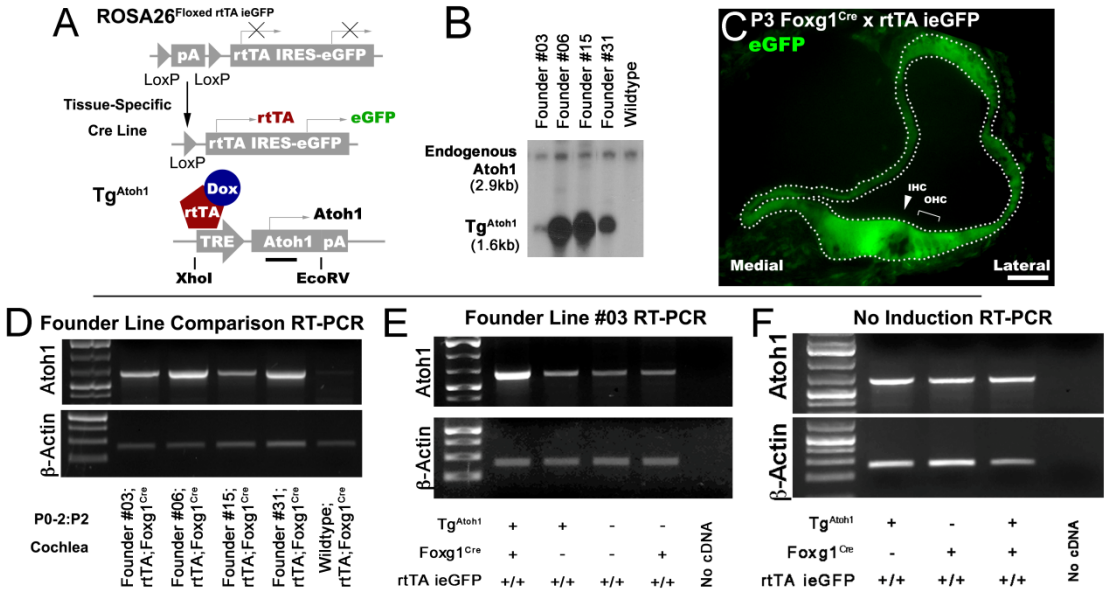

Fig. 8 - Inducible Transgenic Atoh1 Mouse Design

(*A*) Schematic diagram of the triallelic Tg^{Atoh1} mouse line. When crossed to a Cre mouse line, expression of rtTA (and an eGFP reporter) is tissue-specific determined by Cre recombinase-mediated excision of an upstream stop cassette. Expression of Tg^{Atoh1} is regulated by a tetracycline response element (TRE), which is activated when rtTA is bound to Dox. Restriction enzyme sites and the probe location for Southern blot are shown the diagram of the Tg^{Atoh} locus. (*B*) As confirmed by Southern blot using an *Atoh1* probe, four transgenic founders carry Tg^{Atoh1}. In comparison to the endogenous *Atoh1* DNA fragment (2.9kb), line #3 is estimated to have 2-3 copies of the Tg^{Atoh1} transgene (1.6kb). (*C*) The expression of eGFP and, presumably, rtTA is ubiquitous in cochlear epithelial cells (*marked with dotted lines*) when $Foxg1^{Cre}$ line is crossed to animals carrying the rtTA (and an eGFP reporter) transgenic locus. Triallelic Tg^{Atoh1} animals, or $Foxg1^{Cre}$;rtTA;Tg^{Atoh1} animals would direct *Atoh1* transgene expression within this domain following Dox-induction. Scale bar: 20 μm (*D*) Following 2 days of Dox administration, $Foxg1^{Cre}$;rtTA;Tg^{Atoh1} animals from each of the four founder lines, but not wildtype controls showed strong up-regulation of *Atoh1* transcript as shown by RT-PCR. Different founder lines

showed variations in the amount of up-regulation following Dox-induction that did not necessarily match transgene copy number. (*E*) A similar RT-PCR experiment using founder line #03 shows that only in animals carrying Tg^{Atoh1} and an allele for $Foxg1^{Cre}$, the *Atoh1* transcript is highly up-regulated in the cochlear epithelium. (*F*) P2 triallelic Tg^{Atoh1} animals not administered Dox had similar levels of *Atoh1* transcript compared to littermate controls as shown by RT-PCR, illustrating that *Atoh1* up-regulation is also dependent upon Dox-induction.

B. *In Vivo* Generation of Functional Ectopic Hair Cells

Forced ectopic delivery of Atoh1 to cochlear epithelial cells has been shown previously to generate cells expressing hair cell markers (Zheng and Gao 2000; Kawamoto *et al.* 2003; Shou *et al.* 2003; Woods *et al.* 2004; Izumikawa *et al.* 2005; Gubbels *et al.* 2008), but limited numbers of transfected cells and an inability to direct transfection to certain cells types did not allow a direct comparison of Atoh1's effects within all cell types of the cochlear epithelium.

Using triallelic $Foxg1^{Cre}$;rtTA;Tg^{Atoh1} mice to direct Atoh1 expression to the entire cochlea, I confirmed that our model of Atoh1 delivery could generate new hair cells *in vivo* and was able to test the relative competency of various cochlear epithelial cell types within the neonatal cochlea. Compared to littermate controls, triallelic Tg^{Atoh1} P1 mice treated with Dox for two days and harvested at P6 (P1-3:P6) had a large number of hair cells within the non-sensory Kölliker's organ region, or greater epithelial ridge (Fig. 9*A*,*B*), and isolated ectopic hair cells within the developing spiral prominence region on the lateral wall of the cochlea (Fig. 9*B*, arrow).

Sox2 is normally expressed early in the entire sensory precursor domain and subsequently down-regulated within differentiating hair cells and maintained in non-sensory supporting cells during development (Kiernan *et al.* 2005b; Kelley 2006; Dabdoub *et al.* 2008). Interestingly, Sox2 appeared to be induced in the ectopic hair cell region within the developing spiral prominence upon induction of Atoh1 (Fig. 9*B*, arrow). Furthermore, there appeared to be an inverse relationship between the expression of the hair cell marker MyoVI and Sox2 within ectopic sensory regions (Fig. 9*B*, inset).

This suggests that as new hair cells differentiate, they down-regulate the expression of Sox2 in a manner similar to the development of hair cells within the organ of Corti.

I examined the morphology and physical properties of Atoh1-induced hair cells. I first looked at the apical surface of the cochlear epithelium stained with phalloidin to identify cellular borders and the presence of stereocilia bundles. Ectopic hair cells produced within Kölliker's organ displayed stereocilia, which varied in maturity and had largely randomized orientation (Fig. 9*C*,*D*). However, the ectopic hair cells appeared to have auditory hair cell rather than vestibular hair cell like morphologies (Fig. 9*D,* Fig. 2*E*-*G*). Although a large number of ectopic hair cells were observed, they were surrounded by cells that did not display stereocilia (Fig. 9*D*). These non-sensory cells likely corresponded to the cells observed to maintain high Sox2 expression within cross section (Fig. 9*B*). Ectopic hair cells attracted Tuj1-positive neuronal fibers into Kölliker's organ region, which is normally devoid of these fibers by the P6-stage (Fig. 9*E*,*F*). These Tuj1-positive fibers appear to surround the basolateral surface of the ectopic hair cells in a manner similar to endogenous hair cells within the organ of Corti (Fig. 9*F*).

Unfortunately, $Foxg1^{Cre}$;rtTA;Tg^{Atoh1} mice treated with Dox became sick, with most positive mice dying about a week after the start of Dox treatment. The associated lethality is likely the result of detrimental effects of forced Atoh1 expression within other tissue activated by $Foxg1^{Cre}$ expression. However, this limited the length of our Dox induction protocols and the length of time over which we could let new hair cells differentiate.

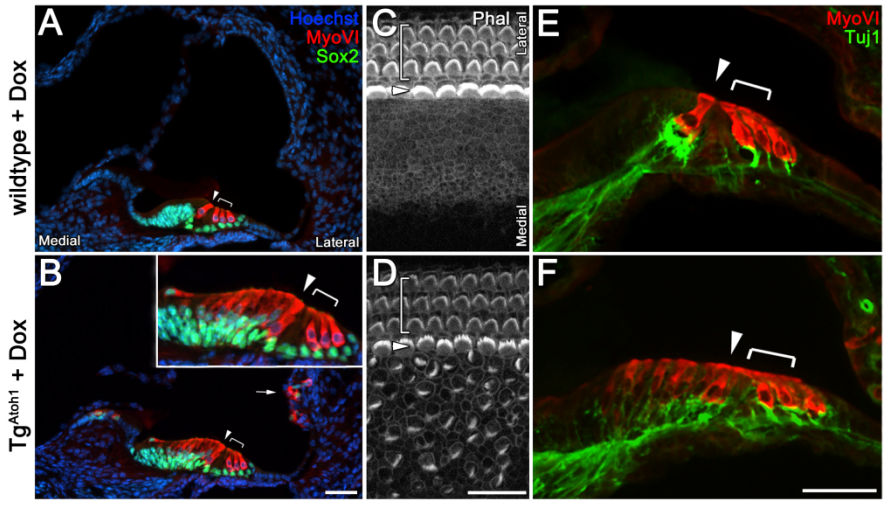

Fig. 9 - Atoh1 Induces Ectopic Sensory Regions with Hair Cells

(*A-B*) Compared to littermate controls administered Dox from P1-P3 and examined at P6 (*A*), cross-sections through the medial turn of triallelic Tg^{Atoh1} cochlea (*B*) show a large number of MyoVI-positive hair cells generated within Kölliker's organ, which is medial to the endogenous one row of inner (arrowheads) and three rows of outer hair cells (brackets). MyoVI-positive hair cells are also observed on the lateral wall of the duct (arrow). Note that Sox2-positive cells remain within Kölliker's organ, but have an inverse relationship with MyoVI-positive expression in the ectopic hair cells (*B*, inset). Sox2 is also induced in the lateral wall of the duct (*B*, arrow). (*C-D*) Phalloidin-stained stereocilia bundles are observed as polarized arrays on the apical surfaces of the inner (arrowheads) and outer (brackets) hair cells within the organ of Corti (*C*). Ectopic hair cells medial to the organ of Corti display similar stereocilia bundles that are randomly oriented (*D*). Ectopic hair cells are surrounded by cells without stereocilia. (*E-F*) Tuj1 immunostained neuronal fibers innervated the sensory hair cells of the organ of Corti in wildtype littermate and have no fibers within Kölliker's organ (*E*), whereas ectopic hair cells in Kölliker's organ have Tuj1-positive fibers surrounding their basolateral surface in triallelic Tg^{Atoh1} animals (*F*). All scale bars: 20 μm.

Despite the limited induction time period and the short timeframe allowed for hair cell differentiation, the morphology of ectopic hair cells and their ability to attract neuronal fibers suggested that ectopic hair cells were relatively mature. Previous reports suggested that delivery of Atoh1 could generate functional new hair cells (Izumikawa *et al.* 2005; Gubbels *et al.* 2008; Schlecker *et al.* 2011), but it was unclear if the ectopic cells generated in Tg^{Atoh1} animals would also be functionally mature. I therefore looked to determine the physiological status of Tg^{Atoh1}-generated ectopic hair cells by collaborating with Qing Chang from Xi Lin's laboratory at Emory to record the passive biophysical properties of these ectopic hair cells.

Whole-cell patch clamp recording allows measurement of a hair cell's current response to various manipulations, such as membrane potential changes. Analysis of these measurements provide insight into the types of ion channels expressed by the cell and how they function as a group to maintain the cell's resting membrane potential (Fig. 3*C*) (Housley and Ashmore 1992). Variations of this technique have been used previously to provide evidence that cells displaying molecular and morphological characteristics of hair cells also function physiologically like hair cells (Gubbels *et al.* 2008; Oshima *et al.* 2010a).

We compared the passive basolateral currents of Tg^{Atoh1}-generated hair cells to endogenous organ of Corti hair cells by whole cell patch-clamp recording technique (Fig. 10*A-C*). Voltage-dependent whole cell currents from patched ectopic or endogenous outer hair cells showed that many characteristics were hair cell-like for the ectopic hair cells, with the overall current response timecourse looking similar (Fig. 10*D-F*). Despite

the similarities, the recorded current amplitudes from ectopic hair cells were noticeably smaller for most of the voltage-steps at which the currents were recorded (Fig. 10*E*-*G*).

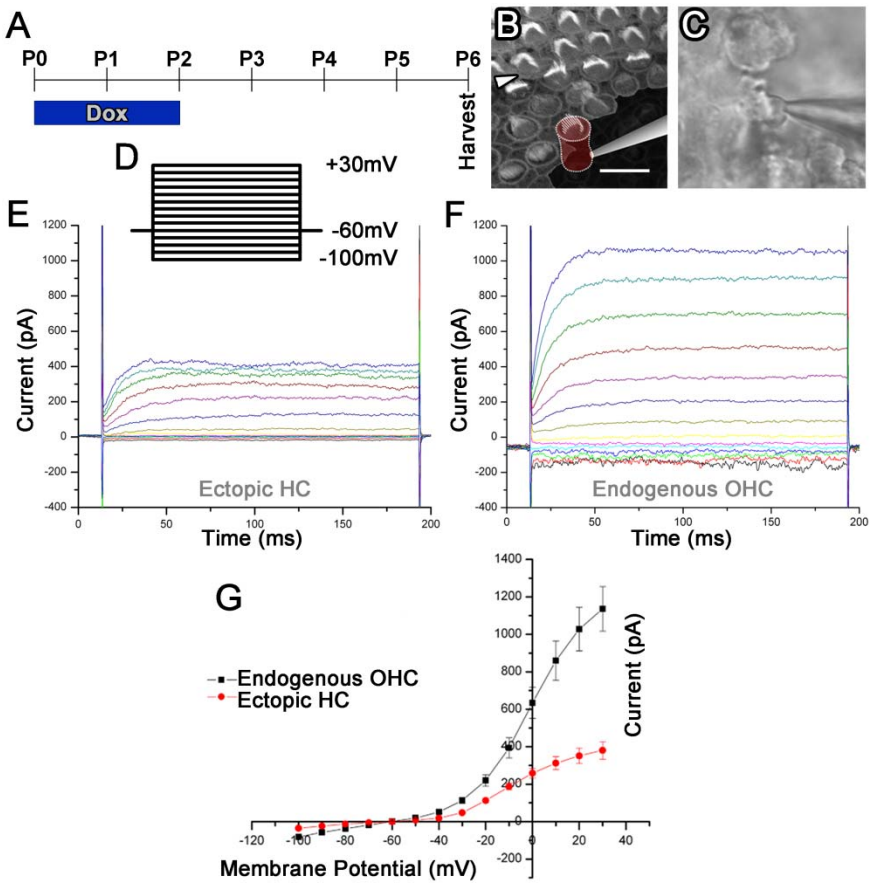

Fig. 10 - Atoh1 Generates Functional Hair Cells

(*A*) Diagram of the Tg^{Atoh1} induction protocol used to generate ectopic hair cells *in vivo* for patch-clamp recordings. (*B-C*) The relative position of the patched basolateral surfaces of ectopic hair cells is illustrated (*B*). An example of an actual patched ectopic hair cell is shown (*C*). (*D*) The voltage steps used for the recording, from -100mV up to +30mV. (*E-F*) A representative example set of current traces from an endogenous outer hair cell (*E*) and an Atoh1-induced ectopic hair cell (*F*) are shown. (*G*) An I-V plot summarizing the voltage-dependent currents recorded from endogenous outer hair cells and ectopic hair cells. More than six individual cells were recorded from tissue from at least two separate animals. Error bars represent standard deviation.

C. Age-Related Changes in Competency

A relatively short, two-day Tg^{Atoh1} induction at the neonatal-stage produced a large ectopic sensory region within Kölliker's organ and a small patch in the lateral wall (Fig. 9, Fig. 11*A,B*). The Atoh1-mediated hair cell differentiation competency of various cell populations in the older postnatal cochlea, however, remained to be tested. Significant molecular and morphological changes occur in the mammalian cochlea during development leading up to the onset of hearing (Hinojosa 1977; Kelly and Chen 2009; Reisinger *et al.* 2010). It was therefore necessary to determine how the competency of these various cell types change within the neonatal and more mature cochlea.

