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Genomic Approaches for Pathway Identification in Regenerating Sensory Epithelia of the Inner Ear

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GENOMIC APPROACHES FOR PATHWAY IDENTIFICATION IN REGENERATING SENSORY EPITHELIA OF THE INNER EAR

by

David Michael Alvarado

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ABSTRACT OF THE DISSERTATION

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Professor Michael Lovett, Thesis Advisor

The inner ear utilizes sensory hair cells as mechano-electric transducers for sensing sound and balance. In mammals, these sensory hair cells lack the capacity for regeneration and if damaged lead to hearing or balance disorders. However, non-mammalian vertebrates such as birds maintain their regenerative abilities throughout their life. We completed a gene expression profiling time course of regenerating sensory epithelia (SE) in avian cochlea and utricle on a custom transcription factor microarray following damage by laser and chemical ablation and identified genes from known signaling cascades differentially expressed during SE regeneration. In the second study, we selected 27 of these genes for knockdown by siRNA or small molecule inhibition to determine their requirement for SE regeneration and identified downstream targets. We assessed affects on proliferation using a 96 well high throughput assay and profiled gene expression changes that resulted from each knockdown. Using these techniques we have determined genes required for SE proliferation and identified novel epistatic relationships between many of these genes.

In a third study we used 3 complimentary approaches to identify downstream targets of GATA3 in the avian utricle. The zinc finger transcription factor GATA3 is required for inner ear development and mutations cause sensory neural deafness in humans. In a previous study we had observed that GATA3 is expressed throughout the SE in the cochlea; however, expression is limited to the striola of the utricle. The striola corresponds to an abrupt change in morphologically distinct hair cell types and a 180° shif t in hair cell orientation. We used microarray expression profiling of direct comparisons between cells micro-dissected from the striola vs. extra-striola, GATA3 knockdown by siRNA in utricle sensory epithelia and GATA3 over-expression to identify genes potentially regulated by GATA3 in the inner ear. We confirmed the direct *in vivo* interaction of GATA3 with two of these targets (LMO4 and MBNL2) by chromatin immunoprecipitation (ChIP) using GATA3 antibodies and also demonstrated by RNA *in situs* that both these genes exhibit patterns of expression consistent with their direct regulation by GATA3.

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CHAPTER ONE

INNER EAR DEVELOPMENTAL GENETICS

Up to 30 million people in the United States are estimated to have significant auditory impairment, 60% of these individuals being between the ages of 21 and 65. Over the age of 65, 1 in 3 individuals suffers from age related hearing loss (Cruickshanks, Wiley et al. 1998). One in 1000 newborns suffer from congenital hearing impairment, more than half of these cases are due to genetic factors (Morton 1991; Parving 1993; Mehl and Thompson 1998). Though deafness is a component of many syndromes, most genetic causes of hearing impairment are non-syndromic. There are 75 loci for autosomal recessive non-syndromic (DFNB) forms of deafness, 57 loci for autosomal dominant (DFNA) and 5 X-linked (DFN) loci mapped to date. 43 causative genes for deafness have been identified (Van Camp and Smith 2008). The most common cause of genetic deafness is the gap-junction protein connexin 26, responsible for greater than 50% of pre-lingual, recessive deafness and 15-30 % of sporadic cases (Tranebjaerg 2008). The majority of hearing loss cases are due to sensorineural deafness. This sensorineural hearing loss results from damage to the sensory hair cells or nerves of the inner ear.

The inner ear is divided into two functional structures: the vestibular organ, which is responsible for maintaining balance and the auditory organs, which sense sound (Figure 1-1). The vestibular organ consists of three semi-circular canals responsible for sensing rotational acceleration as well as the saccule and utricle, which sense linear acceleration and gravity. The cochlea is a coiled chamber filled with a potassium ion-rich fluid called endolymph. The cochlea's primary function is to sense sound. Both organs of the inner ear utilize hair cells

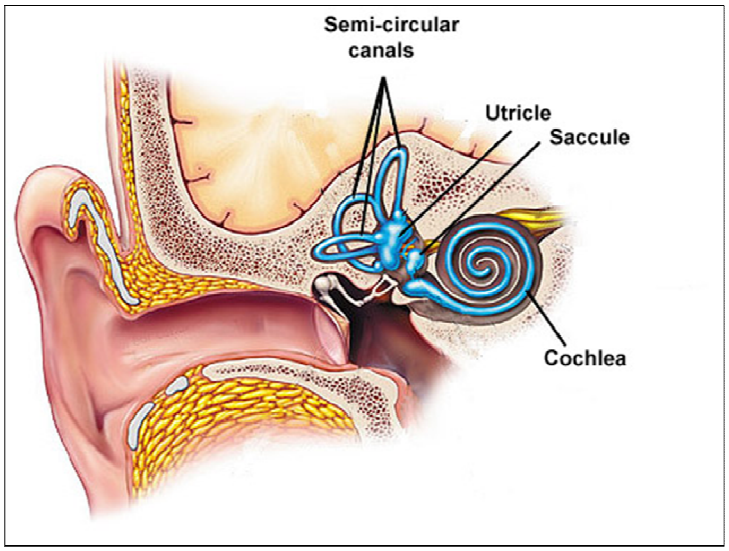

Figure 1-1 Anatomy of the adult inner ear, reproduced from (APTA 2002). Three semi-circular canals sense rotational acceleration, the utricle and saccule sense linear acceleration and gravity. Together these three structures comprise the vestibular organ which primarily functions in maintaining balance. The cochlea is the coiled auditory organ which senses sound.

as mechano-electrical transducers. Movement of the stereocilia of sensory hair cells in the inner ear initiates an action potential, translating mechanical movement into an electrical impulse. Two types of hair cells are used to detect sound in the cochlea. A single row of inner hair cells are responsible for the majority of hearing. Three rows of outer hair cells refine the sensitivity and serve as sound amplifiers (Figure 1-2). High frequency sounds are detected from the shorter stereocilia at the base of the cochlea, while low frequencies are detected from the longer stereocilia at the apex. The sensory hair cells are surrounded by non-sensory supporting cells; together these cells comprise the sensory epithelia. The sensory epithelia is under laid by a basal membrane and overlaid by the tectoral membrane. As sound waves travel through the outer ear, they cause vibration of the tympanic membrane, the eardrum. These vibrations in turn cause movement of the bones in the middle ear. Movement of these bones vibrates the cochlea initiating waves of the endolymph fluid within. These waves cause movement of the basal membrane and the sensory epithelia. Contact between the stereocilia and the tectoral membrane causes the stereocilia to

bend. This bending action opens cation channels allowing an influx of potassium ions from the endolymph. Depolarization of the hair cells initiates neurotransmitter release at the base of the sensory hair cells sending an action potential to the auditory cortex of the brain via the VIIIth cranial nerve (Hudspeth 1997).

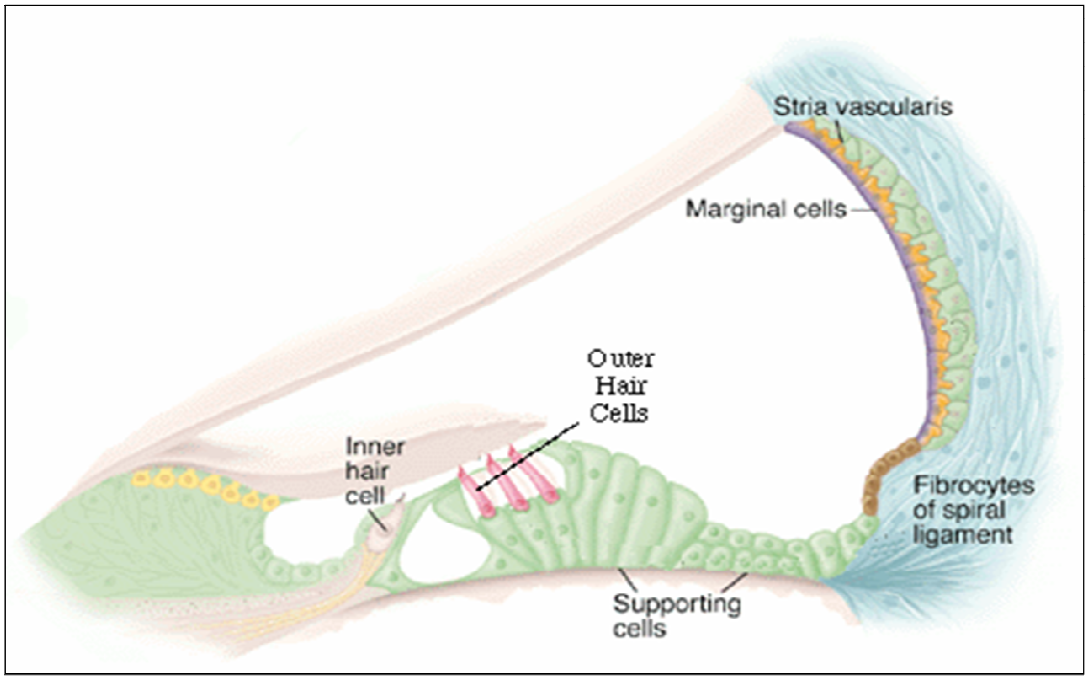

Figure 1-2 Cross section of the Organ of Corti from a mammalian cochlea, modified from (Steel 1999). The cochlea utilizes sensory hair cells as mechano-electric transducers to detect sound. Sensory hair cells are surrounded by non-sensory supporting cells. Both cell types arise from the same cell lineage and together comprise the sensory epithelia.

The hair cells of the utricle operate in a similar manner to the cochlea. The sensory epithelia of the utricle consist of supporting cells and sensory hair cells overlaid by a gelatinous matrix containing small calcium carbonate particles called otoconia (Figure 1-3). During movement, the overlaying matrix stimulates the sensory hair cells activating action potentials that signal linear acceleration and gravity.

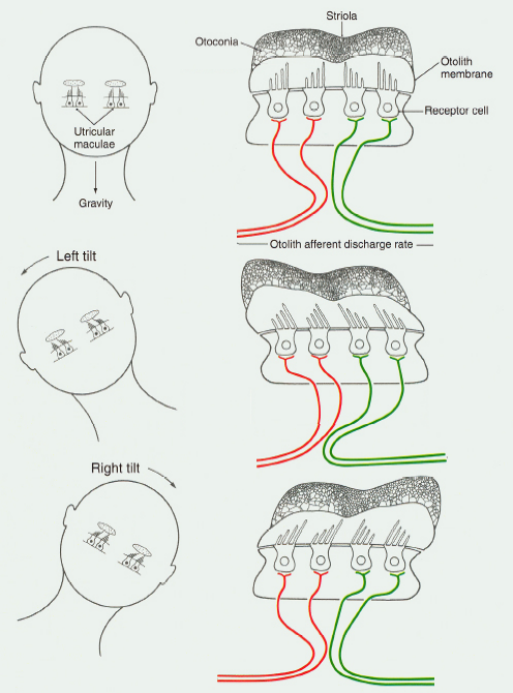

Figure 1-3 The utricle otoconia, modified from (Dickman 2009). The utricle utilizes sensory hair cells to detect linear acceleration and gravity. The utricle sensory hair cells are overlaid by a gelatinous matrix containing calcium carbonate particles called otoconia. As the head tilts left or right, the weighted otoconia shift the gelatinous matrix, deflecting the sensory hair cells.

The vertebrate inner ear originates from the otic placode, a thickening in surface ectoderm that forms above the hindbrain early in embryonic development. The otic placode invaginates into the mesenchymal tissue to form the otic pit. The otic pit enlarges and closes to form the otocyst, also known as the otic vesicle. The resulting structure goes on to form the vestibular (balance) and cochlear (auditory) organs of the inner ear. Terminal mitosis occurs between E14-E15 (Ruben 1967). At this time, sensory hair cells are first detected. These

sensory hair cells are surrounded by non-sensory supporting cells (Adam, Myat et al. 1998). In the cochlea, each hair cell is surrounded by specialized supporting cells: inner phalangeal cells or Deiters' cells which lie beneath inner or outer hair cells respectively. Inner and outer hair cells are separated and supported by inner and outer pillar supporting cells. Sensory hair cells and non-sensory supporting cells originate from the same cell lineage and together comprise the sensory epithelia of the inner ear.

Development of Inner Ear Anatomy

FGF Signaling

Peptide ligands of the Fibroblast Growth Factor family are likely candidates for inducers of inner ear development. In zebrafish, targeted disruption of *fgf3* or *fgf8* cause disruption of the otic vesicle formation, but do not affect otic placode formation (Mansour, Goddard et al. 1993; Whitfield, Granato et al. 1996). These genes may have a redundant function since targeted disruption of both completely and specifically disrupts inner ear development in zebrafish (Phillips, Bolding et al. 2001). In mouse, FGF3 and FGF10 are thought to be the inducers of inner ear induction. FGF10 mutant mice develop smaller otic vesicles (Ohuchi, Hori et al. 2000) and FGF3 mutants do develop an otic placode, however, lateral ear differentiation is disrupted (Mansour, Goddard et al. 1993). Similar to the zebrafish, mouse FGF3 and FGF10 mutants fail to form an otic vesicle (Alvarez, Alonso et al. 2003; Wright and Mansour 2003). In the

reciprocal experiment, FGF3 misexpression in the vicinity of otic tissue was sufficient to induce ectopic otic vesicles in *Xenopus* and chicken embryos (Lombardo, Isaac et al. 1998; Lombardo and Slack 1998; Vendrell, Carnicero et al. 2000). In chicken the first FGF family members detected during inner ear development are FGF8, expressed in the endoderm, and FGF19 first expressed in the mesoderm, followed closely by FGF3 (Ladher, Anakwe et al. 2000; Kil, Streit et al. 2005; Ladher, Wright et al. 2005). While induction of the inner ear appears to be controlled by FGFs in multiple species, the specific FGFs appear to vary and it is still not clear whether these FGFs act directly or indirectly.

The Pax Pathway

The earliest known marker for otic fate is *PAX8*, which is expressed in preotic cells during gastrulation in the mouse (Pfeffer, Gerster et al. 1998; Heller and Brändli 1999). Knockdowns of *PAX8* result in reduced otic placode size and disrupt development of hair cells in zebrafish otic vesicles (Riley and Phillips 2003). The closely related homolog of *PAX8, PAX2,* is also expressed in preotic cells following *PAX8* expression (Pfeffer, Gerster et al. 1998). *PAX2* disruption does not affect otic placode formation, but it does prevent formation of the cochlea in mouse (Torres, Gomez-Pardo et al. 1996). Loss of *PAX8* expression does not affect *PAX2* expression, suggesting that though they are both required for proper inner ear formation, they act in different developmental pathways (Mansouri, Chowdhury et al. 1998). *Drosophila* homologs of Pax genes are well documented for their role in development of the eye (Silver and Rebay 2005).

These highly conserved genes likely act in a similar genetic network to regulate inner ear development.

Notch Signaling and Atoh1

Though the specific signaling pathways required for triggering sensory hair cell regeneration have yet to be identified, several pathways have been implicated as playing a role in hair cell differentiation and proliferation. Progenitor cells of the sensory epithelia acquire either the sensory hair cell or supporting cell fate by lateral inhibition through the Notch signaling cascade (Figure 1-4). Progenitor cells that differentiate into sensory hair cells express elevated levels of Delta (Adam, Myat et al. 1998; Morrison, Hodgetts et al. 1999). This causes neighboring cells to increase Notch expression. Increased levels of Notch inhibit hair cell differentiation in these cells, forcing them to assume a supporting cell fate. *Atoh1* is initially expressed at low levels in all sensory epithelia progenitor cells, but is upregulated in emerging hair cells and increases expression of Delta (Bermingham, Hassan et al. 1999). Over-expression studies of *Atoh1* in immature rat cochlear cultures results in an overproduction of hair cells, whereas *Atoh1* null mice develop sensory epithelia completely lacking hair cells and consisting only of supporting cells (Zheng and Gao 2000). In progenitor cells surrounding emerging hair cells, increased levels of *Notch* expression induces *Hairy and Enhancer of Split* related genes *Hes1* and *Hes5*. Both *Hes1* and *Hes5* negatively regulate *Atoh1* and as expected, knockdown of either gene leads to an overproduction of hair cells (Zheng, Shou et al. 2000; Zine, Aubert et al.

2001). Additionally, adenoviral over-expression of *Atoh1* in mature guinea pigs caused non-sensory supporting cells to transdifferentiate into functional sensory hair cells (Kawamoto, Ishimoto et al. 2003; Izumikawa, Minoda et al. 2005). Though forced expression of *Atho1* has shown limited success in restoring hearing in mature mammals, this method is not a favorable choice for human hearing loss therapy. *Atoh1* over-expression does not induce mitosis, rather transdifferentiation of supporting cells into hair cells. This leads to a depletion of supporting cells and compromises the ability to fully restore hearing.

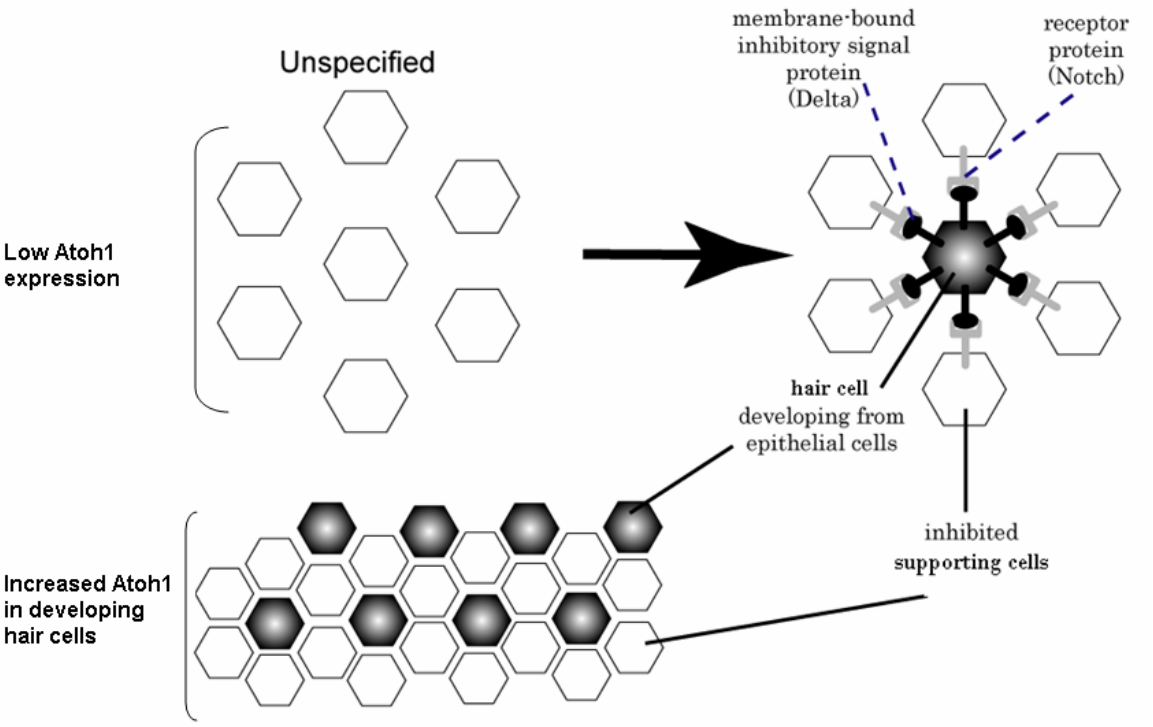

Figure 1-4 Lateral inhibition by Notch signaling. Sensory hair cells and non-sensory supporting cells originate from the same cell lineage. Unspecified cells express low levels of *Atoh1*. Levels of *Atoh1* increase in progenitor sensory hair cells. Progenitor hair cells increase expression of the membrane bound Notch receptor ligand, *Delta.* Activation of the Notch receptors in neighboring cells inhibits expression of *Atoh1* and *Delta*, inhibiting the sensory hair cell fate in these neighboring cells.

Wnt Signaling and Planar Cell Polarity

Modulators of Wnt signaling have been implicated in a variety of inner ear developmental stages from early otic placode induction and sensory specification to planar polarity of stereocilia bundles during cochlea elongation (Hollyday, McMahon et al. 1995; Dabdoub, Donohue et al. 2003; Stevens, Davies et al. 2003; Ohyama, Mohamed et al. 2006; Sajan, Warchol et al. 2007). The Wnt Signaling pathway consists of highly conserved signaling molecules and receptors that regulate numerous developmental processes (for review see (Logan and Nusse 2004). The primary components of Wnt signaling are secreted Wnt ligands characterized by highly conserved cysteine residues, their seven transmembrane Frizzled (Fzd) receptors and LRP5/LRP6 co-receptors (Rijsewijk, Schuermann et al. 1987; Bhanot, Brink et al. 1996; Yang-Snyder, Miller et al. 1996; Pinson, Brennan et al. 2000; Tamai, Semenov et al. 2000).

Wnt signaling is primarily divided into canonical and noncanonical pathways. The canonical pathway functions by controlling protein levels and the availability of the cytoplasmic protein β-catenin (Clevers 2006). In the absence of canonical Wnt signaling, β-catenin is sequestered by APC and Axin facilitating its phosphorylation by CK1α and GSK3 kinases. This phosphorylation initiates ubiquitination and proteosomal degradation of β-catenin. Wnt ligand activation of canonical Wnt signaling induces formation of Frizzled, LRP, Dishevelled (Dsh) complexes at the cellular membrane. Phosphorylation of the Fzd, LRP5, Dsh complex recruits Axin to the receptor complex. Recruitment of Axin to the cell

membrane releases β-catenin, which enters the nucleus and activates transcription of canonical Wnt regulated targets.

The majority of research to date has focused on the canonical Wnt signaling pathway. Wnt components also regulate developmental processes through the β-catenin independent, non-canonical pathway (for review see (Veeman, Axelrod et al. 2003). The majority of research on non-canonical Wnt signaling suggests several overlapping genetic components and functions similar to the planar cell polarity (PCP) pathway. Mutations in *Celsr1, Scribble* and *Vangl*, mammalian homologues of *Drosophila* PCP genes, have been reported to cause defects in mouse cochlea hair cell polarity (Bilder and Perrimon 2000; Curtin, Quint et al. 2003; Montcouquiol, Rachel et al. 2003). Noncanonical Wnt signaling utilizes the same Fzd receptors as canonical Wnt signaling and involves the cytoplasmic signaling transduction protein Dsh. Downstream of Dsh, the noncanonical Wnt pathway diverges from canonical Wnt by mechanisms independent of GSK3 and β-catenin. A variety of intracellular mechanisms have been implicated in noncanonical Wnt signaling, from intracellular calcium release, Rho family GTPase mediated cytoskeletal remodeling and possibly the JNK pathway and Notch signaling.

