ABSTRACT

Title of Document: A COMPARATIVE STUDY OF FGFR3 SIGNALING DURING THE DEVELOPMENT OF THE ORGAN OF CORTI AND BASILAR PAPILLA

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PhD
2008

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Most age-related hearing loss is the result of the accumulated death of inner ear hair cells over a life span. Human hair cells lack the ability to be regenerated once they die and thus there is a need to understand the processes which regulate hair cell formation. Unlike the mammalian ear, the avian cochlea has the ability to regenerate lost hair cells and thus there exists an ongoing race to find the key to regeneration in the mammalian ear. Human hearing is dependent on the interactions between *numerous* cell types yet very little is known about the pathways which regulate the development of the functionally essential support cells of the mammalian cochlear sensory epithelium. This study aims to elucidate some of the genetic pathways involved in hair cell and support cell differentiation in the developing cochlea. Specifically, the role of Fgfr3 signaling in pillar cell and hair cell differentiation will be revealed through the use of an *in vivo* mutant mouse model containing a null *Fgf8* gene and *in vitro* whole organ culturing of

the embryonic cochlear sensory epithelia of mice and chickens. The classic localize, activate, inhibit scheme will be employed. This study will demonstrate that *Fgf8* and *Fgfr3* are expressed by inner hair cells and pillar cells, respectively, and are required throughout development for normal differentiation and pattern formation of the organ of Corti. Inhibition of the receptor or ligand results in the loss of pillar cells and ectopic formation of hair cells, while activation of this pathway inhibits hair cell formation and induces pillar cells or activation of these genes and their proteins have on the formation of hair cell and support cell types. This study also takes a comparative approach by addressing the similarities and differences of the Fgfr3 signaling pathway in the mammalian organ of Corti and the avian basilar papilla. Fgfr inhibition in the developing basilar papilla causes an increase in hair cell density via the direct transdifferentiation of support cells into hair cells suggesting a role for this signaling pathway in the ability to regenerate hair cells.

A COMPARATIVE STUDY OF FGFR3 SIGNALING DURING THE DEVELOPMENT OF THE ORGAN OF CORTI AND BASILAR PAPILLA

By

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Dissertation submitted to the Faculty of the Graduate School of the University of Maryland, College Park, in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
2008

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Dedication

This dissertation is dedicated to
Kahuku High School
without which I would not be here.

Acknowledgements

I would foremast like to thank Dr. Matthew Kelley for all of his hard work and efforts in seeing this project through to completion, for providing guidance when it was needed and for having the keen eye to step back and give me the freedom to think and do. I would also like to thank him for understanding why rubber slippers are OK lab attire.

I would like to thank the members of my committee and Dr. William Jeffery for their willingness to be flexible and for being available when I needed them. I would also like to thank Lois Reid for pushing me through deadlines and keeping me on track.

I would especially like to thank the members of the Kelley lab both past and present for all of their help in scientific discussions, hands on tutoring, and moral support, and especially Drs. Alain Dabdoub, Chandrakala Puligilla, Elizabeth Driver, Weise Chang, Jennifer Jones and Helen May-Simera for teaching me everything I know about bench work. I would like to extend my most heartfelt thanks to Dr. Mireille Montcouquiol who first showed me how to be an independent scientist and thinker, and for pointing out that I was doing my dissections upside down. I would also like to thank Erynn Layman for her technical and not so technical help. Additionally, I would like to thank all of the lab members for switching from pizza to sushi for lunch on Fridays, the only thing that got me through some weeks.

For contributions to chapter one I would like to thank Drs. Alain Dabdoub, Mireille Montcouquiol, Matthew Holley, Karen Avraham and Katy McCabe for

intellectual and/or technical advice, and Drs. Thomas Friedman, Doris Wu, Weise Chang and Thomas Reh for reading an earlier version of the manuscript. The authors would also like to thank Kasey Heintz for technical support.

For contributions to chapter two I would like to thank Mireille E. Montcouquiol, Erynn M. Layman, and Mark Lewandoski, my co-authors. I'd also like to thank Dr. Elizabeth Grove for providing the *Fgf8b*-expression construct, as well as Chad Woods for technical assistance and Drs. Alain Dabdoub and Jennifer Jones for scientific advice.

I must also thank my parents John and Kathryn Jacques for their love and encouragement, my sister Kerry for being such an excellent role model and friend, as well as my grandparents David and Evelyn McPherson and Isabelle Gary for giving me a reason to make them proud.

Most of all I would like to thank William Risser, without whom I would not have been able to complete this task.

Mahalo.

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Chapter 1: Introduction

Hearing is a fundamental sense. The ability to detect sound can be found in a variety of animals from insects to humans and has played crucial roles in adaptation. In the human species, hearing has enabled the evolution of complex communication, speech, and language and as a result we are the only animals with a written recorded history. We now have an even more elaborate recorded history in the music, movies, and television so prevalent in our society. This love of auditory stimulation, however, may have come at a price, and we are now at risk of losing this, one of our most cherished senses. Humans are now experiencing a greater risk for sound-induced hearing damage than ever before. The prevalence of portable music devices with endless amounts of playback time, the accessibility of rock shows and bands with killer amps as well as our love of high speed travel are all hazards for our delicate sense of hearing. It is for this reason, now more than ever, that we must target efforts to find therapies for hearing loss. In order to understand how hearing can be repaired, however, we must first understand how it is created.

Transduction of sound

What we hear as sound is actually oscillations in the density of air or water caused by a vibrating object. Movement of an object causes the compression of atmospheric particles around it which are propagated in a wave away from the object in the same way a boat makes a wake as it moves through and displaces water. These micro-scale "wakes" or changes in atmospheric pressure are what we detect as sound. The volume of a sound

is determined by the height of the wave generated and is referred to as amplitude, while the rate at which waves are produced define the frequency of sound.

Detection, decoding and processing of sound occurs within the inner ear of vertebrates. The outer ear, including the pinna, concha and auditory meatus, collects and amplifies the sound. In humans it amplifies those frequencies important for human speech and funnels it to the ear drum which is sensitive to the tiny pressure changes created in the air by the sound. The tympanic membrane connects to the ossicles of the middle ear. These tiny bones act to translate the vibrations in the air into fluid vibrations of the inner ear via contacts with the oval window of the coiled cochlea. In the cochlea the mechanical vibratory signals are converted into electrical nerve impulses which are sent via the auditory neurons to the central nervous system for processing. The transduced sound travels through multiple regions of the brain where, at each step, the components of sound are further integrated. In ascending order, these include the cochlear nucleus, superior olivary nucleus, lateral lemniscus, inferior colliculus, and medial geniculate nucleus, before the signal reaches the auditory cortex where final processing occurs. In the cortex the auditory scene is created and auditory signals intermix with input from other senses, this is the basis of complex communication and awareness (central auditory processing reviewed by Demanez and Demanez, 2003).

Auditory hair cells

This fine-tuned processing would not be possible, however, without highly sensitive receivers to pick up the detailed components of sound. The primary receivers of the incoming stimuli are mechanosensory hair cells (HCs) found in the cochlea of the

inner ear. Hair cells sit on a basilar membrane that vibrates in response to sound pressure waves entering the cochlea through the oval window. Hair cells are responsible for decoding and transmitting this fluid motion in the cochlea into electrical nerve impulses. Each hair cell has a complex stereocilia bundle at its luminal surface that responds to changes in sound waves by opening and closing ion channels found at their tips (cochlear structure reviewed in Raphael and Altschuler 2003). Hair cells turn the mechanical shearing of the stereocilia relative to one another into a nerve impulse in a process known as mechanotransduction. The individual stereocilia within a bundle are linked together by spring-gated ion channels (Pickles, Comis et al. 1984; Kachar, Parakkal et al. 2000). When incoming vibrations cause the flexion of a stereocilium the force exerted on the tip link causes the ion channels to open or close depending on the direction of the stimulus. When a channel is pulled opened on the cell membrane potassium ions flow in triggering the depolarization of the cell and an influx of calcium ions into the base of the cell. Calcium intake causes the release of neurotransmitters which stimulate the afferent neurons of the spiral ganglion. This signal is then propagated up to the brain.

Decoding begins with the type of hair cell detecting the sound. Hair cells are grouped into a large field with other hair cells in the auditory sensory epithelium. This inner ear epithelium of lizards, birds (Figure 1.2) and mammals (Figure 1.1) is elongate and tonotopically arranged (Fay and Popper 2000). In mammals the cochlear duct forms a tight spiral (Figure 1.1B) while in birds it is relatively straight with a slight curve to it (Figure 1.2B). Tonotopicity means that within the epithelia there are regions that are specifically tuned to have the highest sensitivity to one specific frequency range. The proximal region (referred to as the base) of these epithelia decodes high frequency sound

Figure 1.1

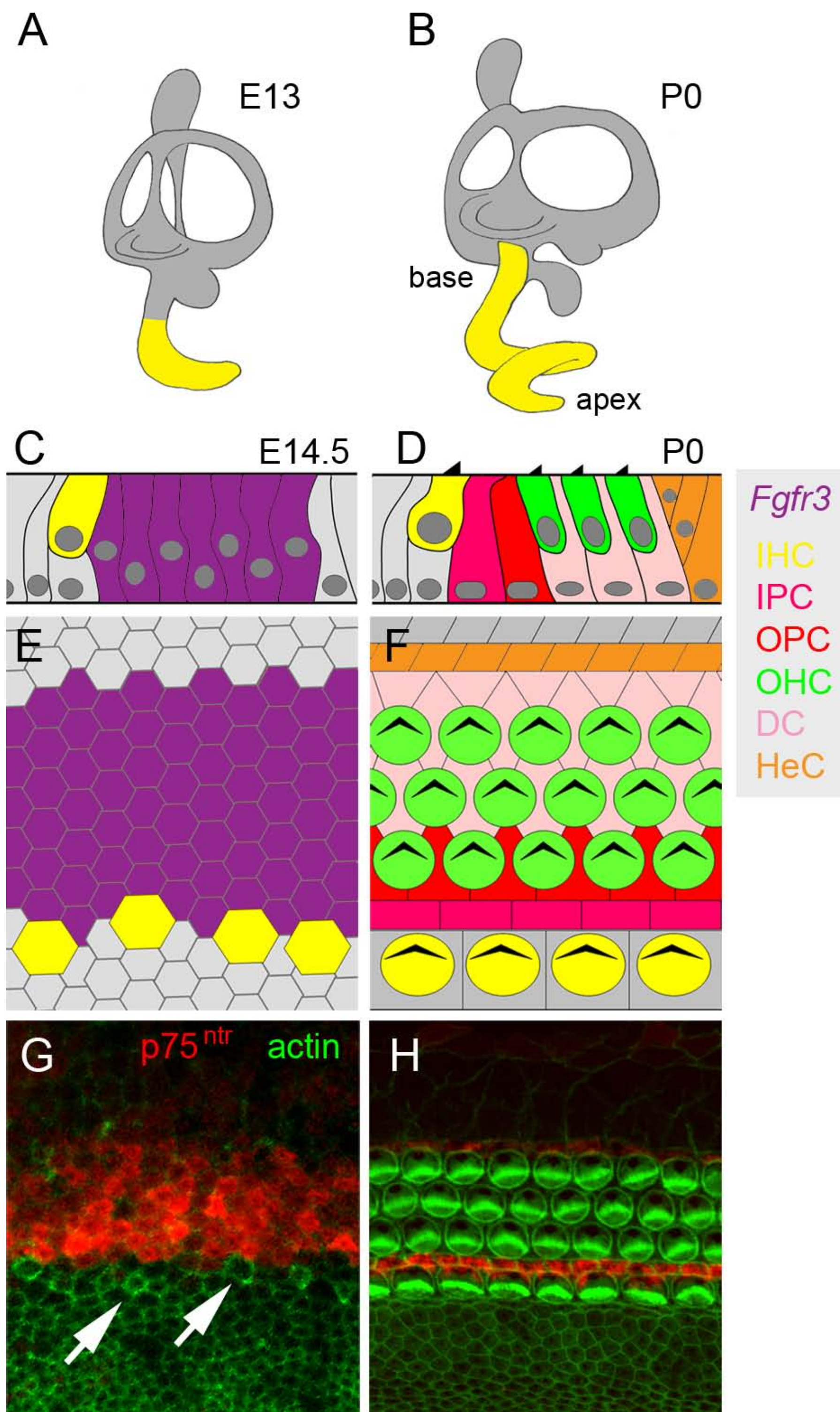

Figure 1.1. Development and structure of the organ of Corti. At E13, the cochlear duct has just begun to form and is a partial turn (A), by P0 it has fully formed its 1.75 turns (B); the vestibular system is colored in gray and the cochlear duct is highlighted in yellow. C and D are cross-sections through an E14.5 (C) and P0 (D) organ of Corti. E and F are the lumenal surfaces of the OC at the same ages, respectively. The large pool of progenitor cells is indicated in purple representing the expression of *Fgfr3* in these cells at this age(C). In D and F the unique differentiated cell types of the OC can be identified: inner hair cells (IHC, yellow), inner pillar cells (IPC, fuscia), outer pillar cells (OPC, red), outer hair cells (OHC, green), Deiter's cells (DC, pink) and Hensen's cells (HeC, orange). G and H are images of the lumenal surface of the OC at E14.5 (G) and P0 (H). $p75^{ntr}$ is a marker of pillar cells late in development (H) and is expressed broadly in the developing epithelium early in development (G), at this stage IHC differentiation has just begun (arrows). Hair cells and bundles can be seen with Phalloidin stain (H).

Figure 1.2

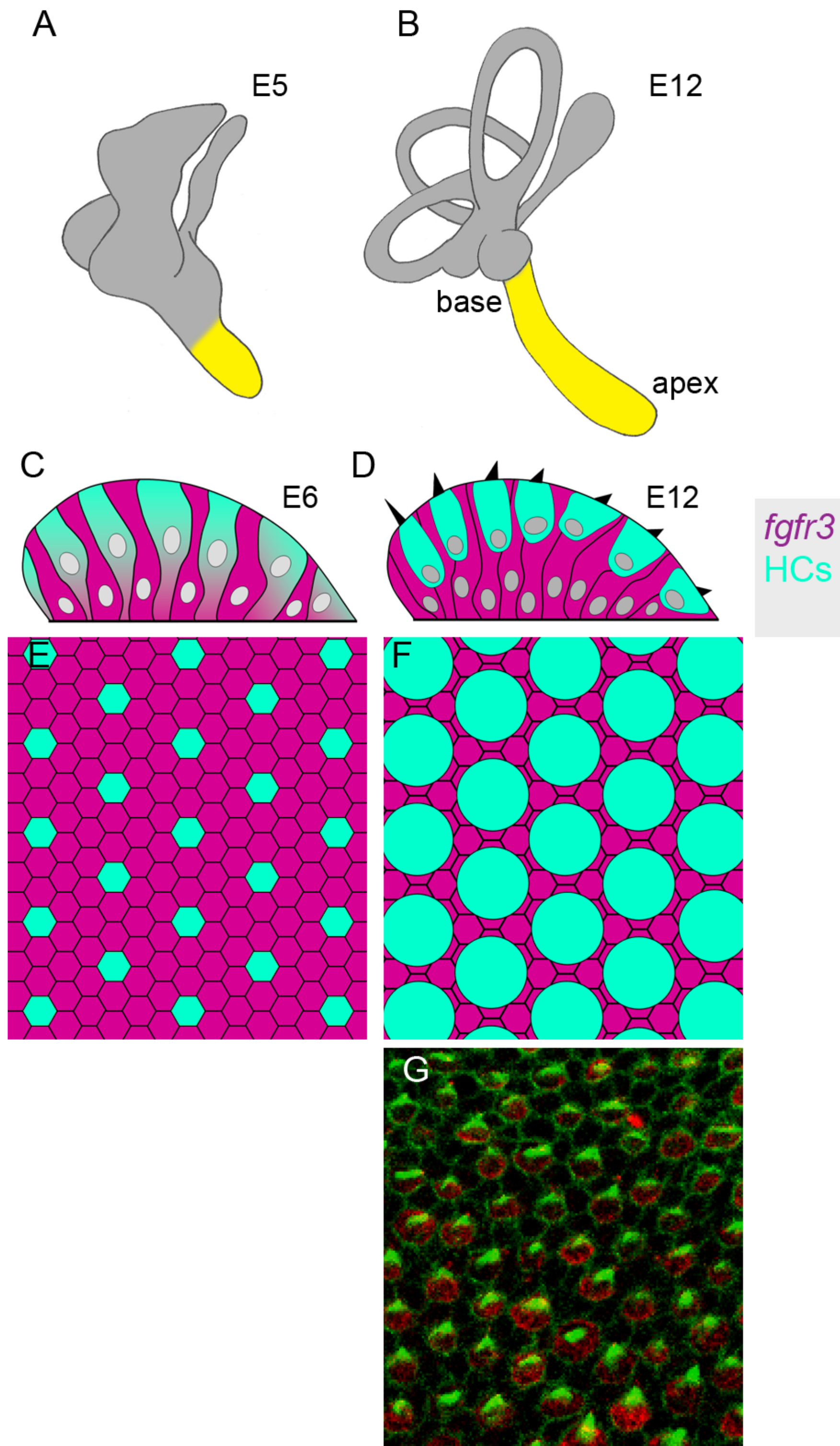

Figure 1.2. Development and structure of the basilar papilla. At E15, the cochlear duct has just begun to form (A), by E12 it has completed its extension to the midline and is slightly curved (B); the vestibular system is colored in gray and the cochlear duct is highlighted in yellow. C and D are cross-sections through an E6 (C) and E12 (D) basilar papilla. E and F are the lumenal surfaces of the BP at the same ages, respectively. The large pool of progenitor cells is indicated in purple representing the expression of *fgfr3* in these cells (C, E), the first sign of HC development is evident at this stage. In D and F differentiated hair cells (aqua) and support cells (purple) can be identified, support cells maintain *fgfr3* expression. HC shape ranges from tall HCs on the neural side (D, right) to shorter HCs on the abneural side (D, left), the relative stereocilia heights of these cells are also indicated. G is an image of the lumenal surface of the BP at E12. HCs are identifiable by their stereocilia and the marker Tuj1, the boundaries of all the cells are labeled with Phalloidin.

while the distal end (known as the apex) decodes low frequency with a gradient of frequencies between. Tonotopic variations include hair cell body size, stereocilia length, mechanotransduction channel composition as well as the thickness of extracellular membranes (Koppl, Manley et al. 2000); these specializations enable the decoding of sound by breaking it up into its component parts.

Most age-related hearing loss is the result of the accumulated death of these inner ear hair cells over a life span. Human hair cells lack the ability to be regenerated once they die and this has spurred much research into hair cell development. Unlike the mammalian inner ear, the avian cochlea has the ability to regenerate lost hair cells. Difference in structure and development of these organs may help explain why mammals lack the ability to repair their hearing (Matsui and Cotanche, 2004). Many of the mutations that cause hair cell abnormalities and consequently deafness have been well characterized (Schrijver, 2004**).** Hearing however, is dependent on the interactions between *numerous* cell types.

Cochlear structure

The hair cell containing epithelium of the mammalian cochlea is called the organ of Corti (OC). It is highly specialized and housed deep within the bony coiled labyrinth of the cochlea (Figure 1.1B). The OC is comprised of over six distinct cell types which must develop in a specific pattern in order for normal auditory function to occur (Echteler, Fay et al. 1994)(Figure 1.1D, F). Typically, there are four rows of hair cells that spiral in parallel up the cochlear duct. Inner hair cells (IHCs) are the primary receptors for incoming stimuli and comprise the first row; they are supported by more basally situated

phalangeal cells. Outer hair cells (OHCs) comprise the last three rows and have underlying Deiter's cells for support. The stereocilia of OHCs are embedded in the tectorial membrane which allows them to better sense and respond to stimuli. Separating the IHCs from OHCs are two rows of pillar cells (PCs), the inner pillar cell and outer pillar cell, which form a triangular fluid-filled space between them, the tunnel of Corti (cochlear structure reviewed inRaphael and Altschuler 2003). Pillar cells have large processes constructed of dense networks of microtubules which give them a rigid structure; these pillar heads extend from the basal lamina up to the lumenal surface of the epithelium and act to separate the inner hair cells from the outer hair cells. Their exact function however is unknown.

The tightly defined rows of hair cells in the OC are flanked by two fields of relatively undifferentiated cells, one of these is Kolliker's organ that lays between the IHCs and the spiralganglion and the other is referred to as the lesser epithelial ridge (LER) which extends beyond the last OHC. These fields of undifferentiated cells have the potential to form hair cells when experimentally manipulated to express hair cell specific genes (Woods, Montcouquiol et al. 2004; Jones, Montcouquiol et al. 2006; Driver, Pryor et al. 2008). Retention of the ability to become a hair cell may, in part, be explained by looking at a homologous organ such as the basilar papilla of the avian cochlea. The basilar papilla (BP), is much broader than the mammalian cochlea and the hair cells are more loosely organized(Koppl, Manley et al. 2000) (Figure 1-2F). The field of hair cells almost entirely fills up the cochlear duct, in contrast to the small strip they occupy in the OC. This suggests that development of the organ of Corti may have evolved to produce hair cells only in a subset of cells within a broader ancestral organ.

Other differences suggest that the OC is the byproduct of a significant amount of evolutionary remodeling. For example, there are two types of hair cells in the BP, short and tall that are arranged in a gradient across the width of the basilar papilla. Shorter hair cells cluster along the abneural edge, while taller hair cells are found more on the neural edge (Koppl, Manley et al. 2000) (Figure 1.2B). There is no distinct boundary line between the two types as is found between the inner and outer hair cells of the OC. Also, avian hair cells perform both amplification and detection roles while in the OC, detection is done mostly by the IHCs and amplification is achieved via membrane-bound motor proteins of the OHCs (Dallos, Wu et al. 2008). The hair cells of the BP are surrounded by a uniform type of support cell (Figure 1.2D, F) that is morphologically different from either pillar cells or Deiter's cells. The specializations seen in the OC can be attributed to changes in genes that regulate the development and differentiation of hair cells and support cells from a common progenitor pool.

Development of the inner ear

The inner ear is derived from a thickening of the head ectoderm called the otic placode which is induced by signals arising from the hindbrain and adjacent mesoderm. In mice, the otic placode is formed around embryonic day 8 (E8) (Ohyama, Groves et al. 2007)[1]. This structure invaginates to form the otic vesicle, which eventually divides into two regions: a dorsal region which forms the gravistatic vestibular system and a ventral region which forms the saccule and cochlea (Bok, Chang et al. 2007), both of which are composed of hair cells that respond to vibratory stimuli.

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[1] For the sake of consistency, development of the inner ear will be described as it occurs in the mouse in which parturition occurs around E19.

The cochlea can be distinguished as a medially-oriented ventral out-pocketing of the otic vesicle around E10 (Figure 1.1A, shown slightly later). As development proceeds, the cochlea continues to grow outward and distinctive coiling is evident by E15; at P0, formation of the 1.75 turns characteristic of the mouse cochlea is complete (McKenzie, Krupin et al. 2004) (Figure 1.1B). In chickens, ventral out-pocketing of the basilar papilla is evident by E5 (Figure 1.2A), after which the cochlea continues to grow out medially and elongate; by E10 the lengthening of the duct is complete (Figure 1.2B).

Sensory structures in the mouse otocyst first begin to differentiate at E11, as indicated by a thickening of the otic epithelium in patches; at this point spiral ganglion neurons are first detectable (Bok, Chang et al. 2007). Innervation to the inner ear arises from the cochleovestibular ganglion which is comprised of two distinct populations of neurons, those in the lateral region (vestibular ganglia) that will innervate the vestibular portion of the inner ear and its hair cells and those situated medially (spiral ganglia) which will go on to innervate the cochlear hair cells (Sanchez-Calderon, Milo et al. 2007).

Development of the organ of Corti

Development of the organ of Corti occurs in two waves. The first wave is a mitotic front which progresses from apex to base beginning around E11, with terminal mitosis occurring lastly in the base by E14. A second wave of differentiation begins at in the base and extends towards the apex to initiate the formation of specific cell types in the OC. In addition to the axial gradient of differentiation there is also a planar gradient beginning with the IHCs and spreading laterally to the Hensen’s cells which sit right

beyond the OHCs. One of the first cues that differentiation has begun is a thickening of the actin ring that surrounds the lateral boundaries of future IHCs; this occurs along with an increase in size and more lumenal positioning of their nuclei (McKenzie, Krupin et al. 2004) . This can be seen as a line of cells that extends from the base which have more pronounced labeling by Phalloidin F-actin than the surrounding cells (Figure 1.1E, G). Adjacent to this row of presumptive IHCs is a large pool of cells that are positive for the neurotrophin receptor $p75^{ntr}$. This pool of $p75^{ntr}$-positive cells begins in the lesser epithelial ridge and terminates at the line of IHCs (Figure 1.1G). These cells will eventually become the pillar cells, OHCs, Deiter's cells and Hensen's cells (Mueller, Jacques et al. 2002) (Figure 1.1H). Differentiation is complete by E17, after which cells grow and attain their proper orientation (Kelley 2007).

Deletions or mutations of any of these cell types during development can result in mild to severe hearing loss (Friedman, Dror et al. 2007). Given the specialized nature of hair cells and support cells of the organ of Corti, it is reasonable to assume that their development and maintenance is dependent on variable gene expression patterns. The highly specialized support cells of the OC are just as vital to normal hearing function as are hair cells, yet very little is known about these genetic pathways which regulate their development, the elucidation of which would greatly aid the understanding of the molecular basis of deafness as well as provide clues for potential therapies.

Fibroblast growth factor signaling

There are many families of genes whose products are secreted that play important conserved roles in the development and morphogenesis of the body's systems. Some of

these include the Fgf, Wnt, Bmp, and Hh signaling pathways. All of these gene networks have been shown to play a role in auditory development as well (Kelley 2007). Of particular interest to developmental auditory research, the Fgf family has been shown to be the prime signaling pathway involved in the early induction of the inner ear (Schimmang 2007).

Fibroblast growth factors (Fgf) are a family of 23 secreted proteins that mediate their response by binding with and activating one of four transmembrane Fgf receptors (Fgfrs). Fgfrs are tyrosine kinase receptor proteins made up of three extracellular immunoglobulin-like domains and a heparan sulfate proteoglycan (HSPG) binding region(reviewed by (Thisse and Thisse 2005)). Each transcribed Fgfr can be alternately spliced in the third immunoglobulin domain to produce multiple variants of the same gene (Miki, Bottaro et al. 1992). Each splice variant shows unique binding properties and affinities for the various Fgf ligands and are differentially expressed by different tissues. Two Fgf ligands bound to an HSPG are required for activation of an Fgfr. Once the Fgf-HSPG binds to the extracellular domain of the Fgfr the receptor undergoes homo-dimerization, bringing together the intracellular TrK regions of the receptors. This triggers cross-activation of the tyrosine kinase domains, which are auto-phosphorylated, and actives down-stream signaling cascades via the MAP kinase signaling pathway (Mohammadi, Olsen et al. 2005) (Figure 1.3).

Fgf signaling also functions in many feedback loops, one of which is the Sprouty pathway. *Sprouty*'s are genes that negatively regulate the activity of Fgf signaling by binding to and blocking the activity of Grb2, a binding partner required during Fgf receptor activation (Hanafusa, Torii et al. 2002). Tyrosine phosphorylation during Fgf

Figure 1.3

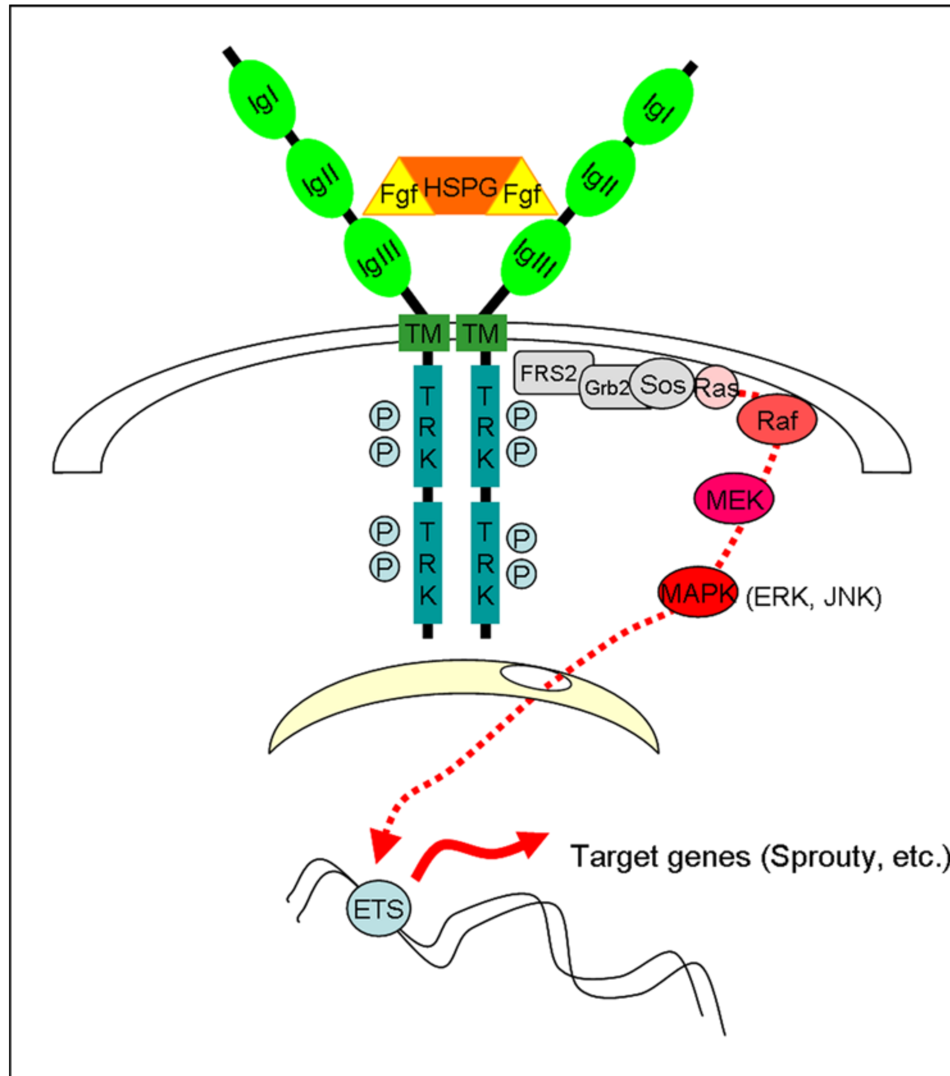


Figure 1.3. Model of the Fgf signaling cascade. Fgf receptors are transmembrane proteins made up of three extracellular immunoglobulin-like domains (Ig, light green), a transmembrane domain (dark green) and intracellular tyrosine kinase domains (TRK, teal). Two Fgf ligands (yellow) bound to an HSPG (orange) can bind to the Fgfr in the IgII and IgIII region. Once the Fgf-HSPG binds to the extracellular domain of the Fgfr the receptor undergoes homodimerization, bringing together the intracellular TRK regions of two receptors. This triggers cross-activation of the tyrosine kinase domains, which are auto-phosphorylated (light blue, P) causing recruitment of multiple signaling modulators (FRS2, Grb2, Sos, and Ras). Activation of Ras starts the MAP kinase signaling cascade (red), which targets and binds to DNA promoter regions influencing transcription of members of multiple gene pathways such as the Sprouty's. Sprouty's negatively regulate Fgf signaling by binding to Grb2 and effectively blocking downstream, activation of MAPK.

signaling induces the expression of Sprouty's thus activating this negative feedback loop (Tefft, Lee et al. 1999).

Fgf signaling in inner ear development and differentiation

Ligands of the Fgf signaling pathway are known to regulate the early development of the inner ear (Schimmang 2007). Of particular interest, *Fgf8* has been shown to play a key regulatory role during otic placode induction (Maroon, Walshe et al. 2002; Sanchez-Calderon, Martin-Partido et al. 2004; Ohyama, Groves et al. 2007) and differentiation of the otocyst (Adamska, Herbrand et al. 2001; Sanchez-Calderon, Martin-Partido et al. 2004; Schimmang 2007). Fgf8 secreted by the developing hindbrain is required for induction of otic pre-placodal cells (Phillips, Storch et al. 2004). Fgf3 and Fgf8 are required together for normal formation of the otocyst (Maroon, Walshe et al. 2002) and, independently, Fgf8 is required for differentiation of the various sensory epithelia within the otocyst (Sanchez-Calderon, Martin-Partido et al. 2004).

While much is known about the factors which regulate otic induction and formation of the gross morphology of the ear there remains much to be learned about the gene networks responsible for hair cell and support cell formation in the organ of Corti. Some key evidence has demonstrated that *Atoh1* is required for specification of HCs (Bermingham, Hassan et al. 1999; Woods, Montcouquiol et al. 2004). While hair cells are directly responsible for the detection of vibratory signals, support cells have been shown to have a vital role in the maintenance of these hair cells thus understanding how they develop is crucial to understanding hair cell viability and cochlear function.

Characterization of mice deficient in the gene encoding fibroblast growth factor receptor-3 (Fgfr3) revealed that these animals have relatively normal hair cells yet were entirely deaf (up to and greater than 60 dB at all frequency ranges tested)(Colvin, Bohne et al. 1996; Puligilla, Feng et al. 2007). The most prominent malformation in the cochleae of these animals, besides an extra row of hair cells, is a complete inhibition of pillar cell development, and consequently the absence of the tunnel of Corti (Colvin, Bohne et al. 1996; Hayashi, Cunningham et al. 2007; Puligilla, Feng et al. 2007).

Fgfr3 is one of the four Fgfrs and is a member of the Fgf8 synexpression group (Tsang, Friesel et al. 2002) (a family of co-expressed ligands, receptors, inhibitors and signal modulators that work in tandem within developing tissues to regulate development). Fgfr3 is most highly activated by the Fgf8 ligand and this signaling pathway has been shown to have unique effects during pattern formation, differentiation, and cell growth throughout the developing embryo and nervous system (Olsen, Li et al. 2006). Deletion of *Fgfr3*, as discussed above, has been shown to prevent the formation of a specific type of support cell, the pillar cell (Colvin, Bohne et al. 1996; Hayashi, Cunningham et al. 2007; Puligilla, Feng et al. 2007). Concurrently, *Fgfr3* has been reported to be expressed early in the domain of the developing OC which will become the pillar cells and outer hair cells (Peters, Ornitz et al. 1993) (Figure 1-1C, E) and then is down-regulated in all but the pillar cells (Pirvola, Cao et al. 1995; Pirvola, Ylikoski et al. 2002) (Figure 1-1D, F). These two lines of evidence point to a direct role for Fgfr3 in pillar cell formation and differentiation, which would make *Fgfr3* the only gene identified to specifically regulate the differentiation of a support cell.