I compared the competency of Atoh1-induced hair cell generation in P0, P8, and P14 cochleae (Fig. 11). In comparison to the ectopic sensory region along the length of the cochlear duct in the neonatal cochlea (Fig. 11 4*A,B*), I observed an ectopic sensory region limited to the most apical regions of the cochlea just medial to the inner hair cell row following a two-day Tg^{Atoh1} induction in P8-stage mice (Fig. 11*C,D,H*). The competency was severely restricted by the P14-stage and Tg^{Atoh1} cochleae were indistinguishable from controls, even with an extended six-day induction paradigm (Fig. 4*E,F*) and clear up-regulation of Atoh1 transcript following just two days of Dox administration (Fig. 11*G*). Unfortunately, more extended induction protocols were prevented by the lethal side-effects of Dox treatment in $Foxg1^{Cre}$;rtTA;Tg^{Atoh1} mice.

I noted that the progressively restricted competency of cells medial to the organ of Corti appeared to correlate with a progressive narrowing of the Sox2-positive domain as Kölliker's organ undergoes its developmental regression to form the inner sulcus (Fig. 12) (Hinojosa 1977). The narrowing of the competency domain and Sox2 expression

followed a basal–to-apical and a mediolateral gradient, leaving a small patch of ectopic hair cells immediately adjacent to the inner hair cell row in apical regions (Fig. 12*E*,*F*) and no additional hair cells in basal regions (*data not shown*) of P8-stage Tg^{Atoh1} induced cochleae. Sox2, which is also expressed within the inner ear sensory precursor domains during normal development and is necessary for the formation of the organ of Corti within the cochlear epithelium (Kiernan *et al.* 2005b), may identify cells competent for Atoh1-mediated hair cell generation.

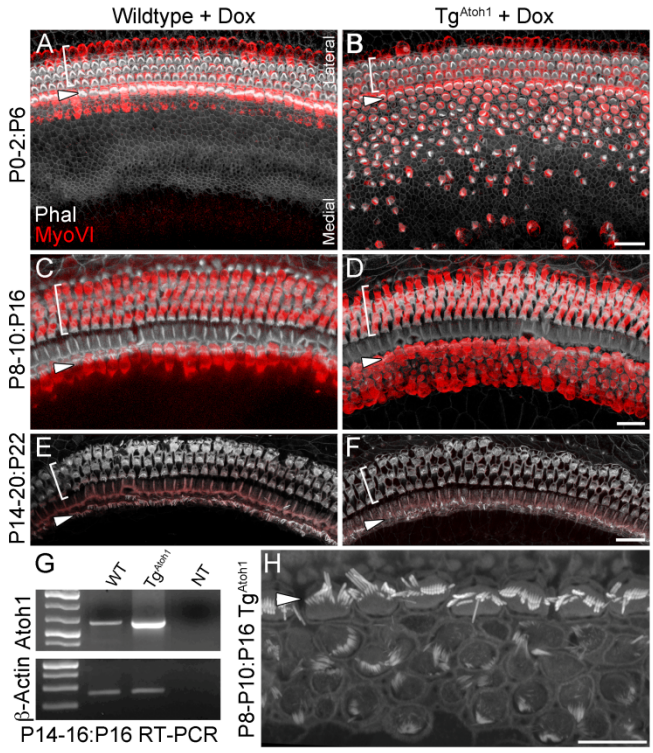

Fig. 11 - Age-Dependent Decreases in Competency

(*A-D*) Apical turn whole-mount cochleae from P6-stage (*A* and *B*) and P16-stage (*C* and *D*) mice administered Dox for two days (P0-2:P6 or P8-10:P16). Control littermates (*A* and *C*) have a normal one row of inner (*arrowhead*) and three rows of outer (*brackets*) hair cells. In comparison, triallelic Tg^{Atoh1} cochleae had ectopic MyoVI-positive hair cells with stereocilia throughout Kölliker's organ medial to the organ of Corti at the P6-stage (*B*) and a region of ectopic hair cells at the P16-stage (*D*). (*E-F*) Whole-mounts of P22-stage apical turn cochlea from mice administered Dox for an extended six days (P14-20:P22) shows no obvious differences between control (*E*) and triallelic Tg^{Atoh1} (*F*) mice. (*G*) Atoh1 is clearly up-regulated in transgenic cochlea at the P14-stage, even following just two days of Dox administration, as shown by RT-PCR (*WT: littermate control, NT: no cDNA template*). (*H*) High-resolution confocal image showing ectopic hair cells at from P8-10:P16 samples with the characteristically round apical membrane and varied stereocilia morphology in a mosaic cellular pattern surrounded by non-sensory cells. Scale bars (*A-F*): 20 μm, (*H*): 10 μm.

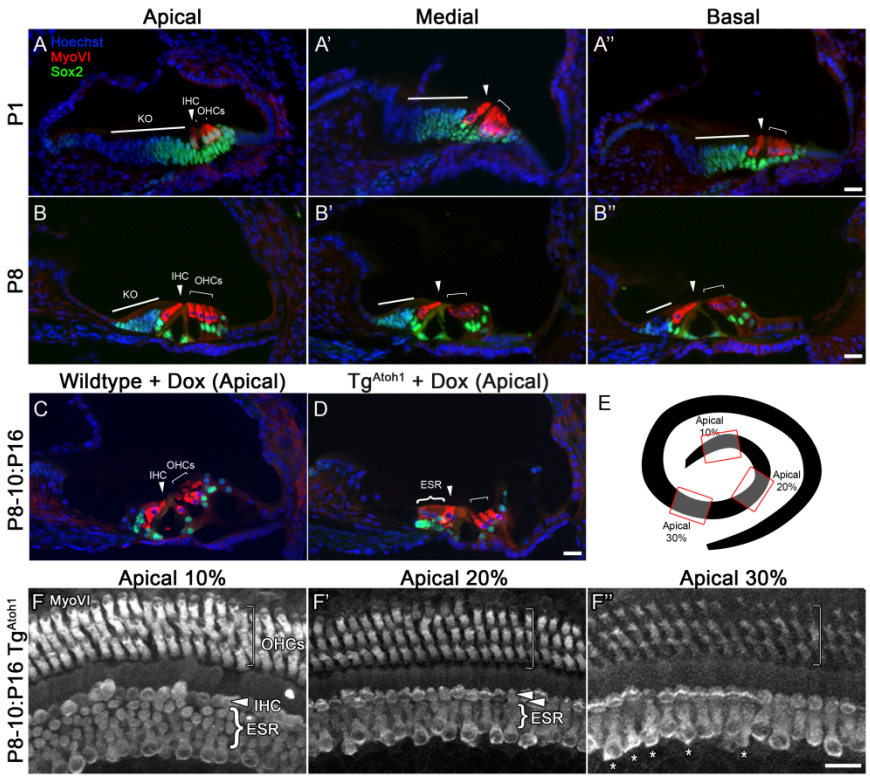

Fig. 12 - Changes in Competency Correlates with Sox2 Domain

(*A*) Tg^{Atoh1}-generated ectopic hair cells are generated in large numbers from the Sox2 expression domain, which extends throughout Kölliker's organ in P1 stage cochleae from apex to base (*A-A''*). (*B*) The developmentally transient Kölliker's organ begins to disappear by P8 in a basal (*B''*) to apical (*B*) gradient that results in a loss of the Sox2-positive cell population. (*C*) P8 mice induced for 2 days and harvested at P16 (P8-10:P16) show ectopic hair cells only in the apical ~30% in a domain that roughly corresponds to the Sox2-positive expression domain of Kölliker's organ at P8 (*B*). (*D*) Littermate control cochleae at P16-stage have Sox2 expression limited to sensory region supporting cells (*D*) and limited hair cell generation competency compared to earlier stages. (*E*) Diagram of regions competent for hair cell generation using the P8-10 induction with analysis at P16 (P8-10:P16). Example regions shown in (*E*) are highlighted. (*F*) Example whole-mount images of P8-10:P16 cochlea from the apical ~10% (*F*), apical ~20% (*F'*), and apical ~30% (*F''*) with ectopic sensory regions demarcated by brackets (*F-F'*) or isolated ectopic hair cells labeled by asterisks (*F''*). Scale bars: 20 μm.

D. Cellular Patterning in Ectopic Sensory Regions

Despite a presumably ubiquitous induction of Atoh1 in the $Foxg1^{Cre}$-expressing cochlear epithelium (Fig. 8*C*), all ectopic sensory regions contained a mosaic pattern of hair cells and non-sensory cells (Fig. 9*D*, Fig. 11*B,H*). These patterned ectopic sensory regions were reminiscent of endogenous inner ear sensory regions with hair cells surrounded by supporting cells (Fig. 2). The existence of non-sensory cells within these ectopic sensory regions suggested that Atoh1 was activating pathways to control cell fate decisions. Notch signaling is known to play an important role during development to define the cellular mosaic pattern of the organ of Corti (Lanford *et al.* 1999; Kiernan *et al.* 2005a; Brooker *et al.* 2006; Takebayashi *et al.* 2007) and was therefore a likely candidate. In order to determine whether Notch signaling was being activated within the cochlear epithelium following Tg^{Atoh1} induction, I treated Tg^{Atoh1} and wildtype neonatal mice with Dox and compared the expression of Notch downstream mediators by RT-PCR. I found that two Notch-associated downstream mediators known to inhibit hair cell differentiation during development, Hes1 and Hes5 (Zheng *et al.* 2000; Zine *et al.* 2001; Li *et al.* 2008), were up-regulated within Tg^{Atoh1} cochleae following two days of induction (Fig. 13*A*).

To further explore the possibility that Notch signaling plays a role in patterning the ectopic sensory regions, I induced Tg^{Atoh1} neonatal cochlear explant cultures while pharmacologically blocking Notch activity (Fig. 11*B*). In drastic comparison to the cellular mosaic pattern in the control cultures (Fig. 11*C,D*), cultures treated with DAPT, which inhibits gamma-secretase dependent cleavage of the Notch receptor required for downstream signaling (Geling *et al.* 2002), had an ectopic sensory region made up almost

entirely of MyoVI-positive hair cells with only a few non-sensory cells throughout (Fig. 13*E,F*). Whereas DAPT-treatment does not generate ectopic hair cells within Kölliker's organ in wildtype cochlea (Doetzlhofer *et al.* 2009), the normal percentage of ectopic hair cells generated within Kolliker's organ of induced Tg^{Atoh1} cochlear explants was significantly increased when cultured in the presence of DAPT (DMSO-treated: 22±11% vs. DAPT-treated: 66±13%, $p < 0.01$, n=3). DAPT-treatment also caused a significant increase in the number of ectopic hair cells that were in direct contact with one another (DMSO-treated: 23±4% vs. DAPT-treated: 95%±5%, $p < 0.001$, n=3).

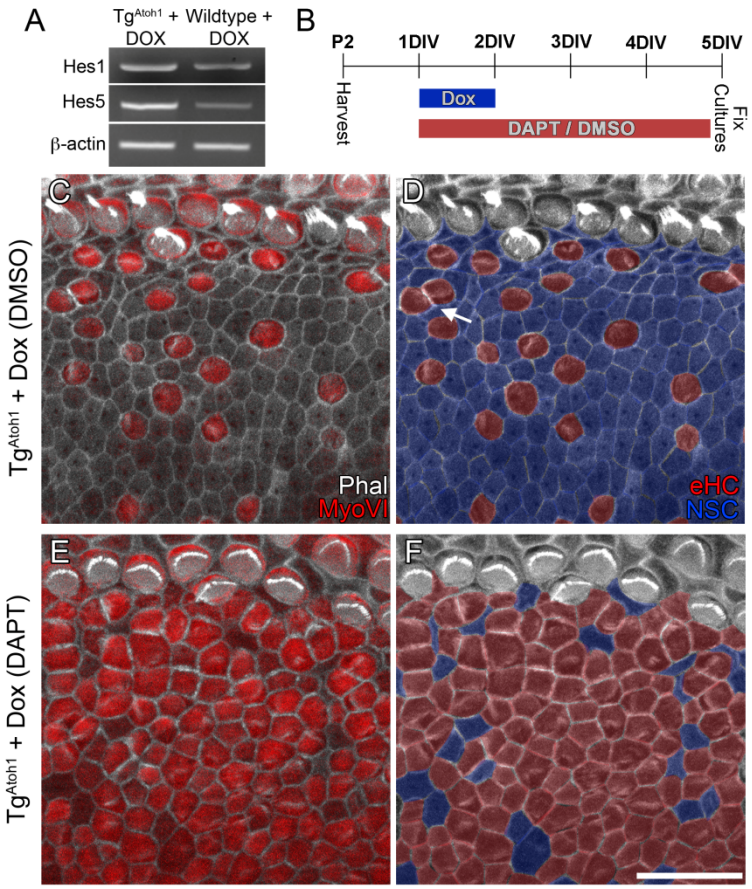

Fig. 13 - Notch Patterns Atoh1-Generated Ectopic Sensory Regions

(*A*) RT-PCR showing up-regulation of Hes1 and Hes5 following 2 days of Dox administration in triallelic Tg^{Atoh1} cochleae compared to controls. (*B*) Diagram of the experimental protocol. (*C-F*) In DMSO-control cultures, Atoh1 induced MyoVI-positive ectopic hair cells with stereocilia surrounding by non-sensory cells (NSCs, blue) (*C-D*). Very few ectopic hair cells (eHCs, red) were observed to be in direct contact with each other (*D*, arrow). Note that the shared borders between two contact eHCs are enriched for actin and that some non-sensory cells are not in direct contact with eHCs (*D*). Cochlear epithelia from contralateral ears of the Atoh1-induced animals were treated with DAPT (*E-F*). Significantly more cells within the ectopic sensory region became MyoVI-positive hair cells (*E*), with few NSCs sparsely distributed throughout (F, blue). (*D*) and (*F*) are corresponding images of (*C*) and (*E*) where eHCs (red) and NSC (blue) are color coded for easy visualization. Scale bar (*C-F*): 20 μm.

E. Effects of Extended Atoh1 Induction in the Mammalian Cochlea

While patterned ectopic sensory regions were readily generated in Kölliker's organ following a two-day Tg^{Atoh1} induction in $Foxg1^{Cre}$;rtTA; Tg^{Atoh1f} triallelic mice, there appeared to be little effect on the non-sensory supporting cells within the organ of Corti (Fig. 9, Fig. 11). No newly differentiated hair cells were observed within the endogenous sensory region (Fig. 9*B,D*, Fig. 11*B,D*). Despite having a shared expression of Sox2 with Kölliker's organ (Fig. 9*A*, Fig. 12*A,B*), the postnatal supporting cells within the organ of Corti represent a distinct and highly differentiated cell population (Henderson *et al.* 1995; Slepecky *et al.* 1995; Kelly and Chen 2007) that are specified and maintained by multiple signaling pathways (Fritzsch *et al.* 2002; Kelley 2006; Doetzlhofer *et al.* 2009). Since these cells are the only ones that remain in the organ of Corti following hair cells loss and represent the most likely targets for Atoh1-mediated hair cell regeneration, it was important to further test their competency.

I explored whether longer Tg^{Atoh1} induction could override the inhibitory signals and differentiated status of the cells that appeared to be less competent for hair cell differentiation by Atoh1, including the supporting cell population. I administered Dox to neonatal $Foxg1^{Cre}$;rtTA; Tg^{Atoh1} triallelic mice for four days and compared to age-matched Tg^{Atoh1} triallelic induced using the routine two-day administration (Fig. 14). Outside the organ of Corti region, I found expanded ectopic patches in Kölliker's organ and the lateral wall, as well as MyoVI-positive cells along Reissner's membrane (Fig. 14). In the most apical region of the cochlear duct of these animals, I observed a cochlear epithelium composed almost entirely of MyoVI-positive hair cells (Fig. 14*C*, inset).

These results suggested that extended Atoh1 expression was capable of promoting hair cell differentiation in less-competent cochlear epithelial cell populations.