Specific Wnt ligands are thought to play an important role during cochlear and vestibular differentiation. *Wnt3a* is first detected in the otocyst from E2.5 to E6. Misexpression of *Wnt3a* gives rise to vestibular patches within the cochlear duct. *Wnt4* is expressed later by E5, bordering sensory vs. nonsensory regions just prior to sensory organ differentiation (Stevens, Davies et al. 2003). Though

specific sensory primordia have already been defined by this time, *Wnt4* may play an important role refining sensory vs. non-sensory boundaries. A more recent study has described specific expression pattern differences of Wnt ligands and Frizzled receptors during vestibular and auditory sensory organ development (Sienknecht and Fekete 2008). During hair cell regeneration in response to injury, new hair cells will be required to properly orient themselves to restore proper hearing. It is likely that components of the Wnt Signaling and PCP pathways will be involved in this process. Recently, several reports have suggested a closely linked relationship between Wnt and Notch ('Wntch') signaling during embryonic development (Hayward, Kalmar et al. 2008). These reports suggest a model in which Wnt Signaling establishes a prepatterned group of cells capable of specific differentiation states. Individual cell fates are then further refined by Notch Signaling. A further appreciation of these pathways and their intertwined relationships will be necessary to understand their roles during inner ear development and hair cell regeneration.

GATA3

In humans, mutations in *GATA3* that disrupt the C-terminal zinc finger result in loss of DNA binding function. These mutations result in hypoparathyroidism, sensorineural deafness and renal anomaly syndrome (HDR) (Van Esch, Groenen et al. 2000), illustrating the sensitivity of these organ systems to *GATA3* haploinsufficiency. *GATA3* is expressed throughout the otic placode, beginning at E8-E9.5 in the mouse embryo (Grace Lawoko-Kerali

2002). *GATA3* is required for otic cup invagination and closure (Lilleväli, Haugas et al. 2006). Homozygous knockout of the *GATA3* gene in mice results in embryonic lethality by E11, due to multiple organ abnormalities, massive internal bleeding, a complete inhibition of T-cell differentiation (Pandolfi, Roth et al. 1995) and abnormal brain morphology. Heterozygote knockouts are viable, but have a progressive degeneration of cochlear sensory hair cells and corresponding hearing loss (van der Wees, van Looij et al. 2004), similar to that observed in the human HDR phenotype. Notably, both *GATA3* heterozygous and null mutant mice also exhibit misrouted axonal projections to the inner ear (Karis, Pata et al. 2001) and elsewhere in the nervous system (Nardelli, Thiesson et al. 1999; Lundfald, Restrepo et al. 2007). These observations suggest a role for *GATA3* in neural development.

GATA3 has been most extensively studied in the development and differentiation of the mammalian hematopoietic system. During differentiation of T lymphocytes from hematopoietic stem cells, naïve CD4+ cells differentiate into either T helper type 1 (Th1) or T helper type 2 (Th2) cells. This switch is tightly regulated by *GATA3* (Szabo, Sullivan et al. 2003; Mowen and Glimcher 2004) and involves the direct transcriptional regulation of IL5 and IL13 by *GATA3* to specify Th2 differentiation (Siegel, Zhang et al. 1995; Kishikawa, Sun et al. 2001; Lavenu-Bombled, Trainor et al. 2002). *GATA3* also plays a significant role in skin development and particularly in specifying inner root sheath cell vs. hair shaft cell differentiation and organization (Kaufman, Zhou et al. 2003). Recently, a direct binding target of *GATA3* has been described in the first intron of the lipid

acyltransferase gene *AGPAT5* suggesting a critical role for *GATA3* in lipid biosynthesis during skin epidermal barrier acquisition (de Guzman Strong, Wertz et al. 2006). Although some *GATA3* transcriptional targets of this type have been described in T-lymphocyte specification, skin differentiation and brain development (Hikke van Doorninck, van der Wees et al. 1999), little is known about its direct targets of action in inner ear development/differentiation.

Although most previous studies of GATA3 in the inner ear have focused on its role in embryonic development, expression of GATA3 is also maintained in the mature inner ear. Our group noted that *GATA3* is expressed throughout the sensory epithelium of the mature avian cochlea, but its expression in the vestibular organs is limited to a 6-10 cell wide region in the striola of the utricle and lagena (Hawkins, Bashiardes et al. 2003) (Figure 1-5). In the utricle, this narrow region of GATA3 expression corresponds to the location at which hair cell stereocilia undergo a 180° shift in orientation (Flock 1 964). Within the striola region, hair cell phenotype changes from so-called type I to type II (Figure 1-6). Type I hair cells are connected to calyx nerve terminals (Lysakowski and Goldberg 1997), these hair cells are morphologically distinct from type II hair cells connected to bouton nerve terminals from afferent and efferent neurons (Jørgensen and Andersen 1973; Jørgensen 1989). Specific roles for type I and type II hair cells have not yet been defined, but their distinct morphologies suggest specialized functions. The relationship between *GATA3* expression and these two morphological changes is not clear. Recent experiments have examined the orientation of regenerated hair cells in explants of the avian utricle

following surgical ablation of the GATA3-expressing region. Such hair cells are normally oriented, suggesting that *GATA3* probably does not specify hair cell reversal (Warchol and Montcouquiol, manuscript submitted). Instead, it is likely that *GATA3* plays a role in specification of hair cell phenotype (as type I vs. type II) and/or axon guidance near the reversal zone.

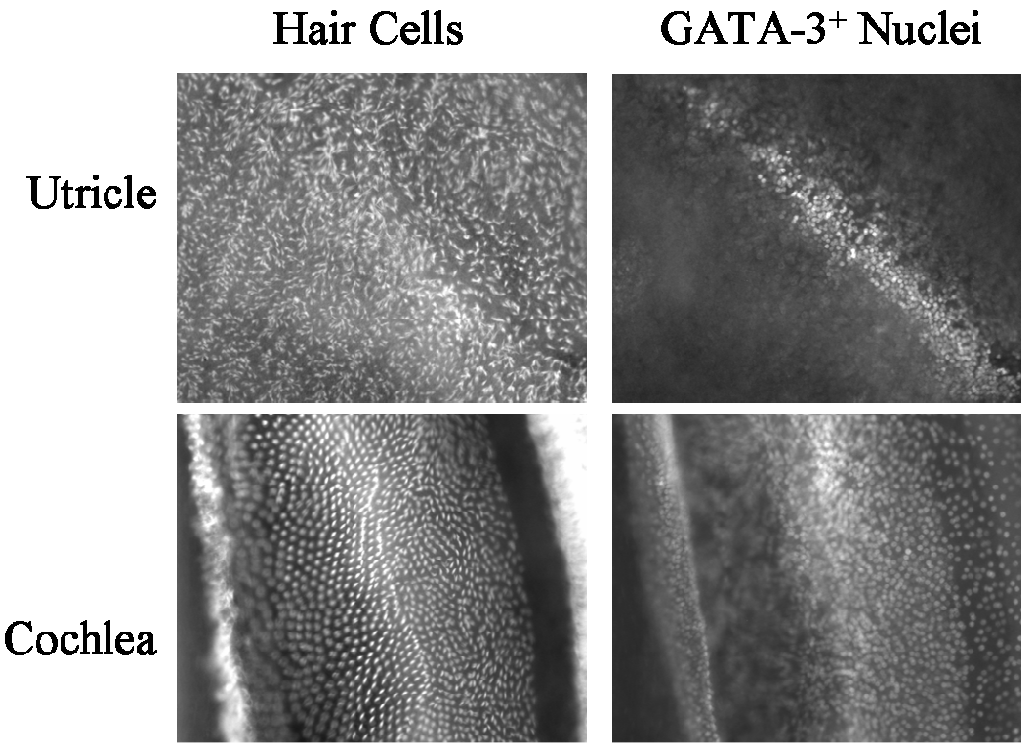

Figure 1-5 Immunohistochemical staining with a GATA3 antibody shows GATA3 expression localized to a small strip of cells in the utricle (top panels) compared to diffuse expression in the cochlea (bottom panels) (Hawkins, Bashiardes et al. 2003)

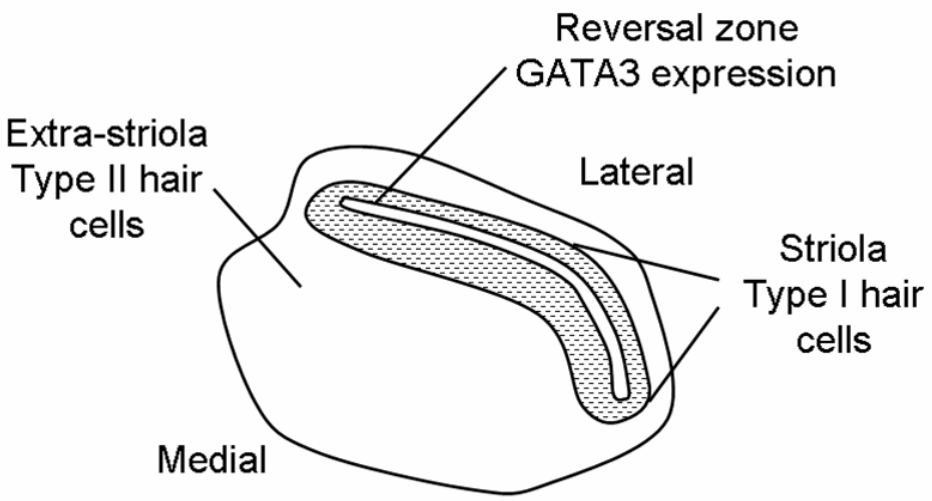

Figure 1-6 Avian utricle hair cell patterns. The striola region contains the Type I hair cells and the extrastriola region is populated by the Type II hair cells. GATA3 is expressed in a 6-10 cell wide strip of cells corresponding to the striola reversal zone. Sensory hair cells undergo a 180° shift in orientation at the striola reversal zone.

Regeneration

Avian hair cell regeneration

Avian hair cell regeneration was first identified in 1987 following acoustic (Cotanche 1987) and chemical (Cruz, Lambert et al. 1987) trauma. Following severe trauma on the stereocilia bundles of the cochlea hair cells, both groups identified signs of new hair cells following recovery. Cotanche (Cotanche 1987) performed a time course of hair cell regeneration response to auditory trauma. Following exposure to 120 decibel for 48 hrs, initial hair cell recovery was first detected by 24 hrs post trauma and by 10 days hair cells had completely recovered. Similarly, a more detailed study of hair cell regeneration in response

to the known ototoxic antibiotic gentamicin was performed by Ryals and Rubel (Ryals and Rubel 1988). Chicks were treated with gentamicin for 10 days and hair cells were counted from 11- 32 days post treatment by light microscopy, a significant increase in hair cells was identified by day 25. Additionally, while sensory hair cells of the avian cochlea remain quiescent until they are damaged, hair cells of the avian utricle were found to undergo continuous turnover even in the absence of trauma (Jørgensen and Mathiesen 1988). Several studies have shown a limited regenerative ability in mammalian vestibular organs (Forge, Li et al. 1993; Warchol, Lambert et al. 1993), however, the regeneration is inadequate to repair any damage that is sustained, though this does provide some evidence that mammals may be capable of hair cell regeneration under the proper conditions. More recently, post mitotic, non-sensory supporting cells from mouse cochlea have been shown to be capable of re-entering the cell cycle up to 2-3 weeks postnatal (Oshima, Grimm et al. 2007). The regenerative ability of neonatal mouse cochlea sharply decreases after 3 weeks due to a loss of the ability to downregulate the cyclin dependent kinase inhibitor, $p27^{Kip1}$ (White, Doetzlhofer et al. 2006).

Avian hair cell regeneration has been shown to occur by two distinct mechanisms. The first mechanism is similar to the process of hair cell differentiation that occurs during inner ear development. Tritiated thymidine incorporation in new hair cells was utilized to show that new hair cell populations arise by mitosis of surviving cells (Corwin and Cotanche 1988; Ryals and Rubel 1988). Later studies also demonstrated that supporting cells that survive the

initial trauma re-enter the cell cycle and that these newly formed precursor cells differentiate into sensory hair cells and supporting cells (Raphael 1992; Hashino and Salvi 1993; Stone and Cotanche 1994; Warchol and Corwin 1996). The second mechanism for generating new sensory hair cells is direct transdifferentiation. Both chickens and amphibians are capable of generating new hair cells in response to either ototoxic or auditory injury in the presence of Aphidicolin, a blocker of S-phase division (Adler and Raphael 1996; Baird, Burton et al. 2000; Taylor and Forge 2005). Through this process, new hair cells are generated without cell cycle re-entry. Supporting cells that survive the initial trauma phenotypically convert to functional hair cells.

Cyclin Dependent Kinase Inhibitors

Cyclin dependent kinase inhibitors appear to play a major role in sensory epithelia maintenance once hair cell/supporting cell differentiation has occurred. Cyclin dependent kinases regulate steps through the cell cycle. Expression of cyclin dependent kinase inhibitors causes cells to exit the cell cycle, rendering them mitotically inactive. Shortly after differentiation, the cyclin dependent kinase inhibitor $p27^{Kip1}$ is highly expressed in cells of the sensory epithelia (Chen and Segil 1999; Lowenheim, Furness et al. 1999). $p27^{Kip1}$ homozygous knockout mice develop with an excessive number of sensory hair cells, but retain a normal number of supporting cells. This suggests that $p27^{Kip1}$ plays a role in preventing hair cell proliferation rather than differentiation. Similarly, the cyclin dependent kinase inhibitor *Ink4D* is expressed in cells that have acquired a sensory hair cell

fate (Chen, Zindy et al. 2003). *Ink4D* knockdowns initially develop normal sensory epithelia. Sensory hair cells begin to progressively re-enter the cell cycle and die through apoptosis at approximately 5 weeks following birth. This evidence suggests cyclin dependent kinase inhibitors play an important role in maintaining mitotically inactive sensory epithelia cells in mammals. Though removal from the cell cycle plays an important role in maintaining functionally active sensory epithelia, this may be an important factor in the lack of regenerative capabilities in the mammalian cochlea.

Genomic Approaches to Hair Cell Regeneration

In a previous study from our group, differences in gene expression between cochlear and utricular hair cells of the avian sensory epithelia were expression profiled on a cross species transcription factor microarray (Hawkins, Bashiardes et al. 2003). Sensory hair cells of the avian cochlea only undergo regeneration when damaged and sensory hair cells of the avian utricle are in a constant state of regeneration. Sensory epithelia of the avian cochlea and utricle were compared to identify differences in mitotically quiescent and regenerating sensory epithelia. Transcription factor gene expression was assayed by comparative hybridization (avian cochlea vs. utricle) on a cross species custom transcription factor gene microarray (Messina, Glasscock et al. 2004). Previous studies have demonstrated that cross-species hybridizations can be reliably used on this type of array platform (Hawkins, Bashiardes et al. 2003; Renn, Aubin-Horth et al. 2004). This study represented the first use of human microarrays to

interrogate chick gene expression. In addition to developing micro-cDNA amplification techniques enabling the study of a small number of cells from the sensory epithelia of the inner ear, this study identified several genes potentially involved in hair cell regeneration. Notably, this study identified up-regulation of known deafness loci, *c-KIT* and *PAX3*, in the utricle and *GATA3* in the cochlea. *In situ* hybridizations confirmed *GATA3* expression throughout the sensory region of the cochlea, but limited to a 6-10 cell wide region in the utricle corresponding to the striola reversal zone. *PAX3* and *GATA3* will be examined in greater detail in Chapters 3 and 4 respectively.

As a follow up study, the first large scale gene expression profiling of avian hair cell regeneration examined expression changes in regenerating avian cochlea and utricle (Hawkins, Bashiardes et al. 2007). Avian cochlea and utricle were separately damaged by either laser or chemical ablation. Samples were then expression profiled on a custom, cross-species transcription factor microarray across a recovery time course. This study identified components of known pathways differentially expressed during avian hair cell regeneration: *TGFβ, PAX, NOTCH, WNT, NFKappaB, INSULIN/IGF1* and *AP1*. Additionally, several genes that had not been implicated in any known pathways, such as *CEBPG,* were also identified as differentially expressed during avian hair cell regeneration. A detailed analysis of specific transcription factors and pathways enriched in regenerating cochlea and utricle will be described later in Chapter 2.

Another study from our group examined expression changes in the developing mammalian inner ear. All stages and substructures of the inner ear were expression profiled from E9-E15 in the developing mouse (Sajan, Warchol et al. 2007). This study identified several genes known to cause inner ear defects in mouse mutants (*e.g.*, *Ctnnb1*, *Eya1*, *Eya4*, *Gja1*, *Gjb6*, *Notch1*, and *Sox10* among others). Interestingly, components of several known pathways such as *Wnt, Notch, FGF* Signaling, were found to be differentially expressed in specific structures and stages of mouse inner ear organogenesis. Though components of several pathways were identified in multiple stages and structures, different components were expressed at particular stages of development. For example, *Wnt7a* expression is specific to the cochlea during later development (E12.5-E15) and *Wnt4* is higher in both the cochlea and the saccule compared to the utricle. Components of pathways that had not previously been implicated in inner ear development, such as the circadian rhythm pathway and estrogen signaling, were also identified. This study represented the most comprehensive analysis of expression changes in the developing mouse inner ear to date and identified several important genetic pathways involved in inner ear organogenesis.

Future Directions

Previous studies have identified several genes that are involved in hair cell regeneration and provided some evidence that the mammalian inner ear is capable of limited regeneration. Unfortunately the extent of mammalian

regeneration is not sufficient to compensate for the damage sustained. The evidence suggests that under the proper conditions mammals may be capable of hair cell regeneration. Our current understanding of genes involved in inner ear development and hair cell regeneration have mostly involved one gene at a time. A full understanding of genetic pathways required for hair cell regeneration will require connecting known pathways with newly discovered, unknown components. The microarray expression profiling of avian hair cell regeneration provided an important dataset to greatly increase our understanding of the genetic wiring utilized during sensory epithelia regeneration. In this thesis, genes involved in avian hair cell regeneration are first identified from this microarray expression profiling dataset. To determine if these genes are required for sensory epithelia proliferation, siRNA knockdowns and small molecule inhibitors were used to disrupt genes identified from the regenerative time course study. Effects on proliferation were determined in a high throughput 96 well assay, and each knockdown was expression profiled to identify genes that act downstream. In addition to understanding the genetic pathways required for hair cell regeneration, it is also important to identify the genes directly regulated by these critical transcription factors. Three complimentary approaches were used to identify genes potentially regulated by a transcription factor required for inner ear development, the zinc finger transcription factor GATA3. Direct *in vivo* interaction of GATA3 with two of these targets (LMO4 and MBNL2) was determined by chromatin immunoprecipitation (ChIP) using GATA3 antibodies and expression patterns consistent with their direct regulation by GATA3 was demonstrated by

RNA *in situ* hybridizations. These studies identified genes involved in avian hair cell regeneration and identified novel epistatic relationships between numerous genes that had not previously been implicated in hair cell regeneration.

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CHAPTER TWO

GENE EXPRESSION PROFILES OF REGENERATING SENSORY EPITHELIA IN THE INNER EAR

Introduction

Loss of inner ear sensory hair cells (HC) is a leading cause of human hearing loss and balance disorders. Unlike mammals, many lower vertebrates can regenerate these cells. In a previous study from the Lovett lab, cross-species microarrays were used to examine the differences between avian sensory epithelia (SE) from the mitotically quiescent cochlea and constantly regenerating utricle (Hawkins, Bashiardes et al. 2003). Two former members of the Lovett lab; David Hawkins and Stavros Bashiardes, conducted a follow up microarray gene expression profiling study of regenerating avian sensory epithelia from damaged cochlea and utricle (Hawkins, Bashiardes et al. 2007). I was a co-author in the study. In this chapter I will focus on the microarray analysis of the dataset generated from this study. Specifically, this study describes the identification of major gene changes and pathways involved in avian hair cell regeneration. This dataset was generated by profiling transcription factor changes in SE from avian cochlea and utricle following two distinct forms of *in vitro* injury: (1) laser 'wounding' of cultured SE or; (2) ototoxic hair cell death caused by treatment with the aminoglycoside antibiotic neomycin. In the first case, cultured SE received linear 'wounds' with a pulsed laser microbeam (Figure 2-1). Creation of the lesion typically required 3–5 min/culture; during this time, control cultures were removed from the incubator and kept under identical conditions, but did not receive lesions. Wounded epithelia were allowed to recover for 30 min, 1 hr, 2 hrs or 3 hrs after the lesions. Equal numbers of lesioned and unlesioned specimens were analyzed at each recovery time point.

For the second injury regimen, utricles or cochleae were cultured for 24 hr in medium that contained 1 mM neomycin (Warchol 1999) (Figure 2-2). A sample of SE was collected immediately after this treatment; this constituted the 0 hr time point for the regenerative time course. Other cultures were rinsed and maintained in neomycin-free medium for an additional 24 or 48 hr. Equal numbers of specimens were cultured under identical conditions, but did not receive neomycin; these served as time-matched controls for comparative gene expression

profiling.

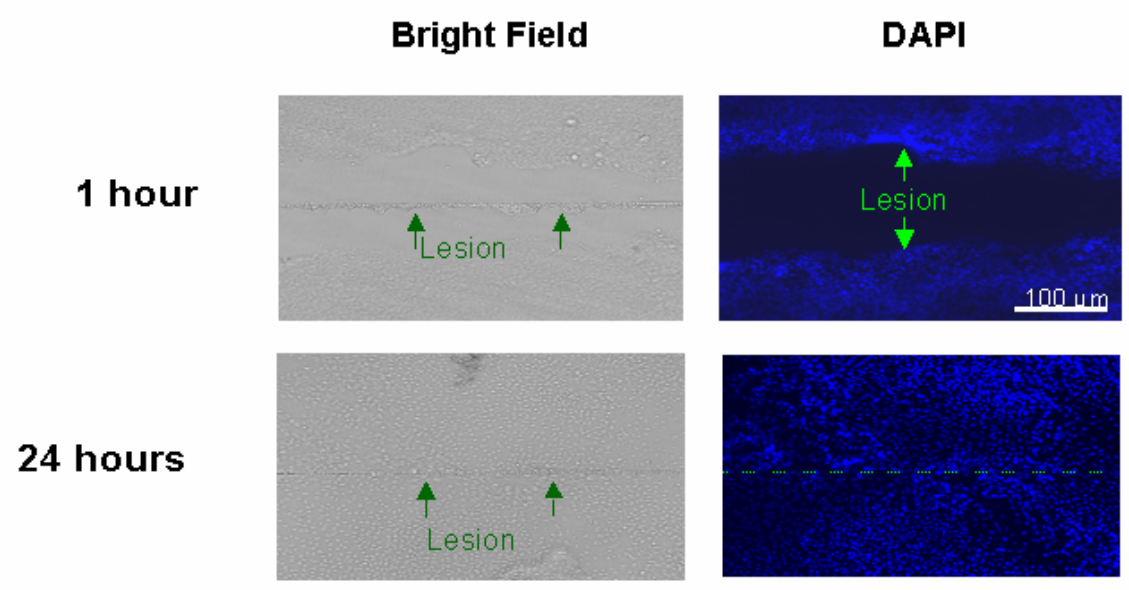


Figure 2-1 Sensory epithelia laser ablation. A laser microbeam was used to damage pure avian sensory epithelia. The laser path can be seen in the bright field image (left) and cell nuclei are shown by DAPI staining (right). After 24 hrs, cells have repopulated the lesion site.