Quantitative RT-PCR analysis has also indicated that *Fgf8* is strongly expressed in the embryonic cochlear sensory epithelium (Pickles 2001). More specifically it has been suggested that *Fgf8* may be expressed in the inner hair cells of the cochlea (unpublished data by Ulla Pirvola), a location in which it would have the potential to play a role in the Fgfr3 signaling cascade. Fgf8 is a non-autonomously acting ligand, meaning it exerts its effects on cells adjacent to those secreting it rather than influencing the cells in which it is transcribed. The positioning of pillar cells directly adjacent to *Fgf8*-expressing IHCs, and the high affinity binding Fgf8 has with Fgfr3 (Ornitz, Xu et al. 1996; Olsen, Li et al. 2006), shown to be expressed within the pillar cell region (Pickles 2001), suggest that this ligand-receptor pair may act in tandem to regulate the differentiation of pillar cells.

The epithelium of the chicken basilar papilla, however, also expresses *fgfr3* in a pattern similar to that of OC yet the BP lacks pillar cells (reviewed by Manley and Koppl, 1998). Specifically, *fgfr3* is expressed in all cells of the BP prior to differentiation at E5.5 (Figure 1.2C, E). During hair cell specification (E6-E8) *fgfr3* is down-regulated in cells that will become hair cells and is expressed only by support cells of the adult chicken (Bermingham-McDonogh, Stone et al. 2001) (Figure 1.2D, F). The fact that *Fgfr3* is down-regulated during organ of Corti development yet is maintained in the basilar papilla suggests that this signaling pathway may have contributed to the divergence of the unique support cell types of the OC which may also explain some functional differences between these two ears.

For example, while avian hair cells are as vulnerable to acoustic or toxic damage as are mammalian hair cells, unlike mammals, birds are able to regenerate and repair the

auditory sensory epithelium when hair cells are lost. The basilar papilla is able to produce new hair cells to replace ones that are damaged and thus hair cell loss in the adult BP is not permanent (Cruz, Lambert et al. 1987; Corwin and Cotanche 1988). Replacement of dead HCs initially involves the direct transdifferentiation of a support cell into a hair cell following HC death which is followed by a round of mitosis in the support cell layer (Cruz, Lambert et al. 1987; Corwin and Cotanche 1988; Cotanche, Lee et al. 1994). Interestingly, *Fgfr3* has been shown to be down-regulated in support cells following HC death, suggesting it may play a role during HC regeneration (Bermingham-McDonogh, Stone et al. 2001).

For reasons unknown, perhaps owing to their highly differentiated state, support cells in the mammalian OC are unable to transdifferentiate or re-induce mitosis following HC damage. It appears that the OC has given up the ability to repair itself in exchange for the high frequency tuning and detection made possible by the presence of these unique support cells (Bermingham-McDonogh and Rubel 2003). The deafness that occurs in mammals when *Fgfr3* is knocked out and pillar cells fail to form suggests that these support cells have evolved a vital role in hearing. Given the similarities of *fgfr3* expression during development and regeneration in the BP, and the concurrence of *Fgfr3* expression in the developing OC, there is a strong potential that this gene may have a conserved role in the development of both the avian and mammalian cochleae and may also play a role in the regenerative process. Thus there existed a clear need to study the role of Fgfr3 signaling within these organs.

Aims and hypotheses

This project aims to elucidate the role of Fgfr3 in the regulation, development and differentiation of hair cells, pillar cells and other support cells from a common progenitor pool. A comparative approach will be taken by analyzing cochlear development in both birds and mammals. Addressing the signaling in two different types of cochleae will provide insights into the conserved mechanisms of hair cell development and formation. Additionally, identifying differences between a regenerative and non-regenerative ear may elucidate some of the molecular requirements for hair cell regeneration. The scope of this research will attempt to experimentally address three main hypotheses.

Hypothesis 1: Fgfr3 regulates the differentiation of pillar cells in the developing organ of Corti.

Hypothesis 2: Fgf8 is responsible for the activity of Fgfr3 in the developing organ of Corti.

Hypothesis 3: Fgfr3 regulates the transition of support cells into hair cells in the basilar papilla.

Experimental Design

In this set of experiments the classic localize, inhibit, activate scheme was used to test the general hypothesis that inductive interactions between Fgf8 and Fgfr3 regulate the formation and differentiation of support cells and hair cells in the mouse organ of Corti and to determine if Fgfr3 plays a similar role in the avian basilar papilla. Two model systems were used to address these underlying questions. An *in vivo* approach was taken by analyzing Fgf pathway-specific knockout mice for defects in the developing organ of Corti. The available data on the fully characterized *Fgfr3* deletion (Colvin, Bohne et al. 1996; Hayashi, Cunningham et al. 2007; Puligilla, Feng et al. 2007) was used as a basis of comparison for analysis performed on mice with an inner ear-specific conditional knockout mutation of *Fgf8* (Meyers, Lewandoski et al. 1998; Hebert and McConnell 2000) the generation of this mutation is described in chapter 3).

The second approach involved an *in vitro* organ culturing technique in which intact cochlear sensory epithelia are isolated from embryonic mice between E13 and E16. These epithelia were grown in culture for 3-7 days during which time various modulators of Fgf signaling can be applied to the culture media to both inhibit and over activate Fgfr3 signaling. After incubation these explant cultures are fixed and labeled with markers for various cochlear cell types. Additionally, square-wave electroporation was used to transfect cochlear cultures with Fgf-expression vectors to drive ectopic expression of transgenes within the sensory epithelium.

The use of tissue culture in developmental studies has a long-standing history. As early as 1949 (Werthessen 1949) *in vitro* culture systems have been published as viable models for understanding how biological processes work *in vitro*. While definite

differences exist between *in vivo* and *in vitro* systems, tissue culture offers the ability to do experiments not possible in living organisms. In particular, the use of whole organ explants for inner ear research has been vital. Culturing offers more precise control over potential variables that are much harder to control for in living animals. The location of the inner ear deep within the bony labyrinth of the cochlea provides a further obstacle by hindering its accessibility in living animals. Embryonic cochlear explants have thus been widely utilized in developmental auditory research (Kelley, Xu et al. 1993; Raz and Kelley 1999; Woods, Montcouquiol et al. 2004; Gross, Machulik et al. 2005; Jones, Montcouquiol et al. 2006; Montcouquiol, Sans et al. 2006; Coleman, Fallon et al. 2007; Hayashi, Kokubo et al. 2008).

Expression analysis

The expression patterns of the components of the Fgf pathway that were hypothesized to play a role in OC development were analyzed by *in situ* hybridization and immunohistochemistry. These included the related ligands *Fgf8* and *Fgf17* as well as *Fgfr3*. Expression of these genes was analyzed at stages of development between E13 and P1.

Additionally, markers for assaying pillar cell growth and development were identified and used to analyze the effects of experiments pertaining to the functional modulation of Fgfr3 signaling such as p75^{ntr}, the low-affinity neurotrophin receptor. Another useful indicator of PC development is the ITO distance: the “Inner-to-Outer hair cell” distance, or the distance between the IHC and first row of OHCs. Larger ITOs are reflective of more mature PCs. A third marker, β-actin, was also used given that it is

expressed strongly in PCs and more weakly in OHCs, Deiter's cells, and Hensen's cells at P0 but is not expressed in any cell types within the OC prior to E17.

Fgfr3 Pathway Inhibition

In order to address the effects of the inhibition of the Fgfr3 signaling pathway, signaling modulation was done *in vivo* and *in vitro* at two different levels: (1) by inhibition of receptor activation and (2) by the functional inhibition of specific ligands. Receptor inhibition will be carried out by treatment of whole organ embryonic cochlear explant cultures with SU5402, a pharmacological agent that inhibits the receptor tyrosine kinase activity of all Fgf receptors. In order to inhibit the specific function of the identified Fgfr3 ligand, Fgf8, cochleae from an inner-ear specific Fgf8 conditional knock-out mouse (Meyers, Lewandoski et al. 1998) were analyzed. Cultured developing OC sensory epithelia were also exposed to an Fgf8 function-blocking antibody to determine if inhibition at the protein level mimics the activity of genetic inhibition.

Fgf pathway ectopic activation

The second major experimental goal is to characterize the effects of the Fgfr3 signaling pathway on pillar cells by looking at the effects of over-activation of the Fgf pathway components. Receptor activation using Fgf2, a non-endogenous broad-acting promiscuous Fgf ligand with high affinity for all Fgf receptors (Ornitz, Xu et al. 1996), was used. In order to identify the endogenous ligands that play a role in activating Fgfr3 and pillar cell development purified protein of the members of the Fgf8 synexpression group were applied to embryonic cultures, including Fgf8, Fgf17 and Fgf18 protein.

Fgf17 protein is known to bind to and activate Fgfr3 in the same domains that bind Fgf8 (Olsen, Li et al. 2006). However, Fgf17 binds Fgfr3 with slightly higher affinity than Fgf8 and thus can function as a strong ectopic activator of the receptor. Transfection of Fgf8 into the sensory epithelium was also done using a viral construct expressing *Fgf8.*

Comparative development

To identify any conserved role Fgfr3 may play in the avian basilar papilla, many of the same experiments described above for the OC were performed on embryonic BP explant cultures. These included inhibition of Fgfr3 with SU5402 and ectopic activation using Fgf17 and Fgf2. Immunocytochemistry was used to identify changes in the cultured versus control epithelia. The antigens HCA and SCA were used to distinguish HCs from SCs, respectively. Serrate-1 (Adam, Myat et al. 1998) and Sox2 (Neves, Kamaid et al. 2007) expression were also used as indicators of SC phenotypes. Tuj1, Myosin VIIa and Math1 antibodies were used to identify developing hair cells.

Chapter Summaries

The results of these experiments will be presented in three main chapters, the summaries of which are below.

Chapter 2: Fibroblast Growth Factor Signaling Regulates Pillar Cell Development in the Organ of Corti

Chapter two is published in its entirety in the Journal of Neuroscience volume 22(21), November 1, 2002, page 9368-9377(Mueller, Jacques et al. 2002). In this chapter

Fgfr3 is identified as a gene that is expressed during early cochlear development in the mouse; this is shown by immunohistochemistry and *in situ* hybridization. *In vitro* functional studies using mouse whole-organ embryonic cochlear explant cultures demonstrate that inhibition of the receptor with SU5402 significantly reduces the ability of pillar cells to develop and differentiate. Conversely, over-activation using ectopic Fgf2 caused a significant increase in the number of cells that developed as pillar cells. This first chapter thus demonstrates that Fgfr3 is important and necessary for normal organ of Corti development and specifically shows that it plays a role in pillar cell formation. It also defines a developmental window between E15 and P0 in which Fgfr signaling is required in the OC.

Chapter 3: Fgf8 induces pillar cell fate and regulates cellular patterning in the mammalian cochlea

Chapter three is published in its entirety in the journal Development volume 134(16), August 2007, page 3021-3029(Jacques, Montcouquiol et al. 2007). This chapter delves further into the role that Fgfr3 plays in the cochlea. *Fgf8* expression by inner hair cells is shown to begin at E15, the same time as *Fgfr3* and adjacent to the pool of *Fgfr3* expressing cells and suggests they may be interacting. *Fgf8* conditional knock out mice are shown to lack pillar cells, very similar to the phenotype of the *Fgfr3* knockouts, a good indication that they are operating in the same pathway. Fgf8 is inhibited *in vitro* using an Fgf8 function-blocking antibody in the media of embryonic OC cultures. As with Fgfr inhibition, and identical to the mutants, inhibition at the protein level causes a complete inhibition of pillar cell development, and demonstrates the need for Fgf8-Fgfr3

signaling to enable formation of pillar cells. Ectopically activating Fgfr3 with Fgf8-expressing constructs via electroporation significantly increases the number of pillar cells adjacent to transfected regions. There is a significant decrease in the number of outer hair cells at these sites as well as in cultures exposed to Fgf17 protein. This chapter thus supports the hypothesis that Fgf8 signaling plays a role in promoting pillar cell formation and activation of Fgfr3 prevents the differentiation of hair cells.

Chapter 4: Fgf signaling regulates development and transdifferentiation of hair cells and support cells in the basilar papilla

Chapter four is a manuscript that is currently being prepared for journal submission. In this final chapter, the role of Fgfr3 signaling in the avian basilar papilla is addressed. Expression of *Fgfr3* in the developing BP suggests that it may play a similar role in support cell and hair cell differentiation as it does in the organ of Corti, given the homologous nature of these organs. Whole-organ embryonic chick cultures are used to understand the effect of inhibiting Fgfr3. Inhibition with SU5402 prior to hair cell differentiation at E6 results in a significant increase in hair cell density. This increase is shown to be the result of direct transdifferentiation of support cells into hair cells. The increase in the number of hair cells following inhibition of Fgfr3 is similar to that seen in mice with mutations in *Fgfr3* or *Fgf8*. In more mature BP cultures SU5402 also causes an increase in hair cell density. In older explants, small young hair cells are identified forming directly adjacent to more mature hair cells. These new hair cells disrupt the regular patterning and ratio of hair cells to support cells and a specific pool of support cells are identified that have the ability to undergo transdifferentiation. The conversion

of support cells into hair cells without a prior round of hair cell death suggests that Fgfr3 is directly responsible for inhibiting hair cell formation and promoting the supportive cell phenotype.

Chapter 2: Fgf Signaling Regulates Pillar Cell Development in the Organ of Corti

Abstract

One of the most striking aspects of the cellular pattern within the sensory epithelium of the mammalian cochlea is the presence of two rows of pillar cells in the region between the single row of inner hair cells and the first row of outer hair cells. The factors that regulate pillar cell development have not been determined, however, previous results suggested a key role for fibroblast growth factor receptor 3 (Fgfr3).

To examine the specific effects of Fgfr3 on pillar cell development, receptor activation was inhibited in embryonic cochlear explant cultures. Results indicated that differentiation of pillar cells is dependent on continuous activation of Fgfr3. Moreover, transient inhibition of Fgfr3 did not permanently inhibit the pillar cell fate, since reactivation of Fgfr3 resulted in the resumption of pillar cell differentiation. The effects of increased Fgfr3 activation were determined by exposing cochlear explants to Fgf2, a strong ligand for several Fgf receptors. Treatment with Fgf2 leads to a significant increase in the number of pillar cells and to a small increase in the number of inner hair cells. These effects were not dependent on cellular proliferation, suggesting that additional pillar cells and inner hair cells were a result of increased recruitment into the prosensory domain.

These results indicate that Fgf signaling plays a critical role in the commitment and differentiation of pillar cells. Moreover, the position of the pillar cells appears to be

determined by the activation of Fgfr3 in a subset of the progenitor cells that initially express this receptor.

Introduction

The sensory epithelium of the mammalian cochlea (the organ of Corti) is comprised of at least six distinct cell types that are arranged in a rigorous cellular pattern (Figure 2.1). One of the most intriguing aspects of this pattern is the presence of the tunnel of Corti, an extracellular space that extends along the basal-to-apical axis of the organ of Corti between the single row of inner hair cells and the first row of outer hair cells(Lim 1986). The walls of the tunnel of Corti are formed by single rows of inner and outer pillar cells that also extend along the length of the cochlea (Figure 2.1). Pillar cells and the existence of a tunnel within the hair cell sensory epithelium appear to be unique to mammalian cochleae (reviewed in(Slepecky and Ogata 1996; Manley and Koppl 1998), suggesting that pillar cells represent a derived cell type. The results of previous studies have demonstrated that the development of pillar cells and the formation of a normal tunnel of Corti are required for normal hearing(Colvin, Bohne et al. 1996; Chen and Segil 1999), however, the factors that play a role in pillar cell development are largely unknown.

One signaling pathway that has been implicated in pillar cell development is the fibroblast growth factor (Fgf) signaling pathway(Ornitz 2000; Ornitz and Itoh 2001). Mice containing a targeted disruption of the fibroblast growth factor receptor 3 (*Fgfr3*) gene are profoundly deaf; however, the only obvious defect in the auditory system of these mice is the incomplete development of the pillar cells and the tunnel of Corti(Colvin, Bohne et al. 1996). In addition, expression of messenger RNA for *Fgfr3* in the developing organ of Corti has been localized to a region of the cochlea that corresponds to the developing sensory epithelium(Peters, Ornitz et al. 1993). These

Figure 2.1

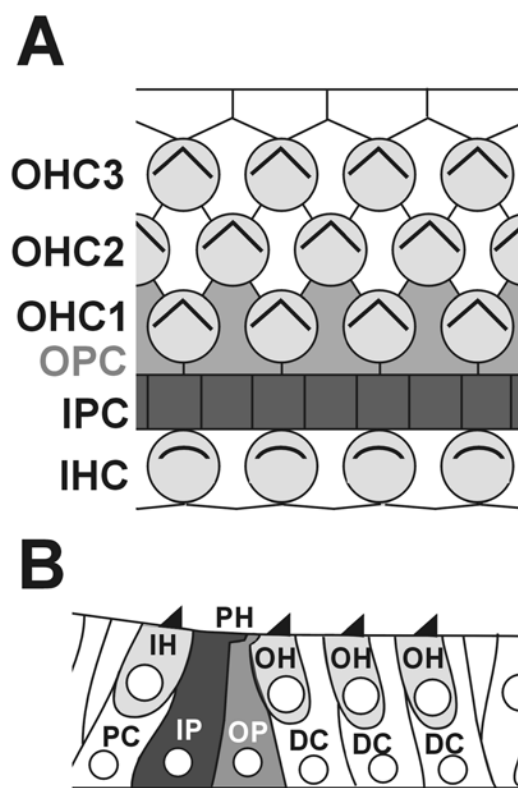


Figure 2.1. Cellular pattern in the organ of Corti. Schematic drawings of tangential (A) and cross-sectional (B) views of the organ of Corti in a mouse at P0. A. Mechanosensory hair cells are arranged in a single row of inner hair cells (IHC) and three or four rows of outer hair cells (OHC1, 2, 3). A single row of inner pillar cells (IPC, dark gray) and a single row of outer pillar cells (OPC, lighter gray) are located in the region between the inner hair cells and the first row of outer hair cells (OHC1). Outer pillar cells also extend cytoplasmic processes that interdigitate between the first row outer hair cells. B. The pillar head is comprised of apical projections from both the inner (IP) and outer (OP) pillar cells. Note that the lumenal surface of the pillar head (PH) is derived

primarily from the inner pillar cell. However, as illustrated in A, the lumenal surfaces of outer pillar cells can be visualized in the interdigitations between first row outer hair cells. As development continues, the tunnel of Corti will form in the region between the inner and outer pillar cells. Abbreviations: IH: inner hair cell, PC: phalangeal cell, OH: outer hair cells, DC: Deiter’s cells.

results suggest that Fgfr3 is required for the development of pillar cells; however, the specific effects of Fgfr3 and the Fgf signaling pathway have not been determined. The results presented here demonstrate that activation of Fgfr3 is required throughout the embryonic period for the ongoing differentiation of the pillar cells. Moreover, increased activation of Fgfr3 by treatment with fibroblast growth factor 2 (Fgf2) leads to an increase in the number of cells that develop as pillar cells. These results demonstrate roles for the Fgf signaling pathway in both the commitment and differentiation of cells as pillar cells.

Materials and Methods

Immunohistochemistry

Cochleae were dissected from mouse embryos of specific ages between E13 and P1 and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Some cochleae were then cryoprotected in sucrose, embedded in TissueTek OCT (Ted Pella, Inc, Redding, CA), and sectioned in a cryostat at a thickness of 12μm. Expression of $p75^{ntr}$ (Chemicon, Temecula, CA) or Fgfr3 (Santa Cruz Biotechnology, Santa Cruz, CA) at different developmental time points was determined in whole-mounts and, for $p75^{ntr}$, cryosections. Briefly, developing scala vestibuli, scala tympani and Reissner's membrane were dissected to expose the developing cochlear sensory epithelium. Samples were then permeabilized with either 0.1% Triton-X 100 in phosphate buffered saline (PBS; $p75^{ntr}$) or 100% acetone (Fgfr3), followed by overnight incubation in the primary antibody at 4°C with rocking. Antibody binding was detected using either a biotin-linked secondary antibody and the Vector Elite ABC (peroxidase) kit (Vector laboratories, Burlingame, CA) or an Alexa-568-conjugated secondary antibody (Molecular Probes, Eugene, OR).

Cochlear explant cultures

Cochlear explant cultures were prepared as described previously (Kelley, Xu et al. 1993; Raz and Kelley 1999). Briefly, timed-pregnant ICR strain mice were deeply anesthetized by inhalation of CO_2 gas and then euthanized by creating a double pneumothorax on gestational day 13 (E13) or 14 (E14). Embryos were removed from the uterus and staged according to Kaufman (1992). All procedures involving animals met the guidelines

described in the *NIH Guide for the care and Use of Laboratory Animals* and had been previously approved by the NIH IACUC.

After removal of embryos, cochleae were dissected and oriented with the lumenal surface of the sensory epithelium facing upwards onto MatTek dishes (MatTek Corporation, Ashland, MA) that had been coated with a 0.01% layer of poly-lysine (Sigma Chemical, St. Louis, MO) followed by a layer of Matrigel (1:70 dilution)(BD Biosciences, San Jose, CA). Cultures were maintained in media comprised of minimum essential medium (MEM), glucose, HEPES, sodium bicarbonate, N1 supplements and 10% fetal bovine serum.

Inhibition of Fgfr3

Activation of Fgfr3 was inhibited using SU5402 (Calbiochem, San Diego, CA), a member of a new class of Fgfr antagonists that block the tyrosine kinase activity of the receptor by interacting with the catalytic domain (Mohammadi, McMahon et al. 1997). A stock solution of SU5402 was dissolved in DMSO and then diluted to specific concentrations between 1 and 50 μM in culture medium. Medium containing either SU5402 (experimental) or an equivalent amount of DMSO (control) was added to explant cultures at specific time points that corresponded with embryonic ages between E14 and E18. Cultures were maintained until hair cells could be identified along the entire length of the developing sensory epithelium, typically six days in vitro (DIV) for cultures established on E13. At the end of each experiment, cultures were fixed in either 4% paraformaldehyde (PFA) or 3% glutaraldehyde, 2% paraformaldehyde for 20 minutes at room temperature.

After fixation, pillar cells were labeled with an antibody against p75^{ntr} (Chemicon, Temecula, CA) and hair cells were labeled with either an antibody against myosin VI (a gift from Tama Hasson, UCSD, Hasson et al., 1997) or VIIa (antibodies kindly provided by both Tama Hasson, UCSD and Christine Petit, Institut Pasteur, (Hasson, Gillespie et al. 1997) (Sahly, El-Amraoui et al. 1997) or with *Griffonia simplicifolia* lectin (Vector Laboratories, Burlingame, CA)(Lanford, Lan et al. 1999; Warchol 2001). Primary antibody labeling was detected using appropriate secondary antibodies conjugated to Alexa-488 (Molecular Probes), Alexa-568 (Molecular Probes, Eugene, OR) or biotin (Vector Laboratories, Burlingame, CA). Binding of secondary antibodies conjugated to biotin was detected using the Vector Elite ABC peroxidase staining kit (Vector Laboratories, Burlingame, CA). *G. simplicifolia* labeling was detected by direct fluorescence or with the Elite ABC alkaline phosphatase staining kit (Vector Laboratories, Burlingame, CA). To visualize cellular borders, filamentous actin was stained with Alexa-488 conjugated-phalloidin (Molecular Probes, Eugene, OR). To examine cellular histology, some cultures were imbedded in immunobed (Polysciences, Warrington, PA) and sectioned at a thickness of 3 μm.

Activation of Fgfr3

Fgf2 (R&D Systems, Minneapolis, MN) was dissolved in culture media containing 2 μg/ml heparin and 1% DMSO (to improve penetration into the epithelium) and then added to cochlear cultures at specific time points. Controls received media containing 2 μg/ml heparin and 1% DMSO. Cultures were maintained until hair cells could be identified along the complete length of the developing organ of Corti, a total of 6 DIV for

cultures established on E13. At the end of the experiment cultures were fixed in 4% PFA. Pillar cells and hair cells were labeled as described above.

Detection of proliferating cells

To identify proliferating cells within cochlear explants, the thymidine analog 5-Bromodeoxyuridine (BrdU)(Sigma Chemical, St. Louis, MO), was added to culture media at a concentration of 3 μg/ml (Montcouquiol and Corwin 2001; Montcouquiol and Corwin 2001). Uptake of BrdU was determined by labeling with an anti-BrdU antibody (BD Biosciences, San Jose, CA), followed by a biotinylated secondary antibody and the Vector Elite ABC staining kit (Vector Laboratories, Burlingame, CA). Since the BrdU antibody used for these studies was generated in a mouse, non-specific labeling was inhibited using the Mouse-On-Mouse kit (Vector Laboratories, Burlingame, CA) prior to addition of the primary antibody.

Determination of numbers of pillar heads, hair cells and ITO distances

Changes in the number of pillar heads (the combined apical extensions of both the inner and outer pillar cells that gives rise to the roof of the organ of Corti), inner and outer hair cells and distances between inner and outer hair cells (ITO (Inner To Outer) distances) were determined as follows: first, the total length of the sensory epithelium was determined based on the extent of inner and outer hair cells. Based on this length, positions that were equivalent to distances of 10%, 20%, 30%, and 40% from the extreme basal end of the epithelium were identified. For each position, the number of inner hair cells, outer hair cells, pillar heads, and ITO distances were determined. The length of

region to be counted always included a minimum of 10 inner hair cells, as determined by counting the number of myosin VI or myosin VIIa positive cells along the inner curve of the sensory epithelium. Outer hair cells within the same region were also counted based on the number of cells that expressed myosin VI or myosin VIIa. The number of pillar heads was determined using one of the two following methods: Cultures were labeled with anti-$p75^{ntr}$ and individual labeled pillar heads were counted at the apical surface of the sensory epithelium. Alternatively, filamentous actin was labeled with phalloidin. As a result, individual pillar heads were outlined in the region between the inner and outer hair cells. For all experiments, a minimum of three independent samples from at least two separate experiments were analyzed.

Results

Expression of Fgfr3 in the organ of Corti

Results of immunolocalization studies indicated no expression of Fgfr3 in the cochlea prior to E16 (data not shown). However, by E16, Fgfr3 is expressed in a band of cells that extends along the length of the cochlear duct (Figure 2.2A). As was reported in Peters et al. (1993), cells that express Fgfr3 are located in the region of the cochlear duct that will develop as the pillar cells, outer hair cells, and Deiter's cells (Figure 2.2A). By P0, expression of Fgfr3 in the sensory epithelium is restricted to pillar cells (Figure 2.2B).

Developing pillar cells express $p75^{ntr}$

The results of previous studies have demonstrated that $p75^{ntr}$ is a specific marker for pillar cells in the organ of Corti of neonatal (P0-P3) mice (von Bartheld, Patterson et al. 1991; Gestwa, Wiechers et al. 1999; Sano, Mukai et al. 2001). At later developmental time points expression of $p75^{ntr}$ is apparently down regulated in pillar cells; however, two published results disagree regarding the timing of this down regulation(Gestwa, Wiechers et al. 1999; Sano, Mukai et al. 2001). To examine the embryonic expression of $p75^{ntr}$, cochleae were dissected from embryos at E15 and expression of $p75^{ntr}$ was determined by immunolabeling. Results indicated that at E15, $p75^{ntr}$ is expressed in a relatively broad band of cells (Figure 2.3A) that extends along the length of the basal-to-apical axis of the cochlea (data not shown). In cross-section, the band of expression of $p75^{ntr}$ correlates with the region of the epithelium that will develop as pillar cells, however, the number of cells expressing $p75^{ntr}$ appears greater than the number of cells that will

Figure 2.2

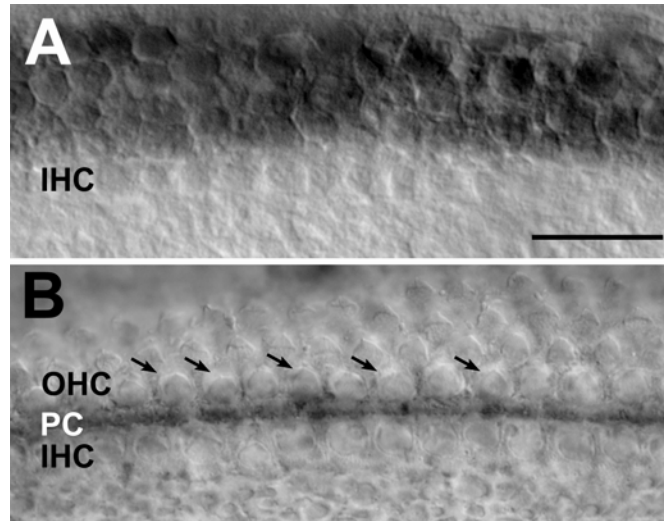


Figure 2.2. Expression of Fgfr3 in the embryonic cochlea. A. Lumenal surface of the developing organ of Corti in the middle turn of the cochlea at E16. The band of expression of Fgfr3 (dark region) is located adjacent to the row of developing inner hair cells (IHC) and appears to correspond with the region of the epithelium that will develop as pillar cells, outer hair cells and Deiter's cells. B. Lumenal surface of the developing organ of Corti in the middle turn of the cochlea at P0. Fgfr3 expression is restricted to the developing pillar cells (PC) located between the single row of inner hair cells (IHC) and the first row of outer hair cells (OHC). Stereociliary bundles are evident in the outer hair cell region (arrows) but are not in the plane of focus for inner hair cells. Scale bar in A (same in B), 50 μm.

Figure 2.3

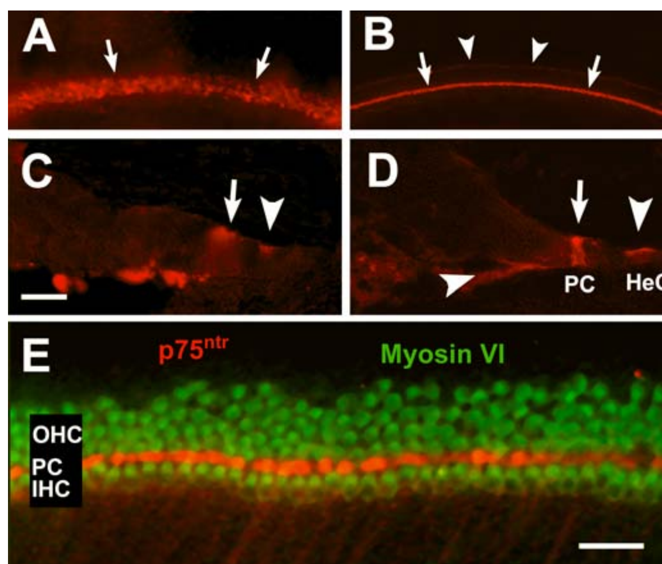


Figure 2.3. Developing pillar cells express p75^{ntr}. Expression of p75^{ntr} at E15 (A and C) and P0 (B and D). A. Low magnification image of the lumenal surface in a whole mount of the cochlear at E15. p75^{ntr} is expressed broadly in a band of cells that correlates with the position of developing pillar cells, outer hair cells and Deiter's cell (arrows). B. Low magnification image of the lumenal surface of the organ of Corti in the basal turn at P0. At this stage, p75^{ntr} is expressed intensely in the row of pillar heads (arrows). A second band of p75^{ntr} expression is present at the lateral edge of the sensory epithelium (arrowheads). C. Cross section of the developing sensory epithelium from the middle turn of the cochlea at E15. p75^{ntr} is expressed diffusely within a group of cells that correlates with the position of developing pillar cells (arrow). p75^{ntr} is also expressed in a more lateral region of the epithelium that appears to correlate with the development of Hensen's cells (arrowhead). D. Cross section of the organ of Corti from the middle turn

of the cochlea at P0. p75^{ntr} is strongly expressed in the inner and outer pillar cells (arrow, PC) and more diffusely in the Hensen's cells (arrowhead, HeC). There is also expression of p75^{ntr} in neurites extending from the spiral ganglion to the sensory epithelium (arrowhead). E. Lumenal surface of the sensory epithelium in a cochlear explant culture established on E14 and fixed after 5 DIV. A single line of P75^{ntr}-positive pillar cells (red, PC) are located between the single row of myosin VI-positive inner hair cells (green, IHC) and the first row of myosin VI-positive outer hair cells (green, OHC). Spiral ganglion neurites that innervate inner hair cells are also labeled with p75^{ntr}. Scale bar in A, 500 µm, scale bar in B, 500 µm, scale bar C (same in D), 50 µm scale bar in E, 50 µm.

ultimately develop as pillar cells (Figure 2.3C). In addition, a second, less intense, region of $p75^{ntr}$ expression is present in a position within the epithelium that correlates with developing Hensen's cells (arrowhead in Figure 2.3C). By P0, as has been reported previously, $p75^{ntr}$ is expressed intensely in a narrow band (Figure 2.3C) that extends along the length of the cochlea (data not shown). Analysis of cross-sections indicates that this intense band of expression of $p75^{ntr}$ correlates with expression in both the inner and outer pillar cells (Figure 2.3D); however, a second, less intense band of staining is also apparent in the region of the Hensen's cells (arrowheads in Figure 2.3B, HeC, arrowhead in Figure 2.3D). Finally, double-labeling of cochlear explant cultures with antibodies against myosin VI and $p75^{ntr}$ demonstrates that the band of $p75^{ntr}$ expression is located between the single row of inner hair cells and the first row of outer hair cells (Figure 2.3E). Based on these results, expression of $p75^{ntr}$ in the region between the row of inner hair cells and the first row of outer hair cells was used as a marker for pillar cell development at P0.

Inhibition of Fgfr3 disrupts pillar cell development

To determine the effects of Fgfr3 during the development of the embryonic organ of Corti, cochlear explant cultures were established on E13 or E14. After 18 hours in vitro, either 10 μM SU5402 or a vehicle control was added to the culture medium. SU5402 was maintained in the culture medium for the duration of the experiment. SU5402 has been shown to inhibit the tyrosine kinase activity of all four Fgfrs by interacting with the catalytic domain(Mohammadi, McMahon et al. 1997). The results of previous studies have suggested that Fgfrs 1, 2 and 4 are not expressed in the cochlear

sensory epithelium(Pirvola, Cao et al. 1995), however, recent unpublished findings have suggested that Fgfr1 may be present in the developing cochlea (U Pirvola, personal communication).

Explants maintained in control media developed a single row of $p75^{ntr}$-positive heads that appeared similar to the pattern of $p75^{ntr}$ expression at P0 in vivo (Figure 2.4A). Analysis of double-labeled samples indicated that the row of $p75^{ntr}$-positive cells was located between the single row of inner hair cells and the first row of outer hair cells (Figure 2.4C). Although expression of $p75^{ntr}$ at the apical surface appeared as a single line, analysis using confocal microscopy indicated that both inner and outer pillar cells expressed $p75^{ntr}$ (data not shown). In contrast with controls, $p75^{ntr}$ -positive cells were absent in explants exposed to SU5402 (Figure 2.4B). A single row of inner hair cells and three to four rows of outer hair cells were still present in these cultures; however, the distance between the row of inner hair cells and the first row of outer hair cells was reduced (Figure 2.4D).