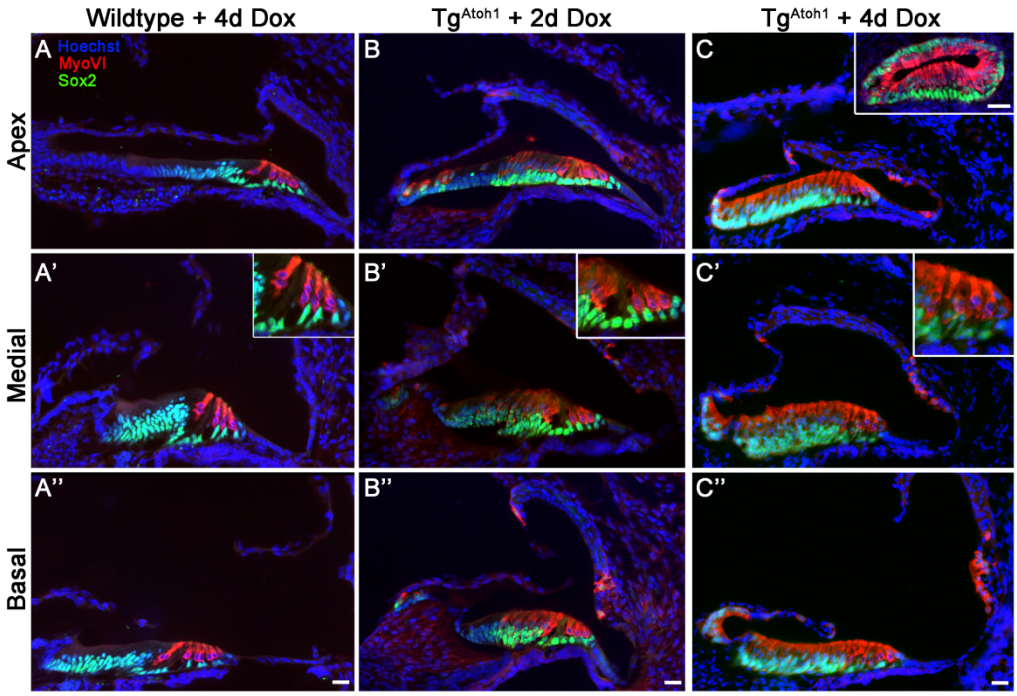

Fig. 14 - Extended Induction Expands Regional Competency

(*A-A")* P5/6-stage control cochlea show a stereotyped cellular architecture with one row of inner hair cells and three rows of outer hair cells that run the length of the cochlear duct from apex (*A*) to base (*A"*). Sox2 expression remains limited to the organ of Corti supporting cells and cells within Kölliker's organ. (*B-B")* P5/6-stage cochlea from a triallelic Tg^{Atoh1} mouse administered Dox for 2 day (P0-2). Ectopic hair cells are mainly limited to an ectopic sensory region within Kölliker's organ and a small patch on the lateral wall that will develop into the spiral prominence region. (*C-C")* P5/6-stage cochlea from a triallelic Tg^{Atoh1} mouse administered with Dox for 4 days (P0-4). The competency region within Kölliker's organ is expanded along the length of the cochlea. The very apical turn (*C*, inset) has a duct lined with MyoVI-positive hair cells. Note, however, that a layer of cells that are positive for Sox2 appear to remain (*C*, inset). Scale bars: 20 μm.

Within the organ of Corti, extended induction Tg^{Atoh1} samples had significantly disrupted patterning within the apical turn of the cochlea (Fig. 15*A,B*), with immature hair cells arising from between endogenous hair cells and immediately lateral to the third row of outer hair cells (Fig. 15*B, arrows*). Interestingly, additional cells were sometimes observed within the organ of Corti region (Fig. 15*A*, Fig. 14*C*). These additional cells were presumably the product of either recruitment or induced cell proliferation in the normally postmitotic cochlear epithelium (Ruben 1967; Chen and Segil 1999; Chen *et al.* 2003).

To determine if proliferation contributed to the additional number of cells within the sensory region, Tg^{Atoh1} neonatal mice were treated with Dox for four days and injected with BrdU to label S-phase nuclei. Surprisingly, BrdU-positive nuclei were observed within the Hensen's or Claudius cells of the organ of Corti (Fig. 15*C,D*), as well as in non-sensory regions of the normally post-mitotic cochlear epithelium (Ruben 1967). These results suggested that extended Atoh1 expression caused a disruption of normal cell cycle control.

One mechanism thought to maintain the postmitotic state of certain cochlear epithelial cells is the expression of cyclin-dependent kinase inhibitor Cdkn1b, formerly p27/Kip1 (Chen and Segil 1999). In order to determine if Atoh1 exerted its mitotic effects by causing a down-regulation of Cdkn1b, I compared its expression pattern in the four-day induced Tg^{Atoh1} and wildtype cochleae. The expression of Cdkn1b appeared to be greatly reduced in the same Hensen's and Claudius cell populations where ectopic cell proliferation is observed (Fig. 15*E,F, arrow*). Abnormal Cdkn1b expression was also observed within Kölliker's organ (Fig. 15*E,F*), though it is unclear if this represents a

change in cell cycle control or just an induction of non-sensory cells into the supporting cell molecular phenotype, which includes the continued expression of Cdkn1b (Fig. 15*E*) (Chen and Segil 1999).

I explored the phenomenon of Atoh1-induced proliferation further using the *in vitro* cochlear explant model with extended Dox treatment. Cochlear explants were incubated with media containing Dox and Brdu for several days. Tg^{Atoh1}-stimulated proliferation was particularly apparent in these cultures, where BrdU-positive nuclei were observed throughout the normally post-mitotic sensory region domain (Fig. 15*G*,*H*). In Tg^{Atoh1} organ cultures, Sox2-positive supporting cells within the sensory region also expressed the metaphase marker phospho-histone H3 (PH3) in patterns consistent with late-stage mitotic progression (Fig. 16) (Hans and Dimitrov 2001). Therefore, it appears that Tg^{Atoh1} induction can not only induce hair cell formation but also promote mitotic events within the postnatal mammalian cochlear epithelium.

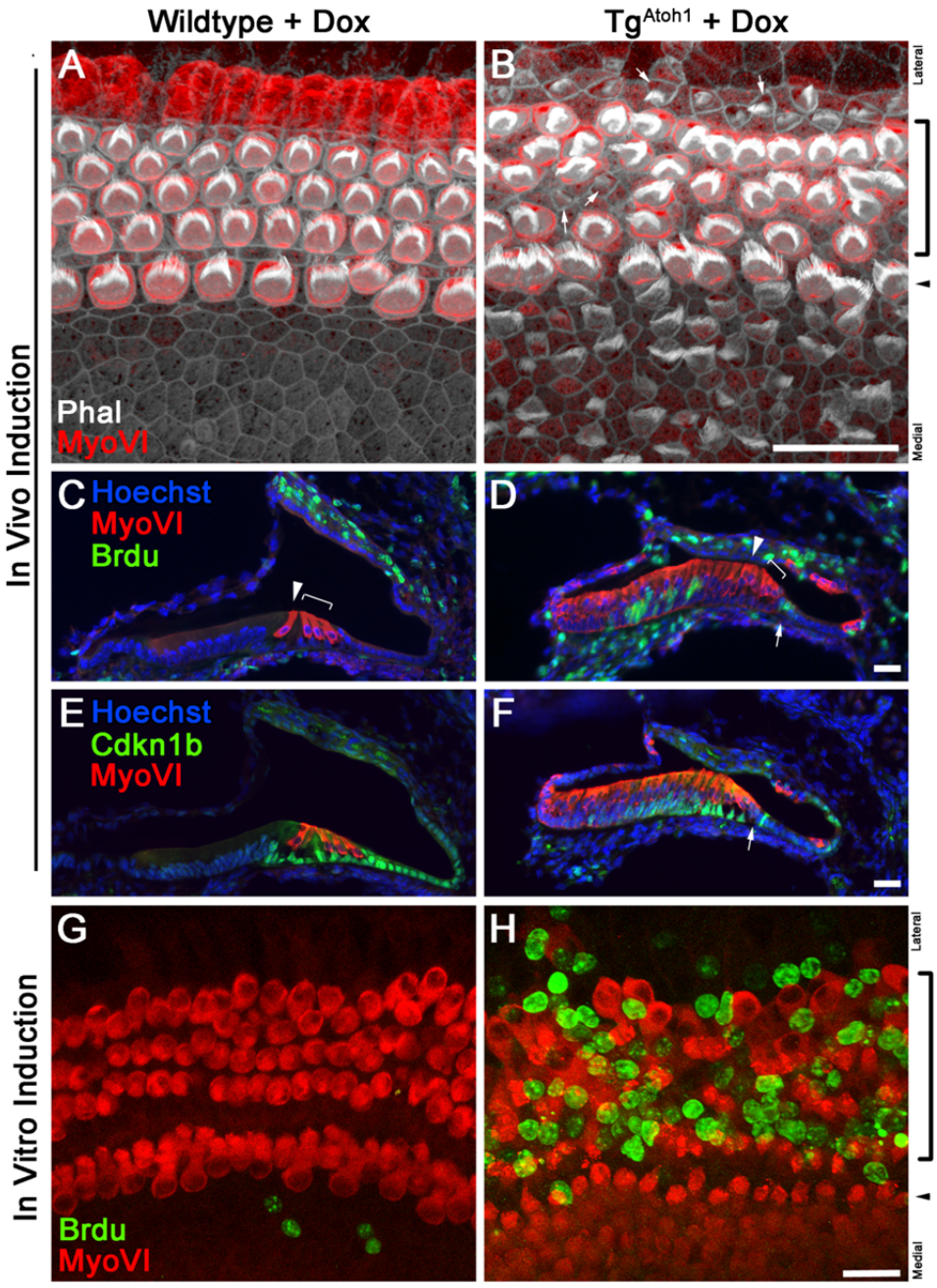

Fig. 15 - Extended Induction Causes Disruption of Patterning and Proliferation

(*A*-*B*) Whole-mount confocal images of apical turn cochlea from P5-stage mice administered Dox for 4 days (P0-4). Compared to the stereotyped patterning of control littermates (*A*), the sensory regions of Atoh1-induced mice show severe disruption (*B*). Cells with immature hair bundles appear within the sensory region (*A*, arrows). In addition, ectopic hair cells are induced in

the region immediately lateral to the third row of outer hair cells (*A*, arrows). (*C-D*) Cross-sections through the apical turn of cochlea from P5-stage triallelic Tg^{Atoh1} mice administered Dox for 4 days (P0-4) and injected with BrdU daily. In comparison to the normally post-mitotic cochlear epithelium at P5 (*C*), the triallelic Tg^{Atoh1} mice (*D*) have a large number of BrdU-positive cells in Kölliker's organ and in the Hensen's and Claudius cell population (*arrows*). (*E-F*) Adjacent sections to those shown in (*C-D*), with changes in Cdkn1b, or p27/Kip1, expression in triallelic Tg^{Atoh1} cochlea (*F*), compared to littermate control cochlea (*E*). (*G-H*) P4-stage cochlear organ cultures induced with Dox for 4 days in the presence of BrdU shows no proliferation occurring in the sensory region of control cochleae (*G*), but robust BrdU incorporation in the normally post-mitotic sensory region of Tg^{Atoh1} cochleae (*H*). Brdu-positive nuclei from the underlying mesenchymal cell layer are shown in (*G*) as a positive control for Brdu incorporation and immunostaining. All confocal z-stacks below the MyoVI-positive hair cells were removed from (*H*) to ensure only Brdu-positive epithelial cells were shown in this panel. All scale bars: 20 μm.

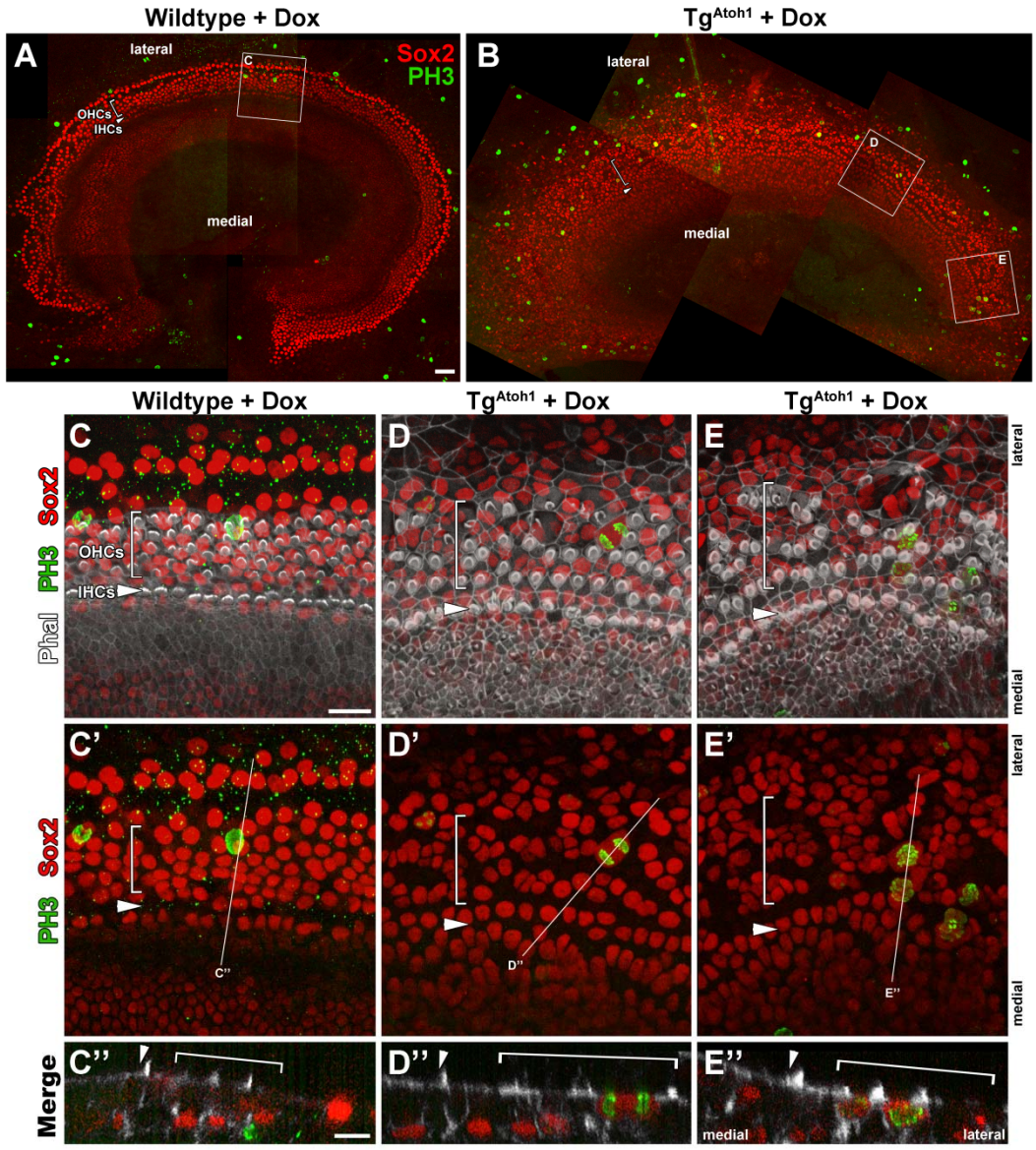

Fig. 16 - Dividing Supporting Cells Survive and Enter Metaphase

(*A-B*) P1-stage apical turn cochlear explant cultures from wildtype littermate control (*A*) and Tg^{Atoh1} (*B*) treated with Dox for 4 days. Epithelial and underlying mesenchymal cells in metaphase are labeled with phospho-histone H3 (PH3), including some cells within the sensory region (OHC region marked with brackets, IHC row marked with arrowheads). (*C-E*) Higher magnification confocal scans of regions identified with white outlines shown in (*A-B*). No Sox2-

positive supporting cells are observed to be PH3-positive within the control sensory region, although clear staining can be seen within the underlying mesenchyme (*C-C''*). (*D-E*) In contrast, many Sox2-positive nuclei have patterns of PH3 localization that show supporting cells in various stages of metaphase and beyond, including cells in anaphase (*D-D''*) and pairs of PH3-positive suggesting completed mitosis (*E-E''*). Orthogonal cuts through confocal scans show PH3-positive cells in control cultures are in Sox2-negative underlying mesenchymal cells (*C''*) and lumenal-localized Sox2/PH3-positive nuclei in Tg^{Atoh1} samples (*D'', E''*). Scale bars (*A-B*): 50μm, (*C-E*): 20μm.