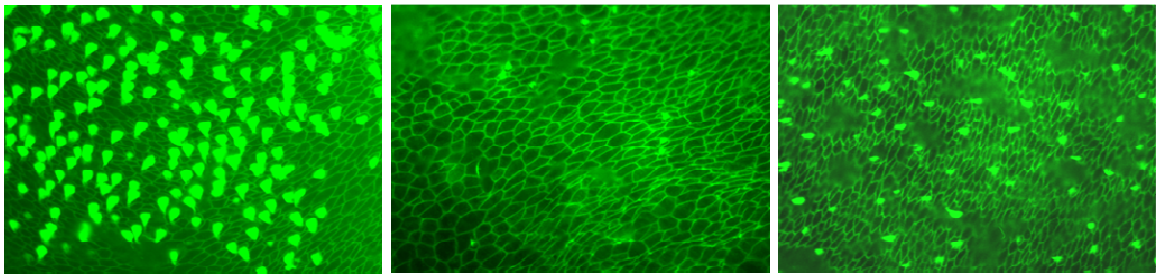


Figure 2-2 Sensory epithelia chemical ablation. The ototoxic antibiotic neomycin was used to damage pure avian sensory epithelia. Using the hair cell specific marker, phalloidin, sensory hair cells can be seen in the untreated control (left) and absent following a 24 hr neomycin treatment (middle). New hair cell populations can be seen following recovery (right).

Transcription factor gene expression was assayed by comparative hybridization (injured specimens vs. time-matched controls) on cross species custom transcription factor gene microarrays (Messina, Glasscock et al. 2004). Previous studies have demonstrated that cross-species hybridizations can be reliably used on this type of array platform (Hawkins, Bashiardes et al. 2003; Renn, Aubin-Horth et al. 2004). This study represented the first large scale gene expression profiling of regenerating sensory epithelia of the inner ear. In the current study I describe multiple components of known signaling pathways that were clearly identifiable: *TGFβ, PAX, NOTCH, WNT, NFKappaB, INSULIN/IGF1* and *AP1*. Numerous components of apoptotic and cell cycle control pathways were differentially expressed, including $p27^{KIP}$ and TFs that regulate its expression. A comparison of expression trends across tissues and treatments revealed identical patterns of expression that occurred at identical times during regenerative proliferation. Network analysis of the patterns of gene expression in

this large dataset also revealed the additional presence of many components (and possible network interactions) of estrogen receptor signaling, circadian rhythm genes and parts of the polycomb complex (among others). Equal numbers of differentially expressed genes were identified that have not yet been placed into any known pathway. Specific time points and tissues also exhibited interesting differences: For example, 45 zinc finger genes were specifically up-regulated at later stages of cochlear regeneration. These results were the first of their kind and provided the starting point for more detailed investigations of the role of these many pathways during sensory hair cell recovery.

Results

Array analysis

In order to quantify gene expression changes, along with associated statistical confidence limits, all expression data were analyzed as described below (see Materials and Methods). Briefly, array data were first normalized by LOWESS, a locally weighted linear regression model, to compensate for dye effects. To assess the similarity and reproducibility of data across multiple biological samples and technical replicates, data from multiple hybridization time points were hierarchically clustered together. Control probes were used to determine a background intensity threshold. Oligonucleotides that fell below this intensity threshold were removed from the dataset. To determine the statistical significance of differentially expressed genes, a one sample t-test was used to calculate a p-value for each gene across all replicate experiments from a particular time point. Self-organizing maps were generated to identify genes with similar expression patterns across multiple regeneration time points. In several cases, genes did not pass the filtering steps in both time courses across all time points. In these cases we extracted the missing values from the primary data and "filled in" the values to construct the patterns of gene expression across all seven time points. In general the vast majority of TFs showed relatively modest gene expression fold changes. This may be due to a compression of the dynamic range in cross-species hybridizations (Hawkins, Bashiardes et al. 2003). The study described here was embarked upon before the recent publication of the

draft chicken genomic DNA sequence (Hillier 2004) or the availability of commercial chicken gene chips. With the release of most of the chicken genomic DNA sequence it is possible to assess sequence identity between our human probes and their chicken orthologs. An analysis of this type indicates that ~98% of our probes have >70% sequence identity with the correct chicken ortholog (data not shown). Our prior experience in employing this array platform for cross-species hybridizations indicated that changes as low as 1.2-fold frequently reflected higher changes when assessed by q-PCR (Hawkins, Bashiardes et al. 2003).

Differential gene expression in the four time courses

In the antibiotic damage regime the 24 and 48 hr time points reflected gene expression changes within supporting cells, as the majority of hair cells had been killed by the ototoxic antibiotic (Warchol 1999; Warchol 2001). By 48 hrs many of the supporting cells had progressed into the S-phase of the cell cycle (Matsui, Gale et al. 2004). By contrast, the laser damage regime resulted in a 100–200 µm-wide 'wound' in the cultured sensory epithelia. The wounds typically closed within 16–24 hrs of recovery time. The initial phase of wound repair was due to cell migration, but elevated levels of cell proliferation were also observed at the wound sites (but not at distant, uninjured regions) at 16–48 hrs after injury. For the utricle, after the data analysis steps described above 143 TFs had passed through the data filters for differential gene expression (>1.2-fold change at one or more time point and a p-value of <0.05) over the three neomycin

damage times. Gene expression in laser damaged SE was compared to time-matched controls at 30 min, 1 hr, 2 hrs, and 3 hrs after laser lesions. For the utricle, a total of 66 TFs were differentially expressed across the four laser time points.

Analysis of the cochlear treatments revealed a much larger number of significant changes in TF gene expression than were found for the utricle. A total of 484 genes were differentially expressed (>1.2-fold change and p-value of <0.05) across the cochlear neomycin time course. Analysis of the cochlear laser comparisons revealed a total of 217 differentially expressed genes. Overall, when overlaps between the various lists of genes were taken into account, a total of 605 TFs accounted for all of the statistically significant changes in gene expression observed across the two cochlear time courses, and a total of 188 TF genes were differentially expressed across the two utricle time courses. It is possible that these apparent differences in numbers of differentially expressed genes between the two epithelia reflect more synchronization of regenerative signaling events in the cochlea when compared to undamaged controls. It is notable that the undamaged avian utricle is in a continual low-level state of hair cell turn-over (Jørgensen and Mathiesen 1988). This process may result in asynchronies in gene expression between injured and uninjured utricles. This might lower apparent fold-changes or increase variability (leading to higher p-values) when the damaged utricles are compared to the undamaged (but constantly regenerating) utricles. It is also possible that the larger number of

expression changes in the cochlea reflect a more robust regenerative program in this particular sensory epithelia.

Identification of known pathways and processes among the differentially expressed genes

The comparative expression profiling data were manually curated via interrogation of Gene Ontology databases as well as Medline literature citations. This served to identify multiple components or “signatures” of seven distinct signaling pathways within all four regenerative time courses. The identified pathways were those previously shown to be mediated by; *TGFβ, PAX, NOTCH, WNT, NFKappaB, Insulin/IGF1*, and *AP1* signaling. All of these have been implicated, in one way or another, in the normal development of the vertebrate inner ear. Again, as with the common genes described above, even within one identified pathway, the profiles of changes in each time course were frequently quite different. Nevertheless, some commonalities could be discerned; for example, the homeobox gene *TITF1/NKX2.1* (a component of both the *TGFβ* and *PAX* pathways) which interacts with both *SMAD3* and *PAX8* (Li, Zhu et al. 2002; Di Palma, Nitsch et al. 2003; Trueba, Auge et al. 2005) showed a similar profile in both neomycin time courses.

Not surprisingly, an additional grouping of genes fell within a set that we termed cell cycle/apoptosis genes. Of interest among this set of genes were three that have been implicated in the regulation of $p27^{KIP}$, a cyclin dependent kinase inhibitor that is a key regulator of cell proliferation during cochlear development

(Chen and Segil 1999). Although *p27^{KIP}* is expressed in supporting cells and may act as a block to cellular proliferation (White, Doetzlhofer et al. 2006), a probe for this gene was not included on our array. Therefore, we conducted a semi-quantitative PCR analysis of the chicken *p27^{KIP}* gene in the utricule neomycin specimens. This is shown in Figure 2-3 and indicates that *p27^{KIP}* transcription was down-regulated after utricle SE damage and then returned to normal levels by 48 hrs after the removal of the antibiotic. Figure 2-3 also shows microarray data for four other genes that have been previously shown to regulate *p27^{KIP}*. These are: *COPS2*, a component of the *COP9* signalosome (Yang, Menon et al. 2002), that can inhibit G1-S transition through interactions with *p27^{KIP}*; *CUTL1* a transcription factor that inhibits *p27^{KIP}* transcription (Ledford, Brantley et al. 2002); *SIX6* within the *PAX* pathway which also represses *p27^{KIP}* transcription (Li, Perissi et al. 2002); and *DACH1* (a component of both the *PAX* and *TGF–* pathways) which interacts with *SIX6* to repress *p27^{KIP}* transcription (Li, Perissi et al. 2002). It is interesting to note that for the *COPS2* and *SIX6* genes the microarray data were consistent with their previously described interactions with *p27^{KIP}* (i.e. *SIX6* transcript levels decreased over the time course and *COPS2* levels initially declined and then increased). *CUTL1* (a putative repressor of *p27^{KIP}*) also appeared to increase in expression level over the time course and *DACH1* transcript levels did not significantly vary through the time course. This set of five genes is just one example of the many changes in known pathway components that can be constructed into mechanistic and testable hypotheses from this dataset.

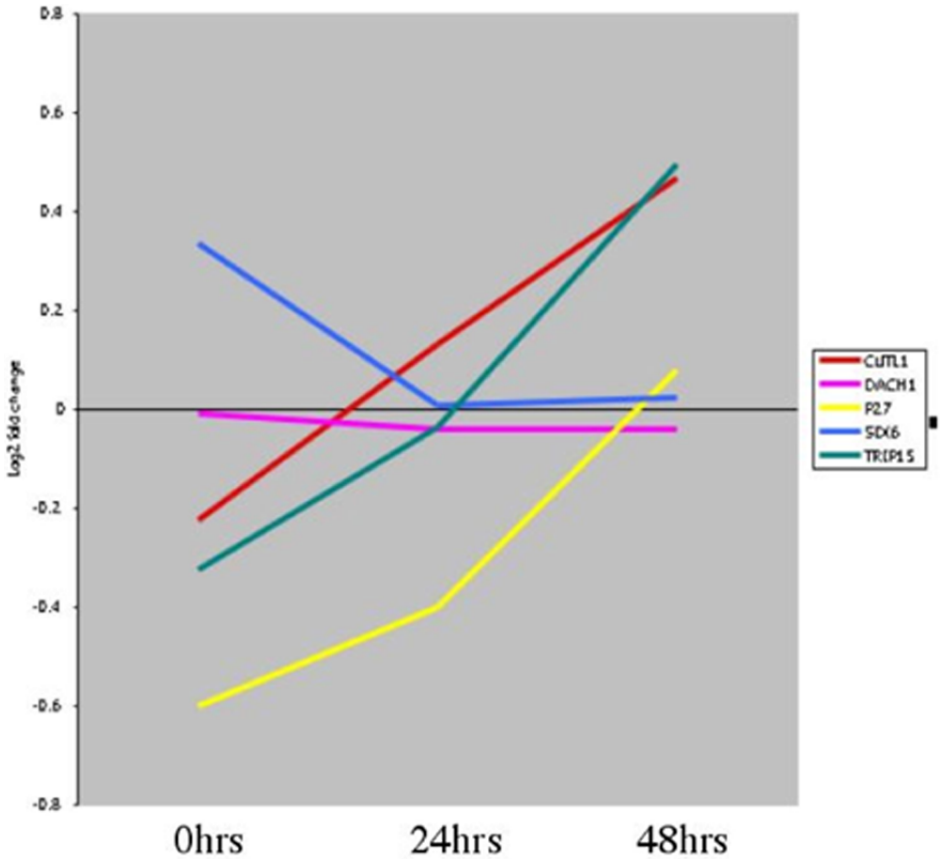


Figure 2-3 Gene expression changes in $p27^{Kip}$ and four genes that may regulate its expression. This diagram shows a combination of semi-quantitative PCR data (for $p27^{Kip}$) and microarray data for the other four genes conducted on the utricle neomycin time courses. Each gene expression profile is color coded with the key to the right of each figure. The X-axis lists time points and the Y-axis is the $\log_2$ fold-change at each time point. Expression values are derived from differentially expressed gene in the utricle, except for *DACH1* which is detectably expressed, but is not significantly differentially expressed across the time course.

Clustering with self organizing maps

As described above, literature/database searches plus manual curation of the data assisted us in placing a total of 70 known TFs into possible interactive pathways. However, the vast majority of the TFs in our set have no known function or correlations with known pathways. In order to potentially identify these relationships and to better discern possible patterns of co-expression within these data, we derived self-organizing maps (SOMs) by combining all differentially expressed genes across both time courses for each tissue type. This form of unsupervised clustering (Tamayo, Slonim et al. 1999; Reich, Ohm et al. 2004) produces clusters of genes (with upper and lower limit bars) that show similar patterns of expression across a time course or set of treatments. In this case the situation is somewhat artificial, since in building these graphs we made the arbitrary choice that the 3 hr laser time point would precede the neomycin zero time point changes on the X-axis, whereas in reality the laser time course probably overlaps the early stages (0 hr to 24 hr) of neomycin recovery. Nevertheless, the purpose of these clusters was to visualize apparent patterns and potential clusters of genes within the data. Figure 2-4A shows a group of 16 SOM centroids (clusters of genes that show similar patterns of differential expression across all the time points) constructed using Genecluster 2 (Tamayo, Slonim et al. 1999; Reich, Ohm et al. 2004) for the utricle time courses. Figure 2-4B shows sixteen centroids for the cochlea data. Some clusters exhibited relatively large temporal fluctuations in gene expression across both time courses. One example of this is centroid 3 in Figure 2-4A which includes a total

of 14 genes such as *CEBPG, JUND, FOXP1*, and *HOXA13*. By contrast, centroids 8 and 12 in Figure 2-4A illustrate genes that show relatively small changes in expression, except at the 48 hour neomycin time point where they were all up regulated. These were the predominantly late genes in the utricle regenerative time course. These two centroids together comprised 19 genes and included *POU4F3* (previously implicated in hearing loss (Vahava, Morell et al. 1998)), *CTNNB1* and *PPARGC1* (both in the *WNT* pathway). At the other end of the spectrum were the 11 genes in centroids 0 and 4 of Figure 2-4A that appeared to be activated early and peak in expression at the first or second laser time point. Among these are the nuclear hormone receptor *NR1I3,* which plays a role in transcriptional activation of genes involved in drug metabolism (Ikeda, Kurose et al. 2005; Thompson, Kuttab-Boulos et al. 2005) *SIX3*, a homeobox gene that regulates *PAX6* and *SOX2* in the developing eye (Liu, Lagutin et al. 2006) and *LOC51637*, a TF of unknown function, that we previously found to be up-regulated in the chicken utricle (Hawkins, Bashiardes et al. 2003) relative to the cochlea.

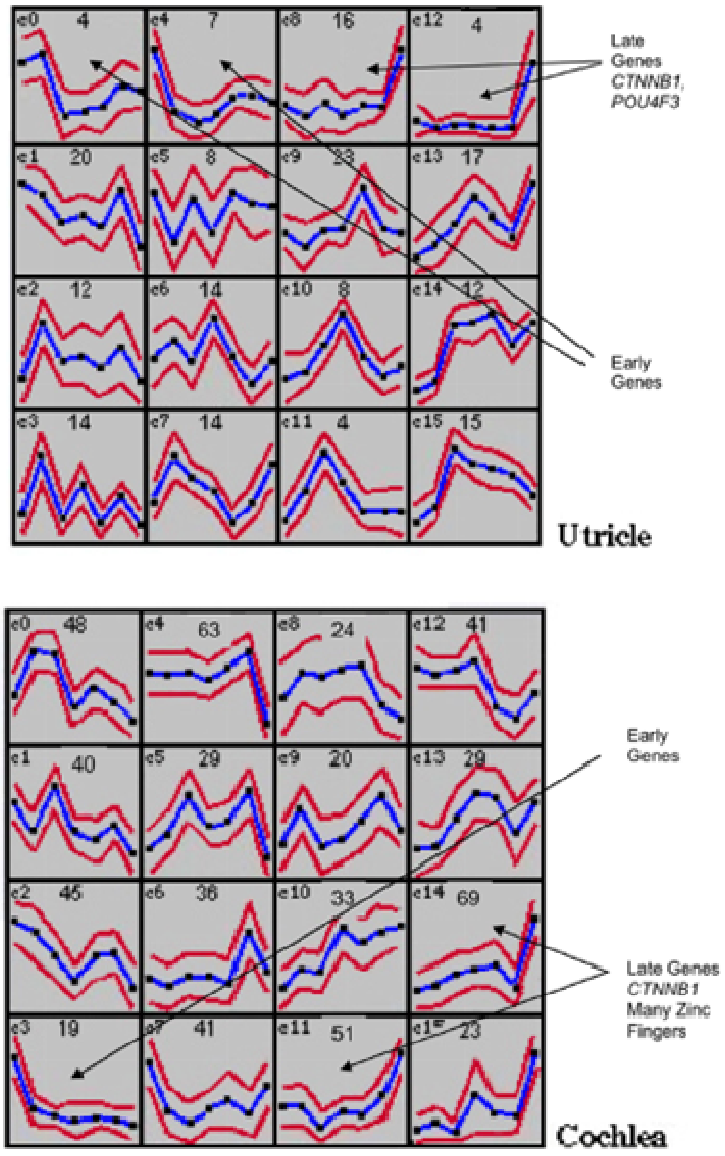


Figure 2-4 Analysis of the datasets by Self Organizing Maps. All of the differentially expressed genes were uploaded into Genecluster 2, 16 centroids per organ were generated. Each box (centroid) in this figure is numbered from C0–C15 and they reflect common patterns of expression for clustered groups of genes within the dataset. The X-axis for each centroid consists of each time point and runs from the laser 30 min time point through 1 hr, 2 hrs, 3 hrs and then into the neomycin 0 time point followed by the neomycin 24 and 48 hr time points. The Y-axis indicates expression level (fold-change). The number in the top left of each centroid indicates the number of genes that fall into this cluster of co-expression. The top line indicates the upper boundary of expression for all of these genes and the lower line indicates the lower boundary. The middle line is the mean. Figure 2-4A shows the clustering for the utricle time points and Figure 2-4B shows the clustering for cochlea time points. Arrows indicate various patterns or genes within specific centroids that are described in the text.

The cochlea regenerative SOMs (16 in total) are shown in Figure 2-4B. In this case the predominantly late genes fall into centroids 11 and 14 and total 118 genes. However, additional examples of gradual up-regulation occur in centroids 10 and 15 (55 additional genes). Interestingly, of the 118 genes in centroids 11 and 14, a total of 45 are zinc finger transcription factors (as defined by being either ZF or ZNF family members). The vast majority of these are of unknown function and unknown target specificity. If the genes in centroids 10 and 15 are included, the total number of zinc finger TFs peaking in expression at the 48Hr time point rises to 61 (35% of the 173 total genes in these centroids). By contrast, the other twelve centroids in Figure 2-4B all together contain 19 zinc finger transcription factors (4% of a total of 432 genes in these centroids). Therefore, it appears that a dramatic burst of zinc finger gene expression occurs specifically at these late stages of regenerartive proliferation in the cochlear SE. This contrasts with the utricle SOMs where zinc finger TFs are distributed fairly evenly through the centroids. In common with the utricle time courses, *CTNNB1* peaks at 48 hrs in the cochlear time courses but, unlike in the utricle, *POU4F3* peaks earlier, at the 24Hr time point (in centroid 4 of Figure 2-4B). The predominately early genes (19 in total) in Figure 2-4B are contained within centroid 3. Of interest within this group are *EGR1*, which can be induced by *IGF* signaling (Jhun, Haruta et al. 1995), *NFIL3* which is a nuclear factor regulated by *IL3* (Zhang, Zhang et al. 1995; Fritzsch 2003) and Neurogenin 1, which is involved in fate choice during inner ear development (Fritzsch 2003).

Contrasting patterns of TF genes that are detectably expressed

In addition to using the normalized intensity values to identify differentially expressed genes, we also used intensity values to determine which TFs were detectably expressed at any given time point, irrespective of any fold-change. This is a useful dataset since, at the level of detection of our microarrays, it defines lists of TFs that specify the normal functioning of the SE and makes no distinction between genes that never vary and those that change in their expression levels. This involved scoring all genes as "on" that reproducibly exceeded a background intensity level (and likewise any gene that failed to meet this cutoff was arbitrarily scored as "off"). This cut-off was based upon control oligonucleotides that were imbedded within our arrays and have no known homologous sequences in the chicken genome. Venn diagrams (Figures 2-5A and 2-5B) illustrate the results of this analysis. It is important to realize the differences between this analysis and the listings of differentially expressed genes. A gene such as *CEBPG* is among those that are differentially expressed in both the utricle laser and neomycin time courses. However, in the Venn diagrams this gene is scored as being detectably expressed at all time points (albeit at different levels between them). In Figure 2-5A it therefore falls among the 367 genes that are commonly present in all time-ponts in the neomycin Venn diagram and the 535 common genes in the laser Venn diagram (Figure 2-5A). Within these two sets of common genes (that are apparently on in either the neomycin or laser time courses) there are 256 that are shared. These comprise a core group of expressed TF genes for the sensory epithelium of the utricle,

irrespective of time point or treatment. Likewise, the cochlea has a core group of 346 TF genes that are common to both time courses at all time points. There are also a group of 134 genes that are detectably expressed at all times in all four time courses. Additionally, the Venn diagrams identify many genes “uniquely” detectable at individual time points. In some cases these may overlap with those scored as being differentially expressed, or they may only just exceed the background threshold level at those particular time points. This analysis also indicates that the largest number of detectably expressed genes occurs at 0 and 48 hr in the Neomycin time course and at the 1 hr time point in the laser time course.