To determine whether exposure to SU5402 lead to the elimination of developing pillar cells, cross-sections from the basal turn of control and SU5402-treated explants were analyzed. In sections from controls, a pair of inner and outer pillar cells was present in the region between the inner hair cell and first row outer hair cell in most sections (Figure 2.4E). In addition, projections from the two pillar cells extended to the apical surface to form a developing pillar head process. Two cells were also present in the region between the inner hair cell and first row outer hair cell in explants that had been exposed to SU5402, however these cells did not give rise to a developing head process

Figure 2.4

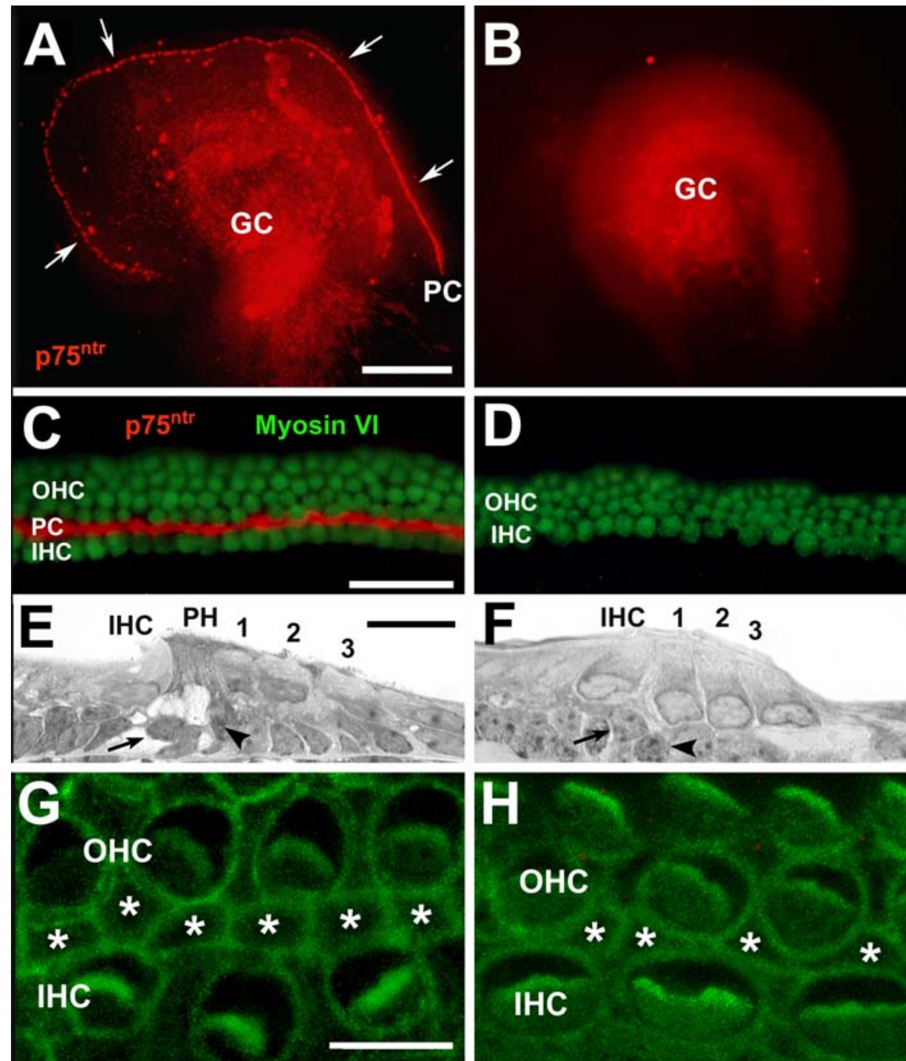


Figure 2.4. Inhibition of Fgfr3 disrupts development of pillar cells. A. Low magnification image of an E14.5 control culture after 5 DIV. Expression of p75^{ntr} is present in the pillar cells (PC, arrows) and spiral ganglion neurons (GC). B. Low magnification image of an E14.5 cochlear culture treated with 10 μM SU5402 beginning after 18 hours in vitro. p75^{ntr} expression is still present in the spiral ganglion neurons (GC), but no expression is detected in the region of the pillar cells. C. High magnification image of the apical surface of the sensory epithelium from an E13 control explant after 6 DIV. The row of pillar cells (red, PC) is located between the single row of inner hair cells (green, IHC) and the first row of outer hair cells (green, OHC). D. High magnification image of an E13 explant treated with 10 μM SU5402 beginning after 18 hours in vitro and maintained for a total of 6 DIV. Cells are labeled as in (C). Note the absence of pillar cell labeling and the close apposition of the row of inner hair cells and the first row of outer hair cells. E. Cross-section of the sensory epithelium from an E13 control explant after 6 DIV. A single inner hair cell (IHC), three outer hair cells (numbered) and single inner and outer pillar cells (arrow and arrowhead, respectively) are present. Note that a pillar head (PH) is present in the space between the inner and first row outer hair cells. F. Cross-section through the sensory epithelium from an E13 explant treated with 10 μM SU5402 beginning after 18 hours in vitro and maintained for a total of 6 DIV. A single inner hair cell (IHC) and three outer hair cells (numbered) are present. Two cell nuclei (arrow and arrowhead) are present in the region between the inner hair cell and first outer hair cell, however, no pillar head is present and neither of these cells appears to contact the lumenal surface. G. Apical surface of the sensory

epithelium from an E13 control explant after 6 DIV. Cell-cell junctions and stereociliary bundles have been stained with phalloidin. A row of pillar heads (asterisks) is present in the region between the row of inner hair cells (IHC) and first row of outer hair cells (OHC). H. Apical surface of the sensory epithelium from an E13 explant treated with 10 μM SU5402 beginning after 18 hours in vitro and maintained for a total of 6 DIV. Cell-cell junctions are labeled as in G. The separation between the single row of inner hair cells (IHC) and first row of outer hair cells (OHC) is noticeably decreased as compared with the control, however a limited number of apical projections (asterisks) are present in the region between the IHC and OHC. Scale bar in A (same in B), 200 μm, scale bar in C (same in D), 50 μm, scale bar in E (same in F), 10 μm, scale bar in G (same in H), 10.

(Figure 2.4F). To determine whether these cells extended apical process, cell-cell junctions at the surface of the organ of Corti were analyzed in control explants and explants that had been exposed to SU5402. In control explants, a row of roughly cuboidal pillar heads was present in the region between the row of inner hair cells and first row of outer hair cells (Figure 2.4G). In contrast, in explants exposed to SU5402 a clear row of pillar heads was not evident and, and as discussed, the distance between the inner and outer hair cells was decreased. However, a small number of apical projections were observed in the region between the inner and outer hair cells (Figure 2.4H), suggesting that some cells within this region did extend limited apical projections. These results are consistent with previous observations from *Fgfr3* mutant mice and support the hypothesis that Fgfr3 is necessary for pillar cell commitment and/or differentiation. In addition, since the overall phenotype in explants exposed to SU5402 appeared to match the phenotype in *Fgfr3* mutants, it seems likely that the effects of treatment with SU5402 in the developing organ of Corti are restricted to inhibition of Fgfr3.

Effects of SU5402 on pillar cell development are dependent on dosage

If Fgfr3 activation is required for the development of cells as pillar cells, then changes in the level of activity of Fgfr3 should lead to proportional changes in the number of cells that develop as pillar cells. In order to examine this hypothesis, cochlear explants were exposed to specific concentrations of SU5402 between 1 μM and 50 μM. As discussed, addition of 10 μM SU5402 lead to the loss of expression of $p75^{ntr}$ along the entire length of the sensory epithelium. In explants exposed to 5 μM SU5402, a 63% reduction (± 17%) in the length of the sensory epithelium with expression of $p75^{ntr}$ was

observed, while exposure to 1 μM SU5402 did not affect the number of cells that developed as pillar cells (Figure 2.5A). Exposure to SU5402 at concentrations greater than 10 μM resulted in general toxicity as determined by the observation of large scale death of multiple cell types including epithelial, neuronal and mesenchymal cells. Most cells appeared rounded and were no longer in contact with substrate. In addition, extensive cell debris was observed in many samples.

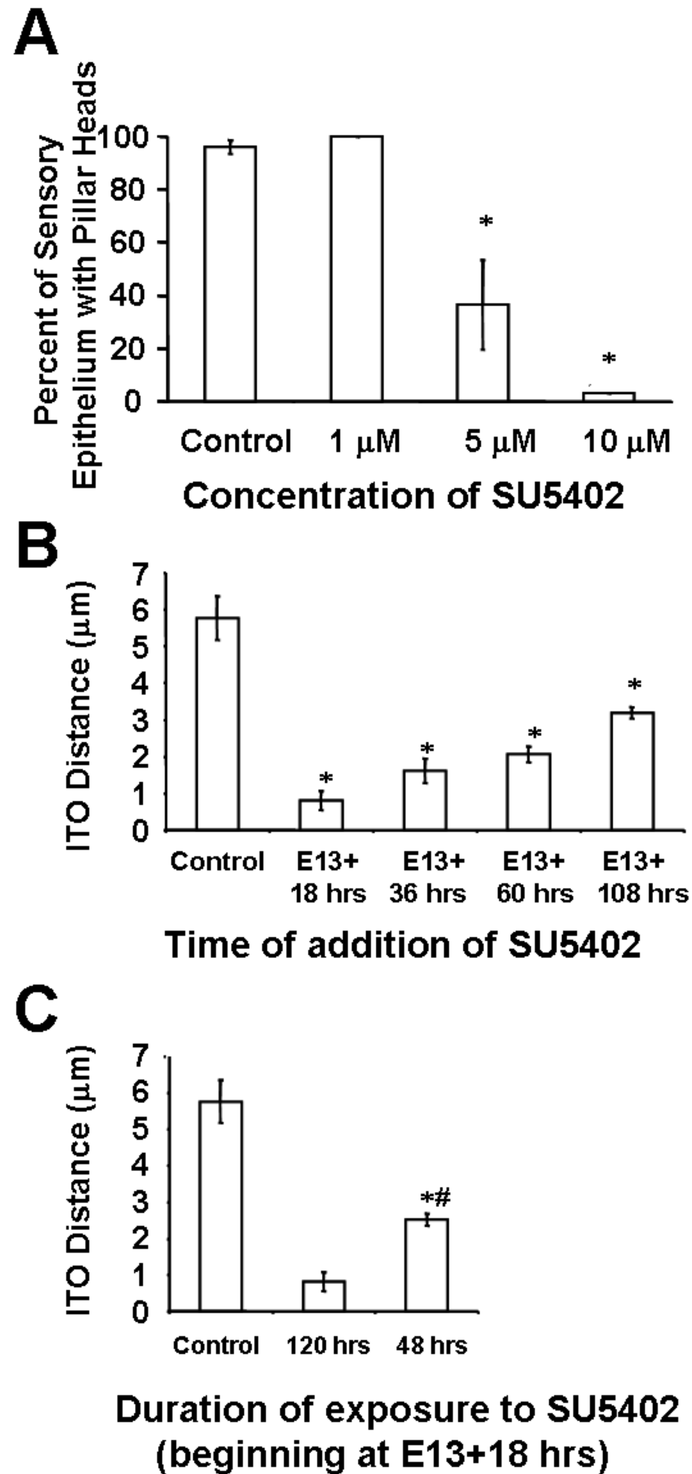
Exposure to SU5402 does not change the number of cells that develop as hair cells

Although two cells were consistently observed in the space between the inner and first row outer hair cells in explants treated with 10 μM SU5402, it seemed possible that progenitor cells that were inhibited from developing as pillar cells might develop as other cell types within the organ of Corti. To examine this possibility, the number of inner and outer hair cells was determined in both control explants and explants exposed to 10 μM SU5402. Results indicated no significant change in the number of inner or outer hair cells in cultures treated with SU5402 (Figure 2.6). This result suggests that inhibition of FGFR3 does not lead to changes in cell fate.

Fgfr3 regulates pillar cell differentiation

The results of the initial experiments in this study confirmed a requirement for Fgfr3 during pillar cell development. To determine whether there is a critical period for Fgfr3 activation during pillar cell development, cochlear explants were established from embryos at E13 as described. SU5402 was then added after 18 hours (equivalent to E14), 36 hours (equivalent to E15), or 60 hours (equivalent to E16) in vitro. All explants were

Figure 2.5



*α=0.001 from Control,
#α=0.001 from E13+18 hrs

Figure 2.5. Effects of SU5402 on pillar cell development are dependent on dose, duration, and time of addition. A. E13 cochlear explants were exposed to different doses of SU5402 for 6 days beginning after 18 hours in vitro. Results indicate a dose dependent decrease in the percentage of the sensory epithelium in which $p75^{ntr}$-positive pillar heads were present. B. E13 cochlear explants were exposed to 10 μM SU5402 beginning at the time points indicated. All explants were maintained for a total of 6 DIV. Exposure to SU5402 induced a significant change in the distance between the row of inner hair cells and the first row of outer hair cells (ITO distance) regardless of the time of addition. However, the effects of SU5402 were clearly dependent on the timing of addition, suggesting an ongoing requirement for activation of Fgfr3. C. E13 cochlear explants were exposed to 10 μM SU5402 beginning after 18 hours in vitro. Removal of SU5402 after 48 hours resulted in a significant increase in the ITO distance as compared with explants maintained in SU5402 for the entire culture period (120 hrs). All explants were maintained for a total of 6 DIV. Values for control and SU5402 exposure for 120 hrs are from the same experiment as in B. Error bars are standard error of the mean (SEM).

Figure 2.6

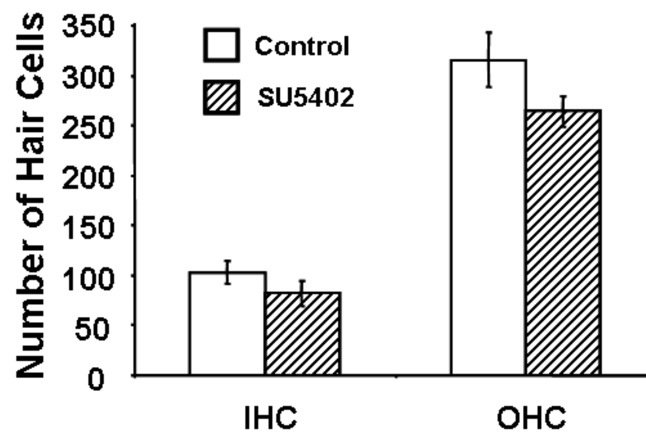


Figure 2.6. Exposure to SU5402 does not affect development of hair cells. The total number of hair cells in the basal 50% of the sensory epithelium was determined for control and SU5402-treated cochlear explants established on E13 and maintained for 6 DIV. Error bars are SEM.

maintained for a total of 6 DIV. Results indicated that expression of p75^{ntr} was disrupted along the length of the sensory epithelium regardless of the timing of addition of SU5402 (data not shown). These results suggest that activation of Fgfr3 is required for pillar cell development throughout most of the embryonic period.

To determine whether the effects of inhibition of SU5402 changed over developmental time, the distance between the row of inner hair cells and the first row of outer hair cells (referred to as the ITO (Inner To Outer) distance) was determined for explants from each time points listed above. In addition, a final time point, 108 hours (equivalent to E18) was also added. Previous morphological studies have demonstrated that the ITO distance progressively increases as pillar cells develop(Ito, Spicer et al. 1995; Kaltenbach and Falzarano 1997), therefore changes in the ITO distance should be reflective of changes in pillar cell development. In addition, since hair cell differentiation along the cochlear duct progresses in a gradient that begins in the mid-basal turn and extends both towards the apex and base, ITO distances for each experimental condition were determined at specific positions along the length of the sensory epithelium. Results for control explants indicated that after 6 DIV (P0 equivalent) the overall average ITO distance from positions located 10%, 20%, 30% and 40% from the basal end of the sensory epithelium was 5.77 μm (Figure 2.5B). Addition of SU5402 beginning after 18 hours in vitro resulted in a mean ITO distance of 0.81 μm (Figure 2.5B), a significant decrease from control. Addition of SU5402 after 36, 60 or 108 hours in vitro also resulted in significant decreases in the mean ITO distance (Figure 2.5B). However, the average change in ITO distance decreased progressively depending on the timing of

addition of SU5402. These results strongly suggest that continuous activation of Fgfr3 is required for ongoing pillar cell differentiation.

To determine whether inhibition of Fgfr3 results in a permanent disruption of pillar cell differentiation, cochlear explants established at E13 were maintained in SU5402 for 48 hours beginning after 18 hours in vitro. At the end of the 48-hour time period, SU5402 was washed out of the culture medium and the cultures were maintained for an additional 4 DIV (P0 equivalent) prior to fixation. Results indicated normal expression of $p75^{ntr}$ along the length of the sensory epithelium (data not shown). In addition, analysis of the mean ITO distance indicated a significant increase by comparison with explants that had been exposed to SU5402 continuously beginning after 18 hours in vitro (Figure 2.5C). These results demonstrate that transient inhibition of Fgfr3 results in a transient disruption of pillar cell differentiation. While the mean ITO distance in explants exposed to SU5402 for 48 hours were significantly greater than in continuously exposed cultures, the distances were approximately half of those in control explants, suggesting that removal of the Fgfr3 antagonist resulted in a resumption of pillar cell differentiation. However, the degree of decrease in ITO distance (approximately 50% by comparison with control) is consistent with a 48 hour inhibition, suggesting that pillar cell differentiation may proceed at a relatively constant rate.

Exogenous Fgf induces an increase in the number of cells that develop as pillar cells

To determine whether increased activation of Fgfr3 would also influence pillar cell development, cochlear explants were treated with exogenous Fgf2. Fgf2 was used because it has been shown to be a strong activator of Fgfr3c(Ornitz, Xu et al. 1996), the

Fgfr3 splice variant that is thought to be expressed in the developing cochlea(Pickles 2001). Analysis of explants established on E13 and exposed to 300 ng/ml Fgf2 for 6 DIV beginning after 18 hours in vitro indicated a marked increase in the number of pillar heads in the region between the row of inner hair cells and first row outer hair cells (Figure 2.7A, B). A similar increase in the expression of $p75^{ntr}$ was also observed in the pillar cell region (Figure 2.7C, D). The effects of Fgf2 were quantified by determining the average number of pillar heads and hair cells at specific positions along the basal half of the sensory epithelium. Results indicated that exposure to Fgf2 induced a significant increase in the number of pillar heads (Figure 2.8A). A significant increase in the number of inner hair cells was also observed (Figure 2.7B), however, the magnitude of this increase was considerably less by comparison with the change in the number of pillar heads (Figure 2.8A). Finally, the number of outer hair cells was unaffected in explants treated with Fgf2 and outer hair cells were separated from one another by supporting cells suggesting that there was no change in the number of Deiter's cells.

To determine whether changes in the number of pillar heads and inner hair cells were also dependent on the level and timing of activation of Fgfr3, the concentration and time of addition of Fgf2 were varied (Figure 2.8B, C). A concentration of 150ng/ml induced an increase of approximately 50% in the density of pillar heads, while explants exposed to 500 ng/ml developed almost six times as many pillar heads as controls (Figure 2.8B). In contrast, inner hair cells were not affected in explants exposed to 150 ng/ml Fgf2 but were significantly increased in explants treated with a concentration of 300 ng/ml or greater (Figure 2.8B). A graded decrease in the density of inner hair cells and pillar heads was observed when the addition of 300 ng/ml of Fgf2 was delayed by either

Figure 2.7

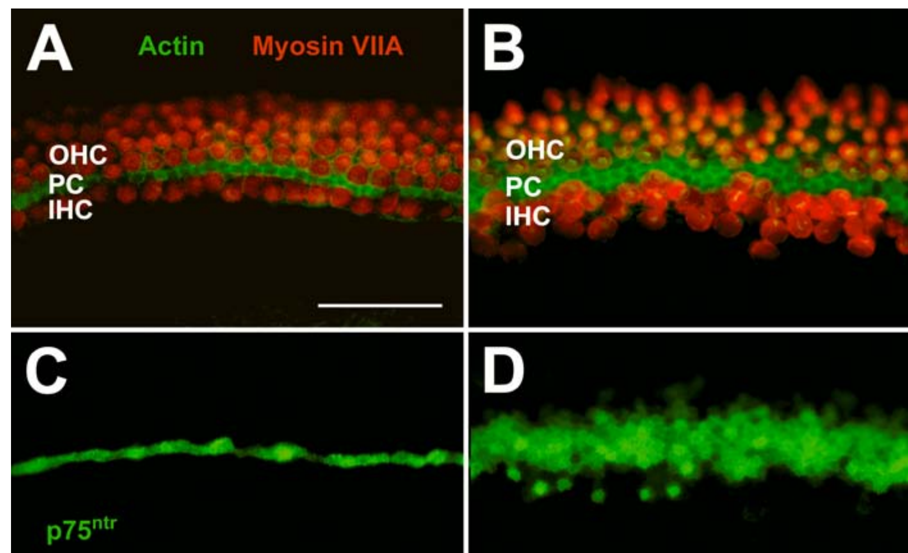
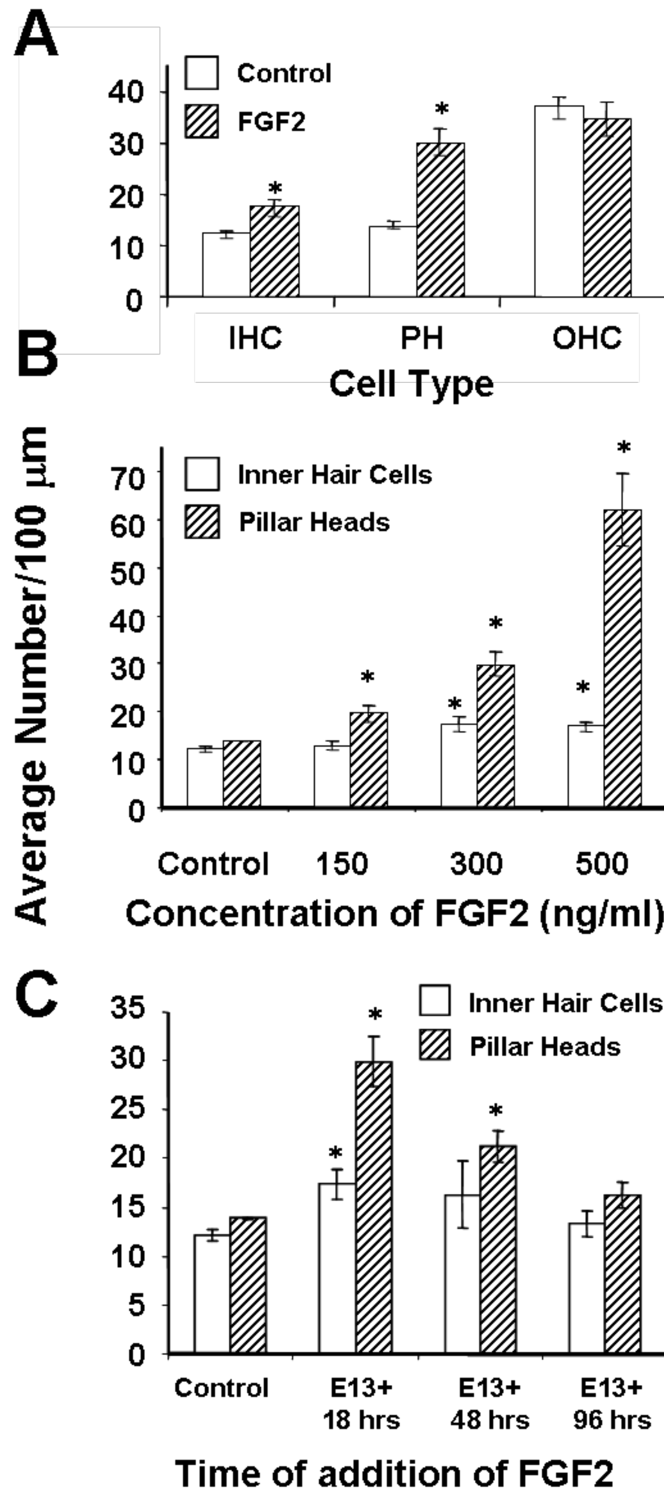


Figure 2.7. Exogenous treatment with Fgf induces an increase in the number of cells that develop as pillar cells. A. Lumenal surface of the organ of Corti from an E13 control explant after 6 DIV. Cell boundaries are labeled with phalloidin in green and hair cells are labeled with anti-myosin VIIA in red. A single row of pillar heads (PH) is present in the region between the single row of inner hair cells (IHC) and the first row of outer hair cells (OHC). B. Lumenal surface of the organ of Corti from an E13 explant exposed to 300 ng/ml of Fgf2 beginning after 18 hours in vitro and maintained for a total of 6 DIV. Labeling is as in A. There is a marked increase in the number of pillar heads (PH) in the region between the inner (IHC) and outer (OHC) hair cells. An increased number of inner hair cells are also present. C. Lumenal surface of the organ of Corti from an E13 control explant after 6 DIV. Pillar cells have been labeled with anti-$p75^{ntr}$. A single band of pillar heads is present. D. Lumenal surface of the organ of Corti from

an E13 explant exposed to 300 ng/ml of Fgf2 beginning after 18 hours in vitro and maintained for a total of 6 DIV. Labeling is as in D. There is a marked increase in the number of individual pillar heads. Scale bar in A (same in B, C, D), 50 μm.

Figure 2.8



* α=0.001

Figure 2.8. Treatment with Fgf2 induces an increase in the number of pillar cells and inner hair cells. A. The average number of pillar heads and inner and outer hair cells was determined at specific locations along the basal half of the sensory epithelium in E13 explants maintained in either control media or media containing 300 ng/ml Fgf2 for 6 DIV. Exposure to Fgf2 leads to an approximate doubling in the number of pillar heads. A significant increase in the number of inner hair cells was also observed, however the magnitude of this increase was considerably lower by comparison with the change in the number of pillar heads. Outer hair cell number was unchanged. IHC: Inner hair cells, PH: Pillar heads, OHC: Outer hair cells. B. Average number of inner hair cells and pillar heads in E13 explants exposed to different concentrations of Fgf2 for 6 DIV. Increased concentrations of Fgf2 lead to a graded increase in the average number of inner hair cells and pillar heads. C. Average number of inner hair cells and pillar heads in E13 explants exposed to 300 ng/ml FGF2 beginning on E14, E15 or E17 and maintained for a total of 6 DIV. The effects of FGF2 on both the number of inner hair cells and the number of pillar heads was dependent on time of administration. Significant increases in inner hair cells were only observed when FGF2 was added on E14. Pillar heads were significantly increased when FGF2 was added on E14 or E15. By E17, treatment with FGF2 did not induce a significant change in either the number of inner hair cells or the number of pillar heads. Error bars are SEM.

48 hours or 96 hours (Figure 2.8C). To determine whether the decreased effects of Fgf2 at later time points were a result of the maturation of the organ of Corti, the changes in the number of pillar cells and inner hair cells were determined for specific positions along the basal half of the sensory epithelium. Results indicated that the effects of addition of Fgf2 after either 48 or 96 hours in vitro were restricted to more apical positions along the sensory epithelium (data not shown). These results are consistent with the hypothesis that there is a critical period for the effects of the Fgf signaling pathway and that this period is related to the developmental progression of the organ of Corti.

The observation that treatment with Fgf2 induced an increase in both inner hair cells and pillar cells suggested that the effects of Fgf2 on pillar cell development could be mediated indirectly through the increased number of inner hair cells. To determine whether an increase in inner hair cells was required to induce an increase in the number of pillar cells, the effects of Fgf2 on inner hair cells and pillar heads were analyzed for different positions along the length of the sensory epithelium (Figure 2.9). Results indicated that Fgf2 induced a significant increase in the number of pillar heads at all 4 positions along the length of the sensory epithelium (Figure 2.9). In contrast, the number of inner hair cells was only significantly increased at the 30% and 40% positions (Figure 2.9). Therefore, at the 10% and 20% positions, Fgf2 induced a significant increase in the number of pillar heads independent of an increase in the number of inner hair cells. These results suggest that the Fgf signaling pathway may mediate multiple events during the development of the organ of Corti including the development of both inner hair cells and pillar cells.

Figure 2.9

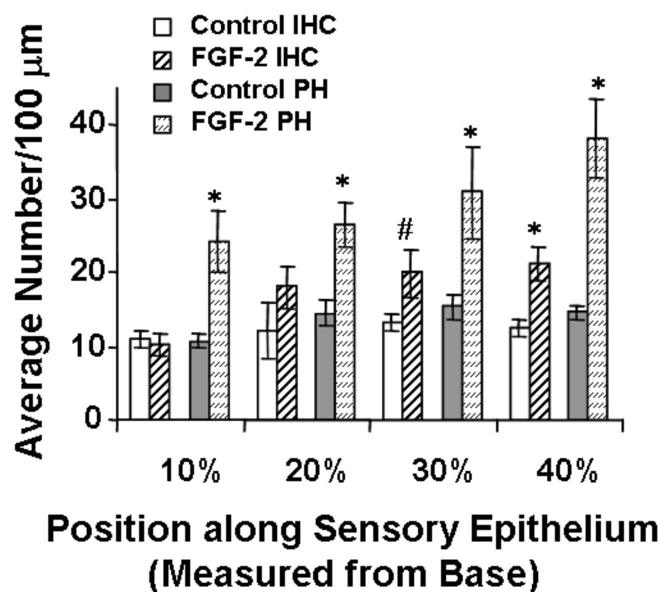


Figure 2.9. Effects of Fgf2 on pillar cell development are independent of changes in inner hair cells. Effects of exposure to 300 ng/ml Ffg2 on E13 explants after 6 DIV were determined at different positions along the length of the cochlea. Significant increases in the number of pillar heads were observed at all four locations. In contrast, inner hair cells were only increased significantly at the 30% and 40% positions. These results indicate that the increase in pillar heads is not dependent on an increase in inner hair cells. IHC: Inner hair cells, PH: Pillar cells, OHC: Outer hair cells.

Fgf2 does not induce proliferation within the developing organ of Corti

To determine whether the effects of Fgf2 on the production of supernumerary inner hair cells and pillar cells were dependent on mitotic proliferation, explant cultures were treated with 300 ng/ml of Fgf2 and maintained in culture media containing 3 μg/ml of BrdU. There was extensive incorporation of BrdU in the nuclei of fibroblasts and other cell types in both control and Fgf2-treated explants. However no BrdU labeling was observed in the nuclei of either inner hair cells or pillar cells in either control or Fgf2-treated cultures (data not shown).

Discussion

Fgfr3 is expressed in the embryonic cochlea

The results of previous studies have demonstrated that *Fgfr3* is expressed in the organ of Corti (Peters, Ornitz et al. 1993; Pirvola, Cao et al. 1995); however, the time course for expression of Fgfr3 protein during embryonic development had not been determined. Consistent with previous findings, the results presented demonstrate that Fgfr3 is initially expressed broadly in progenitor cells that will develop as pillar cells, outer hair cells and Deiter's cells. In contrast, by P0, Fgfr3 was only detected in inner and outer pillar cells, suggesting that activation of Fgfr3 may be required for maintenance of expression.

Fgfr3 activation is required for pillar cell differentiation

Deletion of *Fgfr3* was known to lead to defects in pillar cell development, but the specific role of Fgf signaling had not been determined (Colvin, Bohne et al. 1996). Two rows of cells were observed in the region between the row of inner hair cells and the first row of outer hair cells in *Fgfr3* mutants (Colvin, Bohne et al. 1996), however, whether these cells represented uncommitted progenitor cells or pillar cells that had become arrested in development was not clear. The results presented here support the hypothesis that activation of Fgfr3 is required for pillar cell differentiation. As in *Fgfr3* mutant mice, pillar cell development was disrupted in cochlear explants exposed to the Fgfr inhibitor, SU5402; however, the cells that would have developed as pillar cells did not undergo a change in fate, and instead remained in the epithelium in an apparently

undifferentiated state. Following removal of SU5402, these cells were able to resume their development as pillar cells, but ongoing activation of Fgfr3 was required for their continued differentiation. Fgfr3 has also been shown to control the rate of differentiation in developing lens fibers (Govindarajan and Overbeek 2001) and osteoblasts (Chen, Adar et al. 1999; Funato, Ohtani et al. 2001), suggesting that this may be a conserved function of this receptor.

It is important to consider that Fgfr3 may also play a role in the commitment of progenitor cells to the pillar cell fate. While inhibition of Fgfr3 did not lead to a change in the fates of the cells that would have developed as pillar cells, these cells may have remained in an uncommitted state until Fgfr3 activity was restored. As will be discussed, the effects of treatment with Fgf2 are consistent with a role for Fgf signaling in cell fate.

The Fgf signaling pathway regulates the number of cells that will develop as pillar cells

Treatment with Fgf2 lead to a dose dependent increase in the number of cells that developed as pillar cells, suggesting that Fgf signaling plays a key role in the regulation of pillar cell number. Moreover, the overproduction of pillar cells in response to exogenous Fgf2 demonstrates that the number of cells with the potential to develop as pillar cells is significantly greater than the number of cells that normally assume this fate. These results suggest that the abundance of ligands for Fgfr3 within the developing organ of Corti may be a limiting factor for the determination of cells as pillar cells.

The source of supernumerary pillar cells is not clear. Since BrdU-labeling did not indicate an increase in cellular proliferation, the most likely source of additional pillar

cells appears to be the population of Fgfr3 positive cells that is present in the cochlear duct at E16. As discussed, during normal development of the organ of Corti, the number of cells that express Fgfr3 at E16 is considerably larger than the number of cells that either maintain expression of Fgfr3 or that develop as pillar cells. Increased activation of Fgfr3 in these cells by addition of Fgf2 is apparently sufficient to increase the number of these cells that will become committed to develop as pillar cells.

The population of progenitor cells that expresses Fgfr3 at E16 appears to include cells that will develop as both pillar cells and hair cells(Peters, Ornitz et al. 1993) (data presented here, Figure 4.2). Based on this observation, a second expected outcome of exposure to Fgf2 might be a decrease in the number of cells that develop as outer hair cells. Surprisingly, the number of outer hair cells in explants exposed to Fgf2 did not differ from control. This result suggests that the increased number of pillar cells, treatment with Fgf2, or a combination of these factors, resulted in the recruitment of additional cells into the prosensory domain. In fact, there have been several reports suggesting that the position of the boundary of the prosensory domain at its outer hair cell edge may be variable. Supernumerary outer hair cells have been observed both in vivo and in vitro(Lavigne-Rebillard and Pujol 1986; Lavigne-Rebillard and Pujol 1987; Abdouh, Despres et al. 1993; Abdouh, Despres et al. 1994), and treatment with retinoic acid induces a significant increase in the number of cells that develop as outer hair cells presumably through increased recruitment(Kelley, Xu et al. 1993).

An alternative hypothesis for the source of supernumerary pillar cells could be related to the expression of $p75^{ntr}$ in this cell type. The results of recent experiments have demonstrated that $p75^{ntr}$ can act as a mediator of apoptotic cell death(Chao and Bothwell

2002) and therefore, it seems possible that cell death could play a role in the number of cells that develop as pillar cells. However preliminary results indicate that neither activation of p75^{ntr} by NGF or NT-3 or loss of p75^{ntr} activity by genetic manipulation results in a change in pillar cell number (B.E. Jacques and M.W. Kelley, unpublished results).