3.4 Discussion

Using our novel Atoh1 transgenic mouse line as a stable and flexible system of delivery, I demonstrate Atoh1's profound effects within various cell types of the cochlear epithelium at various stages. I show that a relatively short *in vivo* delivery of Atoh1 to the entire cochlear epithelium at the neonatal stage identifies distinct regions that are highly competent for hair cell differentiation (Fig. 9). Atoh1-generated hair cells are most robustly produced within Kölliker's organ region and have characteristic hair cell properties (Fig. 9*C,* Fig. 10). These results suggest that the cells within Kölliker's organ at the neonatal stage retain competency to undergo an advanced hair cell differentiation program. The production of ectopic hair cells was also observed within in the lateral wall (Fig. 9) with short inductions of Atoh1, and essentially the entire population of cells of the epithelium at the apical region of the cochlea with extended induction of Atoh1 (Fig. 14*C*, inset). These data together demonstrate the potency of Atoh1 in hair cell induction and outline the relative competency of various populations of cells.

Despite the induction of Atoh1 following the ubiquitous expression of rtTA and GFP in the cochlear epithelium (Fig. 8*C*), non-sensory cells persist and surround the ectopic hair cells within Kölliker's organ (Fig. 9*B*, Fig. 11). This cellular patterning appears to be mediated via a Notch signaling-dependent mechanism as Tg^{Atoh1}-induced cochlear explants treated with DAPT produced a field almost exclusively of MyoVI-positive cells (Fig. 13). Although the γ-secretase inhibitor DAPT could theoretically exert this effect by disruption of other signaling pathways, the up-regulation of the expression of Notch downstream mediators (Fig. 13*B*) observed in induced Tg^{Atoh1} cochleae supports the theory that Notch is involved. These results suggest that Atoh1 activates Notch signaling

to pattern ectopic sensory regions and that similar to its role within the development of endogenous sensory regions, Notch plays an important role in hair cell versus supporting cell fate decisions.

Although the effect of blocking Notch signaling via γ-secretase inhibition is clear, there is some evidence that the cellular mosaic within the ectopic regions does not follow a strict model of contact-dependent Notch signaling lateral inhibition. Ectopic hair cells sometimes directly contact each other and not all non-sensory cells surround ectopic hair cells (Fig. 13). As is the case in the development of the organ of Corti, additional pathways, such as *FGF* signaling (Mueller *et al.* 2002; Jacques *et al.* 2007; Puligilla *et al.* 2007) and Nectins (Togashi *et al.* 2006) may also be involved in patterning ectopic sensory regions. Taken together, these results suggest that multiple pathways are activated following Atoh1 delivery to pattern ectopic sensory region.

By comparing cochlear epithelia from Tg^{Atoh1} animals induced at P0, P8, and P14 stages, I show the competency to generate hair cells becomes progressively restricted over the postnatal time period during hearing onset (Fig. 11) and correlates with a regression of the Sox2-positive cells within Kölliker's organ (Fig. 12). These results suggest that the expression of Sox2 within the developmentally transient Kölliker's organ may represent a latent sensory precursor domain that is relatively easily activated by Atoh1 expression. Furthermore, this change in competency illustrates the important distinction between generating new hair cells within the embryonic and neonatal tissue versus the adult cochlea, which presumably would be the primary target for hair cell regeneration.

While the highly competent Sox2-positive cells within Kölliker's organ cells become increasingly limited during postnatal maturation, several possibilities for Atoh1-mediated hair cell generation in the more mature cochlea still exist. In triallelic Tg^{Atoh1} cochleae, in regions where ectopic hair cells were generated outside of Kölliker's organ, ectopic Sox2 expression was also observed (Fig. 9, Fig. 14). Additionally, organ of Corti supporting cells, which continue to express Sox2, but are not as easily converted under short induction protocols (Fig. 9, Fig. 11), appear to be converted with longer Tg^{Atoh1} induction (Fig. 14, Fig. 15). Because of the unexpected lethality associated with Tg^{Atoh1} induction, it remains to be tested whether longer-term Atoh1 induction may further expand the regional and temporal competency of Atoh1-mediated hair cell generation. However, these results, even with a limited timeframe of induction, suggest that longer Atoh1 delivery may be able to generate *de novo* sensory progenitors and convert less competent cell types.

Furthermore, cell cycle re-entry was observed in the normally postmitotic cochlear epithelium following extended Atoh1 induction. BrdU-positive nuclei are present in Hensen's or Claudius cells *in vivo* (Fig. 15*D*, arrow) and within the sensory region *in vitro* (Fig. 15*H*). Dividing supporting cells are capable of progressing through mitosis, suggesting that they are not merely dying cells (Fig. 16). It is possible that the forced expression of Atoh1 in a large domain may be activating pathways not normally observed within the mammalian inner ear, as Atoh1 has varied roles depending on its cellular context (Miesegaes *et al.* 2009; Klisch *et al.* 2011; Lai *et al.* 2011). It is also possible that this phenomenon represents an attempt to repopulate cell populations that have been forcefully converted following extended Atoh1 delivery. This would be an exciting

similarity to the mechanism of self-renewal reported within the avian auditory system, where the conversion of supporting cells by Atoh1 expression is followed by proliferation to generate new supporting cells (Roberson *et al.* 2004; Cafaro *et al.* 2007).

Overall, these findings provide important information about Atoh1's effects within the postnatal cochlear epithelium. I show that the potency of Atoh1 as a hair cell differentiation factor is limited to an increasingly discrete cell population as the cochlea matures, but that extended forced expression of Atoh1 can somewhat expand this populations. In regions and at timepoints when hair cell generation is possible, the ectopic hair cells appear to be relatively mature and recruit innervation. Within Atoh1-generated ectopic sensory regions, signaling helps to pattern a mosaic of hair cells and supporting cells similar to endogenous sensory regions. Furthermore, I discovered that Atoh1 expression could lead to *de novo* Sox2 expression and that when Atoh1 is expressed for an extended time, proliferation is induced in the normally postmitotic cochlear epithelium. These results add to our current understanding of Atoh1's effect within the cochlea and help outline some of the possibilities and limitations associated with promoting hair cell regeneration by *Atoh1*-based gene therapy.

Chapter IV: Targeting Atoh1 to Sensory Region Cells

4. 1 Introduction

Work using inducible expression constructs (Chapter II) and a transgenic mouse model (Chapter III) confirms Atoh1's potency as a hair cell differentiation factor and demonstrates its powerful effects within various cell populations of the cochlear epithelium, including the production of new hair cells in the postnatal mammalian cochlea. However, despite the presumably ubiquitous expression of Atoh1 within $Foxg1^{Cre}$;rtTA;Tg^{Atoh1} cochlea following Dox induction, there are comparatively few hair cells generated within the organ of Corti. Although extended Atoh1 induction appears to produce new hair cells within the sensory region, definitive evidence that they can be converted from the supporting cell population is lacking. Furthermore, it is unclear whether Atoh1 delivery to less differentiated supporting cells or the sensory precursor region from which they arise could lead to more robust hair cell generation.

Supporting cells arise from the same common set of precursors as hair cells, which can be identified early within cochlear development by molecular markers (Kelley 2006) (Fig. 4). Although differentiating hair cells down-regulate many of these sensory precursor markers, supporting cells continue their expression (Chen and Segil 1999; Kiernan *et al.* 2005b; Bermingham-McDonogh *et al.* 2006; Dabdoub *et al.* 2008). This raises the possibility that young supporting cells represent a latent precursor population. In fact, disruption of signals coming from hair cells within the embryonic and neonatal cochlea, either by hair cell ablation (Kelley *et al.* 1995), pharmacological blockade

(Doetzlhofer *et al.* 2009) or the isolation of supporting cells (White *et al.* 2006), leads to the conversion of these cells to new hair cells.

The ability to convert, however, appears to lost well before the onset of auditory function (Kelley *et al.* 1995; Hartman *et al.* 2009; Yamamoto *et al.* 2009) and adult mammalians show no signs of auditory hair cell regeneration (Forge *et al.* 1998). Despite this, an external manipulation, such as forced expression of the hair cell differentiation factor Atoh1, with or without additional manipulations, may be sufficient to promote the conversion of more differentiated supporting cell populations. Support for this idea was given by reports that viral-mediated delivery of Atoh1 to the hair cell ablated guinea pig cochlea led to the generation of new hair cells that were speculated to be converted supporting cells (Izumikawa *et al.* 2005). Because supporting cells are the only remaining cells in hair cell-depleted organ of Corti and represent some of the more likely candidates for Atoh1-mediated hair cell production within the mammalian cochlea, I used alternative transgenic models of Atoh1 delivery to explore these questions more directly.

Here, I show that lineage-traced cells from the differentiated supporting cell population can be converted to hair cells within cultured postnatal cochlear epithelium and that the sensory region expands to presumably recruit new non-sensory cells. Using a bacterial artificial chromosome (BAC) transgene to mis-express Atoh1 within the sensory precursor domain and in supporting cells throughout their development, I show that overexpression of Atoh1 leads to the generation of additional hair cells, but that a supporting cell population remains and cell cycle control may be disrupted. I also introduce and show preliminary data from an additional transgenic model that may allow

a more careful dissection of Atoh1's role within supporting cells at various stages of their differentiation.

4.2 Methods

BAC Modification via Homologous Recombination

Modification of the *Cdkn1b* BAC clone (Clone #485G10) to include an internal ribosome entry site (IRES) expressed enhanced GFP was performed by Neil Segil's lab at House Ear Institute, Los Angeles, CA and is described in previous publications (Lee *et al.* 2006; White *et al.* 2006). Using similar methods, the BAC$^{Cdkn1b:ieGFP}$ was then further modified in our lab by XianKun Li via homologous recombination to replace *Cdkn1b* coding sequence with the entire *Atoh1* ORF (Yang *et al.* 1997; Yang *et al.* 1999). The modified BAC, BAC$^{Cdkn1b:Atoh1\ ieGFP}$, confirmed by Southern blot before being amplified, linearized, and purified in our lab by Dong Qian. The Emory Transgenic core performed pronuclear injection. Positive founders were confirmed by Southern blot using a probe against *Atoh1*.

To modify BAC$^{Cdkn1b:Atoh1\ ieGFP}$ for the generation of the Tamoxifen-inducible Atoh1 transgenic line, the *Atoh1-HA-ER* cassette from our pIRES2-eGFP expression vector was subcloned into a BAC modification PSV RecA shuttle vector. For the right homology arm, a fragment of the BAC$^{Cdkn1b:Atoh1\ ieGFP}$ was PCR amplified and cloned into the shuttle vector downstream of the *Atoh1-HA-ER* sequence. *Atoh1* was used as the left homology arm. BAC$^{Cdkn1b:Atoh-HA-ER\ \ ieGFP}$ was generated via homologous recombination (Yang *et al.* 1997; Yang *et al.* 1999). Positive BAC clones were confirmed by Southern blot before sending to Jian Zuo's lab at St. Jude Children's Research Hospital, Memphis, TN for amplification, linearization, purification, and pronuclear injection. Founders were

confirmed by PCR genotyping and Southern blot (B. Cox, J. Dearman, J. Zuo, *unpublished data*).

BAC Transgenic Sample Generation and Preparation

All BAC$^{Cdkn1b:Atoh1\ ieGFP}$ husbandry, tissue harvesting, and sample preparation was performed in our lab by Sharayne Mark. The E16.5 inner ear cross-section samples used for the immunostaining shown here were prepared in our lab by Sharayne Mark.

BAC$^{Cdkn1b:Atoh\text{-}HA\text{-}ER\ ieGFP}$ husbandry and tissue harvesting was performed by Brandon Cox and Jennifer Dearman from Jian Zuo's laboratory at St. Jude Children's Research Hospital, Memphis, TN. Inner ears were extracted and fixed in 4% paraformaldehyde solution in phosphate buffered saline (PBS) overnight before storage in PBS. Cerebellar tissue samples were flash-frozen in liquid nitrogen. Samples were shipped on ice or dry-ice, respectively.

Western Blot Detection of Cerebellar Atoh1-HA-ER

Whole P1-7 cerebellar protein from BAC$^{Cdkn1b:Atoh\text{-}HA\text{-}ER\ ieGFP}$ animals and littermate controls was prepared and immunoblotted for the presence of the Atoh1-HA-ER fusion protein using standard procedures. Briefly, frozen cerebellar tissue was extracted in RIPA buffer (Upstate Chemicon, Cat: 20-188) by sonication. Total protein concentration was estimated by BCA assay (Pierce, Cat: 23225). 200µg of total protein was on a SDS-PAGE gel and transferred to PVDF membrane. Blocked membrane was incubated overnight with mouse HA antibody (Cell Signaling Cat: 2367S), followed by incubation with an HRP-conjugated secondary (Sigma, Cat: A9044). Chemiluminescent activity

(Millipore, Cat: WBKLS0050) was recorded by standard film (Denville Scientific, Cat: E3018).

Tg^{Atoh1};rtTA;$GFAP^{Cre}$ Cochlear Explant Organ Culture

P0 stage pups were euthanized and cochlear tissue extracted. The lateral wall, Reissner's membrane, and tectorial membrane were removed before plating on poly-D-lysine (Sigma, Cat: P6407) coated glass bottom dishes (MatTek Corp, Cat: P35G-0-14-C) as reported by Qian et al (2007). Tissue was incubated in DMEM/F12 (Gibco, Cat: 11039) with 10% FBS (Atlanta Biologicals, Cat: S11150H), 2% B-27 supplement (Gibco, Cat: 17504-044) and 1% 104U/mL Penicillin G (Sigma, Cat: P3032).

Immunohistochemistry and Tissue Staining

Tissue preparation, immunostaining and imaging were performed as previously described (Li *et al.* 2008). Primary antibodies used were: MyoVI (Proteus Biosciences Cat: 25-6791), PCNA (Santa Cruz Cat: SC-56), HA antibody (Cell Signaling Cat: 2367S), GFP (Millipore, Cat MAB3580). Hoechst stain (Molecular Probes) was used to mark cell nuclei. BrdU and Cdkn1b staining required a 10mM citrate buffer steaming antigen retrieval step (Chen and Segil 1999; Tang *et al.* 2007). Fluorophore-conjugated Phal stains used were: AF488-Phal (Molecular Probes, Cat: A12379), Rhod-Phal (Molecular Probes, Cat: R415), AF635-Phal (Invitrogen, Cat: A34054). Secondary antibodies used were: AF488-conjugated Goat-anti-Mouse (Molecular Probes, Cat: A11001), Rhodamine-conjugated Goat-anti-Rabbit (Jackson IR, Cat: 111-295-144), Cy5-conjugated Donkey-anti-Goat (Jackson IR, Cat: 705-175-147), Cy2-conjugated Donkey-

anti-Mouse (Jackson IR, Cat: 715-225-150). All samples were mounted in Fluoromont-G (SouthernBiotech Cat: 0100-01) and sealed for long-term storage.

Care and Maintenance of Tg^{Atoh1} Animals

Animal care and use was in accordance with NIH guidelines and was approved by the Animal Care and Use Committee of Emory University. E0.5 was determined as the day that a seminal plug was observed. P0 was determined as the day pups were born. Tg^{Atoh1} mice were PCR genotyped for the presence of the pTET-Splice(Atoh1) transgene with fwd-primer: CGCGCAATTAACCCTCACTA and rev-primer: CGGGAGAATGCAGCAGATAC. rtTA mice were genotyped with fwd-primer: GAGTTCTCTGCTGCCTCCTG and rev-primer: AAGACCGCGAAGCGTTTGTC. $GFAP^{Cre}$ mice were genotyped with fwd-primer: TTCATTCTTCGCCAAATTCC and rev-primer: AGGCAAATTTTGGTGTACGG.

Imaging and Figure Compilation

Mounted samples were imaged using either an Olympus IX71 inverted microscope with a Zeiss high-resolution monochrome AxioCam HRm camera or scanned using a Zeiss LSM510 confocal. Images were processed and compiled with Photoshop CS3 (Adobe).