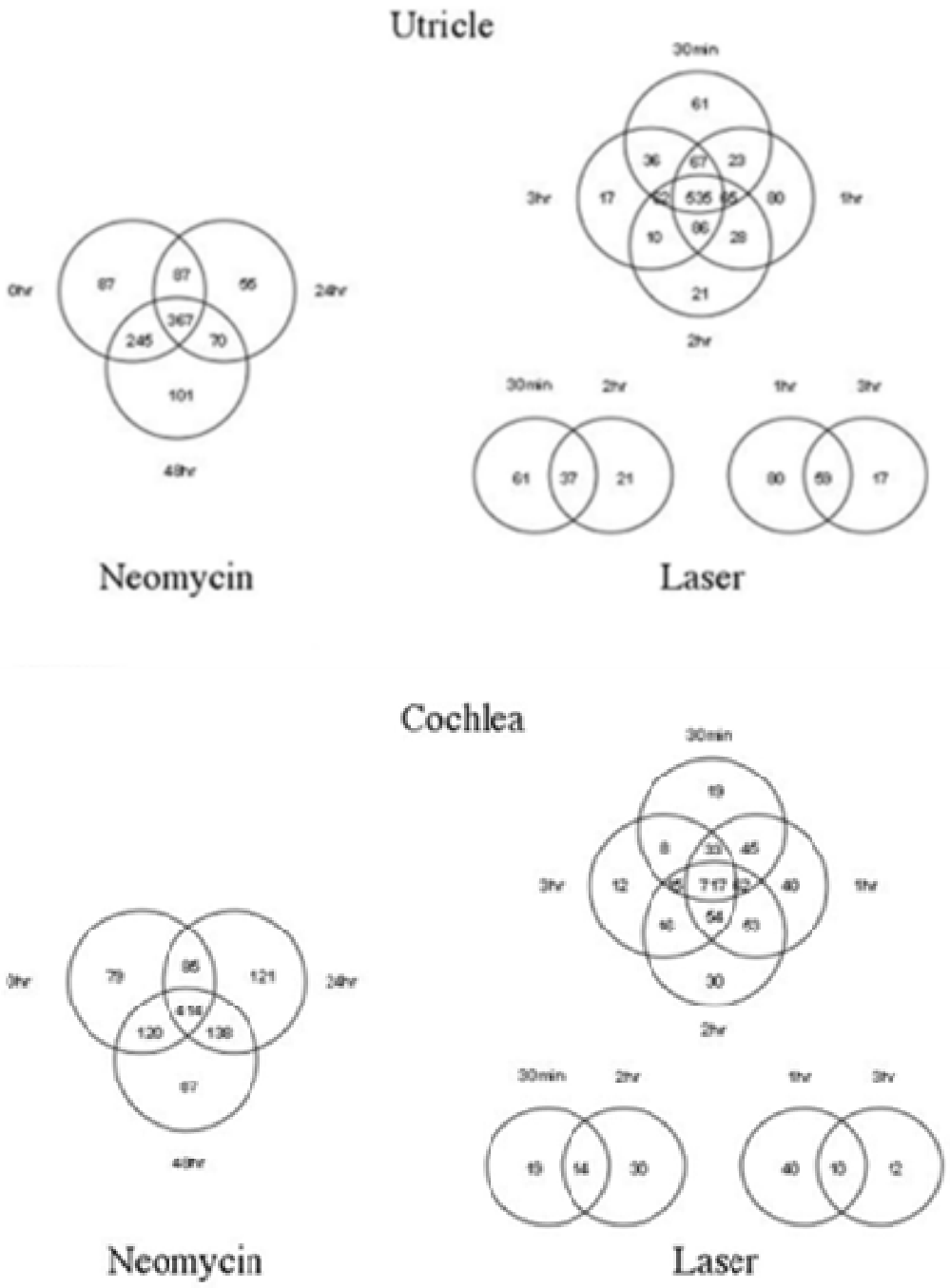


Figure 2-5 Detectably expressed TFs in the four treatment/time course combinations. All TFs that were present (as judged by exceeding a background intensity level) at any stage were considered in this analysis, irrespective of differential expression at any time point. Overlaps between these sets of TFs are illustrated in these Venn diagrams. Figure 2-5A shows overlaps for the utricle time points and treatments. Figure 2-5B shows overlaps for the cochlea time points and treatments.

Conclusions

In this study we report the first large-scale analysis of changes in gene expression during avian hair cell regeneration. We identified components of seven known signaling pathways that are differentially expressed in our microarray gene expression profiling datasets. We also identified specific genes that are common to particular time courses and treatments. Overall, we observed modest fold changes in gene expression. This is most likely due to the cross species microarray platform used for this study and the class of genes we interrogated. Since small changes in transcription factor gene expression levels can have large cascade effects on downstream genes, it is not unexpected to see modest fold-changes in TF genes having significant biological consequences. A previous study examining gene expression changes in ~25,000 genes in mouse organogenesis from E 8.0 to postnatal day 1 identified a total of 160 TF genes differentially expressed > 1.2 fold (Wagner, Tabibiazar et al. 2005). TF changes ranged from 3.66-fold change down-regulation to 3.63-fold change up-regulation, with and average change of 1.63-fold. In addition, the activation of many TFs is mediated by phosphorylation rather than transcription level (Brivanlou and Darnell 2002). In these cases, transcription factors are generally believed to be constitutively expressed. It is interesting to note that in our data set we observed consistent and reproducible changes in gene expression level for genes whose protein products are known to be regulated by phosphorylation, such as *JUND*, *CEBPG* and *CEBPB* (Lacorte, Ktistaki et al. 1997; Brivanlou and Darnell 2002). This suggests that in addition to their known regulation by

phosphorylation, gene expression level is also regulated to control critical transcription factor cascades during avian hair cell regeneration.

We first identified components of known pathways and gene networks that are differentially expressed during avian hair cell regeneration. One such example is changes in the expression of Polycomb complex genes *EZH1*, *EZH2* (enhancer of zeste 1 and 2), *CBX1*, *CBX3*, *CBX4*, *CBX6* and *CBX8* (chromobox genes). We identified consistent changes in these Polycomb complex genes in the regenerative time courses in both the cochlea and the utricle sensory epithelia, suggesting that this pathway may be important during avian hair cell regeneration. Polycomb complex genes are of particular interest because these genes are known to control cell fate decisions during stem cell differentiation (Bracken, Dietrich et al. 2006). One critical role of Polycomb complex genes during stem cell differentiation is to prevent stem cell exhaustion via epigenetic mechanisms (Kamminga, Bystrykh et al. 2006). The maintenance of a stem cell population capable of cell cycle re-entry and differentiation into sensory hair cells and non-sensory supporting cells could be a major difference in the regenerative abilities of mammals and non-mammalian vertebrates.

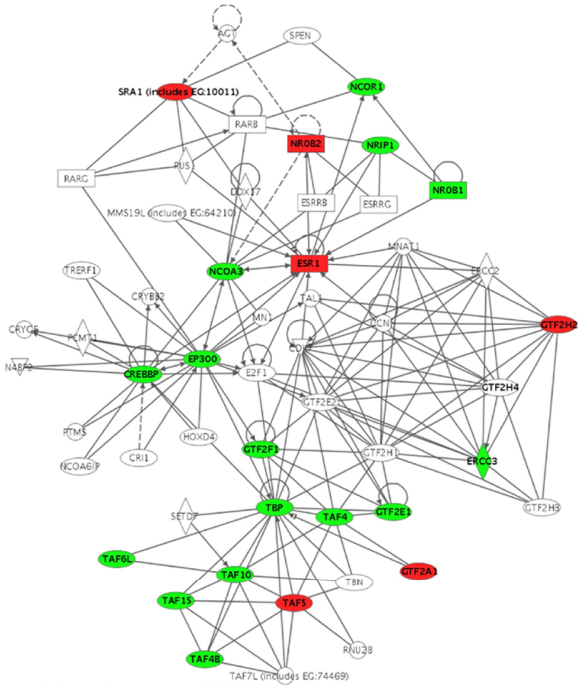
A more global method for interrogating the data presented in this study is to make use of web-delivered tools to discover possible networks or canonical pathways. Ingenuity Pathways Analysis (IPA; Ingenuity® Systems, www.ingenuity.com) is one such set of tools. We uploaded the specific sets of shared genes (fold-changes and p-values) into the IPA application. These genes

were then used to generate biological networks developed form information contained in the Ingenuity Pathways Knowledge Base (IPKB). All connections within the IPKB are supported by at least one reference from the literature (see www.ingenuity.com). IPA also computed a p-value for each generated network derived from a right-tailed Fisher's exact test, which indicates the probability that the focus genes in a network are found together because of chance alone. A complete description of all of these networks is beyond the scope of the current study. One of the highest scoring networks shared between both the cochlea and the utricle during sensory epithelia regeneration involves components of estrogen receptor (ER) signaling (p-value of $2.4x10^{-6}$). Networks of ER components and known gene interactions generated by IPA analysis are shown in Figure 2-6A. Estrogen receptors have been previously been implicated in the developing mammalian inner ear (Stenberg, Wang et al. 2001), though estrogen receptor genes have not previously been implicated in hair cell regeneration. It is still not clear what endogenous ligand(s) are involved in activating this pathway in the inner ear or whether the estrogen receptor signaling pathway acts through the ligand-independent route during hair cell regeneration (Cvoro, Tzagarakis-Foster et al. 2006). Estrogen receptor phosphorylation by various signaling pathways has previously been described in the ligand-independent activation of ER receptor signaling (Sommer and Fuqua 2001).

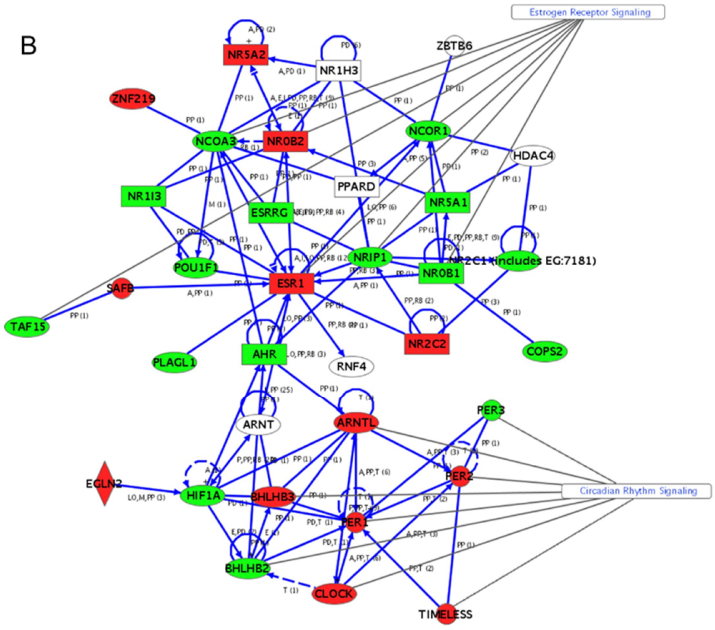
Interestingly, genes involved in the regulation of circadian rhythm are significantly enriched (p-value < $5x10^{-7}$) during cochlear regeneration. Specifically, *BHLB3*, *PER1*, *PER2*, *CREB1*, *TIMELESS* and *CLOCK* are all

differentially expressed specifically during cochlear sensory epithelia regeneration. Circadian rhythm genes were originally identified during the regulation of 24 hour periodicities in gene expression (Hayes, Baggs et al. 2005). Though prior to this study circadian rhythm regulation has not previously been implicated in inner ear development or regeneration, a recent study has described the affects of noise induced hearing loss dependent on circadian changes in serum corticosterone levels (Kim, Kang et al. 2008). Networks of circadian rhythm components and known gene interactions generated by IPA analysis are shown in Figure 2-6B. These networks indicate that circadian rhythm and estrogen receptor signaling pathways may intersect during avian hair cell regeneration in the cochlear sensory epithelia. The circadian rhythm genes *PER1* and *PER2* are known to be regulated by the Polycomb complex gene *EZH2* (Etchegaray, Yang et al. 2006). Taken together, these observations suggest novel pathway intersection between Polycomb complex genes, circadian rhythm genes and estrogen receptor signaling during avian hair cell regeneration.

A


ACT
SPEN
SRA1 (includes EG:10011)
NCOR1
RARB
NR0B2
NRIP1
RARG
RUS1
DDX17
ESRRB
ESRRG
NR0B1
MMS19L (includes EG:64210)
MNAT1
ESR1
TRERF1
NCOA3
ERCC2
CRYBG2
TAL1
CRYGB
PCM1
MN1
ZCCHC8
GTF2H2
NABP2
EP300
CREBBP
E2F1
CUL3
GTF2H4
GTF2E2
RTMS
HOXD4
GTF2F1
GTF2H1
ERCC3
NCOA6IP
CRI1
SETDB1
TBP
TAF4
GTF2E1
GTF2H3
TAF6L
TAF10
TBN
GTF2A1
TAF15
TAF5
RNU2B
TAF4B
TAF7L (includes EG:74469)

B


Estrogen Receptor Signaling
NR5A2
NR1H3
ZBTB6
ZNF219
NCOA3
NR0B2
NCOR1
HDAC4
NR1I3
PPARD
NR5A1
ESRRG
NRIP1
NR2C1 (includes EG:7181)
POU1F1
NR0B1
ESR1
SAFB
TAF15
NR2C2
COPS2
PLAGL1
AHR
RNF4
ARNT
ARNTL
PER3
EGLN2
HIF1A
BHLHB3
PER2
Circadian Rhythm Signaling
PER1
BHLHB2
CLOCK
TIMELESS

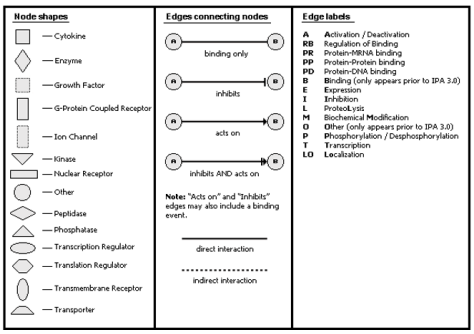

Node shapes
Cytokine
Enzyme
Growth Factor
G-Protein Coupled Receptor
Ion Channel
Kinase
Nuclear Receptor
Other
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
Edges connecting nodes
binding only
inhibits
acts on
inhibits AND acts on
Note: "Acts on" and "Inhibits" edges may also include a binding event.
direct interaction
indirect interaction
Edge labels
A Activation / Deactivation
RB Regulation of Binding
PR Protein-MRNA binding
PP Protein-Protein binding
PD Protein-DNA binding
B Binding (only appears prior to IPA 3.0)
E Expression
I Inhibition
L ProteoLysis
M Biochemical Modification
O Other (only appears prior to IPA 3.0)
P Phosphorylation / Desphosphorylation
T Transcription
LO Localization

Figure 2-6 Two examples of Ingenuity gene networks constructed from cochlear differentially expressed genes. Genes that showed differential expression in both the laser and neomycin cochlear time courses were uploaded to the web-based Ingenuity program (Ingenuity® Systems, www.ingenuity.com) and the network of interactions shown here was generated. Each interaction is shown according to the following legend and is supported by at least one literature citation (available from the Ingenuity website). Figure 2-6A shows the network of interactions for genes specifically identified within the cochlear neomycin time course as being part of Estrogen receptor signaling. Figure 2-6B shows the network of interactions surrounding Circadian rhythm signaling and was generated by uploading all of the cochlear differentially expressed genes (rather than a subset as in 2-6A). Red denotes up-regulation and green down-regulation in at least one time point. Genes shown in bold with no shading vary across a time course (e.g. *GTF2H4* in Figure 2-6A was up-regulated at 24 hrs and down-regulated at 48 hrs). All other genes were either not represented on the microarray or were not significantly differentially expressed. A key to additional Ingenuity labels is listed above.

The majority of gene expression changes we identified in our microarray gene expression profiling dataset have not been correlated with any known networks or pathways. Most of these genes have not been previously implicated in inner ear development or sensory hair cell differentiation. Identifying the role of these genes during avian hair cell regeneration and interactions with other genes involved in this process will be important for describing the genetic programming of the inner ear. Our description of pathways involved in regeneration of the inner ear sensory epithelium and specific gene changes provides a starting point for a systems biology study of the inner ear. One example of a transcription factor identified in our microarray gene expression profiling dataset that had not previously implicated in the inner ear is *FOXP1.* The forkhead transcription factor *FOXP1* is rapidly up-regulated early in the utricle laser time course. Though *FOXP1* had previously been described during cardiac development (Wang, Weidenfeld et al. 2004), prior to this study it had not been identified during inner

ear development or regeneration. Our lab confirmed that this gene is involved during embryonic development of the mouse vestibular organs that give rise the sensory epithelium (Sajan, Warchol et al. 2007) and another group identified *FOXP1* expression in the otic vesicle of developing zebrafish (Cheng, Chong et al. 1997). Another example of a specific gene of interest identified in our dataset is *CEBPG.* Prior to this study, the CCAAT element binding protein *CEBPG* had not been described in the inner ear. We identified *CEBPG* consistently expressed in all time points. However, it was rapidly up-regulated at specific time points in both the utricle laser and neomycin time courses.

In this study, we identified transcription factor pathways and specific genes that are differentially expressed during avian sensory epithelia regeneration. Specifically, we identified components of *Wnt* signaling, Ap-1 pathway, *TGFβ* signaling*, PAX* pathway and cell cycle regulation that are involved in hair cell regeneration. Next, it will be important to identify which of these genes are necessary and sufficient for regeneration and whether they are required for sensory epithelia proliferation, differentiation of sensory hair cells or both of these important steps. This dataset provides an important collection of candidate genes to further explore the complex network of interactions involved in avian hair cell regeneration.

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CHAPTER THREE

AN RNAI-BASED SCREEN OF TRANSCRIPTION FACTOR GENE PATHWAYS DURING INNER EAR SENSORY EPITHELIA REGENERATION

Introduction

In this study we describe the identification of several key transcription factor genes and pathways that are required for avian sensory epithelia regeneration. Though the specific signaling pathways required for triggering sensory hair cell regeneration have yet to be identified, several pathways such as *PAX*, *WNT* and *NOTCH* signaling have been implicated as playing roles in inner ear development and hair cell differentiation. Discernible development of the inner ear begins when ectodermal cells surrounding the neural crest become “placode competent”, having the ability to develop into one of three sensory organs: the nose, lenses of the eyes and the ear. The otic placode invaginates to generate a closed otic vesicle that will later form all of the organs of the inner ear[1]. The earliest known marker for otic fate is *PAX8*, which is expressed in preotic cells during gastrulation in the mouse [2, 3]. Knockdowns of *PAX8* result in reduced otic placode size and disrupt development of hair cells in zebrafish otic vesicles [4]. The closely related homolog of *PAX8, PAX2,* is also expressed in preotic cells following *PAX8* expression [2]. *PAX2* disruption does not affect otic placode formation, but it does prevent formation of the cochlea in the mouse [5]. Loss of *PAX8* expression does not affect *PAX2* expression, suggesting that although they are both required for proper inner ear formation, they act in separate developmental pathways [6]. *Drosophila* homologs of *PAX* genes are well documented for their role in development of the eye [7]. These highly conserved genes likely act in a similar genetic network to regulate inner ear development.

In this study we describe the identification of key transcription factor genes that are differentially expressed during avian sensory hair cell regeneration. These were initially identified in a large microarray-based gene expression study in which we profiled changes in transcription factor gene expression across different time courses of *in-vitro* hair cell regeneration [8]. The design of this study is summarized in Figure 3-1a. We interrogated >1500 transcription factor (TF) genes (out of a total of ~2000 encoded by the human genome) [9] during two different time courses of chicken hair cell regeneration. In the first time course we measured TF gene expression changes in a pure population of hair cells and supporting cells, the SE, as the hair cells regenerated after damage with a laser microbeam. In the second time course we measured TF gene expression changes in SE after the hair cells had been selectively killed by a 24 hour treatment with ototoxic aminoglycoside antibiotic, neomycin. [10], [11]. We conducted these time courses separately on multiple SE biological samples dissected from the cochlea and the utricles of chickens.

In the previous regeneration time course, a total of 683 genes were differentially expressed (> 1.2 fold, P-value < 0.05) in a minimum of one timepoint, treatment or tissue [8]. From this regeneration dataset, seven distinct known pathways were identifiable: *TGF-β, PAX, NOTCH, WNT*, *NFKappaB*, *Insulin/IGF* and *AP1*. In this report we focus upon a subset of transcription factor genes from these key signaling pathways that were reproducibly up-regulated at some point during SE regeneration. We first describe components of “known” pathways that are reproducibly altered during regeneration. We then used

siRNA knockdown and treatment with various inhibitors of specific pathways to interrogate 27 genes. We identified eleven components, from both known and unknown pathways, that are necessary for the early steps in the regenerative process (Figure 3-1b). Finally, by further microarray expression profiling of the SE following siRNA or small molecule inhibitor treatment, we identified novel epistatic relationships between genes that appear to be important downstream effectors of SE proliferation.

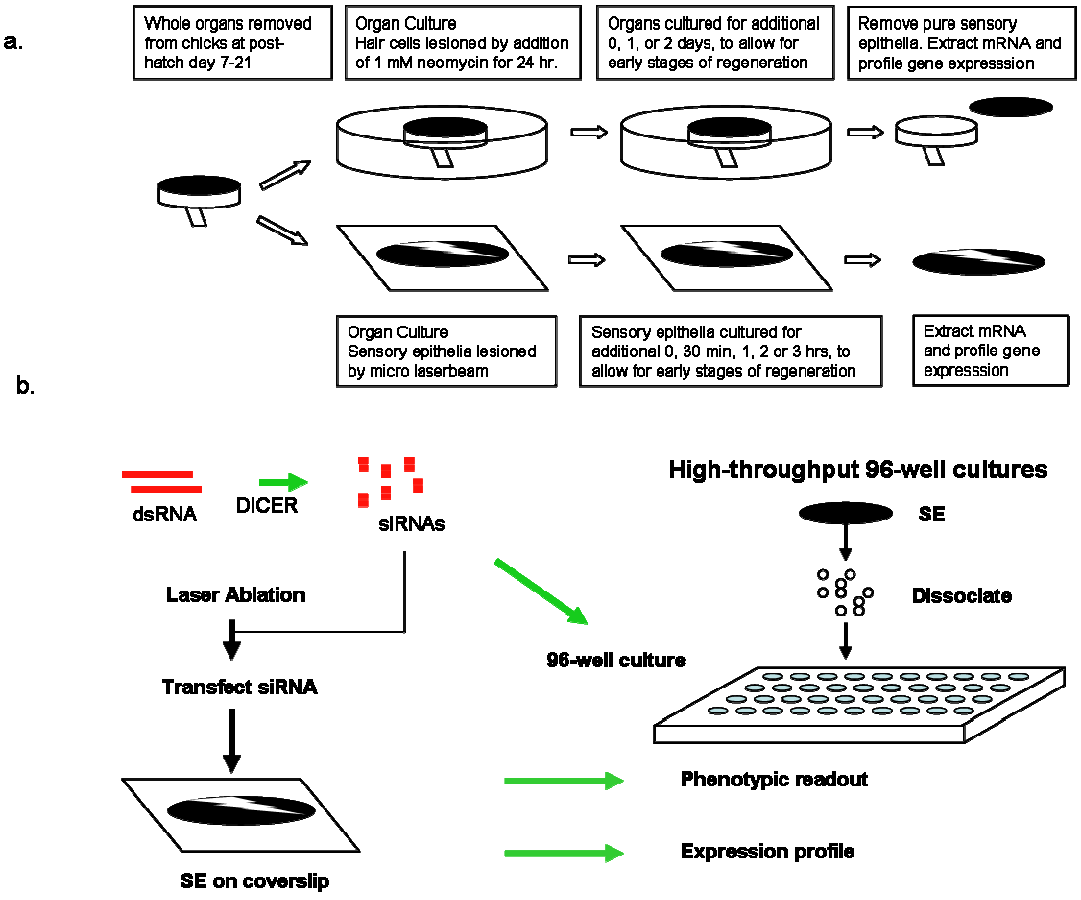

Figure 3-1. Experimental Design. Flow diagram of experimental design scheme for time course profiling in the utricle and cochlea SE and RNAi profiling. (a) Time course of laser and neomycin recovery (b) TFs revealed in the time course of recovery were targeted by siRNA to assess a proliferation phenotype and expression profiled to evaluate knockdown of the target gene and potential epistatic relationships between TF's.