Fgf2 also induces an increase in inner hair cells

In addition to an increase in the number of pillar cells, a more limited increase in the number of inner hair cells was also observed in explants exposed to Fgf2. The basis for this effect is not clear. Fgfr3 is not expressed in the region of the cochlear duct that will develop as inner hair cells, suggesting that the effects of Fgf2 on inner hair cell number are probably regulated through a different Fgf receptor. One possibility would be Fgfr1, which has been reported to be expressed in the embryonic cochlear duct (U. Pirvola, personal communication) and would be activated by treatment with Fgf2 (Ornitz, Xu et al. 1996). However, treatment with SU5402, which antagonizes all Fgfrs (Mohammadi, McMahon et al. 1997), did not lead to a change in inner hair cell number suggesting that the Fgf signaling pathway is not required for inner hair cell development. This result raises the possibility that the effects of Fgf2 on inner hair cell development could be indirect. A potential source of this interaction could be the increased number of pillar cells. If reciprocal signaling interactions between inner hair cells and pillar cells play a role in regulating the ratio of inner hair cells to pillar cells, then an increase in the number of pillar cells could potentially lead to a subsequent increase in the number of inner hair cells. In fact decreases in the number of pillar cells have been reported in

response to the loss of inner hair cells in the Bronx waltzer mutant mouse (Tucker, Mackie et al. 1999). While this does not demonstrate a link in the ratio of the two cell types, it does support the potential for cell-cell interactions.

It is important to consider that the addition of a relatively high concentration of Fgf2, as compared with the physiological concentration, was required to elicit an effect on pillar cell development. The basis for this disparity is not clear. However previous results have suggested that the presence of the developing tectorial membrane(Rau, Legan et al. 1999) along with robust expression of different cadherins (Whitlon 1993) may inhibit the penetration of some molecules into the developing sensory epithelium (Zheng and Gao 2000). In addition, a number of endogenous inhibitors of Fgfr activation have recently been identified (Tefft, Lee et al. 1999; Wakioka, Sasaki et al. 2001; Furthauer, Lin et al. 2002; Tsang, Friesel et al. 2002) and the presence or some of these molecules within the developing cochlea could also play a role in modulating the effective concentration of Fgf2 within individual explants.

Fgf signaling and development of pillar cells

It is not clear which Fgfs might act as endogenous ligands for Fgfr3 in the developing cochlea. Previous reports (U. Pirvola, personal communication) and preliminary data from our laboratory indicate that Fgf8 is expressed specifically in developing inner hair cells, suggesting that it could act as an endogenous ligand for Fgfr3. As discussed, on E16, Fgfr3 is expressed in a band of cells that appears to correspond with the region of developing pillar cells and outer hair cells. Therefore, inner hair cells expressing Fgf8 would be located directly adjacent to the domain of Fgfr3

expression. Presumably, the effective concentration of Fgf8 would be limited to one or two cell diameters from each inner hair cell leading to activation of Fgfr3 and placement of the pillar cells adjacent to inner hair cells. It is not clear why the number of cells that initially express Fgfr3 is considerably larger than the number of cells that ultimately develop as pillar cells, however, the group of Fgfr3-positive cells could serve as a pool of potential pillar cells. This pool could represent a fail-safe mechanism to ensure pillar cell development.

The spatial expression of Fgf8 suggests that it is a candidate for the endogenous ligand for Fgfr3, however, existing data also suggests that other Fgfs are present in the epithelium as well. In particular, Fgf1 and Fgf3 have been reported to be present in the developing cochlea (Luo, Koutnouyan et al. 1993; Pickles 2001) although the specific cellular pattern of expression has not been determined. In addition, preliminary results from our laboratory indicate that Fgf17 is also present in the sensory epithelium and that treatment of cochlear explants with a mixture of Fgf8/17/18 leads to an increase in the number of pillar cells (K. Mueller, B. Jacques, M. Kelley, unpublished results). In contrast, Fgf2 is not expressed in the embryonic cochlea (Luo, Koutnouyan et al. 1993), suggesting that it is unlikely to be an endogenous ligand for Fgfr3.

In summary, the results presented here indicate that the number of cells that develop as pillar cells is regulated through the Fgf-signaling pathway, and specifically through activation of Fgfr3. It seems likely that developing inner hair cells express one or more Fgfs, leading to local activation of Fgfr3 in adjacent cells. Since the number of cells with the potential to develop as pillar cells is considerably greater than the normal

complement, we hypothesize that activation of Fgfr3 is limited to the cells located nearest to the developing inner hair cells.

Chapter 3: Fgf8 induces pillar cell fate and regulates cellular patterning in the mammalian cochlea

Abstract

The mammalian auditory sensory epithelium (the organ of Corti) contains a number of unique cell types that are arranged in ordered rows. Two of these cell types, inner and outer pillar cells, are arranged in adjacent rows that form a boundary between a single row of inner hair cells and three rows of outer hair cells. Pillar cells (PCs) are required for auditory function, as mice lacking PCs due to a mutation in *Fgfr3*, are deaf. Here, using in vitro and in vivo techniques, we demonstrate that an Fgf8 signal arising from the inner hair cells is the key component in an inductive pathway that regulates the number, position, and rate of development of PCs. Deletion of *Fgf8* or inhibition of binding between Fgf8 and Fgfr3 leads to defects in PC development whereas over-expression of Fgf8 or exogenous Fgfr3 activation induces ectopic PC formation and inhibits outer hair cell development. These results suggest that Fgf8/Fgfr3 interactions regulate cellular patterning within the OC through the induction of one cell fate (pillar cell) and simultaneous inhibition of an alternate fate (outer hair cell) in separate progenitor cells. Some of the effects of both inhibition and over-activation of the Fgf8-Fgfr3 signaling pathway are reversible, suggesting that PC differentiation is dependent upon constant activation of Fgfr3 by Fgf8. These results suggest that pillar cells may exist in a transient state of differentiation that makes them potential targets for the development of regenerative therapies.

Introduction

The sensory epithelium of the mammalian cochlea, the organ of Corti (OC), is comprised of at least six distinct cell types arranged into precise rows that extend along the entire length of the cochlear spiral. The OC contains four rows of hair cells (Figure 3.1A): three rows of outer hair cells (OHCs) supported by underlying Deiter's cells (DCs) and flanked on the lateral edge by a several rows of Hensen's cells (HeCs), and one row of inner hair cells (IHCs) with underlying phallangeal cells. Separating the two types of hair cells are parallel rows of non-sensory pillar cells (PCs) (Figure 3.1B). When mature, PCs form the boundaries of a triangular fluid-filled space referred to as the tunnel of Corti (Figure 3.1C) (Raphael and Altschuler 2003). The tunnel of Corti and the pillar cells that form this structure are unique to the mammalian auditory system and are found in no other vertebrate class. Defects in PC development result in significant hearing impairment (Colvin, Bohne et al. 1996).

Despite their crucial role in cochlear function, the factors that regulate pillar cell formation are poorly understood. Previous work has demonstrated that ongoing activation of one of the fibroblast growth factor receptors, Fgfr3, is required for pillar cell development (Colvin, Bohne et al. 1996; Mueller, Jacques et al. 2002). Ectopic activation of Fgfr3 in vitro by treatment with Fgf2 induces an over-production of PCs, suggesting that the relative level of ligand available for Fgfr3 activation plays a key role in regulating PC number and position within the OC (Mueller, Jacques et al. 2002). Fgfr3 is one of four related receptors that bind to members of the fibroblast growth factor family. All Fgf receptors are transmembrane proteins that contain a tyrosine kinase (TK)

Figure 3.1

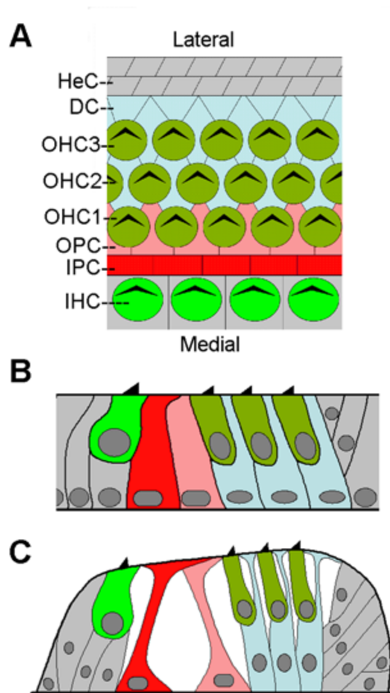


Figure 3.1. Anatomy of the organ of Corti. (A) Diagram of the lumenal surface of the organ of Corti at P0. A row of alternating inner hair cells (light green) and phalangeal cells (gray) are bordered by one row of inner PCs (red) and one row of outer PCs (pink). Outer PCs project between first-row outer hair cells (olive green). Second and third row OHCs are separated by Deiter's cells (blue). Hensen's cells (gray) form the lateral edge of the OC. (B) Cross-sections of the OC at P0 and adult (C), colors same as in A.

domain in their intracellular region. Fgfr activation is mediated through binding of one of at least 23 known Fgfs and a sulphated glycosaminoglycan such as heparin sulphate. Fgf ligand and heparin binding leads to receptor dimerization, cross-activation of the TK domains and down-stream signaling through the MAP kinase signaling pathway (Mohammadi, Olsen et al. 2005).

Within the developing cochlea, Fgfr3 is initially expressed around E16 in a broad pool of progenitor cells located directly adjacent to developing IHCs (Peters, Ornitz et al. 1993; Mueller, Jacques et al. 2002), the first cells to differentiate within the epithelium (Sobin and Anniko 1984). Based on the spatiotemporal pattern of expression, it seems likely that Fgfr3 is expressed in progenitors that will ultimately develop as pillar cells and OHCs, as well as Hensen's cells and Deiter's cells. As development proceeds, Fgfr3 is down-regulated in progenitors that develop as OHCs, HeCs and DCs but is maintained in PCs (Pirvola, Cao et al. 1995; Mueller, Jacques et al. 2002). RNA expression analysis using quantitative PCR has suggested that the *Fgfr3c* splice variant is the predominant isoform expressed in the cochlea (Pickles 2001). In addition, ligand binding assays indicate that the "c" isoform of Fgfr3 binds with the Fgf8b isoform with high affinity (MacArthur, Lawshe et al. 1995; Ornitz, Xu et al. 1996; Olsen, Li et al. 2006). The Fgf8b ligand has been shown to have important regulatory roles during pattern formation, differentiation, and cell growth throughout the developing embryo and nervous system (Olsen, Li et al. 2006). Quantitative RT-PCR analysis has indicated that it is expressed in the embryonic cochlear sensory epithelium (Pickles 2001). Here we demonstrate that *Fgf8* is expressed in a pattern that is consistent with an inductive role in PC development

and that changes in the levels of Fgf8, or in Fgfr3 activation, lead to corresponding changes in the number and differentiation of pillar cells.

Materials and methods

In situ hybridization

In situ hybridization (ISH) was performed as described previously (Wu and Oh 1996) for *Fgf8* and *Fgfr3* on 12 µm frozen sections or whole organs from cochleae isolated at E15, E16 and P0. A probe specific to exons 2 and 3 of *Fgf8* (the region excised by *Cre* in the *Fgf8*$^{\Delta 2,3n/flox}$; *FoxG1*$^{cre/+}$ mutants) was also used on E16-E18 cochleae from *Fgf8*$^{\Delta 2,3n/flox}$; *FoxG1*$^{cre/+}$ mutants and their wild type littermates to demonstrate excision of the targeted region.

*Gene*ration of *Fgf8*$^{\Delta 2,3n/flox}$; *FoxG1*$^{cre/+}$ mutants and analysis of pillar cell defects

Animals with a targeted deletion of *Fgf8* in the forebrain, retina and inner ear were generated by crossing *Fgf8*$^{flox/flox}$ females with *Fgf8*$^{\Delta 2,3n/+}$; *FoxG1*$^{cre/+}$ males. Mice carrying these alleles have been previously described (Meyers, Lewandoski et al. 1998); (Hebert and McConnell 2000). Mutant progeny of the genotype *Fgf8*$^{\Delta 2,3n/flox}$; *FoxG1*$^{cre/+}$ were visually identified based on obvious defects in the development of the forebrain (Storm, Rubenstein et al. 2003) Siblings were of the genotypes *Fgf8*$^{+/flox}$; *FoxG1*$^{cre/+}$, *Fgf8*$^{+/flox}$; *FoxG1*$^{+/+}$ or *Fgf8*$^{\Delta 2,3n/flox}$; *FoxG1*$^{+/+}$ and served as normal littermate controls.

Cochleae were dissected from mutants and littermate controls at E15.5, E16 and E19, and fixed in either 4% paraformaldehyde (PFA) or 3% glutaraldehyde/2% PFA overnight. Following fixation, the cochleae were dissected and the OC were exposed and labeled with cell type-specific antibodies: anti-Myosin VI (Proteus Biosciences) 1:1000, anti-p75^{ntr} (Chemicon) 1:1000, anti-β-actin (Sigma) 1:200. Secondary antibodies were

conjugated to one of the following: Alexa 350, Alexa 488, Alexa 546 or Alexa 633 (Molecular Probes). In addition, filamentous actin was labeled using phalloidin at 1:200 conjugated to either Alexa 488 or Alexa 633 (Molecular Probes). Specimens were then flat mounted and the total length of the cochlear duct was measured. The cochlea was then divided into four equal sections, each representing one fourth of the total length of the cochlear duct, and the distances between the inner hair cells and first row of outer hair cells (ITO distances) were determined in each region (n=5 animals; >50 cells counted per region). All animal care and procedures were approved by the Animal Care and Use Committee at NIH and complied with the NIH guidelines for care and use of animals.

Measurement of ITO distance

The Inner-to-Outer (ITO) distance is defined as the distance between the lateral edge of the IHC and the medial edge of the first row OHC. This is the distance encompassed by the inner pillar head. Digital images of the OC were captured for each sample using a Zeiss 510 LSM confocal laser scanning microscope. Measurements of ITO distances were taken at 3 specific points along the length of the cochlear duct of each sample, roughly at 25%, 50% and 75% of the distance from the most basal region and moving towards the apex. A minimum of 15 ITO measurements were made at each of the three locations. Cell counts were also taken of each cell type in the measured quadrants.

Histological Sections

Temporal bones from control and *Fgf8*$^{\Delta 2,3n/+}$; *FoxG1*$^{cre/+}$ littermates were fixed in 3% glutaraldehyde/2% paraformaldehyde, tissue was dehydrated in ethanol and then embedded in Immunobed (Polysciences Inc.). Cochleae were oriented to generate mid-modiolar sections, cut at 5μm thickness and stained with thionin.

Explant cultures

Explant cultures of embryonic cochleae were established as described previously (Montcouquiol and Kelley 2003) and maintained for 6 DIV. E13.5 explants were incubated for 24 hours before exposure to growth factors or antibodies that were diluted in culture medium to the stated final concentrations along with 0.1% DMSO and 1μg/μl of heparin. Anti-Fgf8b: 75-150 μg/ml; anti-Fgf5: 75-150 μg/ml; Fgf17 (all from R&D systems): 300ng/ml. Antibodies and proteins were used at 100 times the ND_{50} and ED_{50}, to ensure penetration through the reticular lamina, a strong ionic barrier that exists at the lumenal surface of the OC.

Electroporation

Full-length cDNA for murine *Fgf8b* was kindly provided by Elizabeth Grove {Fukuchi-Shimogori, 2001 #38}, University of Chicago. *Fgf8b* was excised from its original vector using BamH1 and then directly ligated into the *pAM/CAG-IRES_EGFP* vector at the BamH1 site. Orientation was determined by sequencing. Empty *pAM/CAG-IRES_EGFP* vector and *pAM/CAG-IRES_EGFP* containing full-length *Fgf8b* in the

reverse order were used as controls. Electroporation of cochlear explants was carried out as previously described (Jones et al, 2006); n>30 for each vector type.

Luminosity measurements

Images of electroporated explants were obtained using a Zeiss LSM510 confocal microscope. All samples were obtained during the same session using the same laser power and detection settings. To quantify the effects of over-expression of *Fgf8*, a rectangle with a long dimension of 225 μm and a short dimension of 110 μm was oriented such that the short dimension of the rectangle was parallel with the line of PCs in the region being measured. The rectangle was positioned so its strial edge aligned with third row OHCs. Thus, the rectangle included a 110 μm stretch of the OC with the adjacent region of the greater epithelial ridge containing transfected cells. Control and experimental regions were obtained and then thresholded for both green and red pixels. The total number of pixels of each color was then determined as a percentage of the total of number of pixels within the entire rectangle.

Results

Expression of *Fgf8* and F*gfr3* in the organ of Corti

Pillar cells develop on the medial edge of an *Fgfr3*-expression domain located directly adjacent to the inner hair cells. Given the crucial role of Fgfr3 in PC formation (Pirvola, Cao et al. 1995; Mueller, Jacques et al. 2002), it seemed likely that hair cells located adjacent to developing PCs might act as a source of ligand for Fgfr3. Furthermore, *Fgf8*, a high affinity ligand for Fgfr3, has been reported to be expressed by the IHCs of the adult cochlea (Pirvola, Ylikoski et al. 2002). To determine whether Fgf8 could play a role in PC development, the expression pattern for *Fgf8* in the embryonic cochlea was compared with that of *Fgfr3* by in situ hybridization. Cochlear development proceeds in a spatially conserved pattern in which cells in the basal region mature prior to those in the apical region, allowing one to visualize multiple developmental stages within the same ear (Sobin and Anniko 1984). No expression of *Fgf8* was observed between E13-E15 (data not shown) or at E16 in the less mature apex (Figure 3.2A). However, *Fgf8* expression was observed in a single cell in the more mature basal region at E16 (Figure 3.2B). *Fgfr3* expression was also first observed in the basal region of the cochlea at E16 in a group of cells that correlates with developing PCs, OHCs, HeCs and DCs (Figure 3.2D). Weak expression of *Fgfr3* was observed in the apex of the same E16 cochlea (Figure 3.2C), suggesting that its onset may slightly precede that of *Fgf8*. By P0, *Fgf8* expression is clearly present in the single IHC (Figure 3.2E, G) while expression of *Fgfr3* has become largely restricted to the developing PCs (Figure 3.2F). Some expression of *Fgfr3* persists in the developing DC and HeC region (Figure 3.2F),

Figure 3.2

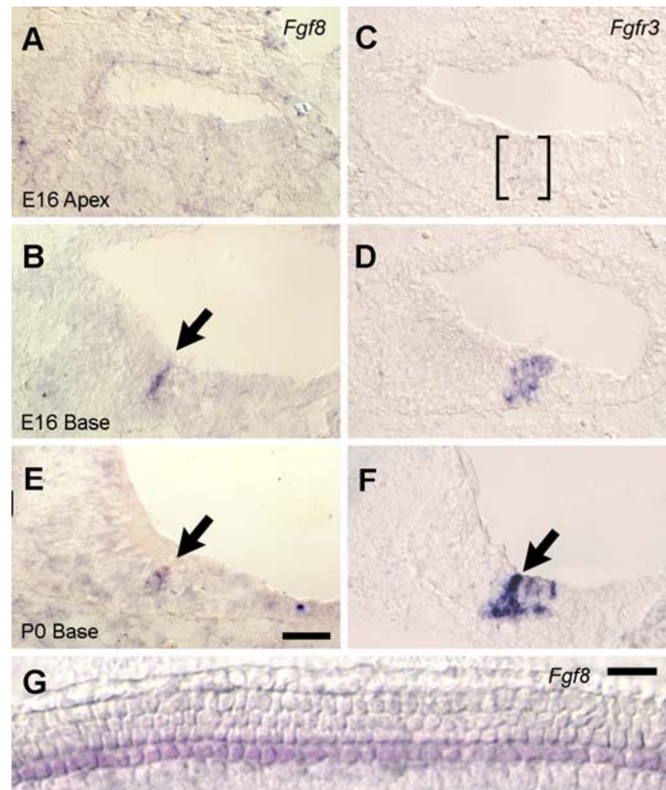


Figure 3.2. *Fgf8* and *Fgfr3* are expressed in the developing organ of Corti. In situ hybridizations for *Fgf8* and *Fgfr3* were performed at E16 (A-D) and P0 (E-G). *Fgf8* is not detected at E16 in the apical region (A) but is seen in developing IHCs at the base (B, arrow). (C) At E16 *Fgfr3* is faintly detected in a group of cells near the apex (indicated by brackets) and shows more robust expression within a similar region at the base (D). (E) Expression of *Fgf8* at P0 is in the IHC (arrow), while *Fgfr3* (F) is restricted to PCs (arrow) and DCs. (G) Whole-mount of the lumenal surface at P0 illustrating expression of *Fgf8* in all IHCs. Scale bar in A-F: 30 µm. Scale bar in G: 20 µm.

however based on immunolocalization, this expression appears to be down-regulated as development continues (Mueller, Jacques et al. 2002). These expression patterns demonstrate that the timing of the onset of *Fgf8* expression correlates strongly with the onset of *Fgfr3* expression and subsequent differentiation of PCs.

Deletion of *Fgf8* in vivo results in defects in pillar cell development

To determine whether Fgf8 is required for pillar cell development, a tissue-specific deletion of *Fgf8* was generated using a Cre-loxP strategy. Briefly, a floxed version of *Fgf8* ($Fgf8^{flox}$) (Meyers, Lewandoski et al. 1998) and the $FoxG1^{cre}$ line (Hebert and McConnell 2000) were used to delete *Fgf8* in a subset of tissues. It has been demonstrated that Cre-mediated excision of the second and third exons of the $Fgf8^{flox}$ allele results in complete inactivation (Meyers, Lewandoski et al. 1998). The *FoxG1* promoter induces expression of *Cre* in a relatively small number of tissues, including the developing otocyst, beginning at E8.5. By E9, the expression of *Cre* is strong in virtually all cells within the otocyst (Hebert and McConnell 2000), well before the normal onset of *Fgf8* expression.

Embryos that were $Fgf8^{\Delta 2,3n/flox}$; $FoxG1^{cre/+}$ (see Material and Methods for specific genetic cross) died at birth as a result of defects in the development of the forebrain (Storm, Rubenstein et al. 2003), however overall development of the inner ear and cochlea appeared normal. To examine the effects of inactivation of *Fgf8* on PC development, cochleae were obtained from mutants at E15 and E18.5. Consistent with the timing of the onset of *Fgf8,* there were no obvious differences in cellular patterning or in the expression of $p75^{ntr}$ (a marker that is co-expressed with Fgfr3 during cochlear

development) (Mueller, Jacques et al. 2002) between mutants and controls at E15 (data not shown). However, while development of inner and outer hair cells appeared normal at E18.5 (Figure 3.3A, 3B), there was a marked decrease in the size and number of PCs as well as a decrease in the overall levels of expression of p75^{ntr} both in the PCs and HeCs (Figure 3.3C, 3D). The effects of deletion of *Fgf8* were quantified by determining the distance between inner and outer hair cells, referred to as the ITO distance (Mueller, Jacques et al. 2002), at different positions along the basal-to-apical axis of the OC. The developing pillar heads expand as the cells differentiate and thus larger ITO distances are reflective of more advanced PC development (Mueller, Jacques et al. 2002). Deletion of *Fgf8* resulted in a significant decrease in ITO distances along the length of the cochlea (Figure 3.3G), similar to that seen in the *Fgfr3* knockout mice (Jacques, Puligilla, and Kelley, unpublished).

Rather than a complete absence of p75^{ntr}-positive PCs, as seen when Fgfr3 is pharmacologically inhibited (Colvin, Bohne et al. 1996; Mueller, Jacques et al. 2002), some p75^{ntr}-positive cells were clearly present in the PC space in *Fgf8*$^{\Delta 2,3n/flox}$; *FoxG1*$^{cre/+}$ mutants (Figure 3.3D). Therefore, semi-thin plastic cross-sections of the cochlea from *Fgf8*$^{\Delta 2,3n/flox}$; *FoxG1*$^{cre/+}$ mice were examined at E15 (n=4) and E18.5 (n=5). As expected based on the timing of onset of *Fgf8* expression, overall structure of the epithelium and putative developing PCs appeared normal in cross-sections from E15 (data not shown). In contrast, at E18.5, two cells were present in the region of the epithelium between the IHCs and OHCs (Figure 3.3E, 3F), but these cells had either weak lumenal projections, or no projections at all. In some sections, the IHCs and OHCs appeared to be in direct contact with one another (Figure 3.3F, magnified in inset with a red line to show the IHC

Figure 3.3

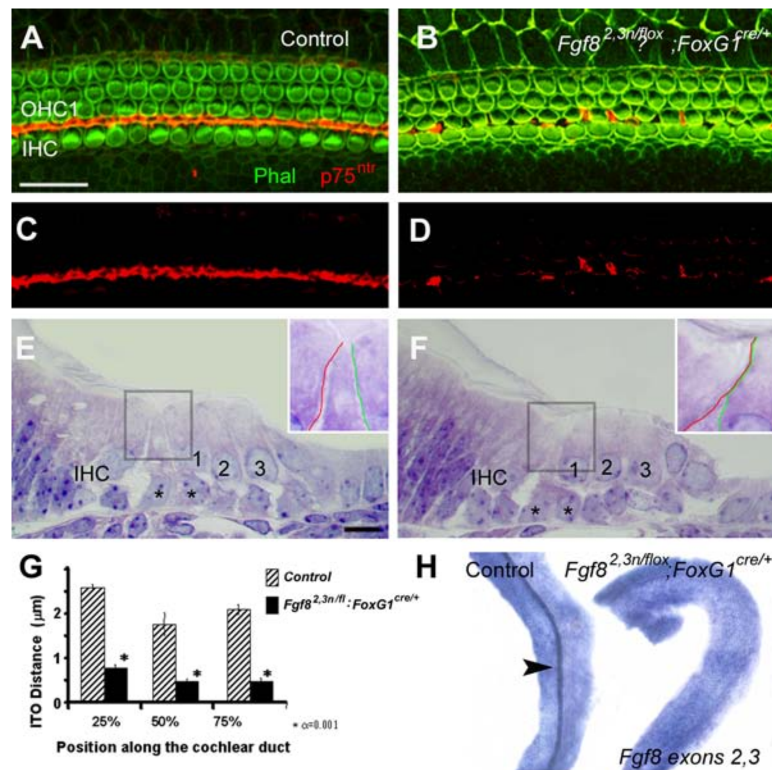


Figure 3.3. Targeted deletion of *Fgf8* leads to a disruption in pillar cell development. (A) Lumenal suface of the OC from a littermate control animal at E18.5. Hair cell stereocilia and cell boundaries labeled with phalloidin (Phal, green) and PCs labeled with anti-p75^{ntr} (red). The row of IHCs and first row of OHCs (OHC1) are indicated. (B) Lumenal surface from an *Fgf8*$^{\Delta 2,3n/flox}$; *FoxG1*$^{cre/+}$ mouse. Note the disrupted growth of the PCs and close approximation of the IHCs to OHCs. (C, D) Red channels from A and B, respectively, to illustrate PC morphology. (D) PCs are missing or under-developed in *Fgf8*$^{\Delta 2,3n/flox}$; *FoxG1*$^{cre/+}$ mice. (E) Cross-section through a control OC at E18.5 showing two PCs (asterisks) extending a lumenal projection between the

IHC and first row OHC (numbered). The morphology of the projection is illustrated in the magnified inset with a red line to indicate the lateral boundary of the IHC and a green line to indicate the medial boundary of the first row OHC. (F) Cross-section through an *Fgf8*$^{\Delta 2,3n/flox}$;*FoxG1*$^{cre/+}$ OC illustrating a stunted lumenal PC projection (magnified in inset). (G) Average ITO distances (see text for details), as a measure of the degree of PC development, in control and *Fgf8*$^{\Delta 2,3n/flox}$;*FoxG1*$^{cre/+}$ cochleae. Error bars indicate standard error of the mean. (H) In situ hybridization using a probe specific to the deleted region of *Fgf8* in control and *Fgf8*$^{\Delta 2,3n/flox}$;*FoxG1*$^{cre/+}$ cochleae. Arrowhead points to the row of labeled IHCs in the control. No similar labeling is present in the *Fgf8*$^{\Delta 2,3n/flox}$; *FoxG1*$^{cre/+}$ cochlea. Both cochleae have been intentionally over-reacted to ensure complete detection of Fgf8 expression. Scale bar in A-D: 20 μm. Scale bar in E-F: 10 μm.

boundary and a green line to show the OHC boundary). Pillar cells with weak or no lumenal projections were observed along the entire length of the cochlea with no region-specific variations. To ensure that *Cre*-mediated excision of *Fgf8* exons 2 and 3 was complete in these mutants, ISH was performed on $Fgf8^{\Delta2,3n/flox}$; $FoxG1^{cre/+}$ mutants and their control littermates (n>3 for each genotype analyzed) at ages E16-E18 using a probe generated from exons 2 and 3 of the *Fgf8* gene. Control and mutant cochleae were processed together and the colorization step was deliberately extended to ensure detection of any residual *Fgf8*. A single row of *Fgf8*-positive IHCs was clearly present in control cochleae, however no expression of *Fgf8* was observed in the mutant cochleae (Figure 3.3H). These results indicate a complete deletion of *Fgf8* in $Fgf8^{\Delta2,3n/flox}$; $FoxG1^{cre/+}$ mutants.

In vitro treatment with an anti-Fgf8 antibody results in complete inhibition of pillar cell development

The presence of some $p75^{ntr}$-positive inner pillar cells in $Fgf8^{\Delta2,3n/flox}$; $FoxG1^{cre/+}$ mutants, compared to the complete absence of these cells in cochlear explants in which Fgfr3 activation has been inhibited (Mueller, Jacques et al. 2002), suggest possible residual Fgfr3 activity in $Fgf8^{\Delta2,3n/flox}$; $FoxG1^{cre/+}$ mutants. This could be the result of functional compensation within the Fgf8-deficient mutant cochleae whereby another endogenously-expressed Fgf may bind to and activate Fgfr3 when no Fgf8 ligand is present. Therefore, we sought to inhibit Fgf8 signaling at the protein level by using an Fgf8-function-blocking antibody (R&D Systems) on cochlear explant cultures. As a control, similar explants were exposed to an antibody that specifically blocks the function

of Fgf5, a ligand not reported to be endogenously expressed within the OC. Explants were established on E13, exposed to anti-Fgf8 beginning after 24-36 hours, maintained for 4 to 6 days in vitro (DIV), then fixed and stained to examine PC development. Exposure to anti-Fgf8 resulted in a complete loss of $p75^{ntr}$ labeling, a lack of obvious pillar heads (Figure 3.4B) and ITO distances of nearly zero (Figure 3.4C) indicating a complete inhibition of PC development. This effect phenocopies that observed in explants exposed to the Fgfr antagonist SU5402 (Mueller, Jacques et al. 2002). In contrast, exposure to anti-Fgf5 had no obvious effect on PC development (Figure 3.4A, C). If addition of anti-Fgf8 was delayed until the equivalent of E17, a reduction in $p75^{ntr}$ staining and ITO distances was observed, however the magnitude of both was decreased compared to exposure with anti-Fgf8 for 6 DIV (data not shown). Similarly, a 72 hour transient exposure to anti-Fgf8 beginning at E14.5 resulted in a decrease in ITO distances that was consistent with a delay in the onset of PC differentiation (data not shown). Based on these results, there appears to be an ongoing requirement for Fgf8 throughout the five day period of embryonic PC development. The complete inhibition of pillar head formation in these explants, in contrast with a small residual development in $Fgf8^{\Delta 2,3n/flox}$; $FoxG1^{cre/+}$ mutants, suggests that either *Fgf8* was not completely deleted in the $Fgf8^{\Delta 2,3n/flox}$; $FoxG1^{cre/+}$ mutants or that the anti-Fgf8 antibody recognizes and inhibits other Fgfs within the epithelium that also activate Fgfr3. The first explanation seems less likely, considering that in situ hybridization indicated no expression of mRNA for *Fgf8* at E16.5. In contrast, *Fgf10* is expressed in the developing inner sulcus (Pauley, Wright et al. 2003), and preliminary results indicate that *Fgf17* is also expressed in the cochlea (Jaques, Mueller and Kelley, unpublished).

Figure 3.4

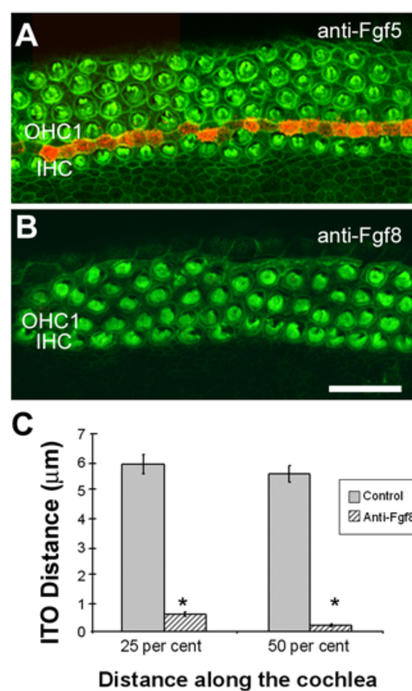


Figure 3.4. Inhibition of Fgf8 signaling disrupts pillar cell development. (A) A control cochlear explant that was treated with a function-blocking antibody directed against Fgf5. Stereocilia and cell boundaries are labeled with phalloidin (green) and PCs with anti-p75^{ntr} (red). IHCs and first row OHCs are indicated. (B) Cochlear explant treated with a function blocking antibody that binds to Fgf8 (labeling as in B). (C) ITO distances for control (anti-Fgf5) and anti-Fgf8-treated explants at basal and midbasal regions of the cochlear duct. Scale bar in B-C: 20 μm.

Ectopic Fgf8 expression results in over-expression of pillar cell markers

It has been shown that addition of exogenous Fgf2 results in the formation of additional rows of PCs (Mueller, Jacques et al. 2002), suggesting that the amount of Fgf within the epithelium could be a limiting factor in PC formation. Therefore, the effects of increased Fgf8 within the cochlea were determined by transfecting cochlear explants with an *Fgf8* expression vector containing *EGFP* as an independent transcript to identify transfected cells (Zheng and Gao 2000; Fukuchi-Shimogori and Grove 2003; Jones, Montcouquiol et al. 2006). For controls, explants were electroporated with a vector that expressed either *EGFP* alone or *EGFP* with *Fgf8* in the reverse orientation. Electroporated explants typically contained large clusters of transfected cells in Kolliker's organ, a population of epithelial cells located adjacent to the developing OC (Figure 3.5A, 5B). In control electroporated explants, no changes in PC number, as determined by expression of $p75^{ntr}$, were observed in regions of the OC located adjacent to large clusters of transfected cells (Figure 3.5A, C). In contrast, there was a marked increase in the number of $p75^{ntr}$-positive cells, and a decrease in the number of OHCs, in regions of the sensory epithelium located adjacent to large clusters of *Fgf8*-transfected cells (Figure 3.5B, D and data not shown). Pillar cells located at a distance from large clusters of *Fgf8*-expressing cells were unaffected, suggesting that Fgf8 has a limited diffusion radius within the cochlear epithelium (Figure 3.5B). However it is also possible that the gradient of Fgf8 is more rapidly decreased because of diffusion into the culture media.