4.3 Results

A. $GFAP^{Cre}$ Directs Dox-Inducible Tg^{Atoh1} to Supporting Cells

To directly test Atoh1's effect within differentiated supporting cells, I used our Dox-inducible Tg^{Atoh1} transgenic line in combination with a supporting cell specific Cre mouse

line and induced expression in cochlear explants from neonatal mice. Glial fibrilary acidic protein (GFAP), which is more commonly known for its increasingly complex role in a certain subclass of glial cells of the nervous system (Middeldorp and Hol 2011), is also expressed within differentiation supporting cells of the mammalian auditory system (Rio *et al.* 2002; Smeti *et al.* 2011). I obtained a $hGFAP^{Cre}$ mouse line created by Ken McCarthy's lab (Casper and McCarthy 2006) and evaluated its ability to activate the rtTA locus and drive Tg^{Atoh1} expression following induction (Fig. 17*A*). Although I found little consistent eGFP expression within $GFAP^{Cre}$;rtTA(eGFP);Tg^{Atoh1} cochlea *in vivo* (*data not shown*), I reliably observed positive Cre-mediated eGFP expression within cochlear explants from P0 tri-allelic animals cultured for several days (Fig. 17*B*). Conveniently, this supporting cell specific recombination not only allowed us to drive Tg^{Atoh1} expression within this specific cell population, it also provided a mechanism to lineage-trace converted supporting cells.

Using this system, I established cochlear explants using cochleae from $GFAP^{Cre}$;rtTA(eGFP);Tg^{Atoh1} and cultured in Dox-containing media for several days (Fig. 17*C*). I found that extended Atoh1 induction within the differentiated supporting cell population led to generation of new hair cells (Fig. 17*D, asterisks*) through the conversion of eGFP lineage-traced supporting cells (Fig. 17*E,F*). Newly generated hair cells displayed expression of hair cell markers, normal morphology (Fig. 17*D,G*) and generated stereocilia bundles on their apical surfaces (Fig. 17*G*). Interestingly, I also observed an expansion of the eGFP domain (Fig. 17*F*), suggesting that an inductive mechanism for the expansion of the hair and supporting cell mosaic directly or indirectly due to the forced expression of Atoh1.

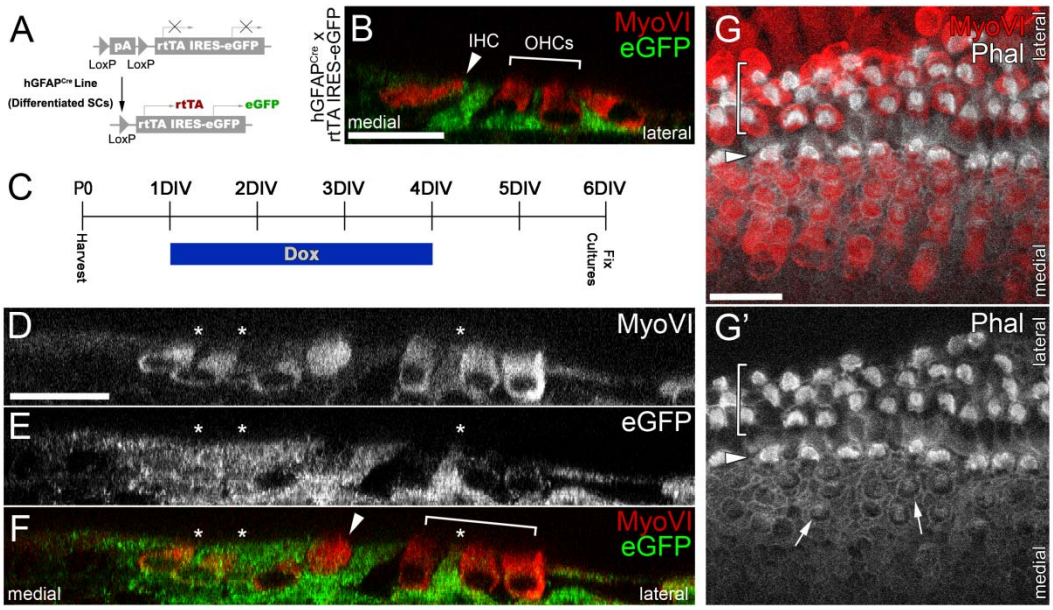

Fig. 17 - Extended Atoh1 Expression Can Convert Supporting Cells

(*A-B*) hGFAP^{Cre} drives rtTA activation (*A*) within differentiated supporting cells. Cross-section of the organ of Corti demonstrates GFP expression strictly in the supporting cells and Myosin VI expression in the hair cells in GFAP^{Cre};rtTA (eGFP) animals. (*C*) Diagram illustrating the induction protocol employed. Doxycycline induction was maintained for three days between 1DIV and 4DIV for GFAP^{Cre};rtTA (eGFP);Tg^{Atoh1} cultures. (*D-F*) Orthogonal confocal sections taken through the cochlea after three days of induction in GFAP^{Cre};rtTA (eGFP);Tg^{Atoh1} animals. Note that a nascent MyoVI-positive cell (asterisk) is also positive for eGFP, indicating its supporting cell origin. In addition, a few eGFP+ and MyoVI+ cells (asterisks) are observed in the medial region of the cochlear epithelium. (*G*) Phalloidin and MyoVI staining of the cochlear whole-mount shows the production of ectopic hair cells with the formation of stereocilia bundles (*G'*, arrows). Scale bars: 20 μm.

B. BAC Transgenic Drives Atoh1 Throughout Prosensory Domain

To determine if forced Atoh1 expression within the undifferentiated sensory precursor domain of the cochlea could generate additional hair cells, a transgenic mouse was generated to reliably direct Atoh1 expression within auditory prosensory cells. Cdkn1b, a cyclin dependent kinase inhibitor, is expressed throughout the precursor domain and clearly marks the prosensory domain early within the embryonic development of the cochlea. Previous work showed that transgenic mouse generate with a bacterial artificial chromosome (BAC) containing the *Cdkn1b* locus and modified to express eGFP via an internal ribosome entry site (IRES), drove reliable reporter expression within early sensory precursor cells of the cochlea (Lee *et al.* 2006; White *et al.* 2006). Using this $BAC^{Cdkn1b:ieGFP}$ construct as a template for homologous recombination, previous work by our lab replaced the *Cdkn1b* coding sequence with the complete *Atoh1* open reading frame (Fig. 18*A*) and a transgenic mouse line, $BAC^{Cdkn1b:Atoh1\ ieGFP}$, was generated to drive Atoh1 expression throughout the sensory precursor domain.

As expected from previous work(Chen and Segil 1999; Lee *et al.* 2006; White *et al.* 2006), expression of Cdkn1b marked the postmitotic sensory primordium in the cochlea E14.5, and $BAC^{Cdkn1b:Atoh1\ ieGFP}$ drove the expression of EGFP in Cdkn1b-expressing domains (Fig. 18*B, D) (*D.Qian and P.Chen*, unpublished data*). Unfortunately, although two $BAC^{Cdkn1b:Atoh1\ ieGFP}$ transgenic founders were confirmed by Southern blot (D. Qian and P.Chen, *unpublished data*), no transgene-positive offspring could be generated due to an embryonic lethality phenotype. A limited number of embryonic samples were generated by time-mating the presumably mosaic $BAC^{Cdkn1b:Atoh1\ ieGFP}$ male founder to

control females, and these previously prepared samples were used for the analysis reported here.

Analysis of E16.5 BAC$^{Cdkn1b:Atoh1\ ieGFP}$ cochlea showed that the organ of Corti had supernumerary hair cells compared to littermate controls (Fig. 18*C,D*). However, not all of the cells within the sensory domain expressed MyoVI, and these MyoVI-negative cell nuclei resided in the supporting cell layer (Fig. 18*D*). This was particularly surprising because the continued promoter activity of the *Cdkn1b* locus within supporting cells should also drive Atoh1 expression. In addition, the width of the BAC$^{Cdkn1b:Atoh1\ ieGFP}$ organ of Corti was noticeably wider and appeared to contain more cells overall (Fig. 18*C,D*). Together, this suggested that although forced expression of Atoh1 within undifferentiated sensory precursor cells could produce more hair cells, the supporting cell population was somehow maintained.

Since cell death is undetectable in the organ of Corti during terminal differentiation (Chen *et al.* 2002; McKenzie *et al.* 2004), the expansion of the organ presumably is the result of either the recruitment of cells from other regions in the cochlea and/or induced ectopic cell proliferation. To determine whether cell proliferation is involved, I analyzed the levels of proliferating cell nuclear antigen (PCNA) (Miyachi *et al.* 1978) in wildtype and BAC$^{Cdkn1b:Atoh1\ ieGFP}$ cochleae (Fig. 18*C',D'*). In E16.5 wild-type control cochleae, PCNA was only detected in the region medial to the organ of Corti but not in the organ of Corti (Fig. 18*C',C''*), consistent with several previous studies and confirming the formation of zone of non-proliferating cells in the organ of Corti (Ruben 1967; Chen and Segil 1999; Mantela *et al.* 2005; Sage *et al.* 2006). In contrast, animals carrying BAC$^{Cdkn1b:Atoh1\ ieGFP}$, cells in the organ of Corti showed elevated levels of PCNA (Fig.

18*D’,D’’*). Interestingly, PCNA appeared to be expressed in both hair cells and non-sensory cells (Fig. 18*D’’*). Furthermore, the pattern of PCNA localization in a subset of hair cells appeared to be devoid of nucleolar labeling (Fig. 18*D’’, inset*), similar to the pattern of PCNA localization in early S-phase or G1-phase (Celis and Celis 1985). These results suggested that forced Atoh1 expression within the sensory precursor cells was causing a disruption of normal cell cycle control.

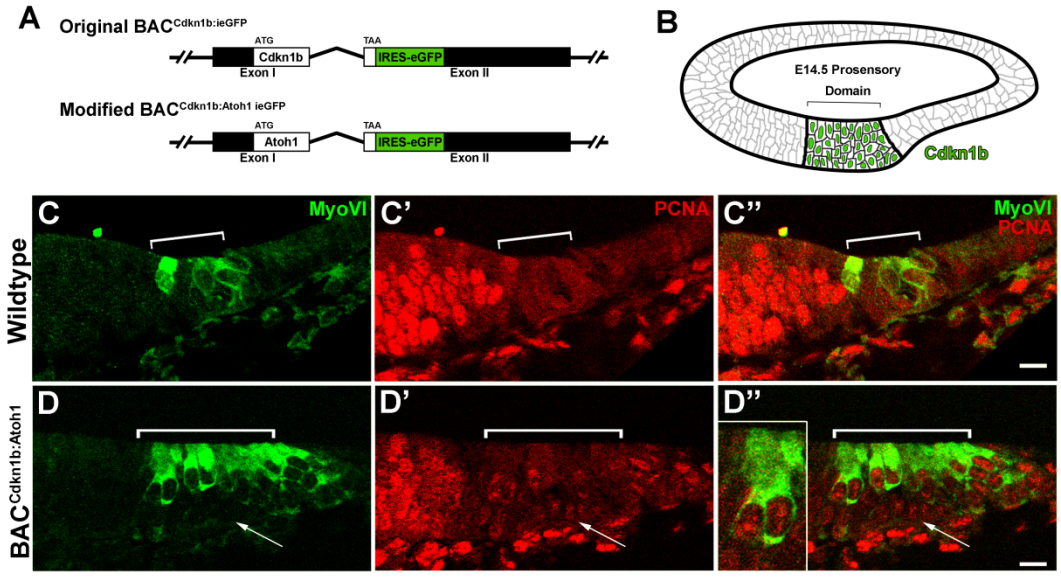

Fig. 18 - Cdkn1b-Driven Atoh1 Generates Supernumerary Hair Cells

(*A*) Construct schematic of the original Cdkn1b locus-containing BAC with inserted internal ribosome entry site (IRES) enhanced GFP, and the modified construct with coding sequence replaced with entire Atoh1 open reading frame. (*B*) Cochlear cross-section diagram of showing Cdkn1b expression domain defining the prosensory domain and region of presumed BAC$^{Cdkn1b:Atoh1\ ieGFP}$ transgene expression. (*C-D*) E16.5 cochleae from BAC$^{Cdkn1b:Atoh1\ ieGFP}$ animals show additional MyoVI-positive hair cells throughout the prosensory region (*D*), whereas wildtype littermate controls have the normal four rows of hair cells (*D*). Some MyoVI-negative cells persist in mutant samples (*D, arrow*). BAC$^{Cdkn1b:Atoh1\ ieGFP}$ also show nuclear expression of PCNA within sensory region cells (*D'*), whereas PCNA is usually absent from this postmitotic region in controls (*C'*). This increased PCNA staining is present in the nuclei of both hair cells and supporting cells and shows lack of nucleolar staining (*D, inset*), consistent with early S or G1 phase of cell cycle (*D''*).

C. Atoh1-ER BAC Transgenic for Targeting Supporting Cells *In Vivo*

To further examine Atoh1's effect specifically on the supporting cell population at various stages of differentiation *in vivo*, I designed and generated an inducible Atoh1 transgenic mouse model with specific expression within these cells. Although Cdkn1b is expressed within the entire precursor domain early on, its expression is downregulated within differentiated hair cells and maintained specifically within supporting cells (Chen and Segil 1999). I modified the BAC$^{Cdkn1b:Atoh1\ ieGFP}$ construct to replace *Atoh1* with the coding sequence for the tamoxifen-inducible Atoh1-HA-ER fusion protein (Fig. 19*A*) that I previously tested *in vitro* (Fig. 6). In collaboration with Jian Zuo's lab at St. Jude Children's Research Hospital, Memphis, TN, five transgenic founder lines containing the modified BAC, BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$, transgene were generated and confirmed by PCR genotyping and Southern blot (B. Cox and J. Dearman, *unpublished data*).

To confirm that BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ mice expressed the fusion protein appropriately within Cdkn1b-expressing cells and to identify the founder line within highest expression, I first analyzed non-treated samples. Non-treated BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ mice appear to be normal and healthy, suggesting that Atoh1-HA-ER function was sufficiently repressed to avoid the detrimental effects of broad Atoh1 activity within the developing embryo seen in the BAC$^{Cdkn1b:Atoh1\ ieGFP}$ transgenic line. Since Cdkn1b is highly expressed within the early postnatal cerebellum (Miyazawa *et al.* 2000), I extracted P5-stage BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ cerebellar protein and immunoblotted for the Atoh1-HA-ER fusion protein. HA antibody detected a unique 76kDa band that corresponded to the expected Atoh1-HA-ER molecular weight within sample from BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ mice, but not littermate controls (Fig. 19*B*). Similar analysis of

cerebellar samples from the four other BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ founder lines showed little or no expression of the fusion protein (*data not shown*). Although I attempted to detect Atoh1-HA-ER expression in BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$), I was unable to confirm expression by Western (*data not shown*), likely because of the small tissue amount and relatively limited number of Cdkn1b-expressing cells within the inner ear.

In order to confirm expression of the Atoh1-HA-ER fusion protein within BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ cochleae, I analyzed tissue from offspring of the same founder line that had strong Atoh1-HA-ER expression in the cerebellum. Cdkn1b is strongly expressed in the early postnatal cochlear epithelium within sensory region in supporting cells and non-sensory cells immediately lateral to the organ of Corti (Fig. 19*C*). Its expression is maintained within these cells at least throughout the first week after birth (Fig. 19*D*). I immunostained inner ear tissue from P7-stage transgenic and wildtype littermates (Fig. 19*E,F*), and detected weak HA immunostaining within BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ supporting cells (Fig. 19*F*). However, significant background within wildtype littermate control supporting cells and surrounding structures makes these results ambiguous (Fig. 19*E*). No additional hair cells were observed in non-treated BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ cochlea (Fig. 19*F*), which further supports that if the fusion protein expressed, Atoh1 activity is repressed without tamoxifen treatment.

Analysis of BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ cochlea from animals treated with tamoxifen has not yet been completed. Following confirmation of Atoh1-HA-ER fusion protein expression and proper nuclear translocation in tamoxifen-induced samples, BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ mice will be transferred to Emory for further experiments.