Results

The Ap-1 Pathway is necessary for sensory hair cell regeneration

The *AP1* Pathway is necessary for sensory hair cell regeneration

The first known pathway that we identified during hair cell regeneration is the activating protein 1 (*AP1*) complex that includes the *JUN* family of transcription factors. *JUN* proteins can be induced by a large number of signaling molecules including growth factors, hormones, and neurotransmitters, as well as by physical or chemical stress [12]. Ten known components of the *AP1* pathway were differentially expressed during SE regeneration [8]. To determine if functional activation of *JUN* is occurring during SE regeneration, we conducted immunohistochemical staining to laser-lesioned utricle SE, using an antibody specific to the phosphorylated form of *c-JUN* (Figure 3-2a). Phosphorylated *c-JUN* is detected at the leading edge of the laser lesion site. To test whether the initial activation of the *JUN* family of transcription factors is necessary for SE proliferation, we treated laser–lesioned utricle SE with a specific small molecule inhibitor (SP600125) of the *JUN* activator, *JUN*-kinase (*JNK*). *JNK* inhibition led to a failure in regenerative wound closure (Figure 3-2b), illustrating that functional *JNK* signaling is necessary for the early proliferative stages in SE regeneration.

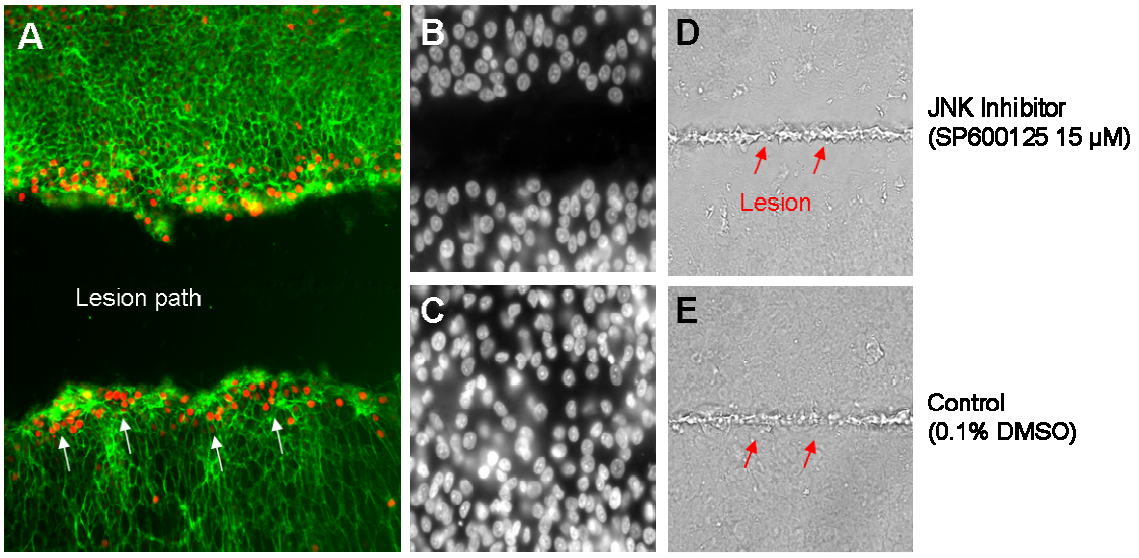

Figure 3-2. *JNK* signaling during SE regeneration. *JNK* signaling is evident at the leading edge of the lesion path in the SE and necessary for proliferative regeneration. SE cultured on a glass coverslip was lesioned by microbeam laser ablation. (A) Phosphorylated *c-JUN* was detected by a phosphorylation specific antibody to the protein (red dots; white arrows). Following laser ablation the cultured SE was treated with (B) JNK inhibitor (SP600125, 15 µM) or (C) 0.1% DMSO (control) and allowed to recover for 24 hrs., nuclei are shown by DAPI staining. The laser lesion path is visible by etching of the coverslip through the phase contrast (D and E, red arrows). Only the *JNK* inhibitor exhibited a failure to close the wound.

<u>High throughput, quantitative measure of sensory epithelia proliferation</u>

In order to determine in a quantitative and higher throughput manner whether specific TFs are necessary for SE proliferation, we used targeted RNAi in dissociated SE from the utricle in a 96-well culture format. Cellular proliferation was assessed by BrdU labeling and counting of labeled nuclei compared to total number of DAPI stained nuclei before the cultures reached confluency. For all RNAi knockdowns, we measured proliferation indexes relative to a *GFP* siRNA control. It should be noted that for all of the RNAi treatments that inhibited repair and regrowth of a laser-lesioned SE, we found similar patterns of proliferative inhibition in our 96 well assays. This suggests that our assay system is correctly identifying a subset of genes that are indeed necessary for proliferative regenerative responses in the intact SE. All RNAi knockdowns were confirmed

by microarray expression profiling and in some cases directly visualized by immunohistochemistry or quantitative PCR. We initially selected genes associated with the following signaling pathways clearly identified during the regeneration timecourse: The *AP1* Pathway, the *PAX* Pathway, Cell Cycle control genes, the Polycomb complex, *SHH* Signaling, *IGF* Signaling, *MAPK* Signaling and *NOTCH* Signaling [8]. We also selected genes that did not necessarily fall within known pathways but were up-regulated during one or more time points of SE regeneration.

As noted above, *JNK* inhibitor treatment prevented SE proliferation in laser-lesioned utricle SE. Therefore, we first focused on members of the *AP1* pathway that are differentially expressed during the SE regeneration time course. Members of the *JUN* family of TF's are normally thought to be constitutively expressed [13] with their activity being regulated by phosphorylation via *JNK*. However, our data suggest some degree of transcriptional regulation during sensory hair cell regeneration, since we observed up-regulation of *JUN* family members during regeneration. To assess whether down regulation of *JUND* or other genes that showed drastic differential expression during hair cell regeneration had similar effects to *JNK* inhibitors, we used RNAi separately targeted to each chicken gene. Individual RNAi knockdowns of *JUND* and the CCAAT enhancer binding protein, *CEBPG*, resulted in reduced proliferation of the SE (Figure 3-3a). Additionally, we tested whether genes that were commonly up-regulated in either treatment or tissue combinations are also required for SE proliferation. Seven known components of *WNT* signaling were differentially expressed in one or more organs or treatments during SE regeneration, including *β-catenin,* a component of canonical *WNT* signaling [14]. *β-catenin* was up-regulated at 48 hrs. in both the cochlea and utricle neomycin regeneration timecourses compared to untreated controls [8]. We also identified *BCL11A* (a zinc finger gene associate with hematopoietic malignancies) [15, 16] and *TRIP15* (a component of the COP9 signalosome that regulates G1-S transition) [17] differentially expressed across all four treatments and tissue combinations [8]. Though *β-catenin, BCL11A* and *TRIP15* were differentially expressed during SE

regeneration, siRNA knockdowns of these genes failed to significantly affect SE proliferation. A complete list of siRNA and small molecule inhibitor treatments and their affects on SE proliferation can be found in Table 3-1 and will be discussed below.

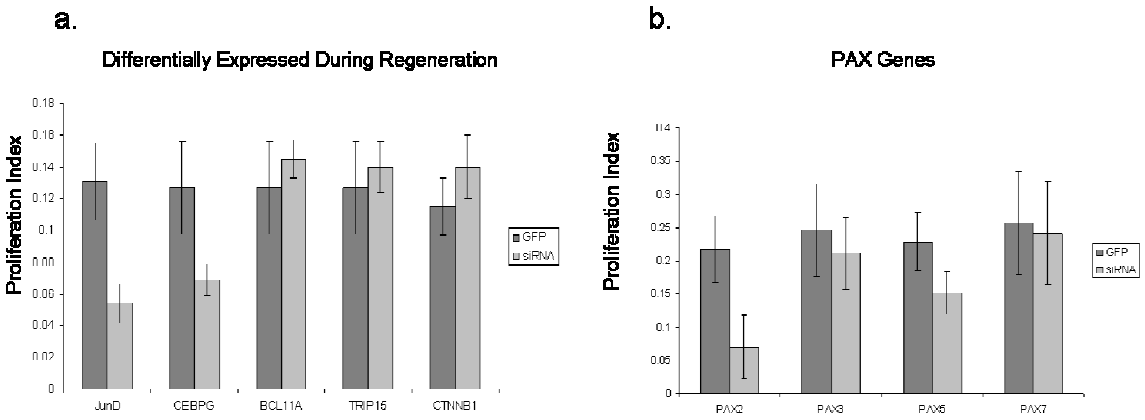

Figure 3-3 Affects of siRNA treatments on SE proliferation. Proliferation phenotypes were quantified for each siRNA knockdown compared to a GFP siRNA control by calculating a proliferation index. BrdU labeled proliferating cells were compared to the total number of DAPI stained cells to calculate a percent proliferation for (a) genes differentially expressed during hair cell regeneration and (b) *PAX* genes that were up-regulated during hair cell regeneration.

Table 3-1. Effects of siRNA/Inhibitor treatments on sensory epithelia proliferation

siRNA/Inhibitor Treatment	Inhibit Proliferation	Regeneration Pathway/Category
CEBPG	Yes	AP-1 Pathway
JNK inhibitor	Yes	
JUND	Yes	
BTAF1	Yes	AP-1 siRNA Commonalities
LRP5	Yes	
RARA	Yes	
PAX2	Yes	Pax Pathway
PAX3	No	
PAX5	Yes	
PAX7	No	
MYT1L	No	AP-1/Pax siRNA Commonalities
WNT4	Yes	
CUTL1	Yes	Cell Cycle
p27KIP	No	
ID1	No	
CBX3	No	Polycomb Complex
CBX4	No	
EZH2	No	
IGF inhibitor	No	Pathway Inhibitors
MAPK inhibitor	Yes	
SHH inhibitor	No	
HRY	No	Notch Signaling
BCL11A	No	Common to all tissues/damage
TRIP15	No	
CTNNB1	No	Common to cochlea and utricle
TIME	No	Early regeneration
PPARGC1	No	Neomycin specific

Proliferation phenotypes were quantified for each siRNA knockdown. Inhibition was determined as a significantly lower proliferation index as compared to a GFP siRNA control (p value < 0.05).

TGF-β signaling and cyclin dependent kinase regulation of sensory epithelia proliferation

One of the most widely studied roles of *TGF-β* is in controlling cell growth and differentiation by blocking cell cycle progression through the G1/S transition [18]. Nine known components of transforming growth factor beta signaling and seventeen regulators of cell cycle/apoptosis were differentially expressed during hair cell regeneration [8]. Degradation of the cyclin dependent kinase (*CDK*) inhibitor, $p27^{Kip1}$, is required for the cellular transition from quiescence to the proliferative state [19]. We independently measured the gene expression of this *CDK* inhibitor within our time courses and found that it decreased in expression one hour after laser lesioning. Likewise, *CUTL1* (a homeobox containing CCAAT displacement protein) and itself a $p27^{Kip1}$ repressor [20], is differentially expressed across the regenerative time course. To determine if *CUTL1* regulation of G1/S transition are important regulators of inner ear SE proliferation, we used siRNA individually targeted to each and measured the effects on utricle SE proliferation. Given the known role of $p27^{Kip1}$ as an inhibitor of proliferation we reasoned that even further inhibiting its levels might lead to hyper–proliferation of the dissociated SE. Conversely, we reasoned that inhibition of *CUTL1* would lead to a release of $p27^{Kip1}$ repression and consequently a decrease in proliferation. In agreement with this model, our siRNA treatments demonstrated that knockdown of the $p27^{Kip1}$ repressor, *CUTL1*, inhibits SE proliferation. We also detect increased expression of $p27^{Kip1}$ in gene expression profiling of *CUTL1* siRNA treated SE (1.68 fold-change, P-value < 0.0176). siRNA knockdowns of $p27^{Kip1}$ had no apparent effect on proliferation (Table 3-1). Our failure to observe hyper proliferation in the case of $p27^{Kip1}$ RNAi may well be attributable to the very high rate of cell division occurring in these cultures already being close to maximal. Overall, these data are consistent with the known roles of *CUTL1* and $p27^{Kip1}$ regulation of the cellular transition from quiescence to the proliferative state. siRNA knockdown of ID-1 has been shown to up-regulate $p27^{Kip1}$ and inhibit proliferation of mammalian tumors [21, 22]. However, our knockdowns of *ID-1* had no affect on

SE proliferation in the utricle. Of course, negative RNAi results of this type are always open to the caveat that none of our knockdowns were taken to zero expression levels. Theoretically, some small level of the gene product will still be present and may be sufficient to maintain proliferation.

PAX genes required for sensory epithelia proliferation

A third known pathway identified from our regenerative expression profiling data involves a cascade of TF genes induced by *PAX* gene expression; the *PAX-EYA-SIX-DACH* pathway. We identified eighteen known components of the *PAX-EYA-SIX-DACH* pathway differentially expressed during sensory hair cell regeneration. Notably, five *PAX* genes (*PAX2, PAX3, PAX5, PAX7* and *PAX8*) were up-regulated during cochlea regeneration [8]. To determine if components of the *PAX-EYA-SIX-DACH* pathway are necessary for SE proliferation, we used RNAi to knockdown *PAX* genes that are up-regulated during sensory hair cell regeneration. An exact chick ortholog for *PAX8* could not be unequivocally identified and it was therefore not targeted for knockdown. Approximately 10% of the chicken genome is missing from the published or web-accessible DNA sequence [23]. This includes many genes that lack clear orthologs such as *PAX8*, but are likely present in the chick genome. Although *PAX2* fell just below the rigorous statistical filtering thresholds in the utricle regenerative time course, we included it as an RNAi knockdown because of its known role in inner ear development. From these four Individual siRNA knockdowns, two (*PAX2* and *PAX5)* inhibited SE proliferation. Knockdowns of *PAX3* and *PAX7* did not have a significant effect on proliferation (Figure 3-3b).

Effects specific to the SE of the inner ear rather than affecting all epithelia

To determine if genes identified as necessary for SE proliferation are elements of epithelial regeneration in general, or specific to the SE of the inner ear, we performed RNAi knockdowns in chick eye retinal epithelia (Figure 3-4). Since it is the most broadly expressed transcription factor of the *AP1* pathway, it is not surprising to observe that siRNA knockdown of *JUND* also inhibits proliferation of

chick eye retinal epithelia. Additionally, siRNA knockdowns of the widely expressed transcription factor *PAX2* also inhibited proliferation of chick eye retinal epithelia, suggesting that *JUND* and *PAX2* may be general factors of epithelia proliferation. However, siRNA knockdowns of *CEBPG* and *LRP5* had no affect on retinal epithelia proliferation suggesting they may be specifically required for SE proliferation in the inner ear. Since up-regulation of *CEBPG* is only detected in the regenerating utricle and no change is detected in the cochlea [8], it is still not clear whether *CEBPG* is also required for cochlea regeneration or specific to the avian utricle.

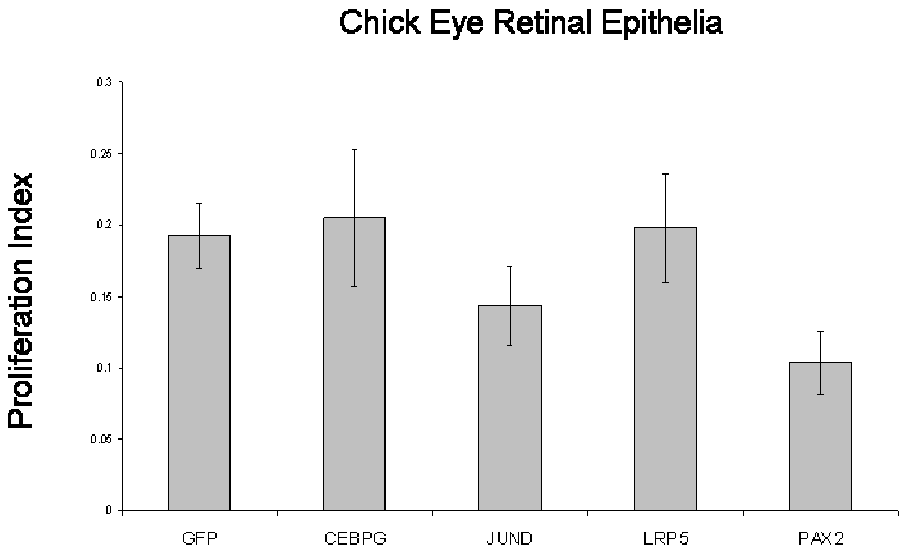

Figure 3-4 Percent proliferation was quantified for each siRNA treatment compared to a GFP control in chick eye retinal epithelia. *CEBPG* and *LRP5* siRNA treatments inhibited chick sensory epithelia proliferation, but had no affect on eye retinal epithelia proliferation.

Identification of downstream effectors of sensory epithelia proliferation

We conducted TF microarray expression profiles on all samples treated with either RNAi or small molecule inhibitors. This served the dual purpose of confirming knockdown of the siRNA target gene and identifying TF genes that showed consistent expression changes in response to RNAi knockdown or inhibition of the target gene. To infer novel epistatic relationships and potential pathway intersections involved in SE proliferation, we next looked for overlapping

expression changes between various RNAi and inhibitor treatments. One example of such an intersection is shown in Figure 3-5a; illustrating the TF expression changes for 3 treatments, all of which individually inhibit SE proliferation: *JNK* inhibitor, *JUND* RNAi and *CEBPG* RNAi. While there are numerous expression changes that are unique to each treatment or shared between pairs of treatments, most significantly we have identified 4 genes that are commonly down-regulated in all three treatments (fold change > 1.3, p-value < 0.05). One of the commonly down-regulated genes is *CEBPG*; this appears to place *CEBPG* downstream of *JUND* and *JNK* in this pathway. In addition to *CEBPG*, the low density lipoprotein receptor-related protein 5 gene (*LRP5*), the B-TFIID transcription factor-associated RNA polymerase (*BTAF1*) and the zinc finger protein 44 (*ZNF44)* were commonly down-regulated in all three treatments (JNK inhibitor, *JUND* and *CEBPG* RNAi) suggesting that *LRP5*, *BTAF1* and *ZNF44* act downstream of *CEBPG* in the JUN signaling cascade (Figure 3-5b). To determine if these commonly down-regulated genes are also required for SE proliferation, we conducted further siRNA knockdown. Individual siRNA knockdowns of *LRP5* and *BTAF1* both significantly inhibited SE proliferation (Figure 3-6). An unequivocal chicken ortholog of *ZNF44* could not be identified. As previously mentioned, approximately 10% of chicken orthologs are still missing from the *Gallus gallus* genome [23]. In the remainder of this study, we have omitted probes with unclear orthologs in the *Gallus gallus* genome.

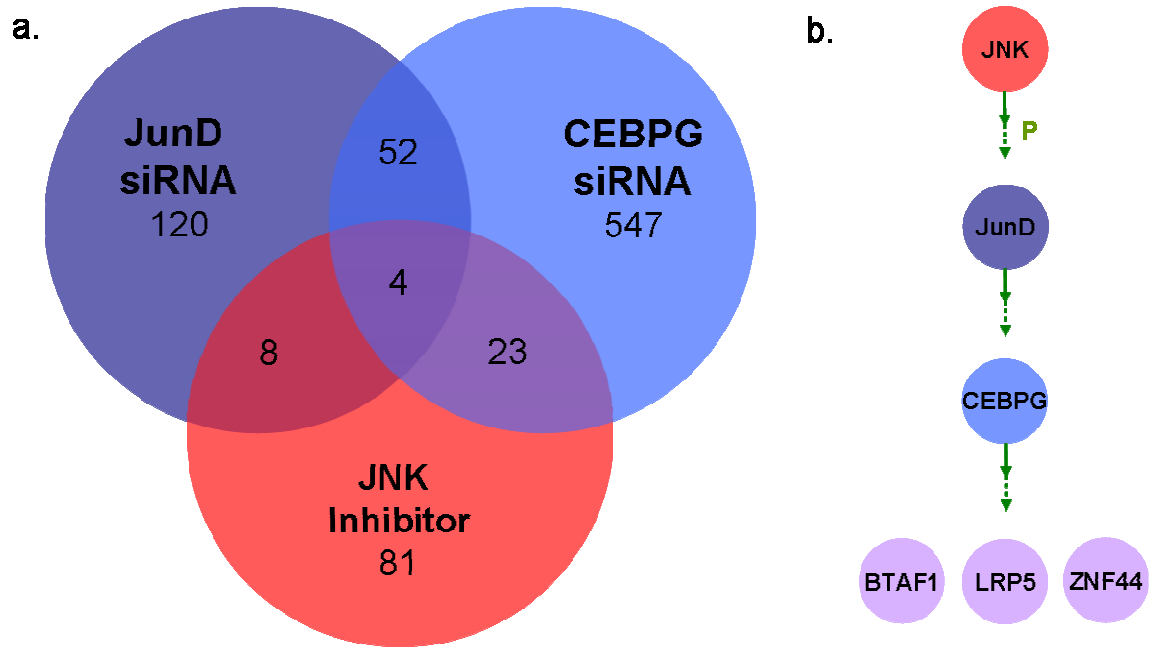

Figure 3-5. Analysis of overlapping expression profiles and novel epistatic relationships between genes that are required for SE proliferation. siRNA and inhibitor treatments were expression profiled to identify downstream effectors of SE proliferation. a) 4 genes are commonly down-regulated in 3 treatments that each individually inhibit SE proliferation, 1 of which is *CEBPG*. b) Novel epistatic relationships can be inferred from TF expression profiling siRNA and inhibitor treatments. *CEBPG* can be placed downstream of JNK and *JunD* and the other commonly down-regulated genes, *BTAF1*, *LRP5* and *ZNF44* can be placed downstream of *CEBPG* in the SE proliferation pathway.

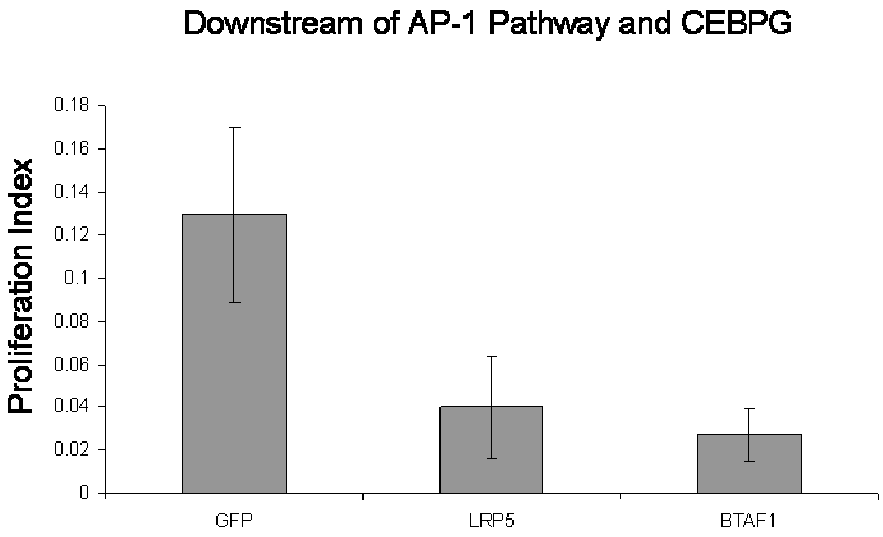

Figure 3-6 *LRP5* and *BTAF1* were commonly down-regulated *JNK*, *JUND* and *CEBPG* treatments that inhibit SE proliferation. Individual siRNA knockdowns of *LRP5* and *BTAF1* also inhibited SE proliferation compared to a GFP control.