Figure 3.5

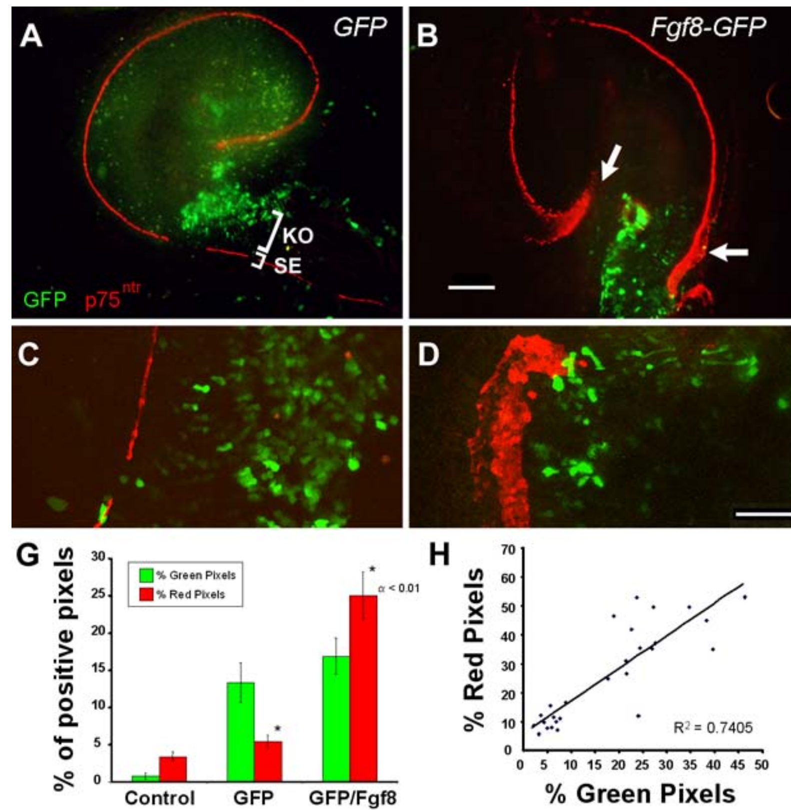


Figure 3.5. Over-expression of *Fgf8* induces an increase in p75^{ntr}-positive cells. (A) Low-magnification image of a cochlear explant transfected with a control vector expressing GFP (green). The sensory epithelium (SE) is indicated by expression of p75^{ntr} (red) in the PCs. Kolliker's organ (KO) is located medially to the SE. (B) Similar view as in A, from an explant transfected with an Fgf8/GFP-expressing vector (green). p75^{ntr}-positive PCs appear normal except in regions located near a cluster of Fgf8-transfected cells (arrows). (C) High-magnification view from a control with a large number of transfected cells. (D) High-magnification view of an Fgf8-transfected cochlea. Note the increased number of p75^{ntr}-positive cells. (G) Quantification of effects of expression of

GFP or Fgf8 (green pixels) on p75^{ntr} expression (red pixels, see text for details). Control data represents regions with no electroporation in comparison to regions with high GFP or Fgf8/GFP electroporation. Error bars indicate standard error of the mean. (H) A scatter plot of the relationship between the overall level of transfection (% Green Pixels) and the overall increase in p75^{ntr} expression (% Red Pixels). Best fit linear regression line and correlation coefficient (R^2) value are indicated. Scale bar in A-B: 100 μm. Scale bar in C-D: 20 μm.

To quantify the effects of over-expression of Fgf8 on p75^{ntr} expression, the relative level of p75^{ntr} (as a measure of number of PCs) was determined for measured sections of the sensory epithelium located adjacent to regions of Kolliker's organ that were comparably transfected with either the *Fgf8* or control vectors. As a further control, the level of p75^{ntr} in comparable sections of the sensory epithelium was also determined in untransfected explants, or in untransfected regions of transfected explants. Untransfected regions and regions transfected with the control plasmid had similar levels of expression of p75^{ntr}, indicating no effect of transfection, or of expression of EGFP, on PC development (Figure 3.5G). In contrast, there was a significant increase in the level of expression of p75^{ntr} in regions of the OC located adjacent to *Fgf8*-transfected cells (Figure 3.5G). To determine whether a direct correlation exists between the level of *Fgf8* transfection and an increase in p75^{ntr} expression, relative levels of p75^{ntr} were determined for sections of the OC located adjacent to regions of Kolliker's organ with variable levels of transfection. The results indicate a strong positive correlation between increasing levels of *Fgf8*-transfection and the number of p75^{ntr}-positive cells (R^2= 0.7405; Fig. 5H), consistent with a dose effect for Fgf8.

Ectopic activation of Fgfr3 increases the expression of pillar cell markers at the expense of outer hair cells

The results presented above are consistent with the hypothesis that an increased level of Fgfr3 activation leads to a greater number of Fgfr3-positive progenitors becoming committed to develop as PCs. Therefore, we sought to fully activate Fgfr3 throughout the developing OC by exposing cochlear explants to Fgf protein. Treatment

with Fgf8 protein had no apparent effect on the development of the OC. While the basis for this is unknown, we were able to obtain a strong effect with Fgf17 protein. To initially confirm that Fgf17 activates Fgfr3 in cochlear explants, explants were exposed to the anti-Fgf8 function blocking antibody and Fgf17 protein. The presence of Fgf17 was sufficient to rescue pillar cell development in these explants (data not shown).

Treatment with Fgf17 resulted in a conversion of the OHC region of the OC into a band of cells that were positive for $p75^{ntr}$ (Figure 3.6A-D). Absence of expression of the hair cell marker myosin VI (Figure 3.6E, F) and lack of stereocilia (Figure 3.6C, D) indicated that these $p75^{ntr}$–positive cells were inhibited from developing as OHCs. When a few OHCs were present in Fgf17-treated explants, each was surrounded by a group of $p75^{ntr}$-negative cells, suggesting that the presence of an OHC was sufficient to cause a local down-regulation of $p75^{ntr}$, even in the presence of Fgf17 (Figure 3.6G-I). The effects of Fgf17-treatment appeared to be restricted to the PC/OHC region as Myosin VI-positive IHCs were still present in all explants (data not shown). In addition, Hensen's cells located lateral to the OHC domain were also present in Fgf17-treated explants.

Considering that expression of $p75^{ntr}$ is a marker for undifferentiated cells at very early stages of OC development we wanted to determine whether the large number of $p75^{ntr}$-positive cells in Fgf17-treated explants represented induction of ectopic pillar cells, maintenance of undifferentiated cells, or both. β-actin is expressed strongly in PCs and more weakly in OHCs, DCs, and HeCs at P0 (Figure 3.8A-F) but is not expressed in any cell types within the OC prior to E16. Cells which are positive for both $p75^{ntr}$ and β-actin can thus be classified as differentiated PCs. In control explants (established on E13 and maintained for 7 DIV) only the cells located directly adjacent to the IHCs were positive

Figure 3.6

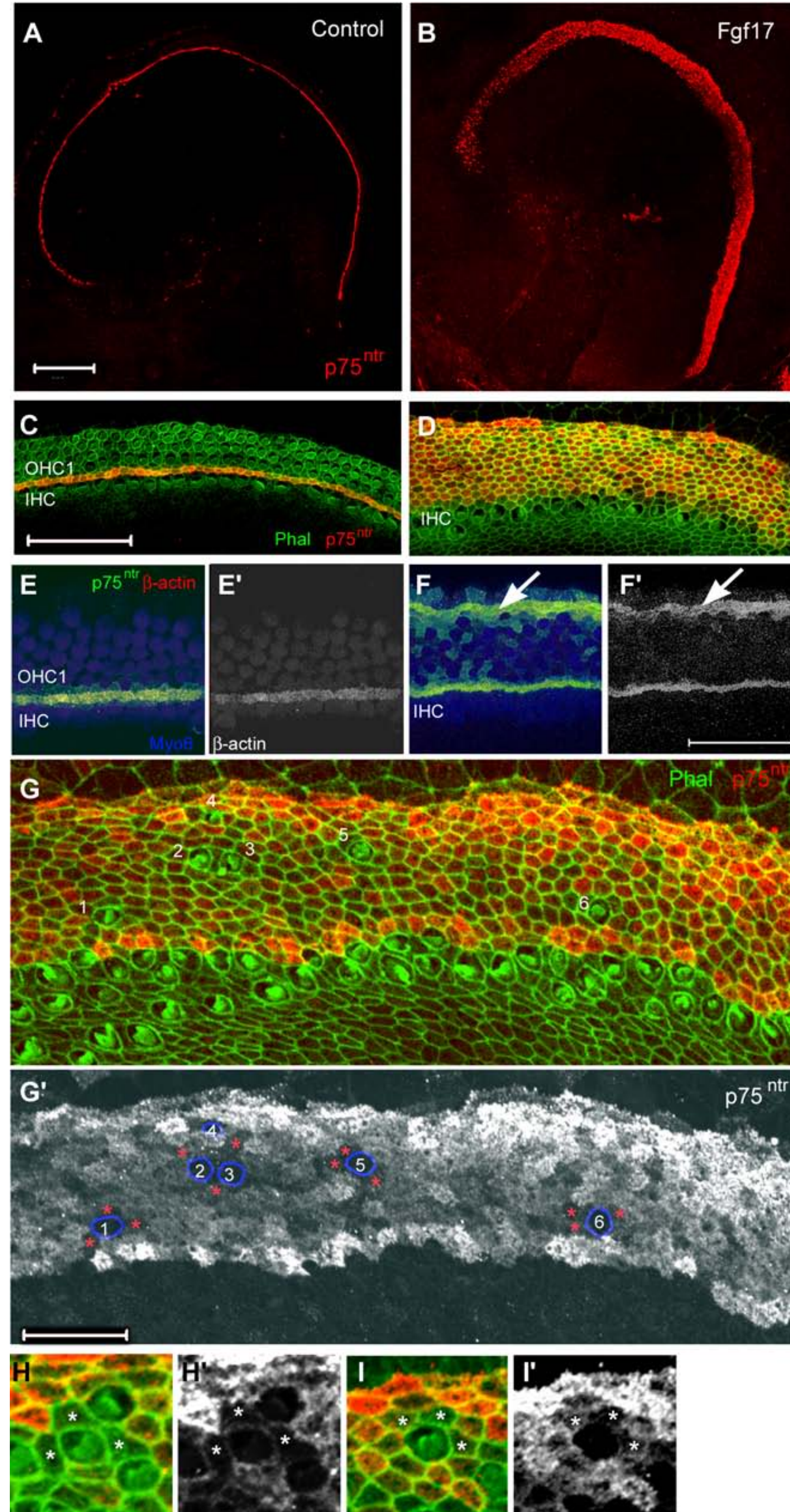


Figure 3.6. Fgf17 inhibits development of outer hair cells and Deiters cells. (A) Low-magnification view of p75^{ntr} expression (red) in a control explant and (B) in an explant treated with 300 ng/ml Fgf17 for 5 days. (C) High-magnification of a control OC. Cell surfaces and stereocilia labeled with 94halloidin (green), p75^{ntr}-positive PCs (red). (D) High-magnification of an Fgf17-treated explant, IHCs are present, but the OHC region appears undifferentiated and is positive for p75^{ntr} (red). (E-F') Lumenal views of the OC from E14 cochlear explants after 6 DIV. (E) PCs, positive for both p75^{ntr} (green) and β-actin (red), appear as a yellow line between IHCs and OHCs (Myo6 in blue). (E') β-actin alone. (F) OHC numbers are reduced and p75^{ntr} expression is increased (green) in the OHC region in Fgf17-treated explants. At the extreme lateral edge a second band of β-actin expression (red in F, arrow) appears. (F') β-actin alone. (G) An Fgf17-treated explant with few OHCs (numbered). Most cells in the OHC region are positive for p75^{ntr} (red). (G') p75^{ntr} expression alone. OHC positions are indicated with numbered circles. (H, I) High-magnification images of individual examples of OHCs from Fgf17-treated explants. Stereocilia and cell boundaries are indicated in green, p75^{ntr} is indicated in red. Asterisks indicate cells adjacent to OHCs that have down-regulated expression of p75^{ntr}. Scale bar in A,B: 200 μm. Scale bar in C,D: 50 μm. Scale bar in E-F': 20 μm. Scale bar in G, G': 25 μm. Scale bar in H-I': 20 μm.

for both p75^{ntr} and β-actin (Figure 3.6E). Treatment with Fgf17 resulted in a marked increase in the number of p75^{ntr}-positive cells with many located throughout the OHC region, however, expression of β-actin was restricted to the cells directly adjacent to the IHCs (Figure 3.6F, F') and to a band of cells located on the lateral edge of the OC, which normally develop as Hensen's cells (Figure 3.6F, arrows). The presence of strong p75^{ntr} expression in the absence of β-actin suggests that the effect of activation of Fgfr3 within the OHC region is to inhibit differentiation rather than to induce a PC fate. To confirm this, explants were treated with Fgf17 for 72 hours followed by a 72 hour recovery period. In contrast with continuous application of Fgf17(Figure 3.8G), the patterning of the OC developed normally in explants in which Fgfr3 had been transiently activated, however OHC development in these explants appeared to be delayed by approximately 72 hours based on the differentiation of OHCs in the second and third rows (Figure 3.8H, I). These results strongly suggest that activation of Fgfr3 inhibits progenitor cells from developing as hair cells.

To determine whether Fgfr3 activation also promotes PC commitment or differentiation, we examined the effects of treatment with Fgf17 on the development of endogenous PCs. In the presence of Fgf17, PCs developed more rapidly as indicated by larger pillar heads, wider foot plates (compare Figure 3.7A to B, and 3.7C to F), and a significant increase in ITO distances versus controls (Control, 3.985 ± 0.124 µm; Fgf17-Treated, 5.046 ± 0.202 µm, $\alpha=0.0004$).

Treatment with Fgf17 also induced a marked increase in the expression of β-actin (Figure 3.6F) and p75^{ntr} (Figure 3.7B) in a lateral band of cells that would normally develop as HeCs, suggesting that these cells might assume a PC fate in response to

Figure 3.7

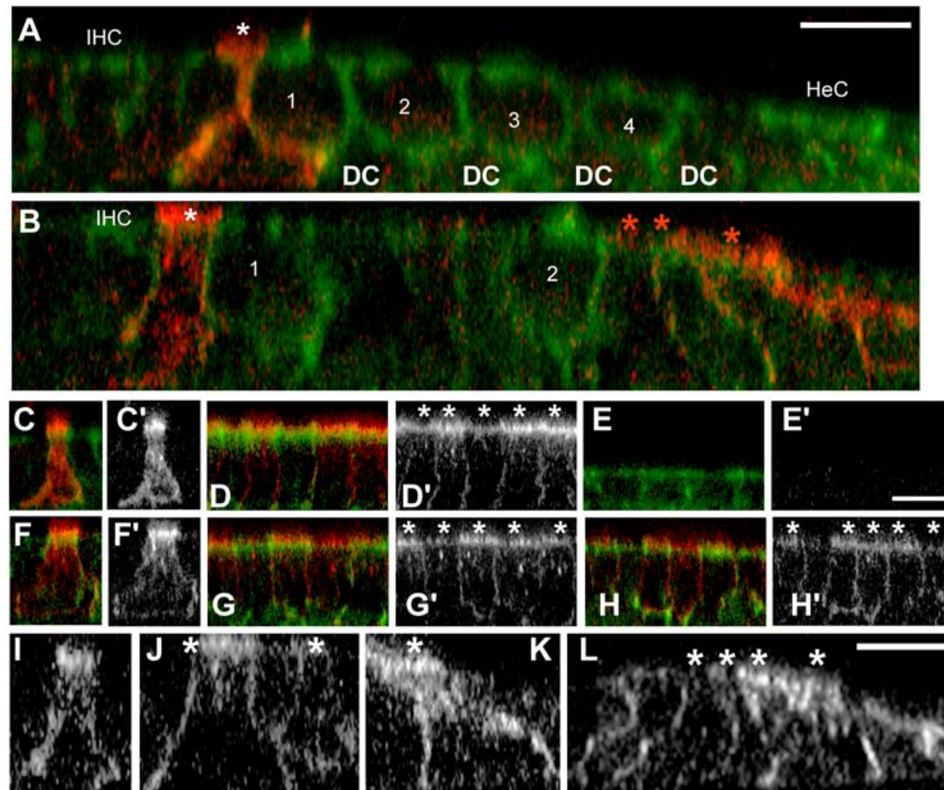


Figure 3.7. Fgf17 treatment promotes PC differentiation and induces ectopic PCs. Cross-sections were generated using confocal reconstruction. (A) Cross-section through a control explant. Cell boundaries labeled with phalloidin (green), developing PCs (red) and pillar head (asterisk); OHCs are numbered. HeCs located adjacent to OHC4 are short and have weak $p75^{ntr}$ expression. (B) An Fgf17-treated explant has a broader pillar head (asterisk), reduced OHC numbers, and three rows of PC-like cells in the HeC position (red asterisks). (C, C') High-magnification cross-section of an inner PC (red in C, and shown alone in C') from a control explant. The pillar head narrows as it approaches the lumenal surface. (D, D') Cross-section along the mediolateral plane illustrating the row

of inner PCs in a control explant. (E, E') Cross-section along the mediolateral plane illustrating the row of HeCs in the same control explant. Note the shortness of the HeCs and lack of $p75^{ntr}$ expression. (F) Cross-section through an inner PC from an Fgf17-treated explant. Note the increased width of the pillar head. (G, G') Cross-section of PCs from an Fgf17-treated explant, same view as in D. (H, H') Cross section of the HeC region from an Fgf17-treated explant. HeCs are taller, are strongly positive for $p75^{ntr}$ and are morphologically similar to PCs (compare with D and G). (I-K) Examples of PC (I, J) and HeC (K, L) morphology in control (I) and Fgf17-treated (J-L) explants. All images illustrate expression of $p75^{ntr}$. Note the increased thickness of the pillar head in J versus I and the similarity between endogenous PCs (I, J) and ectopic PCs located in the HeC region (K, L). Asterisks indicate pillar head-like structures. Scale bars in A-B, C-H, and I-L all 10 μm.

Figure 3.8

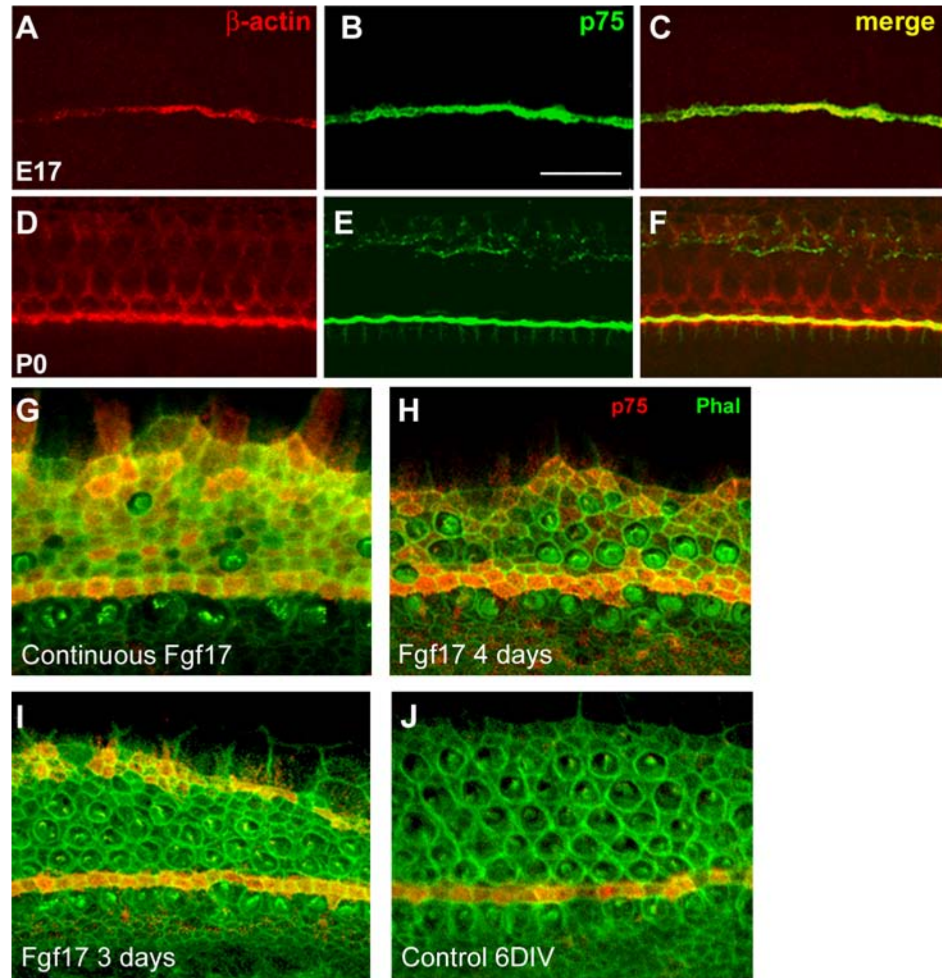


Figure 3.8. Expression of β-actin in the embryonic organ of Corti. Development of the organ of Corti after transient exposure to Fgf17. (A-C) β-actin (red) expression is first observed at E17 and initially co-localizes with $p75^{ntr}$ (green) in the pillar cells. (D-F) At P0, expression of β-actin is still present in the pillar cells but has also expanded to inclue Deiters' cells. Expression of $p75^{ntr}$ (green) is still largely restricted to pillar cells. Merge (F) illustrates strong overlap (yellow) in pillar cells. Scale bar in B, 20 mm. (G-J) Lumenal surfaces of E14 cochlear explant cultures maintained in either control media or in media containing Fgf17 for the times indicated. All explants were fixed after 6 DIV. Hair cell stereocilia and cell boundaries are illustrated in green and $p75^{ntr}$ is shown in red. (G) Explant treated with Fgf17 continuously. The number of OHCs is markedly reduced and there are high levels of $p75^{ntr}$ expression in the OHC region. (H) Explant treated for 4 days with Fgf17, followed by a 48 hour recovery period. First and second row OHCs have begun to develop. (I) Explant treated with Fgf17 for 3 days followed by a three day recovery period. Complete first and second rows of OHCs are present, and $p75^{ntr}$ expression is being down-regulated throughout the outer hair cell region. (J) A control explant maintained 6 DIV with no exposure to Fgf17. The normal pattern of IHCs, PCs and OHCs is present. $p75^{ntr}$ expression is restricted to PCs with low levels of expression in the Hensen's cells.

increased activation of Fgfr3. Confocal analysis indicated a marked change in these cells, including increased height, decreased width and maintenance of expression of $p75^{ntr}$ (Figure 3.7E, H). Many of these cells developed lumenal projections similar to those of PCs (Figure 3.7I-L). Thus it seems that increased activation of Fgfr3 induces progenitor cells that would have developed as HeCs to form as PCs instead.

Discussion

The tunnel of Corti and pillar cells are unique mammalian structures not found in the elongated cochleae of either birds or reptiles. Although the specific role of the tunnel has not been determined, the shape of the pillar cells and the position of the tunnel have led to the suggestion that it plays a role in the vibrational isolation of the inner hair cells. Since a tunnel is only present in the inner ears of mammals, it is generally assumed that the evolution of this structure occurred in response to selective pressures related to increased auditory acuity and perception of high frequencies. The results presented here demonstrate that the development and placement of pillar cells, and therefore of the tunnel of Corti, is dependent on an inductive interaction between Fgf8, expressed exclusively in inner hair cells, and Fgfr3, expressed in a domain of progenitor cells located directly adjacent to inner hair cells. Although Fgfr3 is expressed in progenitors that will develop as OHCs, Deiter's cells and Hensen's cells, as well as cells that will develop as pillar cells, the existing data suggest that the normal range of Fgf8-dependent activation of Fgfr3 is probably limited to the one or two progenitor cells located directly adjacent to the inner hair cell. In addition to the effects of ectopic activation of Fgfr3 by Fgf2(Mueller, Jacques et al. 2002), Fgf8 or Fgf17, deletion of *Sprouty-2*, an Fgf signaling pathway antagonist that is expressed in a similar domain to Fgfr3, results in an extra row of pillar cells(Shim, Minowada et al. 2005). These results are consistent with the hypotheses that normal Fgfr3 activation is limited to the cells located directly adjacent to the IHCs and changes in the spatial activation of Fgfr3, either through increased

availability of ligand or through decreased receptor antagonism results in defects in cellular patterning.

The results presented here coupled with previous findings suggest that Fgfr3 mediates two different aspects of the development of the OC. First, activation of Fgfr3 inhibits the differentiation of cells as outer hair cells. As discussed, the presence of pillar cells creates a disruption in the normal alternating cellular mosaic of hair cells and supporting cells. The developmental and evolutionary mechanisms that generate this disruption are unknown, but the data presented here suggest that inhibition of OHC formation through activation of Fgfr3 could represent an import aspect of this regulatory mechanism. This hypothesis is supported by the recent demonstration of an increase in the number of OHCs in Fgfr3 mutants (Hayashi, Cunningham et al. 2007); Puligilla et al., In Press). *Fgfr3* is also expressed in the developing chick basilar papilla (cochlea) but is down-regulated in developing hair cells (Bermingham-McDonogh, Stone et al. 2001). *Fgfr3* expression is maintained in basilar papilla supporting cells throughout life, and only becomes down-regulated during a regenerative response to hair cell loss(Bermingham-McDonogh, Stone et al. 2001). Once hair cells have been replaced, *Fgfr3* expression returns, suggesting that down regulation of Fgfr3 may be necessary to allow new hair cell formation.

The observation that Fgfr3 expression is maintained in avian supporting cells that retain the ability to develop as hair cells, suggests that expression of Fgfr3 may be an indicator of cells that retain a greater degree of developmental plasticity. Within the adult mammalian cochlea, *Fgfr3* expression is only retained in pillar cells(Pirvola, Cao et al. 1995), suggesting the possibility that these cells may retain a higher degree of cellular

plasticity and that ongoing activation of Fgfr3 may be required to maintain PCs in a differentiated state. This hypothesis is supported by the recent demonstration that, in comparison with other cells in the OC, pillar cells appear to possess a comparatively higher level of plasticity (Izumikawa, Minoda et al. 2005; Kiernan, Cordes et al. 2005; White, Doetzlhofer et al. 2006), and may even be able to differentiate into hair cells under some circumstances(White, Doetzlhofer et al. 2006). Based on these results, it seems possible that inhibition of Fgfr3 activation in mature pillar cells could cause these cells to revert to a less differentiated state. Under some circumstances, it might then be possible to induce these cells to adopt an alternative fate, such as differentiation as a hair cell.

The second role of Fgfr3 appears to be to specify the fate and/or subsequent differentiation of pillar cells. Deletion of either *Fgfr3* or its endogenous ligand *Fgf8* leads to a disruption in pillar cell differentiation, while increased availability of Fgfs enhances the pace of pillar cell differentiation. The induction of ectopic PCs is apparently restricted to a band of cells located on the lateral edge of the OC. The reasons for this restriction are unclear, however the position of these cells in somewhat similar to the position of the endogenous PCs in that they are located on a border of the OHC domain. Therefore, it seems possible that cells within the OHC domain might be prevented from developing as PCs. This hypothesis is supported by the observation that OHCs were capable of inducing a local decrease in p75^{ntr} even in the presence of Fgf17, suggesting that OHCs or OHC progenitors might exert a local influence that is not compatible with PC development. This type of interaction, along with the limited

expression of Fgf8, might play a role in ensuring that PCs only develop between the IHCs and first row OHCs.

The limited expression of Fgf8 in the single row of inner hair cells, along with the demonstrated roles of Fgfs in the development of PCs and OHCs, suggest that IHCs act as a global organizing center for the development of the OC. Cellular differentiation proceeds in a gradient that begins with the IHCs and moves laterally through the pillar cells and OHCs. A similar role for Fgf8 has been described during neurogenesis of the chick spinal cord. During development of the neural tube, Fgf8 signals arising from the caudal neural plate act to regulate the timing of neural development within the spinal cord by inhibiting differentiation {DiezdelCorral, 2002 #33; DiezdelCorral, 2003 #34}, thus maintaining a balance between neuronal and glial cell types. When the Fgf8 signal is removed or inhibited, precocious differentiation of spinal cord neurons is observed {DiezdelCorral, 2002 #33}. Based on the above results it seems likely that Fgf8 signaling may prolong the ability of pillar cells to switch fates and undergo mitosis. In addition, the expression of multiple *sprouty* genes within the DCs (Shim, Minowada et al. 2005) can act to inhibit Fgf8 signaling and thus also inhibit the ability of DCs to assume a more plastic role within the organ of Corti. However, the Hensen's cell region never expresses *sprouty* genes and thus, when exposed to ectopic Fgf8 or Fgf17, can develop as PCs.

Thus, it seems likely that Fgf8 expression by IHCs acts to organize the next step in cellular patterning by inducing the development of pillar cells and simultaneously preventing the development of hair cells in directly adjacent cells. Following this induction, subsequent signaling interactions must play a role in specifying and patterning the OHCs and associated Deiter's cells. Whether these events are regulated through long

range signals generated by IHCs or as a result of shorter range signals originating in pillar cells remains to be determined.

Chapter 4: Fgf signaling regulates development and transdifferentiation of hair cells and support cells in the basilar papilla

Abstract

The avian basilar papilla (BP) is a homolog of the mammalian cochlea's auditory sensory epithelium, the organ of Corti. Fibroblast growth factor receptor-3 is known to regulate the differentiation of hair cells and support cells during mammalian development. Fgfr3 is expressed in developing support cells of both the BP and the organ of Corti however its role in BP development in unknown. We utilize an *in vitro* whole organ embryonic culture system to look at the role Fgfr3 plays in the developing avian cochlea. SU5402 (an antagonist of Fgfr signaling) is applied to developing and mature BP cultures and the effects it has on HC growth is assayed. Similar to blocking the *Fgfr3* pathway in mice, SU5402 causes an increase in the amount of HCs seen in the BP, measured as a function of density. With this treatment new young hair cells can be seen intermixed with older more mature hair cells. Lack of any BrdU-positive HCs suggests that the Fgf pathway is involved in the direct conversion of support cells into hair cells, a process known as transdifferentiation, rather than having a mitotic role. Given this role, it suggests that Fgfr3 is a likely candidate to play a central role in the regeneration of hair cells. Here we demonstrate for the first time a signaling pathway which regulates the conversion of support cells into hair cells; Fgfr signaling is needed to maintain the support cell phenotype and inhibit the formation of hair cells. When this signaling pathway is interrupted, inhibition is lifted and SCs are converted into hair cells via direct

transdifferentiation. Recent research has pointed to a missing pathway that acts complementary too and probably upstream of the Notch pathway during regeneration. We suggest that this is the Fgfr3 pathway. This is the first description of a signaling pathway shown to directly regulate the conversion of support cells into hair cells in the basilar papilla.

Introduction

The avian auditory periphery is comprised of the auditory portion of the cochleovestibular ganglion and an elongated mechanosensory epithelium, the basilar papilla (BP). The basilar papilla is an elongated cochlea that projects medially from the external auditory meatus. While mostly straight, there is a noticeable curve to the cochlear duct (Figure 4.1C,E), the superior outer edge is the neural edge as that is where the auditory neurons are clustered, while the inferior edge is referred to as abneural (Figure 4.1E).

The primary detectors of incoming sound are mechanosensory hair cells (HCs); they are arranged across the surface of the cochlear sensory epithelium. Hair cells have bundles of stereocilia on their lumenal surfaces and these bundles are embedded in the mesh of an overlying tectorial membrane. The nuclei of the hair cells sit atop a group of uniform supportive cells whose bases form contacts with a basilar membrane below (Figure 4.1D) and whose apical surfaces articulate between the hair cells at the surface of the epithelium. When sound enters the BP, it causes a flexion of the basilar membrane (BM) creating a shearing force that causes the hair bundles to deflect. Deflection opens spring-gated ion channels at the tips of the stereocilia which depolarizes the cell and triggers an electrical response.

In the BP there are two basic types of hair cells, tall and short, referring to both the height of the stereocilia bundle as well as the length of the hair cell's body. Tall hair cells are arranged along the superior edge, while short hair cells are located on the inferior edge (Figure 4.1D,E), however a gradient of hair cell types exists between the

Figure 4.1

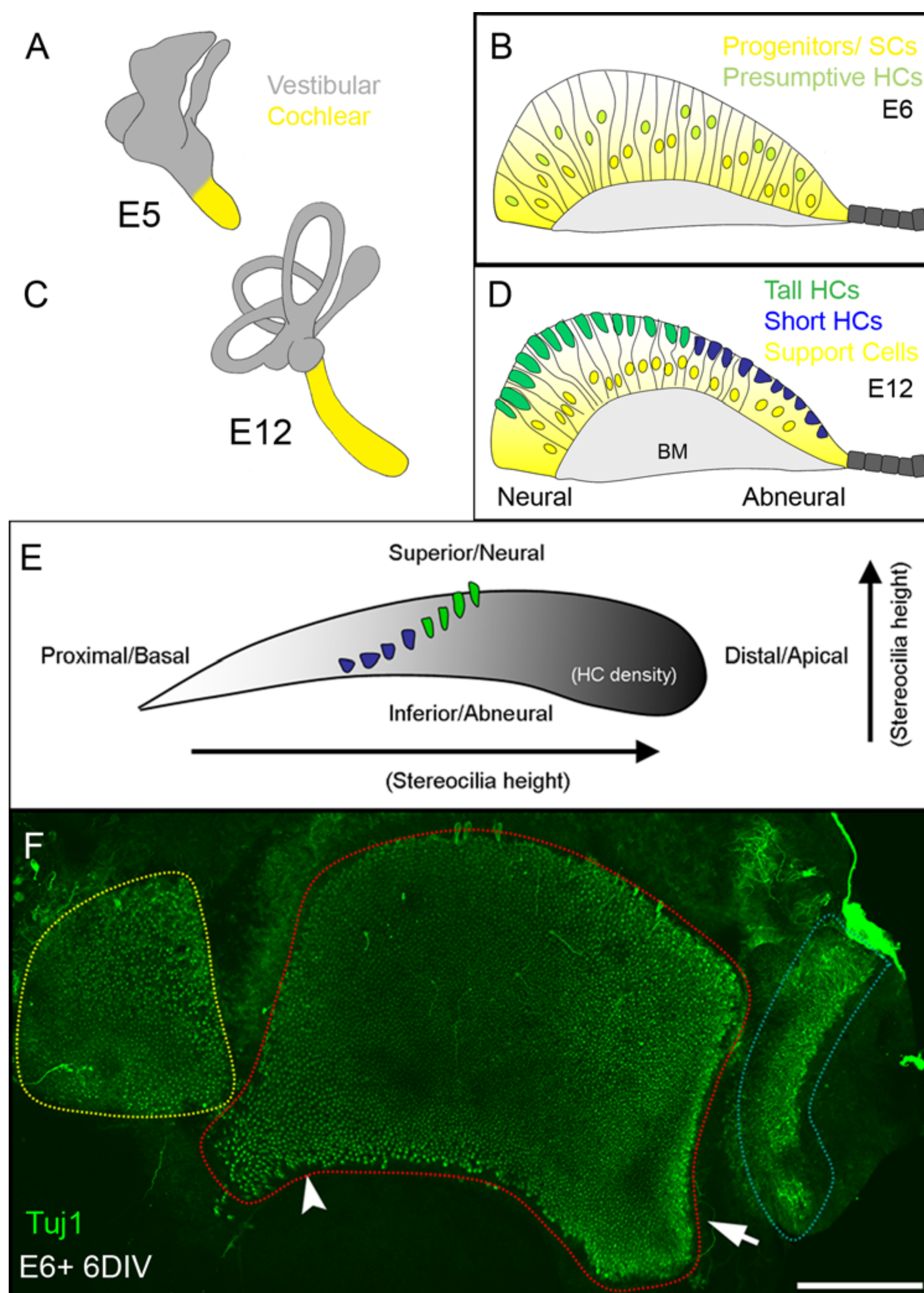


Figure 4.1. Structure and development of the basilar papilla. The gross morphology of the developing avian cochlea at E5 (A) and E12 (B) demonstrates the elongation of the BP during development, the cochlear regions are highlighted in yellow. Schematics of cross sections through the E6 (B) and E12 (D) BP show the delamination of HCs early in development from a large progenitor pool (B, light green) and differentiation of Tall HCs (D, green) and Short HCs (D, blue) positioned above the support cells (B, D, yellow). (E) This schematic demonstrates the two axes of the BP, shading represents the gradient of HC density (darker regions are higher density) the positioning of the different types of HCs across the superior/inferior axis is shown. A basilar papilla from an E6 chicken embryo plated onto a Millipore tissue-tek membrane and maintained for 6 DIV develops relatively normal cellular morphology and patterning (F). The three sensory organs derived from the ventral out-pocketing of the otocyst (A, yellow) can be seen in culture and are labeled for expression of Tuj1 (F, green). The saccule (F, yellow outline), the basilar papilla (F, red outline) and the lagena (F, blue outline). There is a normal distribution of lower HC density in the base (arrow head) and higher HC density in the apical regions (arrow). Scale bar is 200 um.

two extremes. There is also a noticeable difference in distribution of hair cells across the proximal distal axis, with a lower density of hair cells in the proximal region and a higher density in the distal region (Figure 4.1E,F)

During development hair cells begin to differentiate around E6, just proceeding the out-pocketing of the cochlear duct from the otocyst at E5 (Figure 4.1A). Hair cells and support cells are derived from the same progenitor pool of cells that initially have projections that extend from the basal to lumenal surface of the epithelium(Fekete, Muthukumar et al. 1998) (Figure 4.1B). As hair cells differentiate, they delaminate from the basilar membrane and their nuclei shift upward(Daudet and Lewis 2005) above the nuclei of the support cells which remain situated more basally.