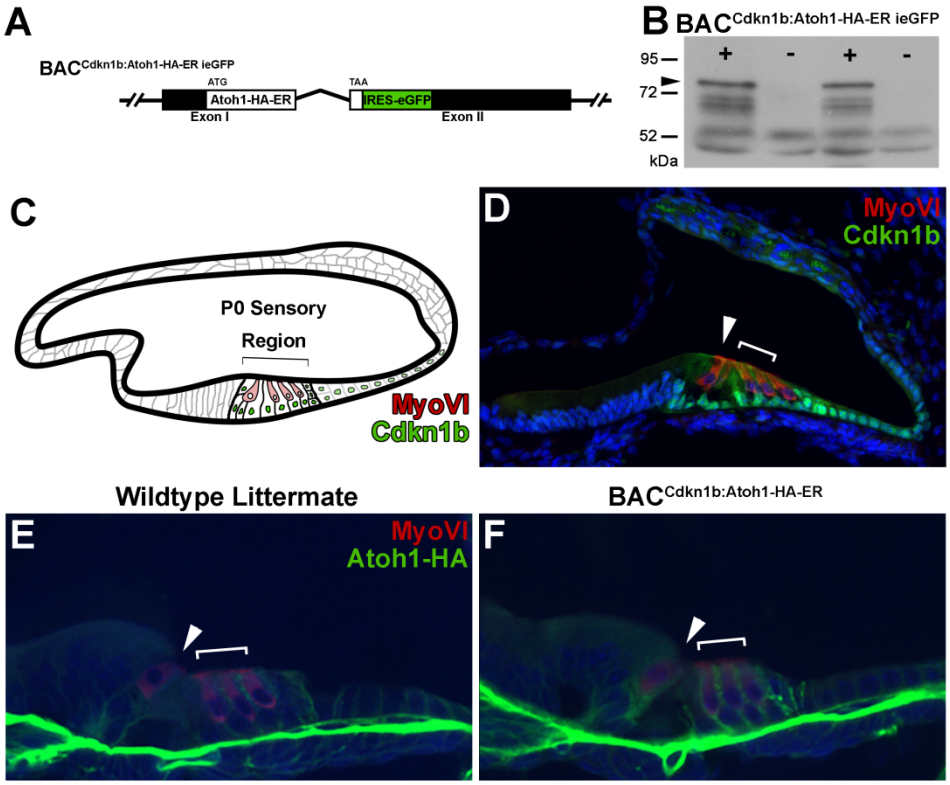

Fig. 19 - Cdkn1b BAC Can Drive Atoh1-HA-ER Expression *In Vivo*

(*A*) Construct schematic of the modified BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ construct containing the coding sequence for the Atoh1-HA-ER fusion protein. (*B*) Western blot of BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ cerebellar tissue from P1 transgenic mice and wildtype littermate controls shows that immunoblotting for the Atoh1-HA-ER detects a protein of the expected 76kDa size (*arrowhead*) in genotyped positive (+) samples, but not negative controls (-). (*C*) Diagram of P0 cochlear cross-section shows Cdkn1b expression domain within supporting cells of the organ of Corti and lateral non-sensory cells, where BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ transgene expression should be expected in postnatal mice. (*D*) Cdkn1b expression is maintained strongly within these cells at least until P5-stage, as shown. (*E-F*) P7-stage BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ may display low HA immunoreactivity within supporting cells (F), although background signal with a similar pattern within wildtype littermate control cochleae (*E*) is also observed.

4.4 Discussion

Using various transgenic mouse models, I have demonstrated that Atoh1 can have multiple effects when it is misexpressed within the developing and early postnatal organ of Corti. The previously-generated BAC$^{Cdkn1b:Atoh1\ ieGFP}$ samples illustrated that forced Atoh1 expression within the entire sensory precursor domain produced a large number of hair cells. Although postnatal differentiated supporting cells required extended Atoh1 induction, the GFAP^{Cre};rtTA(ieGFP);Tg^{Atoh1} transgenic cochlear explant model, which directed Atoh1 to differentiated supporting cells, showed conclusively that lineage-traced postnatal supporting cells could be converted to new hair cells. Our new BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ transgenic mouse line holds promise to more thoroughly test the competency of postnatal supporting cells *in vivo*.

Interestingly, despite forced expression of Atoh1 within presumably all sensory precursor cells and continued expression in remaining Cdkn1b-positive supporting cells BAC$^{Cdkn1b:Atoh1\ ieGFP}$, non-sensory cells appear to persist. Although the underlying mechanism is unknown, this phenomenon hints at important relationships between hair cells and supporting cells. It is possible that expression of the *Atoh1* transgene is lower or non-existent with the remaining non-sensory cells, as this was not tested directly, but similar results in ectopic sensory regions of Foxg1^{Cre};rtTA(ieGFP);Tg^{Atoh1} (Fig. 13) suggest it is not the result of weak Atoh1 expression.

The observed elevated PCNA staining within the normally post-mitotic sensory region of BAC$^{Cdkn1b:Atoh1\ ieGFP}$ (Fig. 18*D'*) suggests the cell cycle control is disrupted when Atoh1 is expressed in the entire population of sensory precursor cells. This too may represent a mechanism to repopulate a depleted supporting cell population, similar to

what I speculate is occurring within Foxg1^{Cre};rtTA(ieGFP);Tg^{Atoh1} cochlea following extended Atoh1 induction (Fig. 15). It remains to be tested whether elevated PCNA levels the hair cell and non-sensory cell nuclei represent truly mitotic cells within the sensory region and if this disruption of cell cycle control is due to an induction of proliferation or merely a failure to properly become quiescent. Again, our BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ transgenic mouse line, which allows temporal control of Atoh1 activity, may be able to answer some of these remaining questions.

Forced Atoh1 expression within the sensory region, either within the precursor domain of the embryonic cochlea (Fig. 18*D*) or within differentiated supporting cells of the cultured postnatal epithelium (Fig. 17*F*), resulted in an expansion of the organ of Corti. Although it remains unclear if elevated PCNA levels represent mitotic events that could generate additional cells within BAC$^{Cdkn1b:Atoh1\ ieGFP}$ sensory regions (Fig. 18*D'*), it is also possible that surrounding non-sensory cells are recruited to replace converted supporting cells. This appears to be the case in GFAP^{Cre};rtTA (ieGFP);Tg^{Atoh1} cultures following extended Atoh1 induction, where the normal eGFP expression domain marking supporting cells expands into the medial non-sensory region (Fig. 17*F*). This likely illustrates a non-cell autonomous effect of Atoh1 to induce supporting cell differentiation in cell adjacent to developing hair cells similar to what has been reported previously (Woods *et al.* 2004).

These results confirm conclusions drawn from our other Atoh1 models, and address an additional question related specifically to Atoh1's effect within the developing organ of Corti. Although I show that hair cells can be generated from this cell population up until the neonatal timepoint, further work is needed to address whether the more highly

differentiated mature supporting cells are also competent for Atoh1-mediated conversion. In addition to our BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ transgenic mouse line, other supporting cell specific Cre mouse lines may be used in combination with our Dox-inducible Tg^{Atoh1} transgenic mouse model to address remaining questions.

Chapter VI: General Discussion of Atoh1's Cochlear Effects

The transcription factor Atoh1 is known to be essential for the differentiation of hair cells within the inner ear and sufficient for the generation of ectopic hair cells within the cochlear epithelium under certain conditions. These studies further characterized the effects of Atoh1 expression within the cochlea by: (1) defining the relative competency various cells types to Atoh1-mediated hair cell generation, (2) determining the changes in competency of the cochlear epithelium to Atoh1-mediated hair cell generation over early postnatal maturation, (3) directly testing the competency of sensory precursor cells and differentiated supporting cells to conversion by forced Atoh1 expression, and (4) determining various effects that forced Atoh1 expression and additional hair cell generation has on surrounding non-sensory cell populations.

Atoh1-Mediated Generation of Ectopic Hair Cells in the Cochlea

Previous reports had shown that Atoh1 could generate new hair cells when delivered to certain populations of non-sensory cochlear epithelial cells. *In vitro* delivery of Atoh1 to cells within the embryonic and early postnatal Kölliker's organ resulted in the expression of hair cell markers and the formation of rudimentary stereocilia bundles in those cells (Zheng and Gao 2000; Woods *et al.* 2004; Qian *et al.* 2006). Experiments with the *in vivo* delivery of Atoh1 into isolated cells using electroporation or viral transfection of the embryonic or more mature cochlea showed even more promising results with the formation of mature and functional hair cells (Kawamoto *et al.* 2003; Izumikawa *et al.* 2005; Gubbels *et al.* 2008). Furthermore, Atoh1-generated hair cells appeared to be able

to attract neurite outgrowth from spiral ganglions (Kawamoto *et al.* 2003; Qian *et al.* 2006; Gubbels *et al.* 2008). These studies suggested an incredible ability of Atoh1 to generate a mature hair cell phenotype when expressed outside its normal developmental context and had important implication for possible hair cell regeneration strategies. However, a low efficiency of transfection and a lack of cell type-specific targeting control somewhat limited the conclusions that could be drawn.

I confirmed the potency of Atoh1 as a hair cell differentiation within the embryonic and early postnatal cochlear epithelium and showed that different cell types have a relatively higher competency to Atoh1-mediated hair cell generation than others. *In vitro* methods of Atoh1 delivery, including the electroporation and viral transfections of postnatal cochlear explants, proved to be difficult and made it clear that a more stable transgenic model of Atoh1 delivery was necessary, though electroporation of Atoh1 could generate ectopic MyoVI-positive cells (Fig. 6*F*). Work using a novel inducible Atoh1 transgenic mouse line, crossed to the $Foxg1^{Cre}$ line to direct its expression to the entire cochlear epithelium (Fig. 8*E*), proved to be a fruitful and informative model. Induction of Atoh1 transgene expression within the entire neonatal cochlear epithelium for a relatively short time lead to the formation of ectopic MyoVI-positive hair cells within Kölliker's organ and the spiral prominence (Fig. 9). Extended induction of Atoh1 expression lead to a slight regional expansion of the cochlear epithelium that was competent for Atoh1-mediated hair cell generation, with additional cells appearing on Reissner's membrane, an epithelium composed of almost all hair cells in the most apical turn, and a conversion of supporting cells (Fig. 14, Fig. 17*G*). Closer examination of ectopic hair cells generated within Kölliker's organ under short induction of Atoh1

induction revealed that they displayed stereocilia bundles and attracted innervation similar to endogenous organ of Corti hair cells (Fig. 9). Furthermore, electrophysiological recordings of the passive membrane currents of ectopic hair cells responding to depolarizing and hyperpolarizing voltage steps showed that their biophysical properties were hair cell-like, but suggested that they were still immature (Fig. 10) (Marcotti *et al.* 2003). Unfortunately, an unexpected lethality associated with Dox treatment in $Foxg1^{Cre}$;rtTA;Tg^{Atoh1} mice did not allow for the analysis of long-term induction of Atoh1 expression or a testing of the functional status of ectopic hair cells left to mature for a longer duration. However, these results showed that, even within a limited timeframe of induction and differentiation, Atoh1 could generate new hair cells with relatively mature morphological and biophysical properties, particularly from within Kölliker's organ region.

The fact that Atoh1 could generate new hair cells with mature phenotypes and recruit innervation was not surprising based on previous reports of Atoh1's effect within the cochlea (Kawamoto *et al.* 2003; Woods *et al.* 2004; Izumikawa *et al.* 2005; Qian *et al.* 2006; Gubbels *et al.* 2008). These results show that Atoh1 is capable of initiating a hair cell differentiation program within non-sensory cells similar to that of endogenous hair cells, including the generation of the specialized stereocilia bundles, the expression of basolateral ion channels, and secretion of appropriate neuroattractants (Fig. 20*A*). Furthermore, the apparently high relative competency of the cells within Kölliker's organ was consistent with the reported success of Atoh1-mediated hair cell generation within this region using *in vitro* transfection methods (Zheng and Gao 2000; Woods *et al.* 2004; Qian *et al.* 2006). Kölliker's organ has been speculated to be an ancestral sensory region

that was once populated with hair cells and supporting cells, but which was restricted to the lateral edge and current location in the evolution of an auditory sensory organ capable of increased sensitivity to high frequency sound (Fritzsch *et al.* 2002). In this case, Kölliker's organ may represent a latent sensory precursor domain where hair cell differentiation is normally repressed, but that Atoh1 expression can reactivate. This theory is supported by disruptions of Notch or Hedgehog signaling, which can cause the generation of ectopic hair cells to spontaneously arise within this region (Zine *et al.* 2001; Driver *et al.* 2008).

The ability of Atoh1 to initiate multiple aspects of sensory hair cell development within certain cochlear epithelial cell types at early postnatal timepoints suggest that the cellular context is appropriate for Atoh1's effects as a hair cell differentiation factor. This in itself is not a trivial point, as Atoh1 is known to have a diverse range of effects throughout the mammalian body besides its role as a hair cell differentiation factor within the inner ear (Ben-Arie *et al.* 1997; Helms and Johnson 1998; Bermingham *et al.* 1999; Bermingham *et al.* 2001; Yang *et al.* 2001; Machold and Fishell 2005; Maricich *et al.* 2009). Atoh1's effects likely depend on its ability to act as a transcription factor and initiate expression of downstream target genes, which have been shown to differ between organ systems (Klisch *et al.* 2011). Some of Atoh1's tissue-specificity may be determined by the presence of cofactors that regulate its prerequisite dimerization with E-proteins, such as E12 and E47, and the binding of these heterodimers to specific sets of gene enhancer elements called E-box regions (Cabrera and Alonso 1991; Jarman *et al.* 1994; Bertrand *et al.* 2002). Expression of appropriate cofactors and an ability to regulate

downstream hair cell differentiation targets may also define the relative competency of cochlear epithelial cell types to Atoh1-mediated conversion (Fig. 20*B*).

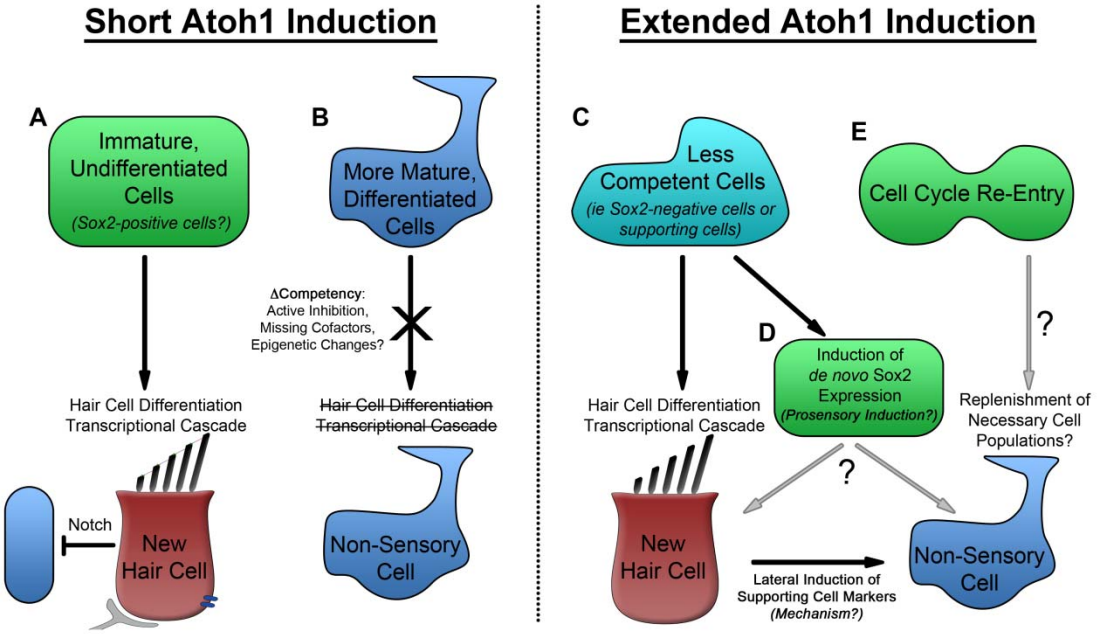

Fig. 20 - Summary Model of Atoh1's Effects in the Mammalian Cochlea

(*A*-*B*) Short-term induction of Atoh1 expression results in the initiation of hair cell differentiation within immature (*A*), but not relatively more mature cochlear epithelial cell types (*B*). Atoh1-directed differentiation cascades lead to cells with hair cell-like morphological, biochemical and biophysical properties (*A*). Within competent cell populations, Notch signaling inhibits Atoh1's effects in some cells, leading to the generation of a cellular mosaic of hair cells and non-sensory cells (*A*). It remains unknown what underlies the change in competency that prevents Atoh1-mediated hair cell differentiation in certain populations of cells following short induction (*B*). (*C*) Extended Atoh1 induction promotes the conversion of less competent cell populations, including differentiated supporting cells. Conversion of existing supporting cells leads to recruitment of adjacent cells to supporting cell phenotypes (*D*-*E*) In addition, extended Atoh1 delivery causes the novel induction of the prosensory marker Sox2 (*D*) and the promotion of cell cycle re-entry (*E*), both of which may work to maintain cellular mosaic patterning through the generation of non-sensory cells to complement new hair cells.