Pathway intersections during SE proliferation

To identify pathways downstream of *CEBPG* and *LRP5*, which we had placed downstream in the *AP1* pathway during SE regeneration, we compared gene expression profiles of *CEBPG* and *LRP5* siRNA knockdowns in dissociated utricle SE. We identified three components of *WNT* Signaling (*WNT4*, *WNT9B* and *WNT16*) that were commonly up-regulated in both siRNA treatments (> 2 fold change, P-value < 0.05). Though these *WNT* Signaling components were not interrogated in our earlier regeneration study, two *PAX* genes that were down-regulated during our previous cochlea regeneration timecourses [8], *PAX1* and *PAX9*, are up-regulated (> 2 fold change, P-value < 0.05) in both *CEBPG* and *LRP5* siRNA treatments that inhibit utricle SE proliferation (Table 3-2). To determine if there are potential pathway intersections downstream of the *AP1* pathway and *PAX* pathways, we next compared gene expression profiles of four siRNA treatments that individually inhibit SE proliferation: *CEBPG*, *LRP5*, *PAX2* and *PAX5* siRNA. We identified two genes that are commonly up or down-regulated across all four siRNA treatments (> 1.3 fold-change, P-value < 0.05).

These are the *WNT* gene family member (*WNT4*) and the myelin transcription factor 1-like (*MYT1L*) (Table 3-3). To determine if *WNT4* and *MYT1L* are also necessary for SE proliferation we used RNAi to individually knockdown each in chick utricle SE. Knockdowns of *MYT1L* did not have a significant affect on SE proliferation, however, knockdown of *WNT4* significantly inhibited SE proliferation (Figure 3-7). A chi-square analysis of WNT4 expression changes in all siRNA knockdowns and their affects on proliferation was P-value < 0.041, further suggesting a critical intersection between the *AP1* Pathway, *PAX* Pathway and *WNT* Signaling during SE proliferation.

Table 3-2. Known pathways commonly differentially expressed in CEBPG and LRP5 siRNA knockdowns

Pathway	Gene	Average fold change: CEBPG siRNA	P-value	Average fold change: LRP5 siRNA	P-value
Wnt Signaling	WNT4	5.48	$1.67x10^{-2}$	4.16	$3.89x10^{-2}$
	WNT9b	4.03	$1.19x10^{-2}$	3.44	$2.48x10^{-2}$
	WNT16	2.93	$1.21x10^{-2}$	2.28	$7.81x10^{-3}$
Pax Pathway	PAX1	2.42	$2.55x10^{-2}$	2.61	$4.90x10^{-4}$
	PAX9	6.5	$8.48x10^{-3}$	4.35	$2.30x10^{-2}$

Average fold changes are displayed as levels in the siRNA knockdown (*CEBPG* or *LRP5*) relative to the control GFP siRNA. Average fold changes > 2-fold and p-value < 0.05 for both siRNA treatments.

Table 3-3. Genes commonly differentially expressed in treatments that inhibit sensory epithelia proliferation.

	Downstream of Ap-1 Pathway				Pax Pathway			
Gene	CEBPG	p-value	LRP5	p-value	PAX2	p-value	PAX5	p-value
MYT1L	-4.27	$7x10^{-3}$	-4.05	$7.00x10^{-3}$	-1.51	$2.00x10^{-3}$	-1.61	$1.40x10^{-2}$
Wnt4	5.41	$1.70x10^{-2}$	4.16	$3.90x10^{-2}$	1.34	$4.70x10^{-2}$	1.37	$7.00x10^{-3}$

Expression profiles of siRNA knockdowns that inhibited sensory epithelia proliferation were compared to identify specific commonalities downstream of the *AP1* and *PAX* pathways. *MYT1L* and *WNT4* were commonly up or down regulated (Fold change > 1.3, p-value < 0.05) in all four siRNA treatments that inhibit proliferation.

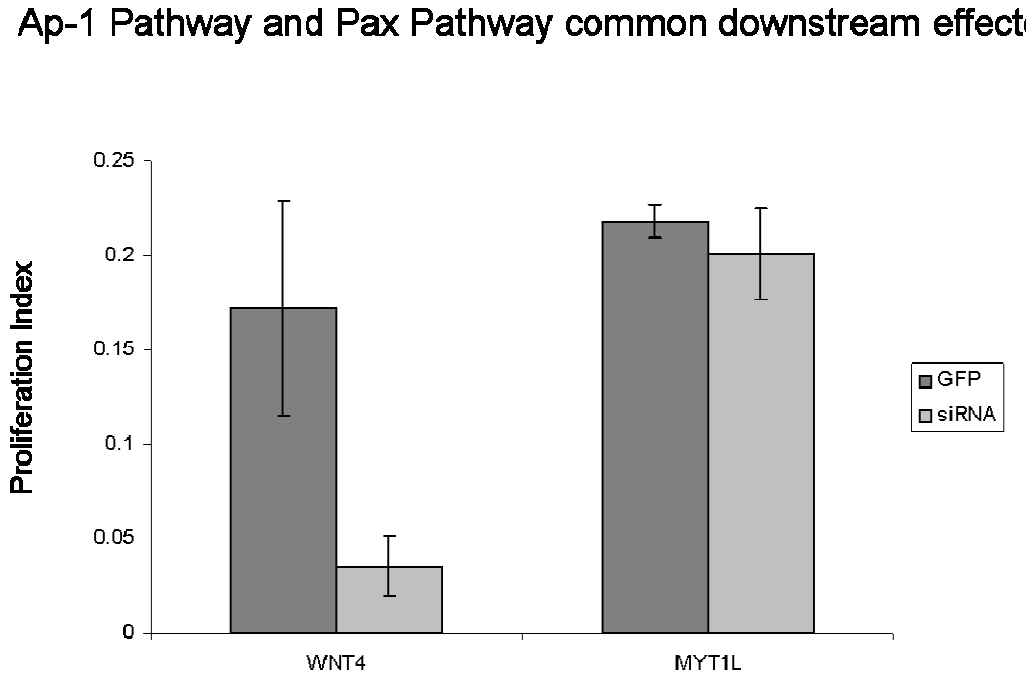

Figure 3-7. *WNT4* and *MYT1L* siRNA phenotypes. *WNT4* siRNA knockdowns inhibited sensory epithelia proliferation compared to a GFP siRNA control while *MYT1L* siRNA did not have a significant effect on proliferation.

Conclusions

In this study we identified two pathways, *AP1* and *PAX*, which are necessary for SE proliferation in the inner ear. We also identified eleven additional genes that are specifically required for SE proliferation. Our data suggests that while the *AP1* pathway and *PAX* pathways have downstream components unique to each pathway during hair cell regeneration, both pathways intersect with *WNT4.* *WNT4* is itself also necessary for optimal SE proliferation, suggesting a critical role for *WNT* signaling during these early events in avian SE regeneration. It is interesting to note that *WNT4* levels increase in siRNA treatments that inhibit SE proliferation, however, siRNA knockdowns of *WNT4* also inhibit SE proliferation. This suggests that while basal levels of *WNT4* expression are required for SE proliferation and regulated by the *AP1* and *PAX* pathways, increased levels of *WNT4* alone is not sufficient to compensate for loss of either pathway. The up-regulation of *WNT*4 in treatments that inhibit proliferation, in siRNA knockdowns from either the AP1 or PAX pathway, is likely due to compensatory mechanisms.

One of the most widely studied roles of *TGF-β* is in controlling cell growth and differentiation by blocking cell cycle progression through the G1/S transition [18]. We identified nine components of the TGF-β pathway differentially expressed during hair cell regeneration. We also determined that siRNA knockdowns of *CUTL1*, a repressor of the cyclin dependent kinase (CDK) inhibitor, $p27^{Kip}$, inhibit SE proliferation. *CUTL1* is also down-regulated in two treatments that individually inhibit SE proliferation, *WNT4* and *BTAF1* (-1.90-fold and -1.38-fold changes respectively, from Supplemental Tables S7 and S8) suggesting some level of cross talk between those genes and cell cycle control. $p27^{Kip1}$ is expressed in the sensory primordia of the mouse cochlea from E12-E14, a time when cellular proliferation is coming to an end and hair cell differentiation is occurring [24]. Its continued expression in the adult inner ear appears to mark the supporting cells in mouse and may reflect the continued inhibition of the cell cycle in these cells. *TGF-β* mediated regulation of $p27^{Kip1}$

may be an important factor in the differing regenerative abilities of mammalian and avian SE.

JUN family TFs play an important role in regulating progression through the cell cycle, proliferation and differentiation. For example, *c-JUN* is required to alleviate the inhibition of p53 on cell cycle entry [25] and *JUND* regulates lymphocyte proliferation in mouse [26]. Additionally, members of the *JUN* family of TFs interact with *FOS* to activate *Cyclin D1* and increase cell proliferation [12]. Ten known components of the *AP1* complex, including *FOS*, were differentially expressed in one or more of our regenerative time points. In addition to our gene expression profiling and phenotype data, the placement of *CEBPG* downstream in the *AP1* pathway during SE regeneration is further supported by evidence that human *CEBPG* is known to interact with *FOS* to activate the *IL-4* gene in Jurkat cells[27]. *CEBPG* belongs to the highly conserved CCAAT/enhancer binding protein (*C/EBP*) family of transcription factors. Members of the *CEBP* family act as master regulators of numerous processes, including differentiation, inflammatory response and liver regeneration [28]. The placement of *CEBPG* downstream of the *AP1* pathway suggests that *CEBPG* may interact with *FOS* or other members of the *AP1* complex to regulate proliferation during avian SE regeneration.

In addition to *CEBPG*, we also placed *LRP5* downstream in the *AP1* pathway during SE proliferation. The *LRP5* gene product is a known co-receptor of *WNT* signaling [29], which connects a component of *WNT* signaling into this pathway. We previously identified the *WNT* Signaling components *β-catenin* and the *TCF/LEF* transcription factors, *TCF7L1* and *TCF7L2*, as being differentially expressed during hair cell regeneration [8]. In the present study, three additional *WNT* signaling components, *WNT4, WNT9B* and *WNT16*, were commonly differentially expressed in siRNA treatments for *CEBPG* and *LRP5*. Canonical *WNT* signaling is generally transduced through the frizzled family of receptors and *LRP5/LRP6* co-receptors to the *β-catenin* signaling cascade [30]. Though *β-catenin* is up-regulated during hair cell regeneration, this occurs at quite a late time point (48 hours) suggesting that it may play a more major role in

differentiation of new hair cells rather than proliferation of supporting cells prior to differentiation. In agreement with that potential role, our siRNA knockdowns of *β-catenin* did not affect proliferation.

We identified seven *PAX* genes differentially expressed during cochlea regeneration; however, only *PAX2* and *PAX5* siRNA treatments individually inhibited SE proliferation in the utricle cultures used here. While most invertebrate genomes posses only a single *PAX2/5/8* gene, early in vertebrate evolution the closely related subclass of paired-box family of transcription factors *PAX2*, *PAX5* and *PAX8* were produced by gene duplication [31-35]. Though a *PAX*8 ortholog could not be identified in chicken, our results suggest the closely related *PAX2* and *PAX5* transcription factors both play an important role during regulation of SE proliferation. We also identified two genes, *WNT4* and *MYT1L,* that are commonly up or down-regulated in siRNA treatments of *PAX* genes (*PAX2* and PAX5) and downstream of the *AP1* pathway (*CEBPG* and *LRP5*), that individually inhibit SE proliferation. Of these commonalities, only *WNT4* was found to be required for SE proliferation. *WNT4* is first detected in the developing chicken otocyst at E5, forming a border between the sensory primordia and nonsensory lateral wall [36, 37] suggesting *WNT4* may play an important role in forming sensory/nonsensory boundaries in the developing inner ear. *PAX2* has been shown to regulate *WNT4* expression during kidney development [38] and our microarray data suggests that *PAX2*, along with *PAX5*, *CEBPG* and *LRP5*, may function as important regulators of *WNT4* in the inner ear connecting the *AP1* and *PAX* pathways to *WNT* Signaling during hair cell regeneration.

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CHAPTER FOUR

DOWNSTREAM TARGETS OF GATA3 IN THE VESTIBULAR SENSORY ORGANS OF THE INNER EAR

Introduction

The inner ear is divided into two functional structures: the vestibular organ, which is responsible for maintaining balance and the auditory organs, which sense sound. The vestibular organ consists of three semi-circular canals responsible for sensing rotational acceleration as well as the saccule and utricle, which sense linear acceleration and gravity. The cochlea is the primary organ of the auditory system which senses sound. Both organs of the inner ear utilize sensory hair cells as mechano-electrical transducers. The transcription factor GATA3 plays an essential role in development of the mammalian ear. GATA3 is a member of the GATA family of transcription factors that contain two highly conserved C2C2 type zinc fingers that recognize the consensus WGATAR sequence (W = A or T and R = A or G) (Ko and Engel 1993; Merika and Orkin 1993). GATA3 is expressed throughout the mouse otic placode from E8-E9.5 and is required for invagination to generate a closed otic vesicle that will later form the vestibular and cochlear organs of the inner ear (Grace Lawoko-Kerali 2002; Lilleväli, Haugas et al. 2006). In humans, GATA3 mutations that disrupt the C-terminal zinc finger result in loss of DNA binding function and have been linked to hypoparathyroidism, sensorineural deafness and renal anomaly syndrome (HDR) (Van Esch, Groenen et al. 2000).

GATA3 influences development of the embryonic ear and brain, among other organ systems. Very little is known about the regulatory role of GATA3 in the inner ear. However, GATA3 has been well studied in hematopoietic induction of Th2 cell differentiation. Naïve CD4 cells differentiate into either T helper type

1 (Th1) or T helper type 2 (Th2) cells. Th2 cell fate is tightly regulated by GATA3 transcriptional regulation of IL5 and IL13 at well defined promoter sites. (Siegel, Zhang et al. 1995; Kishikawa, Sun et al. 2001; Lavenu-Bombled, Trainor et al. 2002; Szabo, Sullivan et al. 2003; Mowen and Glimcher 2004). GATA3 homozygous mutant mice result in embryonic lethality by 11 days post coitum due to multiple organ abnormalities, most notably massive internal bleeding and a complete inhibition of T-cell differentiation (Pandolfi, Roth et al. 1995). Additionally, GATA3 specifies inner root sheath cell differentiation *vs.* hair shaft cells during skin development (Kaufman, Zhou et al. 2003). GATA3 is an important regulator of lineage-specific differentiation in skin development and during hematopoietic induction; it is likely that GATA3 may have a similar role during inner ear development.

In mammals, sensory hair cells of the inner ear lack the capacity for regeneration when damaged. In mouse, GATA3 heterozygous mutant mice have a progressive degeneration of cochlear sensory hair cells (van der Wees, van Looij et al. 2004). In contrast to mammals, non mammalian vertebrates such as birds maintain the ability to regenerate sensory hair cells of the inner ear throughout their lives. GATA3 is expressed in similar expression patterns in the developing mammalian and avian ear. Results from *in-situ* hybridization and immunohistochemical labeling demonstrated that GATA3 is expressed throughout the sensory region of the mature cochlea but is limited to a 6-10 cell wide region in the striola of the utricle (Hawkins, Bashiardes et al. 2003). The striola of the utricle is of particular interest because it corresponds to the reversal

zone in which the sensory hair cells undergo a 180° shift in orientation (Flock 1964). In addition to being a site of hair cell polarity, another interesting feature of the striola is that hair cells undergo an abrupt change in phenotype at this region. The striola is primarily populated by Type I hair cells contacted by calyx nerve terminals (Lysakowski and Goldberg 1997). These hair cells are morphologically distinct from Type II hair cells that populate the majority of the utricle sensory epithelia. Type II hair cells are contacted by bouton nerve terminals from afferent and efferent neurons. Though specific roles for Type I and Type II hair cells are not clear, their distinct morphologies suggest specialized functions.

The genetic mechanisms that regulate Type I vs. Type II differentiation have not been determined, nor is it known how neurons distinguish between Type I and Type II hair cells. After in vivo ototoxic injury, Type II hair cells are the first to repopulate the utricle after 14-20 days (Weisleder and Rubel 1993; Dye, Frank et al. 1999; Matsui, Oesterle et al. 2000; Zakir and Dickman 2006) followed by Type I hair cells 2 months post injury (Weisleder and Rubel 1995). The specific expression pattern of GATA3 in the avian striola is maintained in supporting cells of the utricle following severe ototoxic injury and during subsequent regeneration (Warchol and Speck 2007). GATA3 heterozygous and null mutant mice show misrouted axonal projections in the inner ear, suggesting an important role regulating axon guidance. Recent experiments have demonstrated that avian hair cells regenerate in the proper orientation in the absence of GATA3 expression (Warchol and Montcouquiol, manuscript submitted), suggesting that GATA3 does not regulate hair cell polarity during hair

cell regeneration. Rather, GATA3 may play an important role regulating Type I vs. Type II hair cell differentiation and/or axon guidance to specific hair cell types.

The present study is aimed at the identification of transcription factors in the inner ear whose expression is regulated by GATA3. We specifically focused on the striola of the chick utricle, which is comprised of ~10,000 cells. We used four complementary approaches to characterize GATA3-regulated gene expression. First, we used micro cDNA amplification methods and custom gene microarrays to determine transcription factor (TF) genes specifically co-expressed with *GATA3* in the highly localized striolar region. We next utilized both siRNA knockdown of GATA3 and ectopic over-expression of GATA3 to identify genes that act downstream of *GATA3* in the utricular sensory epithelium. Finally, we confirmed a subset of our microarray observations by RNA *in situ* hybridizations and used chromatin immunoprecipitation (ChIP) to identify direct binding targets of *GATA3* upstream of the LIM domain only 4 (*LMO4*) and muscleblind like-2 (*MBNL2*) transcription factors in the chick utricular sensory epithelium. Our expression profiling data further suggests that regulation of *Wnt* signaling, *FGF* signaling, *Notch* Signaling, *BMP* signaling as well as regulators of neurogenesis and neural survival are differentially expressed in the striolar vs. extra-striolar regions and may play a significant role regulating neuronal differentiation and axon guidance to specific hair cell types within the sensory maculae.

Results

Striola vs. extra-striola microarray comparisons

As an initial screen for genes that are potentially regulated by *GATA3*, we compared gene expression in cells of the striola (which includes the GATA3-expressing reversal zone) to expression in the medial extra-striolar region. Our rationale for investigating just transcription factors and components of known signaling pathways was that changes in these molecules frequently act as important switches in genetic programming. Sensory epithelia from mature chick utricles were isolated and micro-dissected into striolar and extra-striolar portions (Figure 4-1). Both of these are much more accessible than the corresponding samples within the mouse utricle.

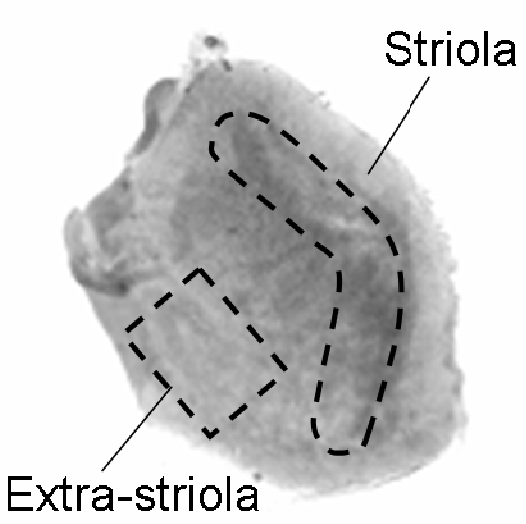

Figure 4-1 Comparison of striola vs. extra-striola. GATA3 expressing cells from the striola sensory epithelia (shown here by whole mount RNA *in situ*) were micro-dissected from avian utricle. These were compared to cells that do not express GATA3, the extra-striola, on a custom transcription factor microarray.

RNAs from these pooled samples were then compared on a custom oligonucleotide microarray that primarily interrogates transcription factor gene expression (Messina et al., 2004), but also includes oligonucleotides specific to

major signaling pathways. All comparative microarray hybridizations consisted of 2 biological samples and 4 technical replicates for each biological sample, including dye switch experiments. To our knowledge this is the first such comparison ever conducted and identified 38 genes that are up-regulated and 45 down-regulated at the striola (Fold change > 2.0 and p-value < 0.05) (Table 4-1). Notably, the four genes that showed the highest relative levels of expression in the striola (*KCNIP4, DKK2, NGN2,* and *HEY2*) have been shown to affect neuronal differentiation (Falk et al., 2002; Sakamoto et al., 2003; Xiong et al., 2004; Guder et al., 2006). For example, the bHLH transcription factor NGN2 can induce neuronal cell fate in mouse neural stem cells (Hu et al., 2005). Expression of NGN2 within the striola was up-regulated by 7.85 fold, compared to the extra-striolar region. In contrast, we observed reduced striolar expression of WNT3A and WNT5 (-7.55 and -5.37 fold changes respectively) and two hairy and enhancer of split (*HES*) paralogs, *HEYL* and *HRY* (-5.02 and -6.99 fold changes respectively). *HES* genes are components of Notch/Delta signaling, and both *HEYL* and *HRY* are known to physically interact with GATA proteins and inhibit transcriptional activity (Kathiriya et al., 2004; Fischer et al., 2005). From this dataset we also identified known components of *WNT*/beta-catenin signaling (DKK2, FZD5, FZD7, WNT3, WNT3A, WNT5A), FGF signaling (FGF16 and FGF20), Notch signaling (HEY2, HEYL and HRY) and BMP signaling (BMP2, BMP4 and BMP15) (Table 4-1). Overall, this comparison revealed a complex pattern of gene expression changes that strongly implicate differential

expression of *WNT*, FGF, Notch and BMP signaling pathways within these distinct regions of the sensory maculae.

Table 4-1. Genes differentially expressed in the striola vs. extra-striola.