The basilar papilla and the mammalian auditory sensory epithelium, the organ of Corti, are considered to be homologous and as such, share many key features (Smith and Takasaka 1971). In particular they both have an elongated cochlear duct with tonotopically organized hair cells, in each cochlea hair cells and support cells are derived from a common progenitor pool(Fekete, Muthukumar et al. 1998) and a similar developmental gradient proceeding along the proximal-to-distal axis(Katayama and Corwin 1989) are among the most striking commonalities between these structures. One notable difference, however, is that mammals are much more susceptible to hearing loss than birds because they lack the ability to regenerate auditory hair cells that are killed by acoustic or ototoxic trauma (reviewed by (Matsui, Parker et al. 2005).

The basilar papilla is able to regenerate hair cells and restore normal hearing function following hair cell loss (Cotanche 1987; Corwin and Cotanche 1988). New hair cells are formed by one of two processes. A support cell can directly transdifferentiate

into a hair cell through up-regulation of *Atoh1* and other hair cell factors (Cotanche, Lee et al. 1994; Cotanche 1997; Stone, Shang et al. 2004) or new hair cells can form via a mitotic route whereby a support cell first undergoes a round of division after which one or both daughter cells can either become hair cells or new support cells(Cotanche, Lee et al. 1994; Stone, Shang et al. 2004). What remains to be known are the factors and pathways that regulate the conversion of support cells into hair cells. It is predicted that HC formation during regeneration occurs in the same way that HCs are formed during development, and recent evidence has shown that at least a few aspects of HC development are recapitulated during regeneration(Levic, Nie et al. 2007; Stone and Cotanche 2007). Thus, fully understanding how hair cells and support cells are formed and which genes are responsible for their differentiation from a common precursor pool will provide much insight into the process of regeneration.

During development of the mammalian organ of Corti, fibroblast growth factor signaling has been shown to regulate the differentiation of hair cells and support cells from a common progenitor pool(Mueller, Jacques et al. 2002; Hayashi, Cunningham et al. 2007; Jacques, Montcouquiol et al. 2007; Puligilla, Feng et al. 2007). Fibroblast growth factor receptor-3 (*Fgfr3*) is initially expressed in a broad pool of cells found adjacent to the developing inner hair cells at E15.5 beginning in the base of the cochlea. As development proceeds, Fgfr3 is down-regulated in developing hair cells but is maintained in pillar cells and Deiters' cells, two specific types of support cells unique to the organ of Corti. By birth, *Fgfr3* expression is lost from all cells except the pillar cells (Mueller, Jacques et al. 2002). If Fgfr3 function is inhibited, either *in vivo* through genetic deletion of *Fgfr3* (Puligilla, Feng et al. 2007; Hayashi, Ray et al. 2008) or *in vitro*

using SU5402, a pharmacological inhibitor of Fgfr signaling, an extra row of outer hair cells forms while the number of pillar cells is decreased and remaining pillar cells fail to develop a mature phenotype (Mueller, Jacques et al. 2002). If Fgfr3 is ectopically activated *in vitro* using Fgf17, Fgf8 or Fgf2, outer hair cells fail to form resulting in ectopic Deiter's cells and pillar cells (Jacques, Montcouquiol et al. 2007). Interestingly, if Fgfr3 is transiently activated for a few days followed by a recovery period, a normal complement of outer hair cells and pillar cells develops (Jacques, Montcouquiol et al. 2007). These data suggest that activated Fgfr3 prevents cells from developing as hair cells while at the same time promoting the differentiation of supporting cells.

The pattern of *fgfr3* expression in the developing and regenerating avian basilar papilla largely parallels the pattern seen in the mammalian cochlea. All precursor cells initially express *fgfr3* at high levels at E5.5 (Bermingham-McDonogh, Stone et al. 2001), it then becomes down-regulated in those cells that will develop as hair cells between E6 and E8. In contrast with the mammalian cochlea, *fgfr3* expression is maintained by all support cells of the BP throughout life. The only exception is during regeneration at which point *fgfr3* is down-regulated by those support cells that are undergoing transdifferentiation into hair cells (Bermingham-McDonogh, Stone et al. 2001). Once regeneration is complete, expression of *fgfr3* returns in all supporting cells. These results are consistent with the hypothesis that Fgfr3 acts to block the development of cochlear progenitor cells as hair cells. The aim of this study is to understand if *fgfr3* is responsible for the differentiation of hair cells and support cells as in the mammalian cochlea and to determine if it also plays a role in the transdifferentiation of support cells.

Materials and Methods

Animals

Fertilized *Gallus gallus* eggs were obtained from CBT Farms (Chestertown, Maryland) at zero, three or six days post-fertilization and incubated until stages E5-E8. Embryos were hatched from their eggs, developmentally staged then decapitated prior to cochlear dissection. Protocols for animal use were approved by the University of Maryland and the NIH ACUC.

Basilar papilla cultures

E5-E8 stage chick embryos were obtained and the sensory epithelia were dissected in HBSS as follows. The cartilaginous capsule surrounding the duct of the basilar papilla is removed, after which the tegmentum vasculosum is peeled away to expose the sensory epithelium. The underlying mesenchyme is then removed and the innervating ganglion is severed in order to free the epithelia and allow flat-plating and proper orientation of the tissue in the culture dish. The dissected sensory epithelia are then placed onto Matrigel-coated (matrigel is diluted to 5% to prevent activity of endogenous aminoglycosides) Millipore tissue-tek inserts that have previously been placed into dishes with 0.8 ml of culture media (Medium-199(Sigma-Aldrich) with 10% FBS, 1% N2 supplement (Gibco) and cipro). This set-up allows tissue to wick media through the porous membrane while maintaining a high surface tension to enable proper growth and orientation of the sensory epithelium. Cultures are maintained for 3-10 days in vitro (DIV). Some cultures are incubated in media that has 10 uM SU5402

(Calbiochem), control media has a proportional amount of DMSO. Tissue is fixed in 4% PFA for 30 minutes then rinsed in PBS. To identify cells undergoing mitosis, some culture sets had BrdU added to the media [1:3000].

Immunohistochemistry

Immunohistochemistry was performed the same for whole mount tissue or fixed explants. First, tissue is incubated in 1% triton-PBS for one hour then blocked in 10% NGS or NHS in PBST. The primary antibodies were used at the following concentrations for 3 hours at room temperature or over night at 4 C: Myosin VIIa [1:1000], HCA and SCA [1:10] (gifts of Guy Richardson, University College, UK), TUJ1 [1:500], anti-goat Sox2 [1:200] (Santa Cruz Biotech), anti-activated caspase 9 [1:100] (Cell Signaling Technology), polyclonal Math1 [1:100] (a gift from Jane Johnson, University of Texas) Phalloidin [1:500]. Secondary anti-mouse, anti-rabbit, or anti-goat antibodies conjugated with 546, 488 or 633 were used at [1:1000]. For identification of BrdU-positive cells, tissue was placed into 1N HCl for 30 minutes following fixation. Anti-BrdU antibody was used at [1:50] in PBST over night.

Quantification of hair cell density

Embryonic BP cultures were established such that one ear from each embryo was maintained under control conditions while the contralateral ear was treated with SU5402. Once fixed, explants were labeled for expression of TUJ1 to mark HCs and Sox2 to identify support cells. Z-stack confocal images were obtained from the base, middle and apex of each sample using a Zeiss LSM510 with the Z-plane beginning at the tips of the

stereocilia and ending below the level of support cell nuclei. A scaled box 50x100 um was then digitally placed over the images that had been compressed in the Z-plane. Three non-overlapping boxes were placed onto each imaged region and the number of HCs and SCs were counted and recorded, n > 3 independent culture sets with at least 5 samples per condition for each set. HC and SC numbers were then pooled for each condition and averaged.

For the quantification of BrdU-incorporation by HCs, samples were labeled for expression of HCA (a HC-specific antigen) and Sox2 as well as for BrdU incorporation with an anti-BrdU antibody. The same imaging process was used as described above. Hair cells were counted to ensure SU5402 treatment had an effect and then the total number of HCs per sample that were both HCA and BrdU-positive were counted.

Measurement of hair cell diameters and surface areas

To determine the cell diameter of HCs in control and SU5402-treated cultures, BP cultures were labeled with Phalloidin to mark the cell boundaries and Tuj1 or HCA to identify them as HCs. Z-stack confocal images were taken beginning at the lumenal surface and extending down to the level of support cell nuclei. For each sample the composed Z-stack was analyzed to find the depth in the epithelium at which hair cell diameters were greatest. Measurements were then taken using software (Zeiss LSM Image Browser) that automatically calculates the diameter of a circle (as the HCs appear in Z-stack) that is superimposed over the Phalloidin-stained perimeter of the HC. Measurements were obtained for multiple regions of the BP in control and SU5402-treated samples.

Results

Inhibition of Fgfr signaling in the developing basilar papilla results in supernumerary hair cells

As discussed, the pattern of expression of *fgfr3* in the developing basilar papilla, along with its known role in the mammalian cochlea suggests that it may play a role in inhibiting progenitor cells from developing as hair cells. To test this hypothesis, a modified *in vitro* culture system for the embryonic basilar papilla was used(Stone and Cotanche 1991). Briefly, the ventral out-pocketing of the otocyst is dissected from E5 or E6 chick embryos and placed in a Millipore filter cup with the lumenal surface oriented up (see Methods and Materials for details). After 5 or 6 days in culture, explants are fixed and hair cells are visualized with either ant-β-tubulin (Tuj1) or other hair cell markers. Tuj1-labeling indicates the development of a relatively normal pattern of hair cells (Figure 4.1F). The sacculus, located at the proximal end, the basilar papilla, and distally-situated macula lagena are easily distinguished from one another (Figure 4.1F). In some explants, such as the one illustrated in Figure 4.1F, the BP does not fully elongate by comparison with *in vivo* development, however hair cells are clearly present along the entire length and the normal proximal-to-distal gradient of hair cell morphologies is evident (Figure 4.1F, arrow; Figure 4.2).

At E6, hair cells and support cells have just barely begun to differentiate from the pool of progenitor cells in the sensory epithelium. To determine whether Fgfr signaling plays a role in the number of cells that develop as hair cells, explants were maintained in media containing 10 μM SU5402, a potent and specific inhibitor of Fgfr signaling.

Figure 4.2

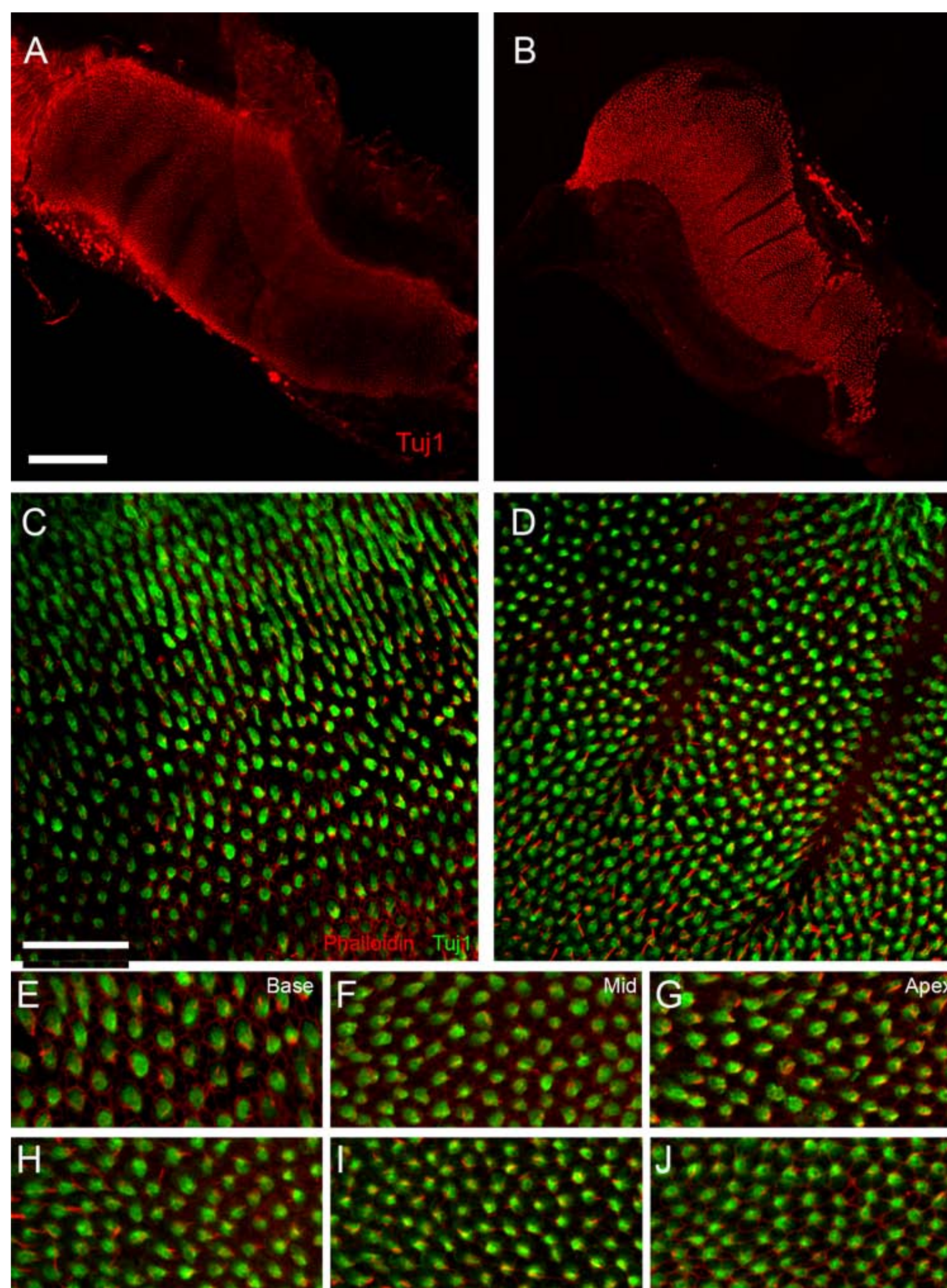


Figure 4.2. Early-stage (E6) inhibition with SU5402 results in an increased hair cell density. Low-mag images of control (A) and SU5402 treated (B) BP explants, started at E6 and maintained for 5 DIV, HCs are labeled with Tuj1 (red). C and D are slightly higher-magnification images of control (C) and SU5402-treated (D) explants. (C-H) High-mag images from basal, middle and apical regions of control (E-G) and SU5402-treated (H-J) BPs. Greater hair cell density is seen in apical regions compared to basal within the same explant, and overall 30% higher HC density is seen in SU5402-treated versus controls (K). Scale bar in A same for B: 200 um, scale bar in C same for D: 50 um.

Treatment with SU5402 beginning on E5 or E6, prior to the onset of HC differentiation, results in a significant increase in HC density along the length of the BP (Figure 4.2B, D, H-J) compared to control explants (Figure 4.2A, C, E-G). Control explants showed a normal range of HC density distribution in the base, middle, and apical regions (Figure 4.2E, F, G). In SU5402 treated cultures HCs were clearly smaller and more closely grouped than in controls in all three regions of the epithelium (Figure 4.2C-J). Quantification of hair cell density indicated an approximately 30% increase in the number of hair cells at each position along the BP (Figure 4.3). All of these increases were significant. Moreover, similar increases were observed when exposure to SU5402 was initiated on E9, a point after which most hair cells have begun differentiation (data not shown).

Supernumerary hair cells do not have mitotic origins

To determine if the increase in hair cell density is the result of renewed mitosis or the result of a greater number of progenitor cells adopting a hair cell fate, the mitotic tracer BrdU was added to the culture media of E6 control and SU5402-treated BP explants. Tissue was fixed after 48, 72 or 120 hours and then stained for incorporation of BrdU into cell nuclei. Results indicate no difference in the number of BrdU-positive hair cells between the control and experimental conditions (Figure 4.4). Many BrdU-positive cells can be seen in the mesenchyme surrounding the BP (Figure 4.4A) however the number of BrdU-positive cells within the region of the sensory epithelium is significantly less. The majority of BrdU-positive nuclei in the sensory regions are not hair cells as they are not found in cells co-expressing Tuj1, a hair cell marker. There is no difference

Figure 4.3

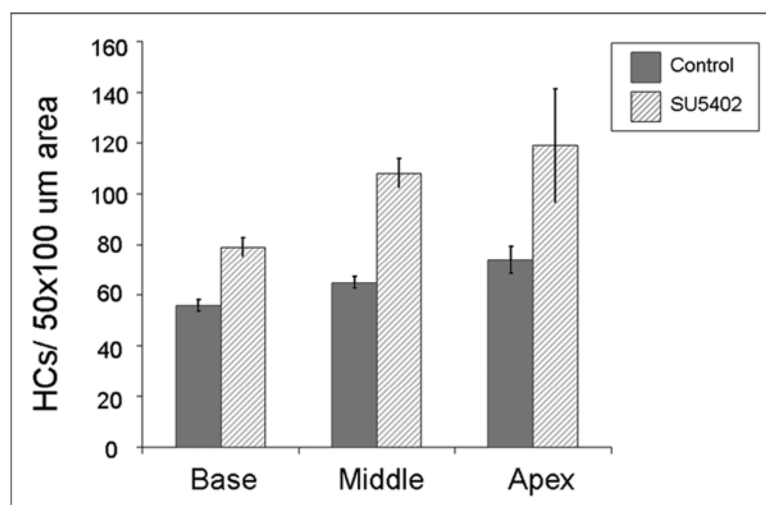


Figure 4.3. Graph of hair cell density in E6 control and SU5402-treated explant cultures. HC density is measured as number of hair cells per 50x100 μm area per region of the BP (base, middle, apical). There is a 30% or greater increase in the number of hair cells per unit area in each region of the BP that was counted in SU5402-treated samples compared to controls.

Figure 4.4

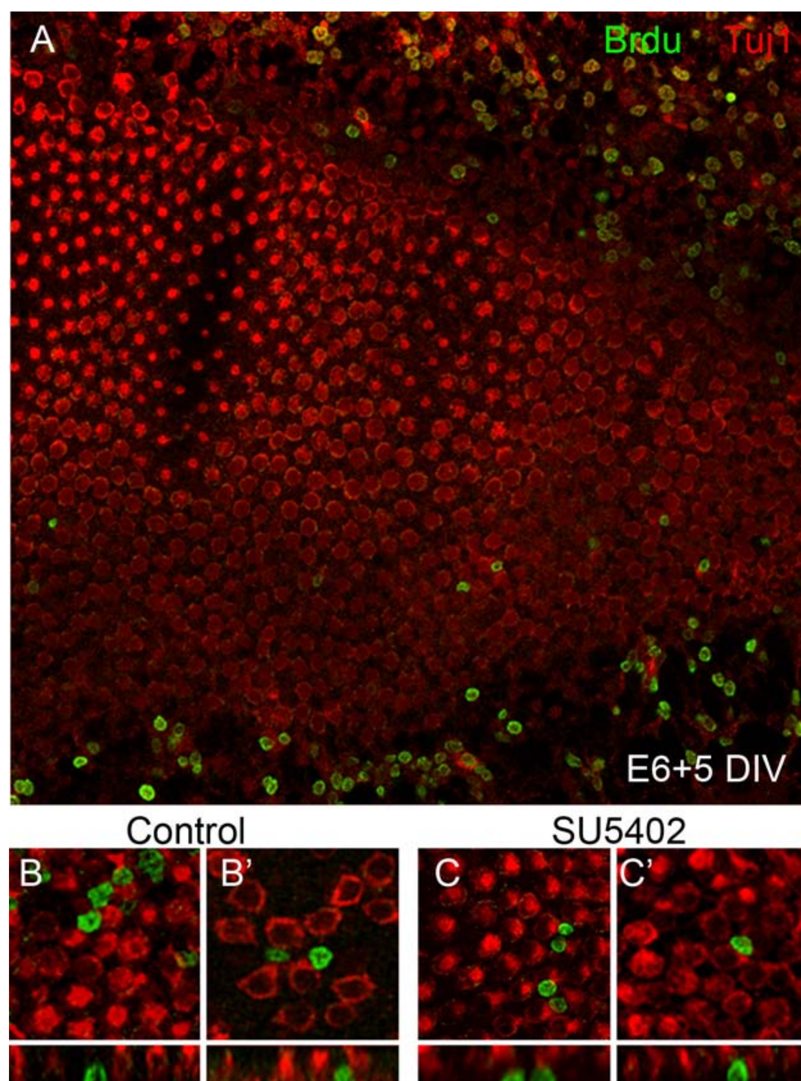


Figure 4.4. SU5402 treatment does not increase the level of BrdU incorporation in developing HCs. A low-magnification view of an embryonic BP culture begun at E6 and maintained for 5 DIV with SU5402 shows high levels of BrdU incorporation (green) in the nuclei of mesenchymal cells outside the epithelium (A), with few BrdU-positive cells in the HC region (HCs identified by Tuj1 expression, red). High magnification images of two control (B, B') and two SU5402-treated (C, C') BP cultures shows that HC nuclei are not positive for BrdU incorporation. Lumenal surfaces are shown above, while confocal generated Z-stack cross sections of the same BrdU-positive cells are shown below.

in control (Figure 4.4B, B') or SU5402-treated (Figure 4.4C, C') cultures in which HCs labeled with Tuj1 can be seen from the lumenal as well as cross-sectional planes. Control samples had an average of only 2.4 brdU-positive HCs per culture, and SU5402-treated samples had an average of 2.2, n>5 samples per condition.

The hair cell and support cell mosaic pattern is altered by inhibition of Fgfr signaling

The normal pattern of hair cells and support cells (as viewed in respect to one hair cell) is a rosette of three concentric rings of support cells (Figure 4.5E). One hair cell (HC) is contacted by approximately six support cells with very small apical projections (Figure 4.5A',E-yellow 1), these projections make complete contact with the HC along one edge. Next is another ring of approximately six support cells with larger apical projections but with very small contact points with the HC (Figure 4.5 A'-dots, E-red 2). A final row of support cells with very small apical projections occurs staggered between the larger support cells (Figure 4.5 E-blue 3). These support cells are completely contacted on one edge by a row of support cells and on the other edge by a hair cell existing outside the original rosette. In control E9 plus 5 DIV BP cultures, this pattern is readily identifiable (Figure 4.5 A'-D') however, in SU5402-treated samples this rosette pattern is disrupted and one hair cell is seen to be completely surrounded by other smaller hair cells (Figure 4.5B',D', F-aqua) and apical support cell projections (from rows 1 and 3 of the diagram in Figure 4.5F) become hard to see. At E9 most hair cells have been formed and the patterning of the cochlea is fairly well established. In SU5402 cultures, new hair cells can be seen in the exact position in which row 2 support cells normally

Figure 4.5

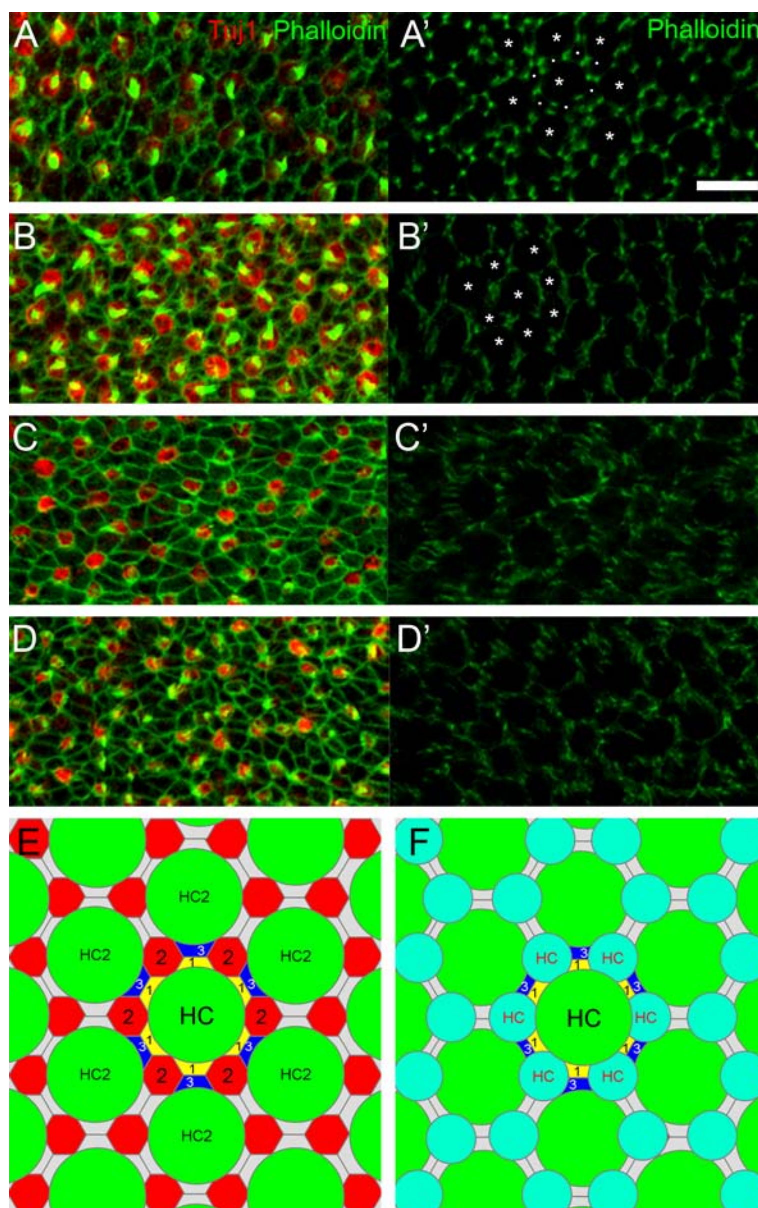


Figure 4.5. SU5402 treatment creates small HCs adjacent to larger ones and alters cellular patterning. Control (A,C) E9 +5DIV BP cultures have lower hair cell density that SU5402-treated (B,D) in both the basal (A-B) and apical (C-D) regions. Support cell apical projections outlined by phalloidin labeled actin (green) are significantly reduced by SU5402 treatment, viewed from the apical surface (B,D) compared to controls (A,C). A'-D' show the same cells as in A-D viewed at a deeper point in the Z-plane of the epithelia (at the point where hair cell diameter is greatest). At this level a reduction in the number of support cells and an increase in the number of small hair cells can be seen in the base of SU5402-treated (B') compared to control (A') explants. The same is true for the apical regions (D' and C', respectively). Hair cells are indicated by asterisks (*) and support cells by dots (.). Scale bar in A' is the same for all: 10 μm. E and F are schematics representing packing and distribution of HCs in control (E) and SU5402-treated (F) explants. In the normal BP (E), HCs (green) are surrounded by three concentric rings of support cells as numbered (1, yellow; 2, red; and 3, blue). Following SU5402 treatment HCs are surround by only two types of support cells and an additional row of HCs (F, aqua).

would be, suggesting these HCs are arising *in situ* and may be the result of transdifferentiation of support cells in this position. This same effect is seen in both basal (Figure 4.5A-B) and apical (Figure 4.5C-D) regions of BP explants.

The positioning of these smaller HCs occurs uniformly throughout the epithelium suggesting that rather than the conversion of HCs occurring in one particular region and having new HCs migrate into place, new HCs are arising *in situ.* Some explants were fixed 48 hours, 72, 96 or 120 hours post application of SU5402 and at all times younger cells are seen throughout the epithelium and never concentrated in one region. At E9, the epithelium is relatively immature however, and it is possible that support cells and hair cells have not fully completed differentiation from the progenitor pool of cells.

SU5402 treatment increases the number of small hair cells in developing BP cultures

While the density of hair cells is significantly greater in SU5402-treated samples, based on qualitative assessment, the overall surface area of the sensory epithelia of the BP was unchanged between control and SU5402-treated explants.

To determine if there were changes in individual cell size or distribution of support cells and HCs, the cellular boundaries of E9 plus 5 DIV epithelia were labeled using Phalloidin. As viewed from the lumenal surface, the surface area of both hair cells and supporting cells seemed reduced in SU5402-treated versus control samples (Figure 4.5). To determine whether the decrease in hair cell lumenal surface area was a reflection of an overall decrease in the size of the hair cells, confocal-generated Z-stack images were generated and used to measure the surface area through the widest region of the hair cells (a few microns below the surface of the epithelium) in SU4502 (Figure 4.5 B, B',

D', D') and control treated samples (Figure 4.5 A, A', C', C'). Because hair cells located in the distal third of the BP are significantly smaller than hair cells located in the middle and proximal thirds, data from distal regions was examined separately (Figure 4.5 B, D). Hair cell bodies located in the middle and proximal thirds of BP explants had an average diameter of 35.49 microns while the average hair cell diameter for cells in equivalent regions in SU5402-treated explants was 23.11, a significant decrease. Similarly, the average diameter of hair cells in the distal region of control explants was 27.80 while in SU5402-treated explants the diameter was 18.79, also significantly decreased. When the data for hair cells located in the proximal and middle regions of the BP was graphed as a frequency histogram, hair cell diameters in control explants showed a relatively normal distribution around the mean value (Figure 4.6 A). In contrast, hair cell diameters in SU5402-treated explants showed a bimodal distribution with approximately 50% of the cells normally distributed around the same mean as in control, but with the remaining 50% of cells normally distributed around a lower mean centered between 10 and 15 microns (Figure 4.6A). The smaller population of cells could be a result of crowding, or alternatively could represent younger, newly formed HCs that have arisen over the course of the experiment and are therefore delayed in their development. Consistent with this hypothesis, cells with transitional diameters located between the two diameter means were observed as well.

Inhibition of Fgfr activity produces new hair cells in the developing BP

To determine whether these smaller cells represented immature hair cells that had formed later in the experiment, we examined the expression of myosin VIIa, a HC marker

Figure 4.6

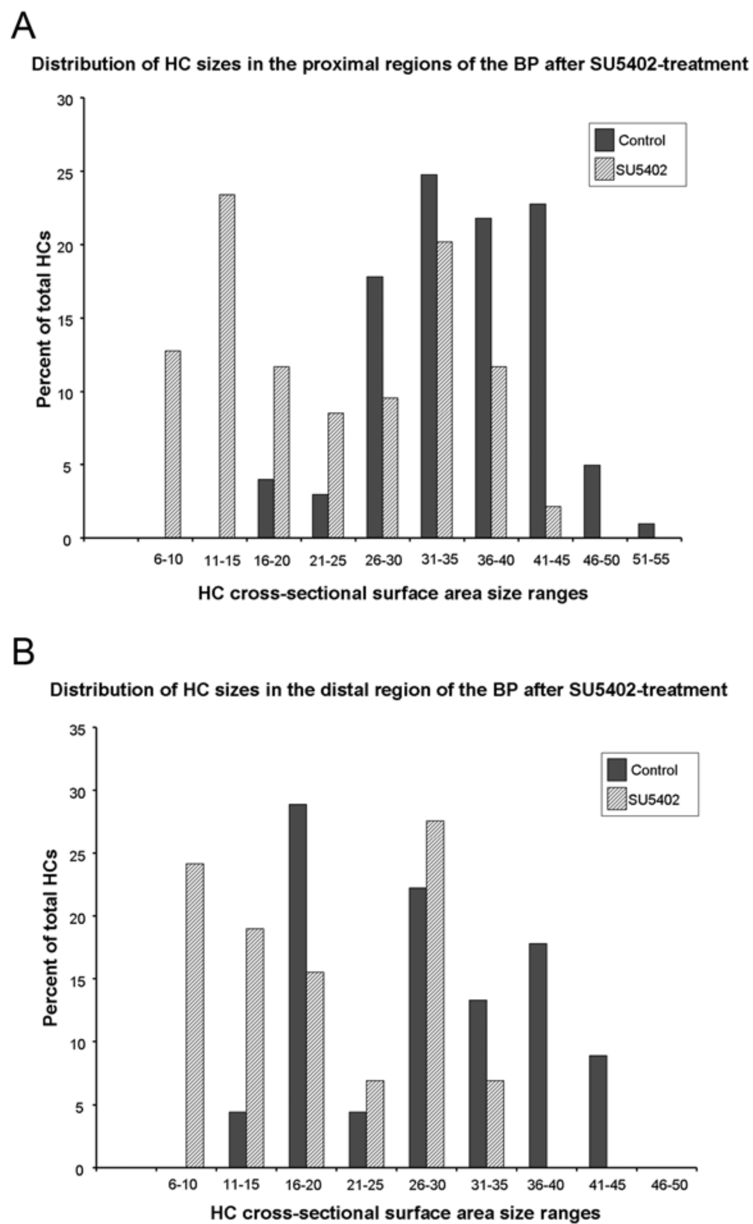


Figure 4.6. Distributions of hair cell sizes in the basilar papilla following SU5402 treatment. HC sizes were measured as the cross sectional surface area through the widest point of the hair cells. HC sizes were measured in control and SU5402 treated samples that were begun on day E9 and maintained for 5 DIV. Hair cells were binned in groups based on the size of their cross sectional surface area, each bin represents a range of 5 μm^2. The number of HCs was then plotted as a percentage of the total number of HCs measured (n= 90 hair cells per region of the cochlea). Distribution of HC sizes in the proximal 2/3 of the cochlea (A) and distal 1/3 (B) are shown.

which turns on approximately 6 hours later than the early hair cell marker Tuj1(Duncan, Mangiardi et al. 2006). BP explants were begun at E6, treated with SU5402 or control media and then a subset of explants were fixed after 24, 48, 72 or 96 hours *in vitro* in hopes of catching newly formed HCs if present. Myosin VIIa expression was not observed in some HCs of SU5402-treated explants while all control HCs were positive for MyosinVIIa (data not shown), this would suggest that they are indeed younger hair cells. However, given the small window of time that exists prior to HCs turning on MyosinVIIa expression, the number of cells negative for this HC marker were very few.