Age-Dependent Changes in Competency

While new hair cells could be readily generated from specific regions within the neonatal cochlea following forced Atoh1 expression, the competency of more differentiated cells of the mature cochlea could not be assumed. The postnatal cochlea undergoes significant morphological and molecular changes in the time period leading up to the onset of hearing (Hinojosa 1977; Reisinger *et al.* 2010), making the cellular context in which Atoh1 would be expressed dynamic over this time period. Despite this, the principles of cellular differentiation have been redefined by the demonstration of efficient generation of induced pluripotent stem cells from mature cells, making the reprogramming of many somatic cell types with forced gene expression now seem plausible (Chambers and Studer 2011). Previous work showed that Atoh1 could generate new hair cells within the adult guinea pig cochlea (Kawamoto *et al.* 2003; Izumikawa *et al.* 2005), but the extent to which this occurred was not clear. Furthermore, a direct comparison of the competency different cochlear cell types at various developmental timepoints had not been critically evaluated.

I tested the effect of Atoh1 delivery to all cochlear epithelial cells at three different developmental timepoints and found drastic decreases in the competency of non-sensory cell types to Atoh1-mediated hair cell generation (Fig. 11). Using the inducible $Foxg1^{Cre}$;rtTA;Tg^{Atoh1} model, Atoh1 delivery at the P8-stage generated hair cells within a small region just medial to the inner hair cell row (Fig. 11*D*,*H*; Fig. 12*D*-*F*) compared to much larger region along the full length of the cochlea when Atoh1 was delivered at the P0-stage (Fig. 11*B*; Fig. 14). Furthermore, no additional hair cells were observed in the cochlea of $Foxg1^{Cre}$;rtTA;Tg^{Atoh1} mice with Atoh1 expression induced at the P14-stage, even with an extended six-day delivery protocol (Fig. 11*F*). Although the lethality

associated with induction prevented an evaluation of the potential effects of longer term Atoh1 delivery, these results defined important changes in the competency of non-sensory cells in the maturing cochlea.

While the results of these studies confirm Atoh1's potency as a hair cell differentiation factor and show that Atoh1 can generate hair cells in even a fairly mature cochlea, there are clearly limitations restricting the competency of various cochlear cells to hair cell generation (Fig. 20*B*). These results highlight the differences between generating hair cells in the immature cochlea, which is populated by relatively undifferentiated cells, and the generation of hair cells within the mature cochlea, which has more highly differentiated cells. It remains unclear what defines the temporal and spatial domains of competency of the cochlear epithelium to Atoh1-mediated hair cell generation and future work will hopefully identify the underlying differential response to Atoh1 in these various contexts. Although previous work suggests that new hair cells can be generated in the adult mammalian cochlea following delivery of Atoh1 (Kawamoto *et al.* 2003; Izumikawa *et al.* 2005), we were unable to observe the generation of new hair cells within our models at the P14-stage even following extended Atoh1 induction. There could be multiple explanations for these differences, a few of which will be discussed below, but the most likely is that mature non-sensory cells require extended Atoh1 expression to be converted. Since our inducible Tg^{Atoh1} model can be used in combination with other Cre mouse lines, in the future, we hope to test longer-term induction using a system that avoids the likely toxic effects of Atoh1 overexpression in other organ systems.

The relative decrease in competency of particular cochlear cell types during their postnatal maturation may be reflecting changes in the expression patterns of positive or negative co-factors and the ability of Atoh1 to regulate its downstream transcriptional targets (Fig. 20*B*). In many ways, the progressive changes in competency for individual cell types over time may be due to similar cell biological mechanisms that underlie differences between high and low competency cochlear cell types at a single developmental stage. It is no surprise that there are significant molecular changes that occur within the postnatal cochlea during its maturation (Reisinger *et al.* 2010), and the loss of necessary co-factors or the expression of active inhibitors may have the effect of decreasing Atoh1's potency. In addition, the epigenetic profile of individual cells may change as they move from multi-potent to differentiation cells (Meissner *et al.* 2008), potentially decreasing Atoh1's ability to bind to its target enhancer elements or blocking transcription of downstream genes (Fig. 20*B*).

Sox2 Expression and Competency

Sox2 plays an essential role in the development of inner ear sensory domains and its relationship to pluripotency makes its expression within the postnatal mammalian cochlea intriguing. Sox2 expression marks the prosensory domains from which the auditory and vestibular sensory regions develop and its genetic disruption can lead to a complete absence of auditory hair cells within the cochlea (Kiernan *et al.* 2005b). Sox2 is known to be an important regulator of pluripotency (Li *et al.* 1998; Avilion *et al.* 2003), and is commonly used as part of the gene set delivered for the generation of induced pluripotent stems cells (Chambers and Studer 2011). Low levels of Sox2 expression within

Kölliker's organ have been noted previously (Dabdoub *et al.* 2008) and the ability to generate ectopic hair cells within this has been reported (Zheng and Gao 2000; Woods *et al.* 2004; Qian *et al.* 2006), but the connection between the two has not be demonstrated clearly. A better understanding of Sox2's role in the competency of cochlear epithelial cells to Atoh1-mediated hair cell generation could possibly explain the regional and temporal changes observed and identify an important upstream factor for hair cell differentiation.

The competency of non-sensory cells to Atoh1-mediated hair cell generation appeared to be strongly correlated with the expression of Sox2. Robust hair cell generation was observed following relatively short expression of Atoh1 within the neonatal Kolliker's organ, which expresses Sox2 (Fig. 9). Similar to what has been reported during the differentiation of endogenous hair cells (Kiernan *et al.* 2005b; Dabdoub *et al.* 2008), Tg^{Atoh1}-generated ectopic hair cells appeared to down-regulate Sox2 (Fig. 9*B, inset*). Furthermore, the competency of this medial non-sensory domain decreased during postnatal maturation in a basal to apical gradient, matching the regression of Kolliker's organ and the decrease in Sox2-positive cells within this region (Fig. 12). Although only correlative, the temporal and spatial pattern the strong association of hair cell generation with Sox2 expression within Kolliker's organ suggested that that prior Sox2 expression might be an important factor in defining the relative competency of non-sensory cells to Atoh1-mediated hair cell differentiation.

These results suggest an interesting relationship between Sox2 expression and Atoh1-mediated hair cell generation (Fig. 20*A*). The postnatal Kölliker's organ shows similarities with the embryonic Sox2-expressing prosensory region, further supporting

the theory that this region represents an ancestral sensory region and a latent source of sensory cells. However, this transient population of cells regresses before the onset of hearing and could not provide a source of new hair cell in the mature mammalian cochlea. Furthermore, while it appears that Atoh1 can relatively easily initiate a hair cell differentiated cascade in non-sensory cochlear epithelial cells that express Sox2, previous work also showed a potentially inhibitory relationship between Sox2 and Atoh1-mediated hair cell generation, when both were delivered ectopically and continuously expressed (Dabdoub *et al.* 2008). The exact connection between competency for Atoh1-mediated hair cell generation and Sox2 expression remains to be directly tested.

Based on the important role that Sox2 plays in maintaining and its cooperative ability to induce pluripotency (Li *et al.* 1998; Avilion *et al.* 2003; Chambers and Studer 2011), it seems likely that Sox2 expression maintains certain cochlear epithelial cell populations in a multi-potent state with high competency to Atoh1-mediated hair cell differentiation. However, continued forced expression of Sox2 may also prohibit the differentiation promoting effects of co-expression factors, such as Atoh1 (Dabdoub *et al.* 2008). Though the exact mechanism by which Sox2 does this is unclear, examination of Sox2's downstream targets include the activation of other pluripotency genes (Masui *et al.* 2007). Sox2 or some of its downstream targets may work to create a favorable molecular environment for Atoh1-mediated hair cell differentiation in some conditions, but may also inhibit cellular differentiation in others. Although the prior expression of Sox2 might increase Atoh1's potency, newly differentiating hair cells will likely also need to down-regulate pluripotency genes, including Sox2.

Atoh1-Mediated Conversion of Sensory Precursors and Supporting Cells

The development of the organ of Corti includes the differentiation of hair cells and supporting cells from a population of sensory precursors. These two cell types are thought to arise from a shared progenitor lineage and multiple signaling pathways control the cell fate decisions to specify the correct ratio and patterning of hair cells and supporting cells (Fig. 4) (Kelley 2006; Kelly and Chen 2009). During embryonic and neonatal development, many supporting cells are actively inhibited from taking on the hair cell fate, but disruption of the pathways involved reveals a degree of plasticity and these cells can spontaneously convert to hair cells (Kelley *et al.* 1995; White *et al.* 2006; Doetzlhofer *et al.* 2009). Furthermore, a previous report suggested that new hair cells could be generated in the hair cell ablated mammalian cochlea following viral transfection of Atoh1 (Izumikawa *et al.* 2005). Since supporting cells share this unique heritage with hair cells and are the only remaining cochlear epithelial cells within the sensory region following the loss of hair cells, it was important to examine the effect of forced Atoh1 expression within the sensory region of the embryonic and postnatal cochlea.

Specific targeting of Atoh1 to the embryonic sensory precursor domain and later, the postnatal supporting cell population showed that these cell types could be converted to hair cells under certain conditions. Analysis of samples from the $BAC^{Cdkn1b:Atoh1\ ieGFP}$, which directed Atoh1 expression within the entire precursor domain and maintained it within differentiating supporting cells showed that many of the cells within this domain could be directed to the hair cell fate (Fig. 18). In neonatal $GFAP^{Cre}$;rtTA(ieGFP);Tg^{Atoh1} cochlear explants, more differentiated supporting cells could also be induced to express

the hair cell marker MyoVI and form stereocilia bundles, but this conversion required extended Atoh1 expression (Fig. 17). Though an examination of Atoh1 effects within supporting cells *in vivo* at various developmental timepoints may be helpful in determining the changes in competency more clearly, these studies give some initial insight.

These results indicate that forced Atoh1 expression can generate additional hair cells from within the sensory domain even in the postnatal organ of Corti and with existing hair cells present (Fig. 20*C*). This supports the belief that new hair cells could be generated from supporting cells in the mature mammalian cochlea with forced Atoh1 expression, possibly using viral delivery methods (Izumikawa *et al.* 2005). However, the limited conversion of supporting cells seen in $Foxg1^{Cre}$;rtTA;Tg^{Atoh1} and the extended induction required in $GFAP^{Cre}$;rtTA(ieGFP);Tg^{Atoh1} cochlear explants suggests that the competency of these cells to Atoh1-mediated hair cell differentiation is relatively low in the postnatal cochlea. It is unclear what inhibits postnatal supporting cells from Atoh1-mediated conversion, but it is likely a combination of intrinsic factors and cues coming from existing hair cells are involved. Loss of existing hair cells does not cause the spontaneous conversion of postnatal supporting cells (Forge *et al.* 1998), as it does in the embryonic organ of Corti (Kelley *et al.* 1995). Interestingly, postnatal supporting cells continue to express the prosensory marker Sox2, which is required for hair cell generation during normal development (Kiernan *et al.* 2005b) and appears to identify Kölliker's organ cells as highly competent latent sensory precursors. However, Sox2 expression in this context may also represent the previously reported inhibitory effects on hair cell differentiation (Dabdoub *et al.* 2008). Ongoing work aims to test whether the

ablation of existing hair cells increases the competency of supporting cells to Atoh1-mediated conversion. It also remains to be seen whether more adult stage supporting cells can be converted to new hair cells and if they can take on a mature hair cell phenotype. Our new BAC$^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$, which expresses an inducible version of Atoh1 within Cdkn1b-positive cells *in vivo* (Fig. 19), may be able to answer some of these remaining questions.

Atoh1 Induction of New Prosensory Regions

Hair cells normally arise from within a predefined post-mitotic prosensory region. The intersecting expression of Cdkn1b, Jagged1 and Sox2 clearly defines this domain within the floor of the developing embryonic cochlea (Fig. 4). Atoh1 expression, and hair cell differentiation, is generally thought to be downstream of the specification of this prosensory domain (Chen *et al.* 2002). Since bHLH transcription factors, including Atoh1, have diverse functions depending on cellular context (Dambly-Chaudiere and Vervoort 1998; Guillemot 1999; Bertrand *et al.* 2002; Powell and Jarman 2008; Klisch *et al.* 2011), it was important to determine the effects of forced Atoh1 expression within other non-sensory cochlear epithelial cells.

The observation of MyoVI-positive cells outside the normal sensory domain following Atoh1 expression suggested that additional non-sensory regions could take on at least initial stages of sensory hair cell differentiation. Although Atoh1 often had its most robust effects within Sox2-positive Kölliker's organ domain, which could be considered an extension of the normal sensory region, MyoVI-positive cells could also be observed on the lateral wall and roof of the cochlea (Fig. 9; Fig. 14). A closer examination revealed

that in regions where hair cells were generated, Atoh1 appeared capable of inducing *de novo* Sox2 expression within the spiral prominence region following relatively short Atoh1 expression (Fig. 9*B*; Fig. 14*B*) and within the entire cochlear epithelium in more apical regions following extended Atoh1 expression (Fig. 14*C, inset*). These results showed that ectopic delivery of Atoh1 could promote the expression of Sox2.

This finding may allude to a possible additional role for Atoh1 in induction of new prosensory regions in the postnatal mammalian cochlea (Fig. 20*D*). It is unclear if this is represents a general role of Atoh1 within the cochlear epithelium, or an artifact of its ectopic expression. Although Atoh1 protein expression has only been detected in the subset of sensory precursor cells and normal sensory domain markers remain in the Atoh1 null mouse (Chen *et al.* 2002; Dabdoub *et al.* 2008), Atoh1 promoter activity and transcript has been reported in broader domain earlier in cochlear development (Bermingham *et al.* 1999; Woods *et al.* 2004). While Atoh1 does not appear to be required for sensory domain formation in the mammalian cochlea, it seems possible that it could function to promote prosensory domains in some circumstances. In fact, in other organisms, homologues of Atoh1 can have a dual role in the initial induction of a proneural field and the later differentiation of an individual cell type (Jarman *et al.* 1994; Hsiung and Moses 2002; Millimaki *et al.* 2007). Atoh1 delivery to the entire cochlear epithelium at embryonic stages and a further analysis of the maturity of MyoVI-positive cells within the lateral wall and roof may provide more information as to the potency of Atoh1 prosensory-inducing effects.

Atoh1 and Sensory Region Cellular Patterning

Auditory and vestibular sensory regions are composed of a patterned mosaic of hair cells and supporting cells (Fig. 2). The ratio of these two cell types and the overall patterning of the sensory organ are controlled by the coordination of inductive and inhibitory signaling pathways during development (Fig. 4). Previous work has shown that within clusters of non-sensory cells transfected with Atoh1, Notch signaling can suppress the hair cell differentiation effects of Atoh1 in adjacent transfected cells (Woods *et al.* 2004). Furthermore, these studies also showed that Atoh1-mediated hair cell differentiation led to the induction of supporting cell markers in surrounding cells (Woods *et al.* 2004). The ability of Atoh1-generated hair cells to pattern surrounding cells would suggest a powerful role for Atoh1 in normal development and a promising additional role for possible hair cell regeneration strategies. Our *in vivo* Atoh1 delivery models provided a unique opportunity to test the effect of forced Atoh1 expression within a large number of adjacent cochlear epithelial cells to determine the ability of ectopic sensory patches to self-pattern.