Gene	Average Fold Change	P-value	Gene	Average Fold Change	P-value
KCNIP4	11.19	7.32×10^{-03}	KLHL9	-19.34	1.75×10^{-02}
DKK2	10.92	2.68×10^{-04}	MGC16733	-15.25	3.81×10^{-03}
NEUROG2	7.86	1.17×10^{-02}	ENO1	-10.46	4.33×10^{-04}
HEY2	6.53	5.81×10^{-03}	WNT3A	-7.55	2.62×10^{-04}
BRD9	5.66	2.35×10^{-02}	CYLD	-7.44	2.90×10^{-04}
GATA3	5.45	1.12×10^{-02}	HRY	-6.99	1.23×10^{-03}
NR0B1	5.04	1.52×10^{-02}	RORA	-6.63	1.26×10^{-03}
LOC90322	4.55	2.51×10^{-02}	FEZL	-6.06	3.44×10^{-04}
RAX2	4.09	2.49×10^{-02}	BMP4	-5.63	1.46×10^{-02}
MYT1L	4.00	1.11×10^{-02}	WNT5A	-5.37	9.07×10^{-03}
TCEA2	3.93	3.13×10^{-02}	PMX1	-5.27	9.96×10^{-03}
FGF20	3.92	2.00×10^{-02}	FHL2	-5.26	1.32×10^{-02}
IHH	3.55	5.90×10^{-03}	HOXD8	-5.14	5.17×10^{-03}
SATB1	3.50	3.41×10^{-04}	HEYL	-5.02	2.36×10^{-03}
MLL3	3.38	6.15×10^{-03}	ZNF652	-4.74	2.86×10^{-02}
KIAA0293	2.94	3.32×10^{-02}	BAPX1	-4.69	2.71×10^{-03}
PDEF	2.92	2.59×10^{-03}	NRTN	-4.61	2.68×10^{-03}
HOXA6	2.83	4.93×10^{-03}	BTBD5	-4.60	1.76×10^{-02}
TBX22	2.69	1.75×10^{-02}	MTF1	-4.34	6.23×10^{-04}
TCFL1	2.68	2.54×10^{-02}	RXR	-4.32	3.72×10^{-04}
SUV39H1	2.64	1.74×10^{-02}	GLI3	-4.18	9.42×10^{-04}
LOC416414	2.56	8.85×10^{-03}	FGF16	-3.64	4.75×10^{-02}
SCA2	2.53	3.75×10^{-05}	SP4	-3.51	1.18×10^{-03}
PAX3	2.50	4.72×10^{-02}	CDK5RAP1	-3.42	1.81×10^{-02}
BS69	2.49	1.07×10^{-02}	HNF1	-3.21	7.33×10^{-04}
NCOR2	2.42	1.12×10^{-02}	ACVR1B	-3.12	1.72×10^{-02}
FOXL2	2.38	3.65×10^{-03}	BMP2	-3.12	1.20×10^{-02}
JAG1	2.33	1.70×10^{-02}	FZD7	-3.01	1.48×10^{-02}
HMGB3	2.28	1.06×10^{-02}	MADH9	-3.00	1.26×10^{-02}
STAT5A	2.28	4.09×10^{-02}	BMP15	-2.99	3.49×10^{-02}
LMO4	2.28	4.26×10^{-02}	DACH1	-2.92	3.23×10^{-02}
IRX1	2.26	5.27×10^{-03}	FZD5	-2.82	3.99×10^{-03}
CLOCK	2.24	3.63×10^{-02}	CDK9	-2.80	1.51×10^{-02}
PMX2B	2.22	9.35×10^{-04}	IKZF2	-2.78	4.56×10^{-02}
LHX3	2.17	7.11×10^{-04}	TRAF4	-2.77	1.41×10^{-02}
RBBP5	2.13	6.59×10^{-03}	WNT3	-2.72	2.24×10^{-03}
LARP1	2.11	4.20×10^{-02}	PLTP	-2.71	4.25×10^{-02}
TCF1	2.10	3.21×10^{-02}	HOXB9	-2.69	1.20×10^{-04}
			TGIF	-2.45	4.13×10^{-03}
			BUB3	-2.28	3.79×10^{-02}

GDF8	-2.22	4.59×10^{-02}
ARNTL2	-2.16	2.63×10^{-02}
BANP	-2.15	2.25×10^{-02}
RNF12	-2.07	1.66×10^{-02}
MADH7	-2.00	1.53×10^{-02}

Average fold changes are displayed as levels in the utricle striola relative to the extra-striola. Values are > 2-fold and p-value < 0.05.

GATA3 RNAi knockdown comparisons

To better discriminate between gene expression changes within the striola that are associated with *GATA3* expression and those that might be coincidental, we utilized RNAi knockdowns in cultured chick utricles (the entire utricle including striola plus extra-striola regions) to identify genes that potentially act downstream of *GATA3*. Since *GATA3* expression is maintained in the adult utricle, it very likely plays a critical and active role regulating direct targets in the adult striola. We compared gene expression profiles of pure sensory epithelia from whole, explanted utricles transfected *in vitro* via electroporation with siRNAs for either *GATA3* or a GFP control. In order to identify both direct and indirect consequences of *GATA3* knockdown, epithelial cells were harvested 48 hours after RNAi treatment. Immunohistochemical labeling indicated that knockdown of *GATA3* is maintained at the striola 48 hrs. post siRNA treatment (Fig 4-2 A,B). We identified 63 genes that were up-regulated and 10 genes down-regulated (including *GATA3* itself) in response to *GATA3* siRNA knockdowns in the avian utricle sensory epithelia (Fold change > 2.0 and p-value < 0.05) (Table 4-2).

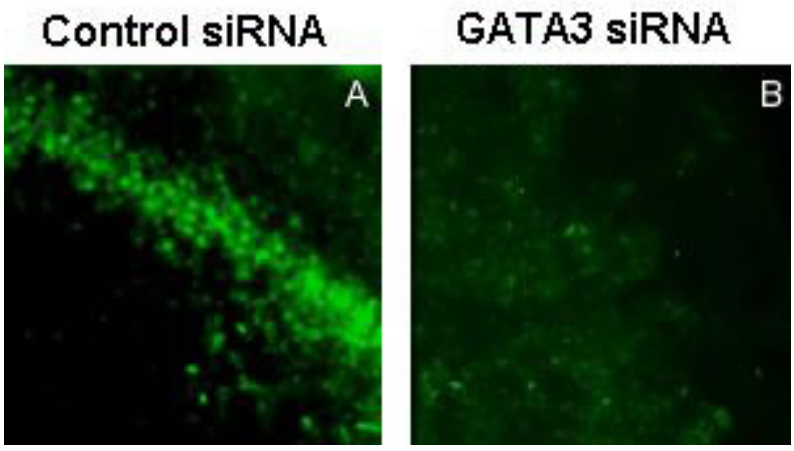

Figure 4-2. Immunohistochemical labeling with a GATA3 antibody (green) in siRNA treated whole avian utricles. GATA3 immunoreactivity is localized to the 6-10 cell wide strip of cells at the striola reversal zone in the A) control GFP siRNA treated sample and undetectable in the B) GATA3 treated siRNA.

The *BAR* homeobox transcription factor 1 (*BARX1*) and *BARH*-like homeobox 1 (*BARHL1*) genes exhibited the largest down-regulation in expression (-9.31 and -6.68 fold changes respectively). *BARHL1* encodes a homeodomain transcription factor involved in sensorineural development. It is expressed in migrating neurons of the CNS as well as in sensory hair cells, where it is required for long-term survival and maintenance (Bulfone et al., 2000; Li et al., 2002). *BARX1* regulates transcription of two *WNT* antagonists, *sFRP1* and *sFRP2* (Kim et al., 2005). Consistent with our earlier observation that *WNT* signaling is differentially regulated in the striola compared to the extra-striola regions, we identified three components of *WNT* signaling that were up-regulated in *GATA3* knockdowns (*WNT3, LRP5* and *FZD5*) and one Wnt gene (*WNT5B*) that was down-regulated. Expression of the Fibroblast Growth Factor *FGF16*,

which was specifically down-regulated at the striola, was up-regulated in *GATA3* knockdowns.

Table 4-2. Genes differentially expressed in GATA3 siRNA treatments.

Gene	Average Fold Change	P-value	Gene	Average Fold Change	P-value
NUP153	12.06	2.01×10^{-02}	MYOD1	2.70	6.75×10^{-03}
HRIHFB2122	7.09	2.75×10^{-03}	MYT1L	2.67	1.23×10^{-03}
VEGFC	7.06	1.84×10^{-02}	NFE2L2	2.62	3.31×10^{-02}
SIAH1	6.94	1.50×10^{-02}	GTF2E2	2.61	2.75×10^{-02}
BRD1	6.29	2.68×10^{-03}	ZFP91	2.55	3.05×10^{-02}
PMX2B	5.28	5.59×10^{-03}	ARNTL2	2.49	2.89×10^{-02}
HOXA7	5.04	9.25×10^{-03}	SHH	2.48	3.56×10^{-02}
ALX4	4.95	3.61×10^{-02}	MTF1	2.45	2.94×10^{-02}
RXRG	4.88	2.42×10^{-02}	RFX3	2.44	1.25×10^{-02}
LHX8	4.86	3.62×10^{-02}	FMR2	2.41	1.13×10^{-02}
BCL11B	4.76	9.73×10^{-03}	MDN1	2.41	4.68×10^{-03}
CHD1	4.56	2.12×10^{-02}	FZD5	2.40	2.56×10^{-02}
CITED2	4.52	3.62×10^{-02}	NEUROD6	2.40	2.26×10^{-02}
FOXP1	4.50	8.76×10^{-03}	CYLD	2.38	8.00×10^{-04}
MYBL1	4.47	3.22×10^{-02}	NHLH2	2.34	1.74×10^{-02}
SPOP	4.37	1.47×10^{-02}	HSF2BP	2.33	4.04×10^{-02}
ARTN	4.26	2.87×10^{-02}	TRIM50A	2.29	2.15×10^{-02}
PCMT1	4.24	2.15×10^{-02}	FOG2	2.27	5.94×10^{-03}
FGF16	4.14	1.45×10^{-02}	MORF4	2.23	2.08×10^{-02}
FEZL	4.08	1.63×10^{-02}	SOX21	2.13	3.91×10^{-02}
FOXH1	4.01	2.07×10^{-02}	SOX1	2.09	3.55×10^{-02}
FELZ	3.86	1.51×10^{-02}	TITF1	2.03	3.43×10^{-02}
EEF1A1	3.84	1.95×10^{-02}	MLLT6	2.03	2.64×10^{-02}
PMX1	3.73	2.82×10^{-02}	CREB3L2	2.01	4.64×10^{-02}
ID3	3.71	1.35×10^{-02}	BCL6B	2.01	1.87×10^{-02}
DAZAP1	3.63	4.14×10^{-02}			
IVNS1ABP	3.57	2.67×10^{-02}			
KLHL3	3.51	3.17×10^{-02}	Gene	Average Fold Change	P-value
ZBTB26	3.44	1.38×10^{-02}	BARX1	-9.31	3.28×10^{-02}
DEAF1	3.38	2.64×10^{-02}	BARHL1	-6.68	2.44×10^{-02}
ALDH3A2	3.11	1.31×10^{-02}	JUN	-3.64	1.57×10^{-02}
TNRC9	3.08	2.06×10^{-02}	POU1F1	-3.53	2.19×10^{-02}
SUPT4H1	3.03	1.91×10^{-02}	WNT5B	-2.67	3.18×10^{-02}
WNT3	3.01	4.11×10^{-02}	CSRP1	-2.25	2.71×10^{-02}
LRP5	2.97	1.12×10^{-02}	ATF7	-2.21	1.11×10^{-02}
MADH7	2.86	1.17×10^{-02}	ETV4	-2.16	1.94×10^{-02}
LAF4	2.77	2.13×10^{-02}	PPARBP	-2.05	1.77×10^{-02}
ZNF71	2.71	4.33×10^{-02}	LMO4	-2.01	3.28×10^{-02}

Average fold changes are displayed as levels in the GATA3 siRNA knockdown relative to the control GFP siRNA. Values are > 2-fold and p-value < 0.05.

GATA3 over-expression microarray comparisons

As a reciprocal experiment to our siRNA knockdowns, we next identified genes differentially expressed in response to *GATA3* over-expression. Using a pMES vector expressing *GATA3* and eGFP under the control of a chick beta-actin promoter we over-expressed *GATA3* in dissociated epithelial cells from the chick utricle (Figure. 4-3). Transfection efficiency was determined to be 24% by comparing eGFP expression to total DAPI stained nuclei (n = 136). We quantified changes in gene expression between the *GATA3* over-expressing samples and those transfected with an eGFP/pMES (empty) vector. We identified 12 genes that are up-regulated and 11 down-regulated in response to *GATA3* over-expression (Fold change > 2.0 and p-value < 0.05) (Table 4-3). The genes showing the most dramatic up-regulation in response to *GATA3* over-expression are the *WNT*/beta-catenin signaling modulators *WNT9A* and *SFRP2*.

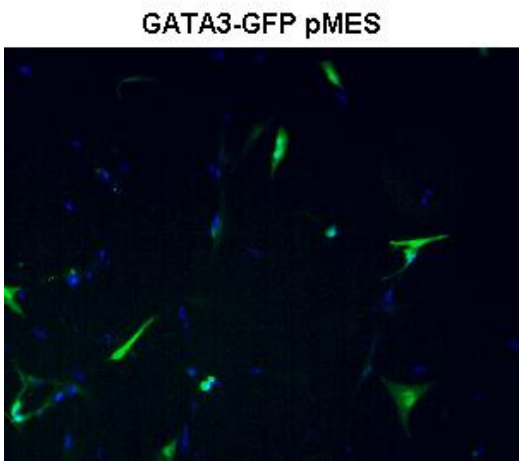

Figure 4-3. Dissociated utricle sensory epithelia transfected with a GATA3-GFP pMES expression vector. Approximately 24% of cells over-expressed GATA3 shown by GFP (green) compared to DAPI labeled nuclei (blue).

Table 4-3. Genes differentially expressed in GATA3 over-expression.

Gene	Average Fold Change	P-value	Gene	Average Fold Change	P-value
GATA3	7.28	2.71×10^{-04}	WDTC1	-3.68	2.40×10^{-03}
WNT9A	6.24	1.28×10^{-02}	H2AFY	-3.04	1.18×10^{-03}
SFRP2	5.84	4.61×10^{-02}	CDKN2C	-2.6	2.08×10^{-03}
TAF-172	3.59	1.83×10^{-02}	WNT4	-2.5	1.38×10^{-03}
BMP15	2.68	3.83×10^{-02}	MADH2	-2.48	2.00×10^{-02}
SOX8	2.67	3.90×10^{-02}	MTA1	-2.28	4.21×10^{-03}
TCF7	2.55	1.97×10^{-02}	TAF2D	-2.16	5.17×10^{-03}
EDAR	2.49	4.40×10^{-02}	SOX10	-2.15	1.28×10^{-02}
PXN	2.33	1.91×10^{-02}	ALDH4A1	-2.04	2.07×10^{-02}
GTF2E2	2.17	4.20×10^{-03}	MADH3	-2.03	2.95×10^{-02}
MADH9	2.16	5.95×10^{-05}	FARSL	-2.03	4.08×10^{-05}
HNK1	2.01	2.05×10^{-02}			

Average fold changes are displayed as levels in the GATA3 over-expression experiment relative to the GFP vector only control. Values are > 2-fold change p-value < 0.05.

Downstream effectors of GATA3 expression

To identify genes that are potentially directly regulated by *GATA3* we compared expression changes across all three conditions. This is a particularly conservative approach given that *GATA3* can in various circumstances act as either an activator or a repressor (Siegel et al., 1995; Lavenu-Bombled et al., 2002; Mantel et al., 2007). It is quite possible that dramatic down-regulation or up-regulation of GATA3 may not have immediately reciprocal effects on actual *in vivo* target genes in a simplistic model of target selection. Nevertheless, we adopted this filtering approach to identify a set of genes that would be strong candidates for direct regulation. We identified 4 genes with similar or reciprocal

expression patterns to *GATA3*: *BMP2, FKHL18, LMO4* and *MBNL2* (Table 4-4). For this comparison, we expanded our datasets to include more modest fold changes (>1.3 fold, p-value < 0.05) across all 3 conditions. As described below, it is clear that at least two of these genes are indeed *in vivo* targets of *GATA3*.

Table 4-4. Genes with similar or reciprocal expression patterns to *GATA3* across all three conditions.

Gene	Striola	P-value	GATA3 Knockdown	P-value	GATA3 Over-expression	P-value
Bmp2	-3.12	1.20×10^{-2}	1.44	2.15×10^{-2}	-1.79	1.52×10^{-2}
Fkhl18	1.57	1.68×10^{-2}	-1.66	4.38×10^{-2}	1.44	1.41×10^{-2}
Gata3	5.45	1.12×10^{-2}	-1.53	6.13×10^{-5}	1.84	1.42×10^{-2}
Lmo4	2.28	4.26×10^{-2}	-2.01	3.28×10^{-2}	1.42	1.43×10^{-3}
Mbnl2	-1.47	2.84×10^{-2}	1.85	1.35×10^{-2}	-1.58	4.44×10^{-2}

All values are derived from striola vs. extra-striola, GATA3 siRNA knockdown and GATA3 over-expression microarray data. Average fold changes > 1.3 and p-value < 0.05 in all three conditions.

We next employed chromatin immunoprecipitation (ChIP) to confirm the direct interaction of *GATA3* with two of the four predicted target genes. This experiment was conducted using dissociated epithelial cells from the chick utricle that had been transfected with the pMES vector, to over-express *GATA3*. Putative GATA binding sites were computationally identified, using TF Search (Heinemeyer et al., 1998), upstream of the transcription start sites of *LMO4* and *MBNL2*. Searches within the regions surrounding the other two genes did not reveal convincing putative *GATA3* targets. However, as previously noted, biologically functional *GATA3* sites are not strictly confined to promoter-proximal sites and broader search parameters revealed numerous potential *GATA3* sites. Primers were designed surrounding the putative GATA binding sites adjacent to

the *LMO4* and *MBNL2* genes. These were used to PCR amplify those regions after ChIP pull-down with a *GATA3* polyclonal antibody. PCR products for each candidate region were compared to products from a mock antibody pull-down, in order to identify enrichment by *GATA3* ChIP (Figure 4-4). We identified enrichment of 1 region upstream of the *LMO4* transcription start site, -627 to -818, containing two putative GATA binding sites and another region upstream of the *MBNL2* transcription start site, -1574 to -1950, containing 8 putative GATA binding sites. These data strongly support the classification of these two genes as being directly regulated by *GATA3.*

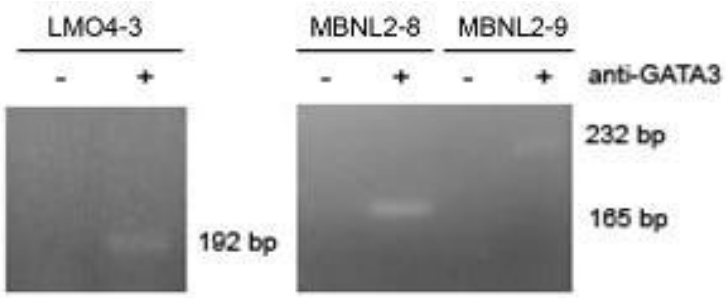

Figure 4-4. Direct *in-vivo* interactions with GATA3 were demonstrated by ChIP in dissociated sensory epithelia over-expressing GATA3. PCR amplification with primers flanking putative GATA binding sites identified enrichment in the anti-GATA antibody (+) ChIP compared to a mock antibody control (-) in a 192 bp product, LMO4-3, from -818 bp to -627 bp upstream of LMO4 and two overlapping 165 bp and 232 bp products from -1738 bp to -1574 bp (MBNL-8) and -1950 bp to -1719 bp (MBNL-9) upstream of MBNL2.

To independently verify the expression patterns predicted by our microarray comparisons and confirm that those expression patterns were consistent with direct regulation by *GATA3*, we conducted RNA in situ hybridizations on whole mount chicken utricles (Figure 4-5). In agreement with our microarray data we found that the area of *LMO4* expression surrounds and

encloses the striolar region. Our microarray data indicate that *MBNL2* exhibits a reciprocal pattern of expression to that of *GATA3*. In agreement with this, we found that *MBNL2* is not expressed at the striola, but is confined to the medial region of the utricle bordering the striola. This is consistent with a model in which *GATA3* acts to repress *MBNL2* expression, but positively regulates *LMO4* expression at the striola.

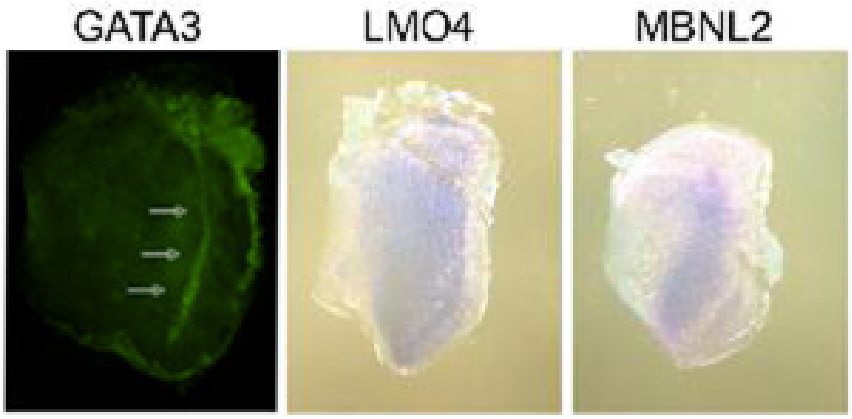

Figure 4-5. *In situ* hybridizations confirm expression patterns of LMO4 and MBNL2 predicted by our microarray and ChIP data. Immunohistochemical labeling with a GATA3 antibody (green) and RNA *in situ* hybridizations with antisense probes to LMO4 and MBNL2 in whole mount chick utricles. GATA3 and LMO4 are expressed at the striola reversal zone and MBNL2 is expressed in the medial region of the utricle bordering the striola reversal zone.

Conclusions

In this study, we report the first large scale analysis of regionalized gene expression differences in the vestibular sensory organ. In addition to identifying transcription factor gene pathways required for inner ear development and regeneration, it is also important to determine direct DNA binding targets of key transcription factors that regulate these processes. Though GATA3 has been well characterized during the development and differentiation of the mammalian hematopoietic system, little is known about downstream effectors and direct DNA binding targets in the inner ear, brain and other organ systems. We focused on genes that are differentially expressed in the striolar vs. extra-striolar regions of the chick utricle which are associated with the expression of the zinc finger transcription factor GATA3. Most notable among the differences are those involving *WNT* signaling and neurogenesis. Our data indicate that the neurogenesis regulators, KCNIP4, NGN2, and HEY2, are correlated with the presence of type I vestibular hair cells. *KCNIP4* is particularly interesting, as it encodes a potassium channel-interacting protein that regulates membrane excitability (Holmqvist, Cao et al. 2002; Rhodes, Carroll et al. 2004) and has also been shown to inhibit *WNT* signaling by promoting presenilin (PS1) mediated degradation of β-catenin (Kitagawa, Ray et al. 2007). Consistent with this observation we also identified 6 modulators of *WNT* signaling that are differentially regulated at the striola. Three secreted *WNT* ligands, *WNT3,* *WNT*3a and *WNT*5a, and two *WNT* receptors, *FZD5* and *FZD7*, are specifically down-regulated in the striolar region. In contrast, the striola expresses high

levels of the Wnt modulator Dickkopft2 (*DKK2*). *DKK2* acts as a context-dependant agonist or antagonist of *WNT*/beta-catenin signaling depending on the presence of its co-factor Kremen-2 (Mao and Niehrs 2003). Though there is no known chick ortholog to Kremen-2, interestingly one of the highest up-regulated genes in response to GATA3 over-expression was the *WNT* antagonist *SFRP2* (5.84 fold change). Members of the Sfrp family inhibit Wnt signaling by acting as extracellular decoy receptors that sequester Wnts and prevent binding to Frizzled receptors. In mouse gut mysenchymal cells, Sfrp2 is induced by Barx1 to reduce local Wnt activity at endodermal cells to direct stomach epithelial differentiation (Kim, Buchner et al. 2005). In our GATA3 siRNA treatments, Barx1 had the highest down-regulation in response to GATA3 knockdown. Together, our data suggests a model in which *GATA3* expression leads to an overall down-regulation of *WNT* signaling at the striola. In addition to *WNT* signaling modulators, we also see several components of *Notch*, *FGF* and *BMP* signaling that are differentially expressed at the striola compared to the extra-striola. A previous study has also identified overrepresentation of *WNT* signaling, *Notch* signaling, *FGF* signaling and *BMP* signaling genes differentially expressed in *GATA3* conditional knockouts of mouse skin epidermis and hair follicles (Kurek, Garinis et al. 2007). *GATA3* is required for differentiation and organization of hair follicles during skin development and regeneration (Kaufman, Zhou et al. 2003). Our data suggests that *GATA3* may also regulate these signaling pathways in the inner ear.