As another indicator of HC age the depth of HC projections were analyzed using confocal Z-stack projections. During normal HC development, undifferentiated cells have projections contacting both the lumenal and basal surfaces and have centrally placed nuclei. As differentiation proceeds HC nuclei migrate towards the lumenal surface(reviewed by (Stone and Cotanche 2007)). While control explants had HCs of uniform depth, in SU5402 treated samples smaller HCs were often viewed to have processes that extended deeper into the epithelium than larger adjacent HCs suggesting they may be younger and have yet to finish their migration to the lumenal surface.

There is an ongoing window of Fgfr activity in the developing basilar papilla

To determine if there is an ongoing window of Fgfr activity in hair cell development in young BPs, expression of sox2 was examined in SU5402-treated explants. Sox2 is a transcription factor that is initially expressed by all progenitor cells in the developing BP. As development proceeds, sox2 is down-regulated in HCs but its

expression is maintained in support cells (Uchikawa, Kamachi et al. 1999). If there is a limited window of activity, sox2 should be found only in support cells and all hair cells should have shown down-regulation. If any sox2 positive cells are found in the HC layer this would suggest that they are still in the process of transitioning from a progenitor cell into a hair and thus it can be concluded that they have differentiated more recently suggesting an ongoing window of Fgfr activity. Sox2 expression was examined in control and SU5402 treated samples. In controls, sox2 expression is strictly restricted to the support cell layer (Figure 4.7A), whereas in SU5402-treated samples, cells that are positive for sox2 are observed in both the support cell and the hair cell layer (Figure 4.7B and C). Strong and weak staining of sox2 in different HCs is consistent with the hypothesis that these cells are newly transitioning into hair cells, and were created at some point following the differentiation of the other, sox2-negative HCs. Given that these cultures were fixed on equivalent day E11, a time point in which initial HC development should be completed, the presence of sox2 positive HCs in SU5402 cultures suggests that support cells may be undergoing transdifferentiation into hair cells as a result of Fgfr inhibition.

Late stage (E12) inhibition of Fgfr signaling results in ectopic hair cells via transdifferentiation

Fgfr3 expression is maintained in support cells in adult chickens in the same pattern observed in embryonic and newly-hatched animals (Bermingham-McDonogh, Stone et al. 2001). Given this, it would seem possible that Fgfr3 functions similarly in both young and mature tissue. As discussed, hair cell loss in the BP induces a

Figure 4.7

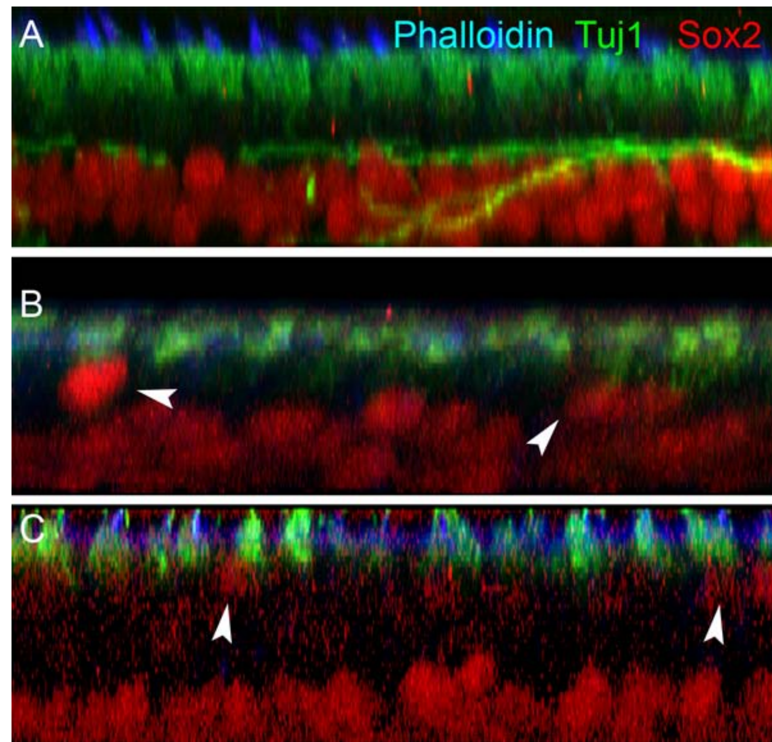


Figure 4.7. Sox2-positive cells are found in the hair cell layer in SU5402-treated samples. Confocal microscope generated optical Z-cross sections of control (A) and SU5402-treated (B, C) E6 BP explants maintained for 6 DIV. Hair cells are labeled with Tuj1(green) and Sox2 is in red, hair bundles are seen with actin (Phalloidin, blue), arrow heads point to sox2-positive nuclei in the HC region.

regenerative response that leads to the replacement of most of the lost hair cells through either transdifferentiation or mitotic proliferation of supporting cells. To determine whether Fgfr signaling might play a role in preventing spontaneous transition of support cells into hair cells, Fgfr activation was inhibited in more mature papillae.

By E12 all HCs are present in the BP and begin to show signs of advanced maturity such as polarization of stereocilia bundles(Cohen and Fermin 1978) and activation of mechanotransduction channels(Si, Brodie et al. 2003). Attempts to establish explants at this late stage result in massive hair cell death. An alternative approach whereby explants are established at a young age (E6-E8) then maintained *in vitro* until relative age E12 at which point the media is changed to experimental conditions proves highly successful. Explants fixed at E12 equivalent after 5 DIV but prior to experimental treatment look healthy (Figure 4.8A), showing strong labeling for HC markers and normal distribution and patterning of HCs in both the basal and apical regions (Figure 4.8A-C). When compared to BPs maintained until E12 *in vivo,* cultured BPs are significantly shorter in length as the method of culture does not enable normal spreading and outgrowth. The relative densities of the apices and basal regions in cultured BPs compared to *in vivo* epithelia remains the same, however. These cultured HCs also show an absence of the cell death marker activated-caspase-9(Kaiser, Chapman et al. 2008)(data not shown) confirming the health of the epithelium. At this age, the tectorial membrane is almost fully developed and shows robust growth *in vitro* making visualization of HCs somewhat difficult; this is especially true in the middle region of the BP where the tectorial membrane appears as a centrally located dark patch in the field of HCs (Figure 4.8A, D, G).

When treated with SU5402 for 5 days beginning at E12 equivalent (4-6 days in vitro beginning on E6 – E8), explants show a 30% increase in HC density in the distal regions compared to controls. The average number of HCs per 50 X 100 µm area in controls is 62.9 in the base and 86.6 in the apex; in SU5402 treated samples the base

Figure 4.8

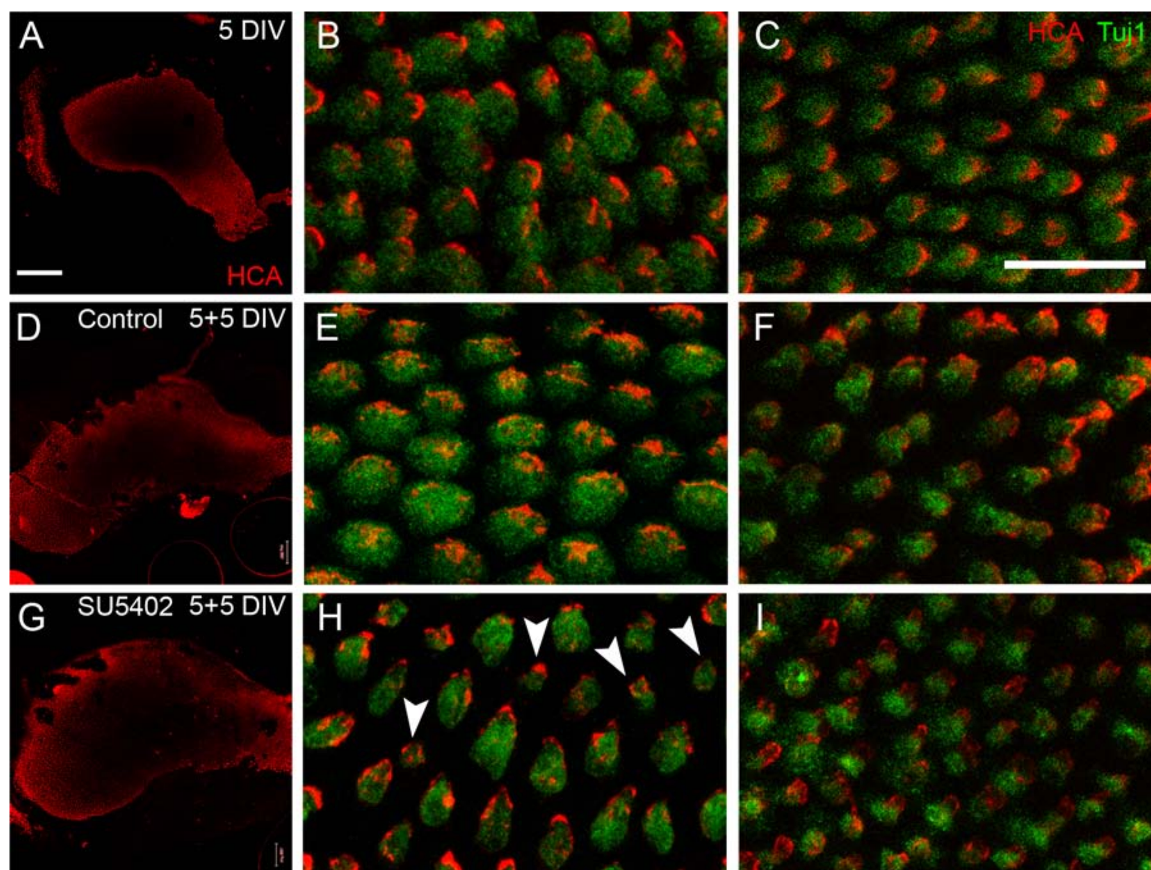


Figure 4.8 Inhibition of Fgfr activity promotes HC formation in mature basilar papillae. (A) Whole mount image of an E7 BP maintained for 5 DIV. At this point in the culture process HCs are healthy and appear to be relatively mature as seen in both the base (B) and apical (C) regions. (D) Whole mount image of an E7 BP from the same culture set as the sample shown in A, "pre-incubated" for 5DIV then maintained in control media for an additional 5 days prior to fixation. Both the base (E) and apex (F) have similar HC densities and sizes as in the sample above. An SU5402-treated BP from the same culture set (G) has significantly higher HC densities in the basal (H) and apical (I) regions as well as smaller hair cells found adjacent to larger ones (H). Scale bar in A same as D and G is 200 um. Scale bar in C same as F and I is 20 um. Contrast and brightness were altered to bring up very low-levels of Sox2 expression in some nuclei of the HC layer (C).

averages 86.6 HCs and the apex has an average of 112.4 HCs per unit area (n=6 for both conditions). An increase in HC density occurs in both the base (Figure 4.8E, H) and apical regions (Figure 4.8F,I), although in the proximal basal region the increase in HC density is less and is only 16.5%, however this is still a significant increase. This 30% increase seen in the proximal region is similar to that seen in young BP explants. In E12 SU5402 treated explants, small HCs can be seen intermixed with large HCs (Figure 4.8H, arrow heads) whereas all HCs in control explants are of a uniform size (Figure 4.8E).

The expression pattern of the hair cell marker HCA in very small HCs of late stage SU5402-treated explants is similar to that of young immature HCs. However, we wanted to confirm that these cells were newly formed HCs and not artifacts of dead or dying HCs. The main mode of cell death in the chick BP is apoptosis(Matsui, Ogilvie et al. 2002), therefore we chose to use an antibody directed against the activated form of Caspase-9(Kaiser, Chapman et al. 2008). We co-labeled late-stage BP cultures with activated-caspase-9 and HCA or Math1 and found that HCs with an immature morphology were indeed young HCs. They expressed the early-onset HC marker Math1 and were negative for activated-caspase-9 expression (data not shown). While some HCs show activated caspase-9 staining, there is no difference in the amount of these cells in SU5402-treated samples compared to controls. None of the activated caspase-9-positive cells co-expressed Math1 (data not shown).

Discussion

Most adult-onset deafness is the result of accumulated hair cell death resulting from acoustic or ototoxic trauma. While in the mammalian ear hair cell loss is permanent, the avian basilar papilla is able to undergo regeneration to replace killed hair cells(Corwin and Cotanche 1988). This occurs in a two step process in which a population of support cells directly transdifferentiates into hair cells. Following this, a round of mitosis occurs in some of the remaining support cells. Some of these new cells go on to become additional support cells while others are converted directly into new hair cells(Cruz, Lambert et al. 1987; Cotanche, Lee et al. 1994; Stone and Rubel 2000).

The Notch signaling pathway has been well characterized and shown to regulate the patterning of hair cells and support cells in both the avian and mammalian ears(Lanford, Lan et al. 1999; Daudet and Lewis 2005; Kiernan, Cordes et al. 2005; Brooker, Hozumi et al. 2006). Recent research has identified a role for Notch signaling during regeneration of lateral line hair cells in zebrafish(Ma, Rubel et al. 2008). The role of Notch1 during regeneration has been well characterized and is shown to regulate proliferation and the amount of new HCs that form(Ma, Rubel et al. 2008) This study suggested that there is an as yet unidentified pathway that acts complementary too and probably upstream of the Notch pathway that acts to maintain support cells in a quiescent state and retain the potential to become a hair cell. Here we suggest that Fgfr3 is this missing component.

Fgfr3 is expressed very early in all cells of the developing basilar papilla then is down-regulated by a subset that differentiates into hair cells while support cells maintain

fgfr3 expression throughout life(Bermingham-McDonogh, Stone et al. 2001). As in development, down-regulation of *fgfr3* occurs during regeneration when support cells that are fated to become HCs cease expressing the receptor(Bermingham-McDonogh, Stone et al. 2001). *Fgfr3* null mutant mice produce ectopic HCs at the expense of pillar cells (the only cell type in the mature organ of Corti to maintain *Fgfr3* expression beyond the developmental stages(Hayashi, Cunningham et al. 2007; Puligilla, Feng et al. 2007). Interestingly, pillar cells have also been shown to maintain a slightly higher level of developmental plasticity(White, Doetzlhofer et al. 2006; Jacques, Montcouquiol et al. 2007). This suggests that the presence of Fgfr3 may act as a competency factor which prevents terminal differentiation thus allowing cells to switch fate. It is possible that mammals have lost the ability to regenerate HCs because they have lost the expression of *Fgfr3* by the majority of their support cells.

When developing basilar papillae are grown in vitro and subjected to the Fgfr inhibitor SU5402, a significant increase in HCs is observed. This increased HC density is seen in all regions of the BP and can be induced at any developmental stage (E5-E14). Based on the lack of BrdU incorporation into these new hair cells, it can be assumed that they are arising via direct transdifferentiation of support cells into hair cells in older tissue and in younger ears, a greater percentage of progenitors are becoming HCs without the need for mitosis(Cotanche 1997).

The pattern of new hair cell formation observed in developing BP explants following SU5402 exposure is the same as seen in regenerating sensory epithelia following aminioglycoside treatment(Cotanche, Lee et al. 1994). Small hair cells are seen arising *in situ* and adjacent to larger HCs which supports the idea that the same pathway

regulates both developmental and regenerative differentiation. Additionally, studies into the activation of calcium channels show that HCs undergoing regeneration mimic the activity of young developing HCs(Levic, Nie et al. 2007) suggesting that the mode of HC formation in the two processes is conserved.

The presence of sox2 positive nuclei in some cells of the support cell layer in young SU5402-treated explants suggests that there is an ongoing period in which hair cells can differentiate if Fgfr signaling is inhibited. At younger ages, this causes more progenitor cells to assume a HC fate, while in more mature BPs, at E9 and E12 when HC formation is complete, inhibition of Fgfr signaling causes the transdifferentiation of support cells into hair cells. The ongoing expression of *fgfr3* in support cells is consistent with the hypothesis that blocking Fgfr signaling at any age pushes support cells out of *fgfr3*-induced stasis and causes them to undergo transdifferentiation into HCs. During regeneration about 30% of new HCs are derived as a result of direct trans-differentiation(Roberson, Alosi et al. 2004). This is the same percent increase seen in HC density when developing and mature BPs are treated with SU5402 suggesting that regenerated hair cells may arise in a similar fashion as is seen by inhibition of Fgfr signaling *in vitro*.

This is the first published report to identify a signaling pathway responsible for regulating the conversion of support cells into hair cells in the BP. Given that new hair cells can arise throughout the epithelium it suggests that there is no specific pool of competent cells that are able to undergo transdifferentiation, lending further support for the idea that Fgfr3 is the competency factor which enables a support cell to transition into a hair cell.

The main issue which remains to be addressed, however, is the exact identity of the Fgfr that is regulating the observed effects in the BP. It is possible that another Fgfr, besides Fgfr3, may be responsible for the effect we see in culture given the pan-acting nature of SU5402. The expression pattern in the BP and effects of Fgfr3 in other tissue, such as it's role in regulating lens fiber differentiation in the eye(Govindarajan and Overbeek 2001) as well as its well characterized role in promoting HC differentiation in the organ of Corti of mammalian ears(Hayashi, Cunningham et al. 2007; Puligilla, Feng et al. 2007) strongly suggest, however, that Fgfr3 is receptor responsible for this activity. Given the similarities of the effects of Fgfr inhibition in mouse and chick, which has been attributed to Fgfr3 signaling in mouse, it would seem that Fgfr3 is indeed the signal which maintains support cells in a quiescent state in the basilar papilla, and as such, is a key component in the regeneration pathway.

Another identity which remains to be solved is that of the endogenous ligand responsible for activating Fgfr3 in the BP. In the organ of Corti, Fgfr3 signaling through Fgf8 has been shown to regulate the timing and differentiation of pillar cells and outer hair cells(Mueller, Jacques et al. 2002; Jacques, Montcouquiol et al. 2007). In developing organ of Corti explants treated with high levels of the Fgfr3-specific ligand Fgf17 fail to form outer hair cells and develop ectopic pillar cells. If Fgf17 is washed out of the media and explants are maintained for an additional three days they will eventually form a normal complement of outer hair cells suggesting that maintenance of Fgfr3 signaling in these cultures holds them in developmental stasis/promotes the support cell phenotype. Once this ectopic activation is removed the normal pathway resumes, Fgfr3 is down-

regulated by a subset of cells and normal morphology of the organ of Corti forms(Jacques, Montcouquiol et al. 2007).

Based on this data, we propose the following working model for Fgf signaling during development and differentiation in the basilar papilla: *fgfr3* is initially expressed by all cells of the developing epithelium, some cells (for reasons unknown, possibly related to changes in expression of other Fgfs, such as Fgf3 or 10 surrounding the cochlea duct(Zelarayan, Vendrell et al. 2007)) down-regulate *fgfr3* and become HCs. These HCs begin to produce an Fgf ligand which activates Fgfr3 in underlying support cells preventing their differentiation into HCs. If hair cell death occurs, the source of Fgf ligand is lost causing the down-regulation of *fgfr3* in some support cells, enabling them to transdifferentiate into hair cells to repair the epithelium.

If Fgfr3 is the prime gene responsible for hair cell differentiation in the BP, then identifying the factors that regulate its expression would provide many answers about the overall regenerative process. One candidate is Prox1 which has been shown to be responsible for regulating the expression of *Fgfr3* during cell fate specification in the lymphatic system(Shin, Min et al. 2006). Interestingly, cProx1 is expressed early and maintained in mature support cells of the BP and is highly up-regulated during regeneration(Stone, Shang et al. 2004). In contrast, Prox1 is expressed early in the development of the organ of Corti, is down-regulated in all but the Deiters' and pillar cells late in development and then is dramatically down-regulated in the mature organ(Bermingham-McDonogh, Oesterle et al. 2006). The key to understanding the loss of mammalian hair cell regeneration may be in what lies upstream of *Fgfr3* causing the down-regulation of its expression in mature support cells of the organ of Corti.

Chapter 5: Summary and Discussion

Finding solutions to developmental and adult-onset deafness is going to continue to be one of the driving factors behind auditory research. As the potential for genetic therapies grows, the need to understand the underlying genetic pathways that regulate the formation of specific hair cell and support cell types is heightened. Significant steps have been taken in resolving the genes, pathways and networks that make up the developmental program of the organ of Corti. The scope of this project was to help further characterize these pathways and build on our understanding of the genetic blueprint of the inner ear specifically by studying the role of Fgfr3 signaling during development of the auditory sensory epithelium. The combined results of these experiments provide strong support for three main contentions.

1: Fgfr3 regulates the differentiation of support cells in the developing organ of Corti.

2. Fgf8 is responsible for the activity of Fgfr3 in the developing organ of Corti.

3: Fgfr3 regulates the transition of support cells into hair cells in the basilar papilla.

Fgfr3 regulates the differentiation of support cells in the developing organ of Corti.

The impetus behind hypothesis one was to further elucidate the genetic factors which regulate the differentiation of the multiple unique cell types found in the mammalian organ of Corti. It had been demonstrated that hair cells and support cells share a common progenitor in the inner ear(Fekete, Muthukumar et al. 1998). At the onset of this study, only a few genes had been identified to play a role in hair cell and support cell formation. *Atoh1*, a bHLH transcription factor has been shown to play a role in hair cell specification(Bermingham, Hassan et al. 1999; Woods, Montcouquiol et al. 2004), however little was known about the factors that regulate the commitment of progenitor cells to a specific supporting cell fate. Using the reported defect in pillar cells of the *Fgfr3* knockout mouse(Colvin, Bohne et al. 1996; Hayashi, Cunningham et al. 2007; Puligilla, Feng et al. 2007) as a basis for this research, a series of experiments were devised to pan-inhibit or pan-activate the Fgf signaling pathway in embryonic mouse cochlear cultures; the goal of this being to understand if and to what extent Fgfr3 signaling affects the development of pillar cells within the organ of Corti.

Signaling through Fgfr3 was shown to play a role in the regulation of PC development in the mouse organ of Corti. It was shown that *Fgfr3* is initially expressed in the OC in a common progenitor pool at E15.5, excluding the inner hair cells, and is eventually down-regulated in the outer hair cell region. By P0, *Fgfr3* expression becomes restricted to support cells (both Deiter's cells and pillar cells). Given that the specific Fgf ligand responsible for activation of Fgfr3 was unknown, the initial scope of this study was limited to addressing the overall functions of Fgf signaling. Inhibition of the receptor with SU5402 *in vitro* blocked the differentiation of pillar cells,

phenocopying the *Fgfr3* knockout mutant. *Conversely,* ectopic activation with a highly promiscuous ligand, Fgf2, resulted in a three-fold increase in the number of pillar cells that formed in cultured embryonic cochlear epithelia. These results also demonstrated that there was an ongoing requirement for Fgfr3 signaling throughout the window of pillar cell differentiation from E14 to E18. No significant changes were observed in the formation of the other hair cell or support cell types, suggesting that some other signaling pathway is required for the specification of Deiter's cells and outer hair cells.

Having elucidated a clear role for activation of Fgfr3 during OC development, the next goal was to identify the specific ligand and pathway responsible for this activity. Given that the effect of Fgfr3 signaling is limited to the pillar cell region and expression of the receptor drops off in the lateral region of the OC, it suggested that a secreted molecule may set up a gradient which maintains receptor expression in areas of high concentration near the source and allows expression to be down-regulated in areas of low concentration. The reported expression of Fgf8 by inner hair cells suggested that it could be the secreted factor responsible for Fgfr3 activation and this formed the basis for the next series of experiments.

Fgf8 is responsible for the activity of Fgfr3 in the developing organ of Corti.

Fgf8 expression was confirmed, via *in situ* hybridization, to be expressed exclusively by inner hair cells of the developing OC. Fgf8 is essential for formation of multiple organ systems, most notably it regulates brain formation, and thus a traditional knockout mouse would be embryonic lethal. To analyze the *in vivo* effects of Fgf8 a conditional mutant mouse in which *Fgf8* is rendered null in limited regions of the developing embryo, including the developing otocyst was obtained. The determination of whether these mice are deaf, however, was not possible as other defects are still significant enough that the mice die at birth. Analysis of these *Fgf8* conditional null mice revealed a lack of formation of pillar cells. The distance between inner and outer hair cells of mutant compared to control littermates was reduced to nearly zero, suggesting a complete lack of formation of the pillar head processes. However, no other significant defect was observed in the ears of these mice. When Fgf8 was inhibited *in vitro* with a function blocking antibody specific to this ligand, a phenocopy of the *Fgf8* mutant was observed; pillar cells failed to form in embryonic sensory epithelia. This suggested that Fgf8 is acting directly on the formation of pillar cells at the protein level. The direct correspondence of the *Fgfr3* and *Fgf8* knockout mice as well as the similar effect of inhibiting the receptor or ligand at the protein level *in vitro* provided strong evidence that these genes operate in the same signaling pathway.

Transfection of embryonic cochlear cultures with an *Fgf8* expressing construct resulted in a significant increase in the number of pillar cells and a complete absence of outer hair cells in regions of the developing OC adjacent to pools of transfected cells. This confirmed that Fgf8 is an inductive factor capable of promoting pillar cell fate in

cells exposed to high concentrations of this morphogen. Similarly, applying Fgf17 protein, which has similar binding kinetics with Fgfr3 as does Fgf8, resulted in a significant reduction in the number of OHCs that formed and a significant increase in pillar cells and Deiter's cells. Ectopic formation of pillar cells in the Hensen's cell region was also observed. When Fgf17 was applied to epithelial cultures at E14 for 48 or 72 hours and then removed, there was a delay in the appearance of OHCs proportional to the amount of time the tissue was exposed to Fgf17 protein. These results demonstrate that there is clearly a window of time in which Fgf signaling can promote pillar cell fate, however its influence on OHCs is limited to the period of time in which they express *Fgfr3*. Additionally, it is clear that Fgfr3 is required throughout the entire window of pillar cell formation, this could mean that Fgfr3 has an additional maintenance role, or that pillar cells at this age are still relatively undifferentiated. More importantly, the fact that OHC formation can be reinitiated after their normal developmental window has lapsed suggests a very strong role for Fgfr3 signaling in the maintenance of the ability to differentiate into a hair cell. That the effect of ectopic Fgf8 (exogenously applied protein or electroporation of *Fgf8*-expressing constructs) is limited to those cells that endogenously express *Fgfr3* demonstrates a clear requirement for Fgfr3 in regulating the differentiation of hair cells and support cells. What is unclear is if Fgf17 activation of Fgfr3 keeps these cells in an undifferentiated state or if they are developing as pillar cells and then undergo a fate switch and dedifferentiate when Fgfr3 signaling is removed.

The expression of *Fgf8* in the IHCs and *Fgfr3* in the outlaying progenitor pool suggests the following model for Fgfr3 signaling (Figure 5.1): Fgf8 sets up a gradient beginning at the IHCs and adjacent to the pool of Fgfr3-expressing cells at E15. Cells

adjacent to the IHCs receive a high concentration of morphogen and maintain Fgfr3 expression, these become pillar cells, whereas cells exposed to a lower dose down-regulate *Fgfr3* and become OHCs and Deiter's cells. If Fgfr3 is ectopically activated across the entire expanse of the developing sensory epithelium, the receptor fails to down-regulate its expression and these cells develop into ectopic pillar cells and Deiter's cells while the differentiation of OHCs is inhibited (Figure 5.1). Thus Fgfr3 signaling is responsible for the determination of hair cell versus support cell fates.

The effective range of Fgf8 signaling is probably limited by the inhibitory effects of Sprouty2. This inhibitor is expressed by the Deiter's cells and Sprouty2 inhibition of Fgf8 signaling probably causes the receptor to be down-regulated in these cells during development (Shim, Minowada et al. 2005).

Sprouty2 is an Fgfr antagonist which has been shown to limit the range of Fgf signaling where expressed, thus knocking it out in a tissue should expand the effective range of Fgf activity, which would mimic the effect of ectopic activation of Fgfr signaling. Indeed, knocking out *Sprouty2* in the ear causes the formation of an extra pillar cell. When a double mutant is created with a *Sprouty2-/-;Fgf8+/-* genotype, the effects of the *Sprouty2* mutation are partially rescued(Shim, Minowada et al. 2005), further confirming that the activity of Sprouty2 is to regulate the effects of Fgf8. The *Sprouty2* and *Fgfr3* knockout mice however have a partially shared phenotype in that they both produce extra outer hair cells. In the *Sprouty2* knockout mouse, the effective

Figure 5.1

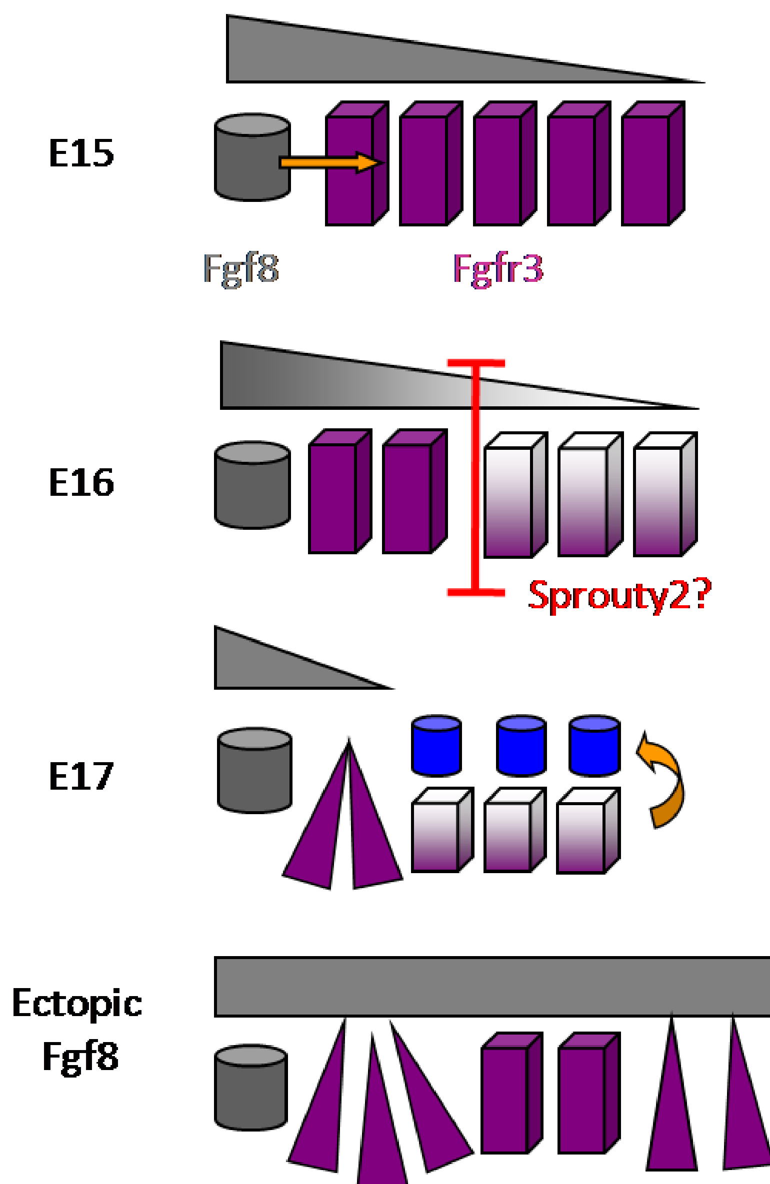


Figure 5.1. Model of Fgfr3 signaling in the developing organ of Corti. At E15 IHCs begin to express *Fgf8* (indicated in gray). The gradient of Fgf8 protein is indicated by the gray shaded triangle. Not long after, *Fgfr3* (purple) begins to be expressed in a pool of cells outside of the IHC. By E16 a gradient of Fgf8 ligand signals across the developing organ of Corti. Cells outside the region of high Fgf8 concentration begin to down-regulate *Fgfr3* expression. It is probable that an Fgf inhibitor such as a Sprouty (red) expressed in the pillar cell region acts to limit the range of Fgf8 activity. Down-regulation of the receptor unblocks the inhibition on hair cells differentiation and by E17 outer hair cells (blue) have begun to differentiate from the pool of precursor cells in the region that had down-regulated *Fgfr3*. If developing organs of Corti are exposed *in vitro* to a uniformly high concentration of ectopic Fgf8 across the entire expanse of the sensory epithelium *Fgfr3* expression is maintained in all cells and OHCs fail to form. If ectopic Fgf is then removed from the media surrounding these developing explants, the E15 condition is reinstated and delayed differentiation of OHCs occurs.

range of Fgf8 is allowed to expand beyond the Deiter's cells and thus an extra pillar cell forms. However, the fact that extra OHCs also form suggests that there may be other Fgfs or related factors which specifically regulate the development of these cells and thus blocking Sprouty2 activity allows over-activation of this pathway as well. Recently it has been shown that Fgf20 may be working through Fgfr1 to promote the differentiation of Deiter's cells and/or outer hair cells and this ligand-receptor pair has been shown to be expressed in the developing OC in this region(Hayashi, Ray et al. 2008). Knocking out *Sprouty2* may allow the range of Fgf8 to expand into the Fgfr1-expressing domain such that Fgf8 also activates this receptor and promotes formation of ectopic outer hair cells.

Another role that has become clear for Fgf8, is that it acts as an organizing center for cellular patterning in the region of the organ or Corti near the inner hair cells. The patterning of the entire epithelium, however, cannot be explained solely by signaling through Fgfr3 given that inhibition of Fgf8-Fgfr3 does not affect this region; clearly other factors are responsible for patterning the lateral regions of the OC. One possibility is BMP4 signaling which is known to antagonize Fgfr3(Massague, Seoane et al. 2005). Bmp4 expression has been shown in the lateral regions of the developing OC. Ectopically activating Bmp4 signaling in the developing mouse causes an increase in outer hair cells. Conversely, inhibiting Bmp4 signaling prevents the formation of outer hair cells(Puligilla, Feng et al. 2007). It is possible that BMP4 signaling through the Smad receptor acts to pattern the lateral domain of the OC, while Fgf8 signaling through Fgfr3 patterns the medial region, and antagonistic interactions between the two pathways may limit the range of their effects.