Within regions that were competent for hair cell generation, interesting non-cell autonomous effects were observed. In electroporated cultures where multiple adjacent cells were transfected with the Atoh1 expression construct, differing levels of MyoVI expression suggested that differentiating hair cells likely inhibited their neighbors from sharing the hair cell fate (Fig. 6*F*). Similarly, in the inducible Atoh1 transgenic model, in regions where new hair cells were generated, non-sensory cells were interspersed between the new hair cells (Fig. 9; Fig. 11). This appeared to be mediated through a Notch-dependent mechanism, as the persistence of non-sensory cells was abolished with

the treatment a pharmacological inhibitor of Notch signaling (Fig. 13). Results of Notch inhibition also confirmed that the mosaic hair cell differentiation was not due to mosaic expression of Tg^{Atoh1} and supported conclusions drawn for *in vitro* electroporations. Additionally, the expansion of the GFP expression domain within $GFAP^{Cre}$;rtTA(ieGFP);Tg^{Atoh1} cultures suggested that the new $GFAP^{Cre}$ supporting cells were being induced adjacent to the normal sensory domain (Fig. 17). This could be the result of inductive cues coming from hair cells and/or representing a mechanism to replace the converted supporting cell population. Evidence for the resilience of the supporting cell population is also given by the remaining MyoVI-negative cells within the supporting cell layer of the $BAC^{Cdkn1b:Atoh1\ ieGFP}$ organ of Corti (Fig. 18). Here, the Cdkn1b-driven *Atoh1* transgene should continue expression within supporting cells that were not previously converted, but non-sensory cells remained despite the production of supernumerary hair cells. Overall, these findings likely represent a robust mechanism for maintaining a cellular mosaic of hair cells and supporting cells that exists within the cochlea following Atoh1 expression, both within the normal sensory domain and in non-sensory regions.

The observed non-cell autonomous effects of Atoh1 within the cochlear epithelium suggests that Atoh1 initiates a complex differentiation cascade that includes the expression of inductive and inhibitory factors that control cell fate decisions in surrounding cells (Fig. 20). While Notch plays an important role during the cell fate decisions of normal (Lanford *et al.* 1999; Zine and de Ribaupierre 2002; Kiernan *et al.* 2005a; Hartman *et al.* 2007) and ectopic (Fig. 13) (Woods *et al.* 2004) sensory regions, and FGF8 expression from inner hair cell induces the production of at least one type of

supporting cell (Colvin *et al.* 1996; Mueller *et al.* 2002; Hayashi *et al.* 2007; Jacques *et al.* 2007; Puligilla *et al.* 2007; Doetzlhofer *et al.* 2009), other signaling pathways may also be involved. An identification of the hair cell-specific targets of Atoh1, similar to what is being accomplished in other organ systems where Atoh1 also plays an important role (Klisch *et al.* 2011; Lai *et al.* 2011) may reveal downstream genes and additional pathways involved in mediating these effects.

Disruptions of Cell Cycle Control by Atoh1 Induction

Normally, cell cycle is strictly controlled within the postnatal mammalian cochlea. In the mouse inner ear, the entire cochlear epithelium becomes post-mitotic by birth (Ruben 1967). Furthermore, the sensory precursor domain exits cell cycle before the onset of differentiation (Fig. 4) (Ruben 1967; Chen and Segil 1999). Whereas the sensory regions of non-mammalian vertebrates often re-enter cell cycle in response to damage in order to generate replacement cells (Stone and Cotanche 2007; Brignull *et al.* 2009) and limited postnatal proliferation has been observed in vestibular sensory regions (Warchol 2011), the organ of Corti is believed to remain quiescent throughout life (Chen *et al.* 2003). It has been speculated that the tight control of cell cycle in the developing and postnatal organ of Corti is necessary for specifying the correct number of cells for maintaining the highly-ordered arrangement of cells important for hearing sensitivity (Lee *et al.* 2006). However, a lack of proliferative response within the organ of Corti following the loss of hair cells presents a theoretical problem to potential Atoh1-mediated hair cell regeneration strategies: direct transdifferentiation of supporting cells would deplete the necessary supporting cell population. It was therefore important to understand the effect

on the cochlea, particularly within the sensory region, when large numbers of non-sensory cells were being forced to convert to hair cells by Atoh1-induction.

Using these novel transgenic models of Atoh1 delivery, surprising effects of extended Atoh1 induction on the cell cycle control of the normally postmitotic sensory region and other regions of the cochlea were demonstrated. BAC$^{Cdkn1b:Atoh1\ ieGFP}$ sensory regions, where Atoh1 expression was presumably initiated at the time of cell cycle exit in all precursor cells, appeared to have elevated levels of PCNA staining and additional number of cells within the sensory region at E16.5 (Fig. 18). It is possible that within this transgenic model the observed up-regulation of PCNA could be due to a failure to exit cell cycle rather than re-entry. Additionally, PCNA immunoreactivity may not always represent actively cycling cells (Harrison *et al.* 1993) and confirmation by BrdU incorporation was not possible due to analysis being limited to previously prepared tissue sections. However, similar cell cycle disruption effects were observed within Foxg1^{Cre};rtTA(ieGFP);Tg^{Atoh1} cochlear explant cultures induced for extended periods. In these Atoh1-induced cultures a large number of supporting cells incorporated Brdu (Fig. 15*H*) and these Sox2-positive supporting cells were shown to complete relatively late stages of cell division (Fig. 16). Though the proliferation in and around the sensory region was not to the same extent in Foxg1^{Cre};rtTA(ieGFP);Tg^{Atoh1} animals *in vivo*, BrdU-positive cells were observed within the normally post-mitotic cochlear epithelium (Fig. 15*D*). Within the cochleae of these animals, significant changes in the expression of the cyclin dependent kinase inhibitor, Ckn1b, were observed to roughly correlate with the cell types that incorporated BrdU (Fig. 15). It remains to be determined how Atoh1's effects on cell cycle are mediated and what underlies the varying degrees in response *in*

vitro and *in vivo*, but Atoh1 clearly has profound effects on cell cycle control in the mammalian cochlea (Fig. 20*E*).

These results show a previously unidentified effect of Atoh1 expression within the mammalian cochlea. Atoh1 has been typically considered a terminal differentiation factor within the inner ear. While Atoh1 generally promotes the differentiation of a specific cell type in other tissues where it is expressed (Klisch *et al.* 2011), it has also been associated with the formation of medulloblastomas (Zhao *et al.* 2008; Flora *et al.* 2009; Ayrault *et al.* 2010). This suggests that Atoh1 expression may promote proliferation in some contexts. Within our transgenic models, Atoh1 was expressed for an extended period within a large population of cells that were in direct contact with each other. In these environments multiple adjacent cells are likely being forced towards hair cell differentiation and differentiating hair cells are attempting to inhibit their neighbors from sharing their cell fate. Differentiating hair cell may also be inducing these same surrounding cells to take on supporting cell phenotypes. Additionally, the pool of non-sensory cells is likely being depleted. All of these abnormal situations may be leading to conflicting signals within cochlear epithelial cells and contributing to the changes in cell cycle control. Further work is needed to determine the mechanism by which Atoh1 causes these surprising effects.

Overall Conclusions

Collectively, results of these studies advance our understanding of Atoh1's effects within the cochlea by further defining its potential and limitations as a hair cell differentiation factor and by demonstrating a diversity of other consequences of forced

Atoh1 expression. Atoh1 can generate hair cells within relatively mature phenotypes within the postnatal mammalian cochlea, but the relative competency of the cochlear epithelium differs across cell types and developmental stages. The Sox2-positive Kölliker's organ region appears to represent a latent prosensory domain that can be efficiently induced to differentiate hair cells following Atoh1-induction, but this cell population completely regresses postnatally and is non-existent in the mature organ of Corti. Interestingly, Atoh1 expression appears to induce the expression of Sox2 *de novo* and generate MyoVI-positive cells in non-sensory regions, suggesting that Atoh1 is capable of promoting the formation of novel sensory domains. While less competent cell types, including differentiated supporting cells, can be converted following extended Atoh1 expression in the neonatal cochlea, the competency of all cell types appears to drop precipitously during postnatal maturation leading up to the onset of hearing. Several mechanisms appear to work to maintain the supporting cell population and the sensory mosaic within the endogenous sensory region and ectopic sensory patches despite pervasive forced Atoh1 expression. This includes the inhibition of hair cell differentiation via Notch lateral inhibition and inductive recruitment of new supporting cells in adjacent cells. Additionally, extended widespread forced expression of Atoh1 caused significant changes to the control of cell cycle within the organ of Corti and surrounding non-sensory regions. These results show that Atoh1 has powerful effects in the development of cochlear sensory regions by controlling cell fate decisions and cell cycle control. Furthermore, these results imply that if Atoh1 can successfully generate of hair cells within the deafened adult cochlea, that necessary cellular relationships will likely also be maintained.

Clinical Relevance of This Work

Considerable interest has surrounded Atoh1 as a potential therapeutic tool to promote hair cell regeneration in humans to treat hearing loss since its essential role in hair cell development and its ability to generate ectopic hair cells was demonstrated over ten years ago (Bermingham *et al.* 1999; Zheng and Gao 2000). Although the work presented within this dissertation is limited to testing Atoh1's effect within undamaged mammalian cochlea at timepoints before the age of hearing onset, results from these studies have clear implications for the evaluation of Atoh1 as a clinical gene therapy.

The generation of new hair cells with morphological, molecular, and biophysical properties similar to endogenous hair cells following Atoh1 induction in neonatal and P8-stage cochlea supports the idea that Atoh1 is potent hair cell differentiation factor capable of directing hair cell differentiation outside of its normal developmental context. This includes the conversion of postnatal supporting cells, which represent the only cell types remaining in the hair cell ablated organ of Corti and most likely target of an Atoh1-based therapy, into hair cells following extended Atoh1 delivery. In addition, within regions where new hair cells are generated, several mechanisms work to maintain the important cellular mosaic patterning of sensory hair cells and non-sensory cells. Atoh1-generated hair cells also appear to be innervated by spiral ganglion neurons, suggesting that once formed, new hair cells may be wired to the central nervous system. These promising results show that there is a great degree of potential in Atoh1-mediated hair cell regeneration strategies to promote functional recovery.

However, the drastic decrease in the relative competency of cochlear epithelial cell types over the period of postnatal maturation illustrates that the mature postnatal mammalian cochlea is not the same as an embryonic or neonatal cochlea and that an Atoh1-based therapy may be less effective in the adult mammalian cochlea. The robust hair cell generation previously reported by other groups is largely at embryonic or neonatal timepoints within the medial non-sensory region (Zheng and Gao 2000; Woods *et al.* 2004; Gubbels *et al.* 2008), when we show that the Sox2-positive Kölliker's organ cells have a relatively high competency to Atoh1-mediated hair cell generation. Unfortunately, this highly competent domain regresses before the onset of hearing and I was unable to demonstrate Atoh1-induced hair cell generation within P14-stage mouse cochlea, a developmental stage that would correlate with prenatal human. Despite the lack of Kölliker's organ cells in the mature cochlea and overall decrease in competency, the ability to convert less competent cell types following extended Atoh1 delivery suggests that longer term forced Atoh1 expression may be able to promote hair cell generation in the mature cochlea.

When attempting to generate new hair cells in the highly organized mature cochlea, the consequence disrupting cellular patterning and the conversion of important non-sensory cell types must also be considered. Following extended induction of Atoh1, the organ of Corti patterning becomes severely disrupted, likely due to the phenotypic conversion of supporting cells into immature hair cells and/or mitotic events without sensory region cells. In addition, newly generated hair cells displayed stereocilia bundles that were of mostly randomized, rather than coordinately polarized, orientations. Since the stereotyped patterned array of cells, including the coordinated polarity of hair cell

stereocilia bundles is important for hearing sensitivity (Shotwell *et al.* 1981; Hudspeth 2000), one can presume that Atoh1-mediated hair cell generation within an undamaged cochlea would actually be detrimental to auditory function. However, within a severely damaged cochlea where only supporting cells remain, the results of the studies presented here suggest that if new hair cells could be generated, several mechanisms may work to generate and maintain a rudimentary sensory mosaic. Although the architecture of these regenerated sensory regions may be less organized than the original organ of Corti, it may be sufficient to improve auditory function (Brigande and Heller 2009; Kwan *et al.* 2009).

The evaluation of Atoh1 as potential therapeutic tool must also consider its possible unintended negative effects. The lethality observed within our Tg^{Atoh1} model, in combination with the $Foxg1^{Cre}$ line, demonstrates the devastating effects of forced Atoh1 expression within other organ systems. Since many gene transfer methods, including viral delivery, may be difficult to target exclusively to the cochlear epithelium, these results suggest that safeguards such as cell-specific tropism and selective promoter design may be necessary. In addition, although the induction of proliferation may represent a mechanism to replace depleted non-sensory cells types, if left unchecked, may also lead to tissue hyperplasia and tumor formation.

Altogether this work provides a more realistic perspective on the potential therapeutic use of Atoh1 for hair cell regeneration to treat hearing impairment. Under the correct conditions, Atoh1 may be able to direct the generation of new functional auditory sensory organs, but its efficacy may decrease with age. As with any treatment, the potential positive effects of Atoh1-mediated hair cell generation must also be weighed against

possible negative side effects. Future studies will hopefully continue to explore the potential and limitations of Atoh1-based regeneration strategies, and the stable and flexible models generated here may serve as useful experimental platforms for this work.

Remaining Questions & Potential Future Directions

While these studies addressed many important aspects of Atoh1's effects within the mammalian cochlea, several important questions are left unanswered. Furthermore, some of the unexpected effects observed following Atoh1 overexpression within our transgenic models lead to additional questions. I will discuss some of these remaining questions, highlight their importance and propose potential future studies.

One very significant and important question that remains is whether longer-term expression of Atoh1 further expands the cell types and developmental timepoints from which hair cells can be generated. In particular, does long-term Atoh1 expression allow the efficient conversion of supporting cells in the mature cochlea? This would be both informative for possible Atoh1-based regeneration strategies and helpful in better understanding Atoh1's potency in mature and highly differentiated cochlear cell types. Though the limitations of current transgenic models prevented a direct evaluation of these questions, the extended Atoh1 delivery that was possible suggested that the length of Atoh1 induction affected the size of the competency domain. The new $BAC^{Cdkn1b:Atoh1\text{-}HA\text{-}ER\ ieGFP}$ model or the Tg^{Atoh1} model in combination with another tissue-specific Cre mouse line may avoid the lethality presumably associated with non-specific expression of Atoh1 in other organ systems and allow the effect of long-term Atoh1 expression to be tested.

Along similar lines, it will also be important to determine the long-term survival and functional status of Atoh1-generated hair cells, as well as the overall effect of adding hair cell number within the postnatal cochlea. Since this would also be a critical component of evaluating an Atoh1-based regeneration strategy, hopefully these issues can be addressed with the alternative transgenic models that avoid the Atoh1-associated lethality.

In addition to testing the effect of extended Atoh1 expression, it may also be helpful to explore the factors that determine a cell's competency to Atoh1-mediated hair cell generation. For example, the prior ablation of existing hair cells may significantly increase Atoh1's effect within the remaining supporting cells by the release from inhibitory cues. This hair cell ablation experiments would also more appropriately model the target tissue of a potential Atoh1-mediated hair cell regeneration strategy and provide further insight into Atoh1 potential and limitations. However, in the event that Atoh1 is shown to insufficient by itself, additional manipulations to increase a target cell's competency could also be tested. One clear advantage of these stable and flexible transgenic Atoh1 models is that they could provide a consistent platform, *in vivo* or *in vitro*, on which to test various other manipulations.

The novel findings of Atoh1's induction of *de novo* Sox2 expression and promotion of cell cycle disruption should also be further explored. The induction of the prosensory marker Sox2 suggests that Atoh1 may have the potential to promote pluripotency and initiate sensory cell formation, identifying a potential role in sensory region formation at a point quite upstream from previously thought possible. Atoh1's disruption of cell cycle control also challenges the current belief that Atoh1 is strictly a terminal differentiation factor within the inner ear. A more complete dissection of the downstream cascades and

signaling pathways involved with these aspects of Atoh1's effects in the cochlea may reveal a much broader network of sensory region induction, patterning and proliferation control. This information would be important for both designing strategies to promote functional regeneration with all the necessary cell types and for understanding the interactions involved during normal development. Comparisons with the roles that Atoh1, or its homologues, play in other tissues and organism may be helpful in identifying candidate pathways involved in Atoh1's induction of prosensory markers and proliferation.

Finally, the utility of these transgenic models may also be extended to examine Atoh1's effects within contexts other than the cochlear epithelium. The competency of Atoh1-mediated hair cell generation within the vestibular epithelium has yet to be analyzed; the competency of non-sensory cells within this region of the inner ear may be quite different from that of the cochlea. In addition, these models may be useful for the study of Atoh1's effects within various other mammalian organ systems.

Final Remarks

This work represents an advancement in our understanding of Atoh1 and its complex effects within the mammalian cochlear epithelium. While Atoh1's role as a hair cell differentiation factor was commonly known within the field, the results presented here clearly delineate its potency and identify previously unreported effects on sensory domain induction and proliferation control.

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