Our microarray gene expression profiling data suggests intersections between several important signaling pathways, most notably *Wnt* and *Notch* signaling. Recently, several reports have suggested an intertwined relationship between *Wnt* and *Notch* ('Wntch') signaling regulation of cell proliferation and differentiation during embryonic development (Hayward, Kalmar et al. 2008). *Wnt* signaling has previously been shown to be involved in early organogenesis during utricle and cochlea differentiation. Specifically, Wnt's are thought to mark specific organ lineages and refine sensory vs. non-sensory boundaries during vestibular and auditory organogenesis (Stevens, Davies et al. 2003; Sienknecht and Fekete 2008). During later vestibular and auditory development (E12.5-E15), sensory hair cell and non-sensory supporting cell differentiation occurs. This process is regulated by the *Notch* signaling pathway. It is not yet known how sensory hair cells of the utricle acquire their specific type I or type II cell fate. The process of differentiation into specific, terminal cell types involves an ordered set of transcriptional regulators. During the final stages of sensory hair cell development in the utricle of the inner ear, this differentiation process involves a series of binary decisions in which cells branch into sensory hair cells vs. non-sensory supporting cells, and finally type I vs. type II hair cells. One of the best studied roles of *GATA3* is during hematopoietic stem cell differentiation. T lymphocytes must traverse a series of binary decisions from pluripotent progenitor cells to T helper type I (Th1) vs. T helper type 2 (Th2) cell types. In the hematopoietic lineage-specific transcription factor model, *GATA3* acts as a master regulator of Th2 specific differentiation. It is not surprising that a similar

lineage specific transcription factor model of *GATA3* regulated differentiation would exist in other organ systems. By comparing gene expression differences between distinct regions of the vestibular organ and in response to expression levels of the transcription factor GATA3, we have identified downstream effectors of GATA3. Specifically, our data suggests a model by which *GATA3* regulated inhibition of *Wnt* signaling may intersect with *Notch* signaling at the striola and play an important role in type I vs. type II hair cell differentiation and/or maintenance.

In addition to implicating candidate effectors within the striola, we also used chromatin immunoprecipitation to identify two direct gene targets of GATA3. Of the confirmed direct targets, the LIM domain only 4 gene (*LMO4*) encodes a cystein-rich transcription regulator containing two LIM domains. The zinc finger binding domains of *LMO4* are structurally similar to GATA zinc fingers (Perez-Alvarado, Miles et al. 1994); however, no specific LIM-DNA interaction has been reported. Rather, LIM family members are thought to act as part of a transcriptional complex that is mediated by protein-protein interactions: LIM family members have been shown to interact with *GATA* transcription factors during hematopoiesis (Osada, Grutz et al. 1995; Wadman, Osada et al. 1997) and in the spinal cord, LMO4 interacts with *GATA* transcription factors to regulate the balanced generation of inhibitory and excitatory neurons (Joshi et al., 2009). *LMO4* is detected in the mouse inner ear during otic placode formation at E8.5 and by E10.5 is expressed primarily in the dorsolateral regions of the otic vesicle that will eventually form the vestibular organs (Fekete and Wu 2002; Burton, Cole

et al. 2004; Deng, Pan et al. 2006). This pattern appears to be coincident with *GATA3* expression, which also occurs throughout the otic placode at E8 and is also restricted to the dorsolateral otic vesicle by E10.5 (Grace Lawoko-Kerali 2002). Our expression profiling and *in situ* hybridization results confirm this pattern of spatial and temporal co-expression. In addition, our ChIP data indicates that *LMO4* is a direct target of GATA3 activity within the utricle and very likely throughout inner ear development.

In addition to *LMO4*, we also identified the muscleblind-like 2 (*MBNL2)* gene as a direct target of *GATA3*. The Muscleblind family of proteins regulate alternative exon splicing during differentiation in many cell types, including muscle, neurons and photoreceptors. *MBNL2* can also function as an RNA binding protein essential for integrin α3 subcellular localization (Adereth, Dammai et al. 2005; Maya Pascual 2006). Interestingly, a role for integrins in hair cell differentiation and stereocilia maturation has previously been described (Littlewood Evans and Muller 2000). Taken together these observations suggest that *GATA3* may play a role in subcellular localization of integrins and in the regulation of alternative splicing during type I vs. type II sensory hair cell differentiation.

We have also identified two genes (*BMP2* and *FKHL18*) with expression that consistently varies with GATA3 levels and localization. Though *BMP2* and *FKHL18* are likely regulated by GATA3 at the striola, it is not clear if this is a direct or indirect regulation. *FKHL18* is a member of the forkhead box (FOX) family of transcription factors. Our expression profiling data suggests that *GATA3*

may induce expression of *FKHL18* at the striola. Though other members of the FOX family of transcription factors have been implicated in the normal development of the inner ear (Hulander, Wurst et al. 1998; Solomon, Kudoh et al. 2003), the structurally unique *FKHL18* has only been described during mouse fetal testis development (Yuko Sato 2008). Our expression profiling data suggests that *GATA3* may also regulate *BMP* signaling in the utricle. Specifically, *GATA3* inhibits *BMP2* at the striola. *BMP2* has previously been shown to act downstream of *Wnt* signaling during osteoblast differentiation (Rawadi, B et al. 2003; Morvan, Boulukos et al. 2006) and our data suggests *GATA3* may regulate a potential intersection between *BMP* and *Wnt* signaling at the striola during inner ear development.

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CHAPTER FIVE

GENERAL CONCLUSIONS

In the previous chapters, I have described the use of genomic microarray expression profiling along with siRNA knockdowns to identify transcription factor pathways critical for sensory epithelia (SE) proliferation in the inner ear. I have also described the use of complimentary genomic techniques to identify pathway intersections in defined regions of the inner ear and direct gene targets of a transcription factors required for inner ear development. These were the first large scale gene expression profiling studies of SE proliferation and regionalized gene expression differences in the vestibular organs of the inner ear.

In the first study, 683 genes from known pathways and genes of previously un-described function were identified during avian sensory hair cell regeneration in the inner ear. This study represented the first large scale gene expression profiling of regenerating SE of the inner ear. We identified multiple components of known signaling pathways that were clearly identifiable: *TGFβ, PAX, NOTCH, WNT, NFKappaB, INSULIN/IGF1* and *AP1*. We also identified transcription factors that had not previously been implicated in known pathways. In addition to identifying genetic pathways involved in avian SE regeneration, in the second study I used a small molecule inhibitor and siRNA screen to determine if specific genes from this regeneration dataset are required for avian SE proliferation. In this study 27 genes and pathways and identified 11 genes/pathways that are required for SE proliferation, including components of the *AP1* complex (*JNK, JUND* and *c*-JUN), *PAX* pathway (*PAX2* and *PAX5*), as well as *TGFβ* signaling and cell cycle regulation (*CUTL*). I also identified 2 genes that we determined act downstream of the AP1 complex and are also required for

SE proliferation; the CCAAT element binding protein (*CEBPG*) and the WNT co-receptor (*LRP5*). These results suggest that components of the AP1 complex and PAX pathway are required for avian SE proliferation and that these pathways intersect with WNT signaling, specifically by regulating expression of Wnt4. The process of SE regeneration involves two major steps; first neighboring cells must re-enter the cell cycle and proliferate. Cells must then differentiate into the proper cell type, either sensory hair cells or non-sensory supporting cells. It is expected that the early stages of proliferation would involve components of apotosis and cell cycle pathways such TFG-β signaling, CUTL1 and $p27^{KIP1}$. It is interesting to find that, PAX2, a component of the PAX pathway involved in the earliest stages of inner ear development and its downstream effector, WNT4, a component of WNT signaling involved in forming sensory/nonsensory boundaries in the developing inner ear, are both required for SE proliferation. This suggests that some of the genetic pathways involved in SE regeneration may be a recapitulation of the early stages of inner ear development.

In addition to identifying transcription factor gene pathways required for inner ear development and regeneration, it is also important to determine direct DNA binding targets of key transcription factors that regulate these processes. In a third study, I described how we can use these previously described techniques to conduct the first large-scale study of regionalized gene expression differences in the vestibular organ of the inner ear. Though *GATA3* has been well characterized during the development and differentiation of the mammalian hematopoietic system, little is known about downstream effectors and direct DNA

binding targets in the inner ear, brain and other organ systems. The organs of the inner ear, vestibular (balance) and cochlea (auditory), are extremely sensitive to *GATA3* expression levels. *GATA3* haploinsufficiency results in hypoparathyroidism, sensorineural deafness and renal anomaly syndrome (HDR) in humans. Though *GATA3* is expressed throughout the sensory region of the cochlea, expression is limited to a 6-10 cell wide region in the striola of the utricle. This region consists of approximately 10,000 cells and corresponds to a 180 degree shift in sensory hair cell orientation and a phenotypic shift in morphologically distinct sensory hair cell types. In this study, I focused on genes that are differentially expressed in the striolar vs. extra-striolar regions of the chick utricle and dependent on levels of expression of the zinc finger transcription factor *GATA3*. I reported the identification of two novel direct gene targets of *GATA3* and two genes whose expression consistently varies with *GATA3* levels and localization. I also reported distinct expression differences in components from known pathways such as *WNT* signaling, *NOTCH* signaling, *FGF* signaling and *BMP* signaling that are potentially regulated by *GATA3*. Specifically, these results suggest a model by which *GATA3* regulated inhibition of *WNT* signaling at the striola may play an important role in type I vs. type II sensory hair cell differentiation and/or maintenance.

These studies have provided novel routes to study pathways involved in inner ear development and hair cell regeneration. Together, these investigations have identified a set of genes that are required for sensory epithelia proliferation in the inner ear and novel interactions between several critical pathways. I have

also completed the first large scale study of regionalized gene expression differences in the avian utricle and identified genes and pathway intersections that potentially regulate specific hair cell fate determination in the inner ear.

These studies have significantly contributed to our understanding of inner ear development and revealed exciting and novel pathways for research into hearing and balance disorders in humans.

CHAPTER SIX

MATERIALS AND METHODS

Sensory Epithelia Isolation

10-21 day post-hatch White Leghorn chicks were CO_2 asphyxiated and decapitated, heads were immersed in chilled 70% ethanol for 5-10 min. Utricles were removed and immediately placed in chilled Medium-199 with Hanks salts. Sheets of sensory epithelia attached to their basal membrane were micro-dissected and pure sensory epithelia was isolated using published methods (Warchol 2002).

Neomycin Time course

Sensory epithelia from 5 utricles were pooled together in 100µl Medium 199. Specimens were treated with 1 mM neomycin in Medium 199 with Earles salts supplemented with 10% FBS for 24 hrs. Specimens were rinsed with fresh Medium199/10% FBS and cultured for an additional 24 hrs to recover. Samples were harvested at 0hr, 24hr and 48hr post recovery. Time point matched control sensory epithelia were cultured following the same protocol with the absence of neomycin.

Laser Microbeam Ablation Time course

Sensory epithelia from the cochlea or utricle were cut into small pieces, and grown for 7-10 days on fibronectin-coated wells (Mat-Tek) that contained 50 µl Medium-199/10%FBS. Semi-confluent cultrures were lesioned via laser microsurgery (Warchol and Corwin 1996). Samples were harvested at 30 min, 1 hr, 2 hr and 3 hr post laser treatment along with time point matched untreated controls.

mRNA Isolation, cDNA Synthesis and Amplification

RNA isolation and cDNA synthesis was carried out using previously published methods (Hawkins, Bashiardes et al. 2003). Briefly, approximately 50,000 sensory epithelial cells from either the utricle or cochlea were suspended in Trizol (Invitrogen) and total RNA was isolated as per the manufacturer's protocol yielding 300-500 ng of total RNA. Polyadenylated RNAs were isolated using 10 µl of oligo dT_{25} streptavidin coated paramagnetic beads (Dynal). Bead linked polyadenylated RNAs were included in a reverse transcription (RT) reaction to synthesize first-strand cDNA on the beads. During this RT reaction, reverse transcriptase adds three C's to the 3' end of the first-strand cDNA. In this same RT reaction, a primer containing T7 viral promoter and three G's complementary to nucleotides added by the reverse transcriptase was included. This additional primer extends the first-strand cDNA synthesis adding a T7 promoter sequence 5' to the poly A tract. An oligo dT primer containing an additional linker sequence was used to generate the second strand by 6 PCR cycles. A PCR reaction using the T7 promoter and linker sequence was used to linearly amplify the cDNA. To prevent skewing during the amplification process, PCR reactions were limited to 10 cycles. Using the Ambion T7 Megascript kit, *in-vitro* RNA runoffs were generated from the T7 promoter using manufacturers protocols.

Microarray Design and Printing

The custom, transcription factor microarray contains ~3,000, 50-70mer oligonucleotide probes designed to the majority of known human transcription factor genes, anonymous ESTs that contain transcription factor motifs,

transcriptional co-activators and components of several known signaling pathways (Messina, Glasscock et al. 2004). The initial design of this array was the work of a former graduate student in the lab, David Messina. The array design has since been updated with addition transcription factor and signaling pathway genes of interest. Tm matched 50-70mer probes were designed using OligoWiz 2.0 design software (Nielsen, Wernersson et al. 2003; Wernersson and Nielsen 2005). Oligonucleotide probes were designed to the more conserved 3' coding regions rather than 3'-UTR to allow for cross-species comparisons. When suitable probes could not be designed to conserved regions, species specific probes were designed. Probes were precipitated and resuspended at a concentration of 60 µM in 6% DMSO and 1.5M Betaine.

Slides for printing were pre-treated with 10% (w/v) NaOH, 57% ethanol solution for 2 hours. Slides were washed in H_2O and then coated in a 10% poly-L-lysine, 10% PBS solution for 1 hour. Slides were washed again in H_2O and dried by spinning in a floor centrifuge at 500 rpm for 10 minutes and baked at 45℃ for 10 minutes. Microarray probes were printed on a GMS 417 arrayer in duplicate. Printed slides were baked at 80℃ for 2 hours and cross-linked at 65mJ prior to sample hybridization.

Microarray Hybridization and Data Processing

Poly(A)$^+$ RNA from *in-vitro* transcriptions were used to generate 1st strand cDNA for the purpose of microarray hybridization using a dye specific oligo-dT primer following 3DNA Array 50 protocols (Genisphere) and hybridized at 42℃.

Following LOWESS normalization (Quackenbush 2002), clustering of experiments and low-intensity filtering to remove probes that fall below the intensity of control spots, fold changes and P-values were determined for 2 biological samples and 4 technical replicates including dye switch experiments for a total of 8 microarrays per treatment. P-values calculated across all replicate experiments using a one sample t-test.

siRNA Generation and Transfection

Predesigned, chemically synthesized 27-mer duplex Dicer-substrate RNAs (DsiRNAs), were obtained from Integrated DNA Technologies (IDT) when available. Otherwise, double stranded RNA (dsRNA) was generated by first PCR amplifying a portion of the gene of interest from chicken SE cDNA. PCR products were amplified using gene specific primers containing the 5' T7 promoter sequence CTCTAATACGACTCACTATAGGG, under the following conditions: 100ng cDNA, 0.2 □M (final conc.) each primer, 10X Advantage Taq Buffer (BD Biosciences), 5U Advantage Taq (BD Biosciences) in a final volume of 50 □L; 95°C-2 min, (95°C-30 sec, 55°C-30 sec, 68°C-2 min)-for 30 cycles. PCR products were DNA sequenced verified.

Promoter containing PCR products were used as template DNA in *in vitro* transcription (IVT) reactions (Ambion). IVT reactions, including post-reaction DNase treatment and precipitation, were performed according the manufacture's protocol for 12 hr. Equal amounts (typically 3□g each) of sense and antisense RNA strands were mixed and heated at 75°C for 10 min and brought to room

temperature on the bench for 2 hr. dsRNAs were treated with RNase ONE (50U, Promega) for 45 min at 37°C. dsRNA was cleaned using RNA Purification Columns 1(Gene Therapy Systems). siRNAs were generated using the Dicer enzyme (Gene Therapy Systems) following the manufacture's protocol. Dicer generated siRNA (d-siRNA) was checked on a 3% agarose gel for ~23bp size. d-siRNA was cleaned up using RNA Purification Columns 2 (Gene Therapy Systems). 50 ng of d-siRNA were transfected in each well of dissociated SE cultures or laser microbeam ablated SE cultures.

Dissociated Sensory Epithelia Transfection

Pure sensory epithelia were physically dissociated and grown in 96 well cultures at a concentration of 0.5 utricle sensory epithelia per well. Dissociated sensory epithelia were cultured for 3 days and transfected prior to confluency with siRNAs (50 ng/well) or inhibitor in 0.1% DMSO (15 µM SP600125 JNK inhibitor) using previously described methods(Elbashir, Harborth et al. 2002).

Dissociated Retinal Epithelia Isolation and Transfection

10-21 day post-hatch White Leghorn chicks were CO_2 asphyxiated and decapitated, heads were immersed in chilled 70% ethanol for 5-10 min. Whole eyes were removed and immediately placed in chilled Medium-199 with Hanks salts. Sheets of retinal epithelia were micro-dissected and physically dissociated. Retinal epithelia were plated in 96 well cultures at 20% confluency. Dissociated

cultures were transfected prior to confluency, 3 days post plating, with siRNAs (50 ng/well) using previously described methods (Elbashir, Harborth et al. 2002).

Proliferation Index Phenotyping

Cells were assayed 24 hrs post-transfection using previously published protocols (Warchol and Corwin 1996). Quantification of cell proliferation was measured by calculating a proliferation index (defined as the number of BrdU+ cells/total cells). Briefly, bromodeoxyuridine (BrdU) was added to S-phase cells in culture for 4 hr. Proliferating cells were labeled with BrdU as viewed with differential interference contrast microscopy. Cells from 10,000μm^2 (100 X 100 µm) regions were counted for total number of cells (DAPI) and the number of BrdU-labeled cells using ImageJ 1.36b software (http://rsb.info.nih.gov/ij/). Calculations from a minimum of 20 regions were combined to obtain a proliferation index for each experimental treatment to determine the affects of siRNA and small molecule inhibitor treatment on utricle or retinal sensory epithelia cell proliferation.

GATA3 siRNA

Whole utricle specimens were treated 1 mM streptomycin in Medium 199 with Earles salts supplemented with 10% FBS for 24 hrs. Specimens were rinsed with fresh Medium199/10% FBS and cultured for an additional 24 hrs to recover. Whole utricle siRNA transfections were performed by electroporation. Utricles were transfected with 21mer synthetic siRNAs (Ambion) at a final concentration

of 100 nM siRNA in 30 µl H_2O under the following conditions: 50 Volts, 30 ms pulse for 10 pulses. Specimens were returned to Medium 199/10% FBS for an additional 48 hrs and pure sensory epithelia were isolated using previously published methods (Warchol 2002).

GATA3 over-expression

Dissociated sensory epithelia were plated in 96 well cultures, 5 wells per sample. 4 days post plating, ~ 30% confluency, cells were transfected with a pMES vector containing an internal ribosome entry site regulating expression of GATA3 and eGFP under control of a chick beta-actin promoter. Controls were transfected with a vector containing eGFP only. Transfections were performed using recommended concentrations for Lipofectamine 2000 (Invitrogen). 24 hrs post transfection cells were harvested in 100 µl Trizol per well, 5 wells were combined for each biological sample.

Separation of striolar and extrastriolar regions of the utricle.

Explanted utricles were placed in Medium-199 with Hanks salts and iridectomy scissors were used to cut away the edges of the sensory organs (which are comprised of no sensory transitional epithelium). Iridectomy scissors were then used to cut the remaining sensory tissue along to anterior-posterior axis, in order to separate the lateral portion of the epithelium (which contains the striola, the GATA3-expressing region and all type I hair cells) from the medial portion (which does not express GATA3 and is populated exclusively by type II

hair cells – Fig. 1). Striolar and extrastriolar regions from 10 utricles were separated into two groups and were incubated for 60 min. in 500 µg/ml thermolysin (at 37^{o}C). A fine needle was then used to remove the sensory epithelium from each of the utricular fragments, and mRNA from the striolar and extra-striolar groups was extracted with 100 µl Trizol.

RNA *in situs*

Primers were designed to generate 200-400 bp PCR amplicons from chicken sequences. A second round of PCR was used to add T7 promoters to either the 5' or 3' end. PCR products were gel purified and verified by DNA sequencing. PCR templates were used to separately generate DIG labeled *in-vitro* transcripts (Ambion T7 MegaScript Kit) for both sense and anti-sense strands. Utricles were obtained from 10-21 day old White Leghorn chicks and processed for whole mount *in-situs* following published protocols (Henrique, Adam et al. 1995). Utricles were labeled and mounted in glycerol/PBS (9:1) and imaged.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed in dissociated sensory epithelia over-expressing GATA. Sensory epithelia from 10 utricles were physically dissociated and plated in 6 well cultures and transfected with a GATA3 expressing pMES vector as previously described. ChIP was conducted following recommended protocols (Active Motif). Specifically, enzymatic shearing was

conducted for 10 min and IP was performed with the GATA3 goat polyclonal IgG sc-22205 (Santa Cruz) at a concentration of 3 µg/100 µl or a mock antibody negative control. Predicted GATA binding sites were identified up to 2000 bp upstream of predicted target genes using TF Search (Heinemeyer, Wingender et al. 1998). Primers were designed to amplify potential sites by PCR (Table 6-1). ChIP pull down products were amplified for 30 cycles with a 55 ℃ annealing temperature followed by an additional 30 cycles and imaged by agarose gel electrophoresis.

Table 6-1. PCR primers flanking putative GATA binding sites upstream of LMO4 and MBNL2.

Primers	Primer Start	Primer Stop	Product Length	Forward Sequence	Reverse Sequence
LMO4-1	-387	-151	237	**TTCGGATAAATGCGATGCTA**	**TGACAGAGCAGAATCCCAACT**
LMO4-2	-735	-510	226	**GGGGAGTCACTTTCTGGTCA**	**CCTGCGCCTTAAATCACTTC**
LMO4-3	-818	-627	192	**TACCGGAGTGCGCCTATTTA**	**CAGCATCCAGTAACCCCATT**
MBNL2-1	-254	-62	193	**AGGACTGCTACGCCTGTGTT**	**CAAGAAAAGCAATGCGTTCA**
MBNL2-2	-478	-324	155	**TCAGCTGGCTATTCCCTTGT**	**TTCACATTCAGCTCGTTTGC**
MBNL2-3	-760	-560	201	**TGGGATTTCTTTGGGAATTG**	**TTAGGCATGCTGGTTGTGAA**
MBNL2-4	-971	-756	216	**AGGCTTTGGTGTTGAACCAT**	**TCCCAAAGAACCACCTTCAC**
MBNL2-5	-1199	-953	247	**CCATCAACTGTTTCTGGCTGT**	**TGGTTCAACACCAAAGCCTA**
MBNL2-6	-1314	-1115	200	**TTATTTTGGCATGGGAAAGC**	**TCACGGTCATGATGTTTCCT**
MBNL2-7	-1593	-1344	250	**TGGACGCATTACTGCATGTT**	**TTAATCGAAGCCAGGATTGTT**
MBNL2-8	-1738	-1574	165	**TGTGGGACAACGTTGGTAGA**	**AACATGCAGTAATGCGTCCA**
MBNL2-9	-1950	-1719	232	**TGGCACTTCTGAATTTGAGC**	**TCTACCAACGTTGTCCCACA**

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