Clearly, one area of research that would be particularly compelling is the identification of the factors which regulate the expression of both *Fgfr3* and *Fgf8*. Retinoic acid has been shown to negatively regulate *Fgf8* expression during somitogenesis(Duester 2007). Interestingly, its expression has been shown in the developing OC(Kelley, Xu et al. 1993). Ectopically adding retinoic acid to auditory epithelial explant cultures resulted in a significant increase in the number of hair cells that formed in the OC(Kelley, Xu et al. 1993). The appearance of additional hair cells when retinoic acid or Fgfr signaling inhibitors are applied to the inner ear suggests that they may operate within the same pathway. More recent data also indicates a role for retinoic acid during OC development and maintenance(Romand, Dolle et al. 2006).

Prox1 has been shown to regulate Fgfr3 expression in the lymphatic system(Shin, Min et al. 2006) and its expression pattern is complementary to that of Fgfr3 in the organ of Corti(Bermingham-McDonogh, Oesterle et al. 2006), suggesting it may play a similar regulatory role in the ear. Interestingly, *Prox1* expression also overlaps with Fgfr3 expression in the basilar papilla(Stone, Shang et al. 2004). In the basilar papilla of birds, *Fgfr3* is expressed in a pattern similar to that of the developing OC(Bermingham-McDonogh, Stone et al. 2001) which suggests it may play a conserved role in the development of both systems.

Fgfr3 regulates the transition of support cells into hair cells in the basilar papilla.

Hair cell regeneration in the basilar papilla is believed to utilize a similar pathway to the one which forms hair cells during development(Levic, Nie et al. 2007). A firm understanding of how hair cells and support cells are formed during avian cochlear

development should provide a few cues as to how regeneration occurs. Levels of *fgfr3* expression rise and fall in a similar fashion during regeneration and development in the basilar papilla(Bermingham-McDonogh, Stone et al. 2001) suggesting it may play a role during both processes.

In this final area of study, Fgfr3 activity in the developing and mature basilar papilla was inhibited using SU5402 at various stages of development in embryonic epithelial cultures. When SU5402 was applied to very young BPs (E5-E6) prior to hair cell formation, a 30% increase in hair cell density was observed. This increase was not accompanied by an uptake of BrdU in either hair cells or support cells, indicating that there was no increase in mitosis as a result of blocking Fgfr signaling. Similarly, at mid (E9) or late stages (E12) of development, an increase in hair cell density was also observed. Close inspection of the epithelia from these cultures revealed the presence of many small hair cells directly adjacent to more mature, larger ones. These smaller hair cells had phenotypic traits characteristic of young immature hair cells, such as more basally displaced nuclei, and retention of sox2 expression at the time of fixation. These small hair cells caused a change in the regular pattern of hair cells and support cells and were seen to arise in locations which previously would contain support cells. New hair cells were seen in both young and mature BP explants suggesting that in the young explants more progenitors were differentiating as HCs, while in the older explants support cells were undergoing transdifferentiation. The overall sizes of the hair cell bodies were measured in control and SU5402-treated explants. A bimodal distribution of hair cell sizes in SU5402-inhibited cultures versus a unimodal distribution in controls suggests the existence of two distinct populations of hair cells in the treated samples. This

confirmed the existence of new hair cells in the BP following inhibition of Fgf signaling and suggested that inhibiting Fgfr signaling can release a population of support cells from their original fate and cause them to directly transdifferentiate into hair cells.

Initial attempts to drive ectopic activation within basilar papilla explants failed to generate any change when compared to controls. Preliminary data, however, suggests that electroporation of Fgf-expressing constructs may work at driving ectopic expression of Fgf genes within the developing BPs, and this line of research is currently being pursued.

Implications for Regeneration

The identification of the Notch pathway and lateral inhibition in the establishment of the precise mosaic of hair cells and support cells(Lanford, Lan et al. 1999) was a key step in understanding how the different types of cells are made and how cell fate is decided. Recently more data has come out to demonstrate a role for Notch signaling in regeneration of the lateral line(Ma, Rubel et al. 2008). Notch signaling has been shown to regulate the number of hair cells that form during regeneration. Blocking Notch activity, however, only has an effect within a damaged epithelium. Quiescent lateral lines do not respond to DAPT inhibition of the Notch pathway(Ma, Rubel et al. 2008). Given the homologous relationship and conserved genetic and developmental pathways of inner ear hair cells and the lateral line, it would argue that a similar requirement for Notch signaling exists in the regenerating basilar papilla. The current model that is used to understand regeneration suggests that there is a factor which conveys an open cell fate on support cells that when removed, allows them to transdifferentiate into hair cells. Notch signaling through Serrate-1 then takes over and is responsible for establishing the mosaic

pattern of hair cells and support cells(Daudet, Ariza-McNaughton et al. 2007). The regenerative and developmental pathways of HC formation are believed to function much in the same way thus the research presented here suggests strongly that the factor enabling transdifferentiation of support cells into hair cells is Fgfr3.
The expression pattern of other Fgfrs in the BP has not been reported, however, thus characterizing expression of other Fgf pathway members in the BP will be critical in our understanding of how regeneration occurs. Given the concurrence of effects of Fgf signaling modulation in both the chick and mouse, where in the mouse they are confirmed to be attributed to the activity of Fgfr3, it would not be unreasonable to hypothesize that the effects seen by blocking Fgfr signaling in the BP are attributable to Fgfr3 as well.

The combined data on Fgfr3 signaling in the organ of Corti and basilar papilla suggests the following model for development and regeneration (Figure 5.2): In the developing BP, *fgfr3* is initially expressed in all cells of the cochlear duct that will become support cells and hair cells(Bermingham-McDonogh, Stone et al. 2001; Jacques, Montcouquiol et al. 2007) (excluding the IHCS of the OC)(Figure 5-2). When differentiation of hair cells begins from this pool of precursor cells, *fgfr3* is down-regulated in those cells fated to become hair cells while all support cells maintain *fgfr3*

Figure 5.2

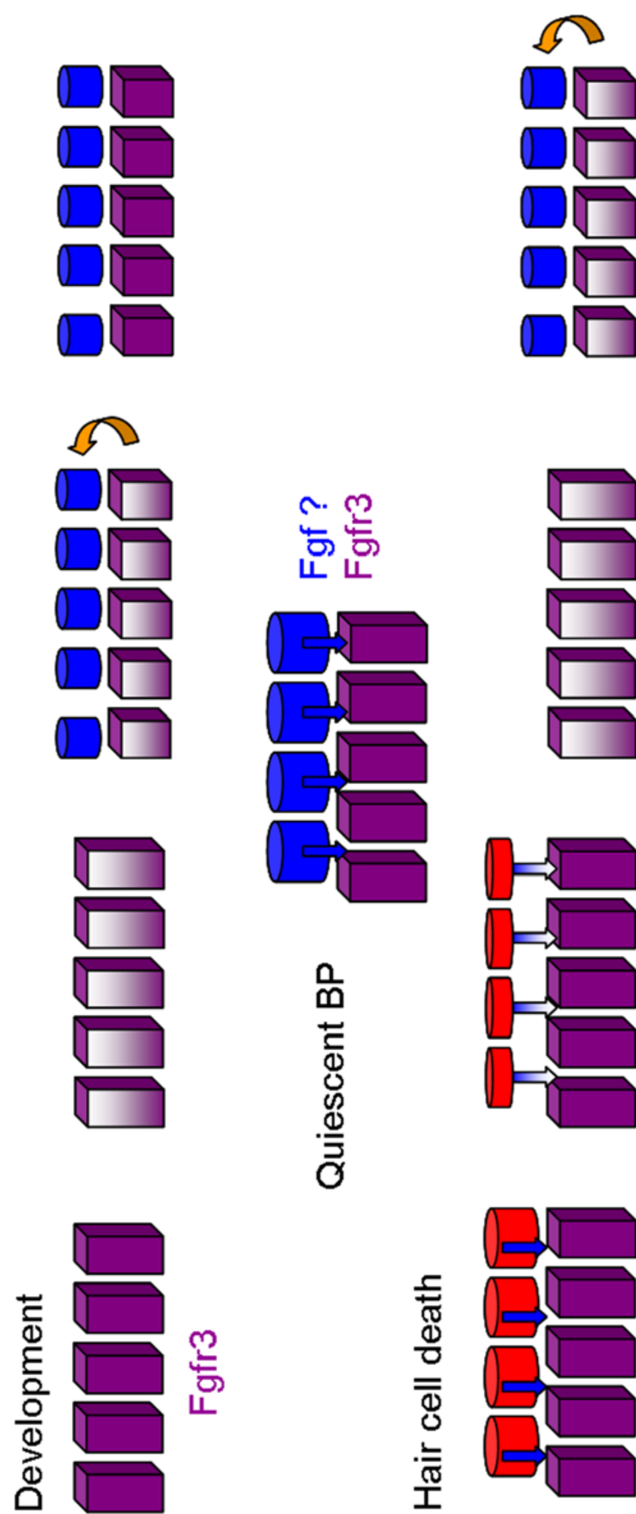


Figure 5.2. Working model of Fgfr3 signaling during development and regeneration in the BP. Early in basilar papilla development *Fgfr3* (purple) is expressed by all cells of the sensory epithelium. *Fgfr3* then becomes down-regulated in a population of these cells that begin differentiation as hair cells (blue). These new hair cells begin secreting an Fgf (blue arrows) which acts to maintain expression of *Fgfr3* in support cells and inhibits further hair cell formation. Following hair cell differentiation the basilar papilla is considered to be in a quiescent state. If ototoxic or acoustic trauma occurs within the BP, rapid hair cell death (red) is triggered. Loss of hair cells results in the loss of secreted Fgf causing *Fgfr3* to be down-regulated in support cells. As in the developing BP, down-regulation of *Fgfr3* removes the inhibition on hair cell formation and a population of support cells transdifferentiate into new hair cells. Following the formation of these new hair cells, the BP returns to a quiescent state.

expression. Applying this same model to regeneration, it can be predicted that BP hair cells secrete an Fgf which is responsible for maintaining Fgfr3 activity in underlying support cells (Figure 5.2). When hair cells are lost, this secreted Fgf signal is also lost, causing a down-regulation of *fgfr3* in the support cells. Down-regulation of *fgfr3* then releases support cells from their differential stasis and enables them to transdifferentiate into hair cells. New hair cells then begin to express Fgf and the system is restored.

Given that Fgf8 is not expressed in the BP(Sanchez-Calderon, Martin-Partido et al. 2002), some other Fgf must be acting endogenously to maintain *fgfr3* expression in the support cells. The elucidation of this gene would greatly contribute to the understanding of the regenerative process. Similarly, understanding the factors which regulate *fgfr3* expression in the BP is of great importance. Identifying both the endogenous Fgf ligand as well as the factor which directly regulates *fgfr3* expression would help to solidify this working model and provide a jump-off point for further studying the regenerative process. The strong empirical evidence provided by the above experiments suggests the possibility that the loss of hair cell regeneration in the organ of Corti is attributable to the loss of *Fgfr3* expression by support cells of the organ of Corti.

Implications for inner ear evolution

In addition to what this research has contributed to the understanding of the developmental signaling pathways of the organ of Corti and basilar papilla, as well as what it may imply about regeneration, this study can also be used to shed some light onto the evolution of the vertebrate ear.

There has been a clear reduction of the width of the mechanotransducing region of the auditory sensory epithelium throughout the evolution of the vertebrate ear. Birds have a very broad field of hair cells that entirely fills up the cochlear duct, while eutherian (placental) mammals have a significantly condensed field of hair cells flanked on either side by large fields of non-mechanotransducing epithelial cells(Raphael and Altschuler 2003). Intermediate to these, the monotreme cochlea is characterized by four rows of IHCs, approximately six rows of OHCs and up to three rows of outer pillar cells(Ladhams and Pickles 1996); thus it is much wider than the OC of eutherian mammals. Monotremes are egg-laying mammals believed to exhibit less divergence from the presumed ancestor of all mammals. They are believed to be extant relatives of a group of animals that branched off very early during the mammalian radiation. This is based on the presence of multiple traits that have both reptilian and mammalian features: they have an interclavicle bone characteristic of the reptilian shoulder girdle, they lay eggs, possess a cloaca and lack nipples.

Not only does the monotreme OC have a wider field of hair cells within the cochlear duct, it contains a few other intermediate traits between eutherian (placental) mammals and birds. Namely, rather than being completely coiled or slightly curved, the monotreme cochlea is “J” shaped. The monotreme cochlear duct also has a macula lagena at its very apical tip(Jorgensen and Locket 1995; Ladhams and Pickles 1996). The macula lagena is a gravistatic sensory patch with hair cells more similar to those of the vestibular rather than the auditory system(Jorgensen and Locket 1995). This organ is found in birds but is completely absent from the eutherian mammal ear. These

similarities and the intermediate nature of the monotreme cochlea provide further support for the organ of Corti being homologous to the basilar papilla.

Reminiscent of the monotreme organ of Corti, the *Sprouty2* mouse mutant has an ectopic row of OHCs in addition to an extra row of outer pillar cells. Extra pillar cells have been proposed to be one reason why the *Sprouty2* mutants have significant hearing impairment(Shim, Minowada et al. 2005). The region of the basilar membrane beneath the outer pillar cell has been shown to exhibit the least amount of flexion, acting to buffer the amplification produced by the OHCs(Nilsen and Russell 1999). The presence of an additional pillar cell could cause additional stiffness of the basilar membrane and impede hearing. *Sprouty2* has been shown to inhibit the range of Fgf8 signaling through Fgfr3 in the OC(Shim, Minowada et al. 2005). It is possible that a shift or expansion of the *Sprouty2*-expressing region in an ancestral mammal could have reduced the effective signaling range of Fgf8, limiting the number of pillar cells, thus driving the reduction in overall size of the HC field in the cochlear duct of eutherian mammals. This compaction of the width of the hair cell field and the general lengthening of the cochlear duct could be related to more refined frequency selectivity, with the need to break up the tuning curve into more precise and finer frequency ranges. Consistent with this, the short monotreme cochlea also has the narrowest hearing range of any mammal(Mills and Shepherd, 2001) suggesting that refinement throughout mammalian history has provided a more fine-tuned auditory organ.

Because Fgf8 is responsible for specifying where pillar cells are found within the epithelium and is responsible for the spacing of inner and outer hair cells, it is not unreasonable to hypothesize that the introduction of *Fgf8* signaling into a more basilar

papilla-like ancestral ear could have initially enabled more fine tuned hearing by physically separating the two types of hair cells. The purpose of the separation of hair cells by the tunnel of Corti may be to buffer the inner hair cells from the movements of the OHCs as they amplify incoming sounds. As mentioned above however, large numbers of pillar cells may have the negative effect of causing too much sound dampening and thus there was an adaptive need for *Sprouty* signaling to reign in this effect.

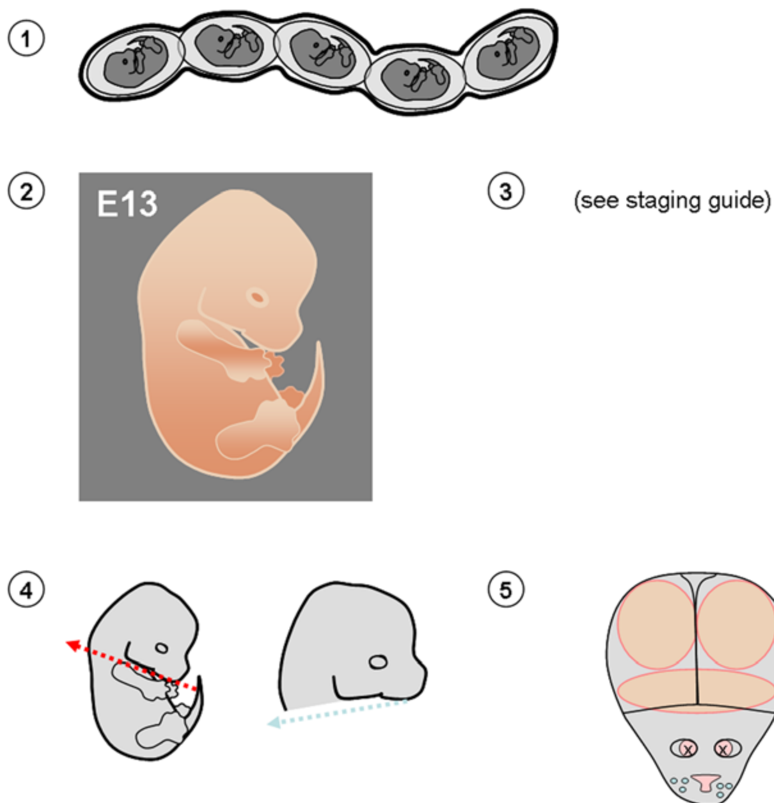
Given the requirement of Fgfr3 signaling for pillar cell development and maintenance, it can be assumed that the additional pillar cells in the monotreme organ of Corti express *Fgfr3*. If Fgfr3 provides cells with the potential to transdifferentiate or undergo mitosis, is it possible that monotremes possess the ability to regenerate hair cells? The very recent publication of the complete monotreme genome(O'Brien 2008) will allow future studies into overall differences in members of these signaling pathways. However, the elusive nature and rarity of the platypus and echidna may prove problematic for obtaining the staged embryos necessary for conducting functional studies and gene expression analysis on the monotreme inner ear.

A final goal of auditory research, as well as developmental biology as a whole, should be the identification of how the individual signaling pathways, such as the Wnts, Fgfs, Hhs, Soxs, and Bmps, to name a few, are interrelated. Understanding the cross activity of these genes will be key in fully elucidating the development of the inner ear, and understanding the loss of regeneration in mammals.

Appendix: Complete Guide to Cochlear Dissection

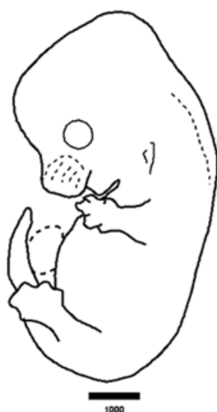
Isolating the Temporal Bone:

1. Once euthanized, open the mom's body cavity, remove uterus intact and place in a dish with chilled HBSS.
2. Remove embryos from uterus, amniotic sac and separate from placenta. Place in new dish.
3. Determine the developmental age of the embryos using a staging guide.
4. Remove heads from embryos and place these in a new dish.
5. To begin the cranial dissection, align the head with the nose toward you, brain-side up. The brain should be clearly visible beneath the skin.



Staging Guide:
(taken from http://genex.hgu.mrc.ac.uk/Databases/Anatomy/MAstaging.shtml)

E12.5



E13.5

E14.5

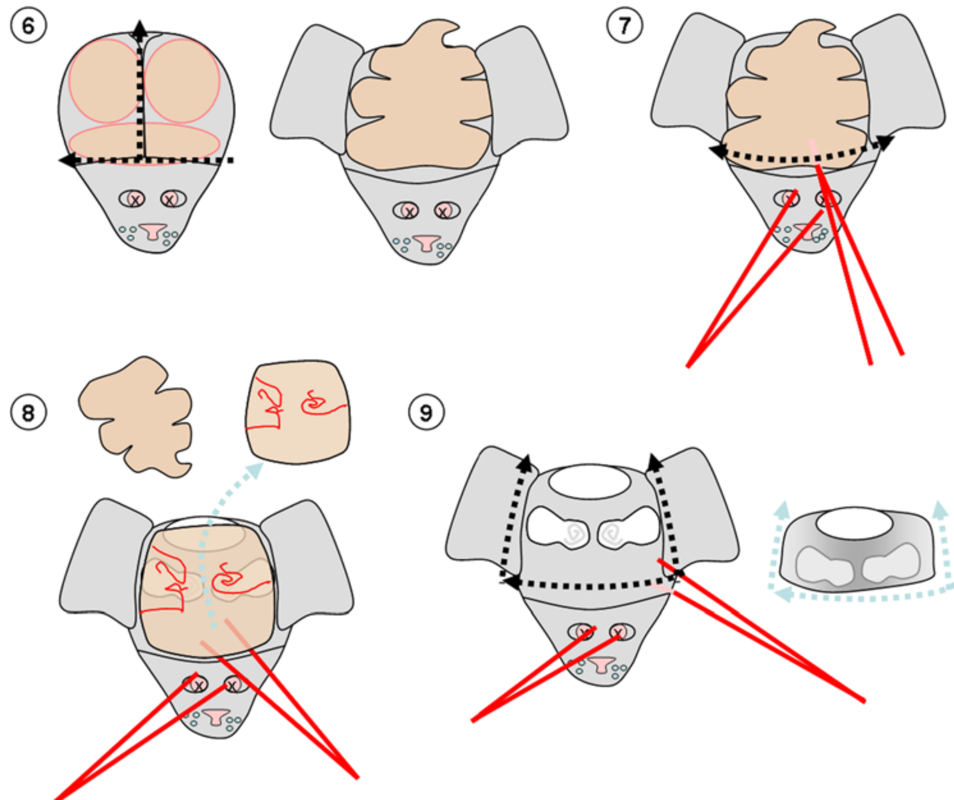
E15.5

E16.5

E17.5

Isolating the Temporal Bone (cont.):

6. Open up the cartilagenous skull to expose the brain.
7. Use your left forcep to stabilize the head by placing it in the region of the eye sockets. Use your right forcep in the closed position to pry the brain upwards by using a left-to-right scraping motion to separate the brain from the underlying connective tissue. Lift the brain from the front towards the back until the entire brain is loosened from the connective tissue below it then remove the brain from the cranial cavity.
8. At early stages (up to E15) it may be necessary to remove a membrane situated beneath the brain in order to expose the skull cavity containing the cochleae. It is easily identified by the blood vessels that run through it and can be grabbed near frontal region and pealed back caudally to remove it.
9. Next, remove the skull flaps and snip off the entire frontal region of the head (from behind the eyes up through the nose, tongue and mouth). ** Once you can easily identify the temporal bones (peanuts), the closer to the peanuts that you snip off the rest of the skull region, the easier it will be to free them from the remaining skull tissue.

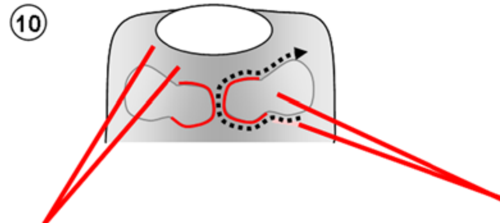


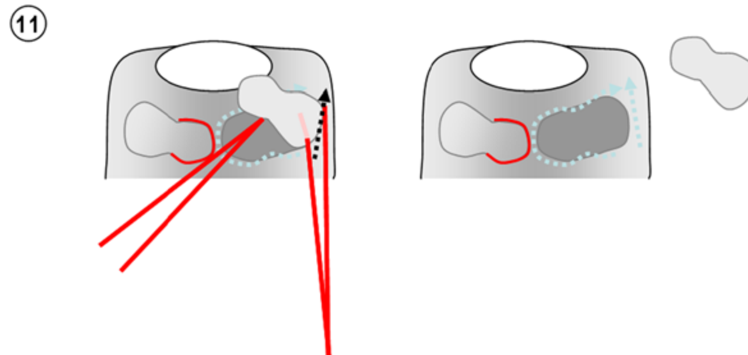
Isolating the Temporal Bone (cont.):

**At this point it may be difficult to discern where the boundaries of the temporal bones (peanuts) are. Often, blood will collect in the small gap at the point of intercalation between the peanuts and the skull. This makes a useful landmark to determine the position and location of the peanuts. so that the tissue may be snipped around the entire temporal bones (cochlear and vestibular parts connected) during the dissection.

10. Use your left forcep to stabilize the cranial piece while using your right forcep to trace around the peanut using a snipping motion. Snip around the entire temporal bone starting near the medial cochlear region which is usually the most easy to detect.
11. After tracing around the cochlea with your forceps, it should be possible to pry up the medial regions which makes it easy to snip across the bottom of the vestibular portion to free the entire peanut. Repeat for the opposite peanut.

**At this point peanuts can be fixed for later processing.

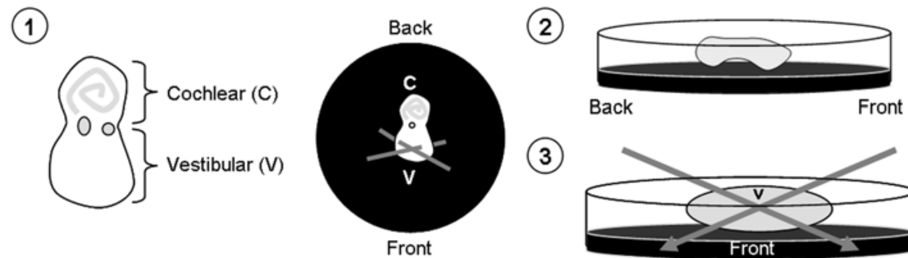




Pinning:

1. Align temporal bone so that the cochlea is pointing towards the back of the Sylgard dish* and the vestibular portion is towards the front.
2. The convex side of the temporal bone should point upwards with the concave side facing the bottom of the dissecting dish. A faint curving line should be visible beneath the surface of the cartilage; this is the developing cochlear duct.
3. Pins should be placed low and horizontal within the plane of the dish (rather than vertical) for maximum stability. Pin one side through to the sylgard before doing the other side. Pins should cross at the midline for maximum stability.

*Silicone (Sylgard, Dow-Corning) is mixed with charcoal powder and set into a glass petri dish to create a dark surface to contrast with the white tissue while dissecting. This also creates a pinnable dissecting pad for tissue stabilization.

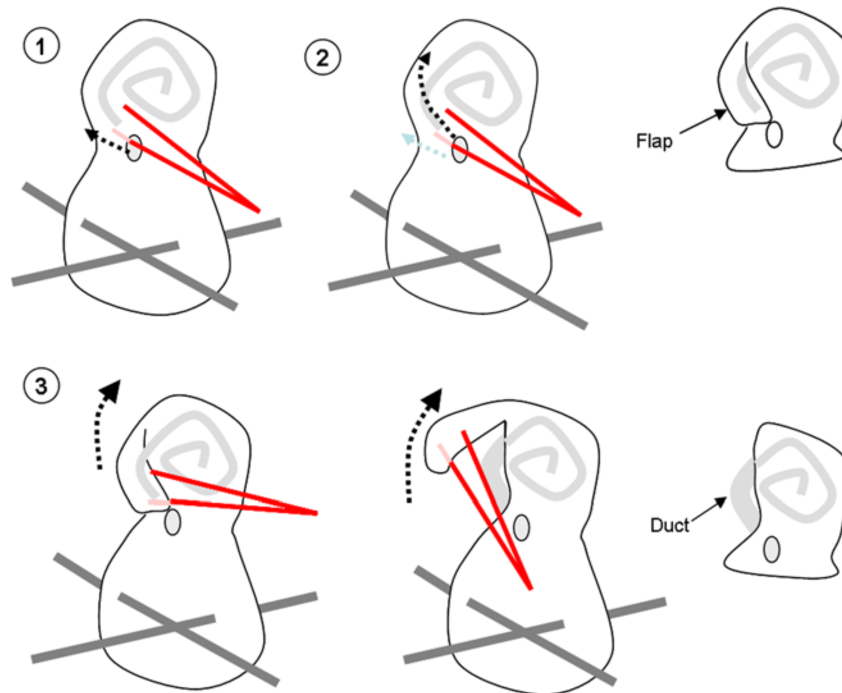


Exposing the Cochlear Duct:

1. Start at the oval window by inserting right bottom forcep tine into hole, snip upwards, try not to insert forcep too deep into the tissue, the tine should be visible right beneath the surface of the cartilage (pink).

2. Next, with forceps in the same position, make a snip from the oval window towards the back of the dish, again making sure that the inserted tine is visible beneath the surface of the cartilage. These cuts will create a flap.

3. Grab the flap that you have created, pinch it between the forceps and pull upwards until it comes off. A portion of the cochlear duct should now be exposed and visible.



Exposing the Cochlear Duct (cont.):

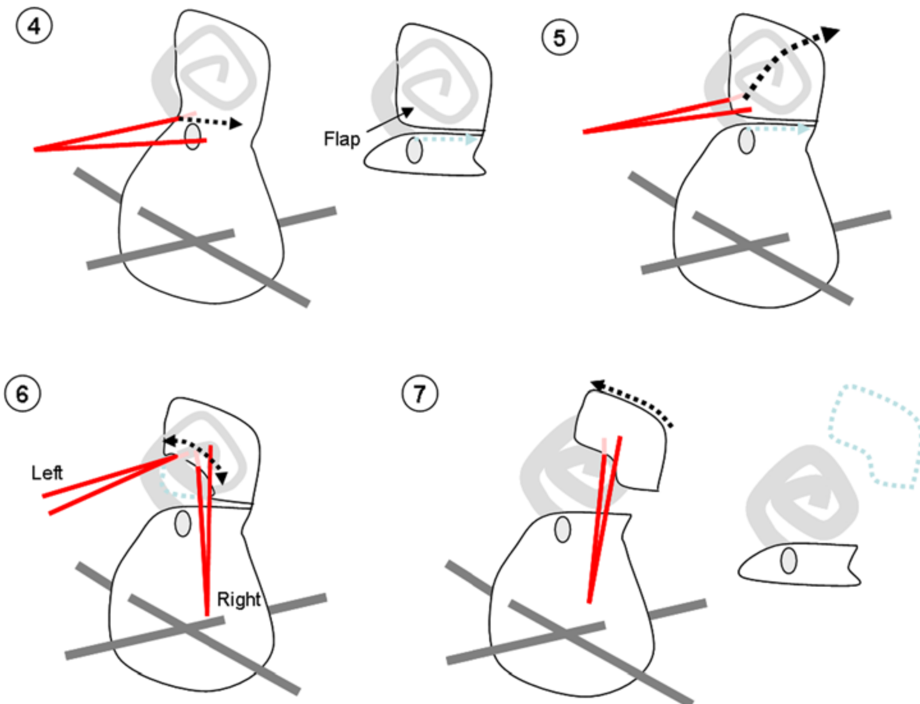
4. Next, just above the oval window, snip in a horizontal plane with one tine of the forcep just below the surface of the cartilage until you reach the opposite side of the cochlea.

5. Now grab the new flap with your right forcep and gently lift upwards.

6. Use your left forcep (keeping the tines pinched together) to carefully pry the apical regions of the cochlear duct from the cartilage using a gentle side-to-side scraping motion right beneath the lip of the cartilage.** Using this same motion, work your way across the cartilagenous "cap" until the apex comes loose.

   ** NOTE: During development, the cochlear duct begins to become more fused and incorporated into the turns of the bony/cartilagenous casing, so in older animals it may be necessary to use more scraping/prying to release the cochlear duct intact from the cartilage, whereas in younger tissue you may not need to do it at all.

7. Pull back further on the flap until it comes off; this may require some snipping at the top edge. At this point the entire cochlear duct should be exposed.

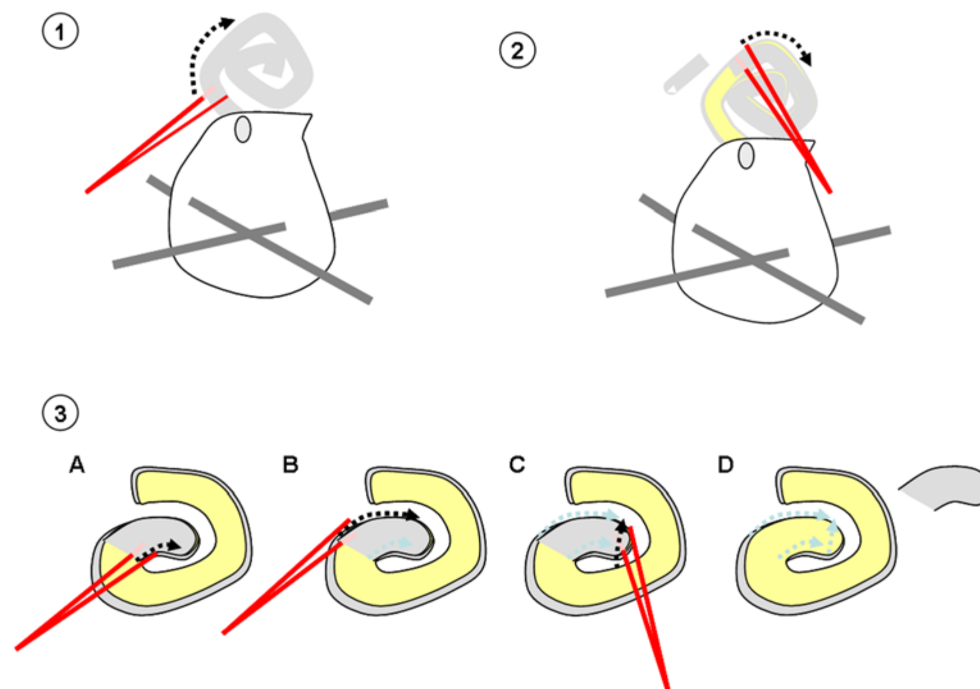


Exposing the Sensory Epithelium:

1. The next step is to remove Reisner's membrane which will open the cochlear duct and expose the sensory epithelium. Use your right forcep to pinch the very surface of the Reisner's membrane at the basal-most region then pull upwards following the turn of the cochlear duct. The Reisner's membrane should pull cleanly away.

2. If the entire membrane does not come off in one piece keep repeating the process pinching each time at the spot where it broke off during the previous attempt until you reach the apex.

3. Be careful when pulling at the Reisner's membrane at the most apical piece as part of the sensory may become detached along with the membrane. It may be best to use some snipping motions in this area to separate the membrane from the cochlear duct.

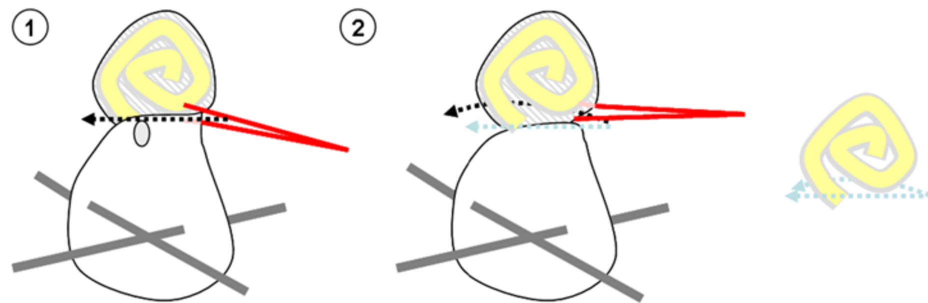


Exposing the Sensory Epithelium (cont.):

At this point the tissue is now ready for processing. To establish cochlear explant cultures or to enable a whole-mount preparation the vestibular region needs to be removed along with underlying cartilage and mesenchyme.

1. Start by snipping away the upper part of the vesitublar system's cartilage.

2. Next, snip around the back side of the cochlea to remove the remaining cartilage. At this point it should be relatively easy to remove the cochlear duct intact.



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