Published in final edited form as:
Annu Rev Neurosci. 2013 July 8; 36: 361–381. doi:10.1146/annurev-neuro-062012-170309.

The Genetics of Hair Cell Development and Regeneration

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Abstract

Sensory hair cells are exquisitely sensitive vertebrate mechanoreceptors that mediate the senses of hearing and balance. Understanding the factors that regulate the development of these cells is important, not only for our understanding of ear development and its functional physiology, but also to shed light on how these cells may be replaced therapeutically. In this review, we describe the signals and molecular mechanisms that initiate hair cell development in vertebrates, with particular emphasis on the transcription factor Atoh1 that is both necessary and sufficient for hair cell development. We then discuss recent findings on how microRNAs may modulate the formation and maturation of hair cells. Lastly, we will review recent work on how hair cells are regenerated in many vertebrate groups, and the factors that conspire to prevent this regeneration in mammals.

Keywords

Cochlea; Atoh1; miRNA; Notch; Cell Cycle

A: Intrinsic and extrinsic signals that drive hair cell induction

The inner ear develops from a thickened patch of cranial ectoderm, the otic placode, and then invaginates or cavitates to form a spherical otocyst. At this point, the ear primordium has already received signals that confer regional identity and polarity to the otocyst (Groves and Fekete, 2012). These signals lead to the formation of multiple prosensory patches, each of which will give rise to a particular inner ear sensory organ - the three cristae at the base of each semicircular canal, the two maculae and the auditory epithelium of the cochlea (Alsina et al 2009, Bok et al 2007). These patches are characterized by the expression of the transcription factor Sox2, and mice carrying hypomorphic mutations for *Sox2* lack all sensory cells in the inner ear (Kiernan et al 2005). These sensory patches then differentiate to produce the hair cells and supporting cells of each inner ear sensory organ.

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A.1: The temporal and spatial regulation of hair cell differentiation

After *Sox2*$^{+}$ prosensory tissue has been induced in each sensory organ, the prosensory domain begins to exit the cell cycle and terminally differentiate into hair cells. In most vertebrates, including the vestibular system of mammals, exit from the cell cycle and the appearance of the first markers of hair cells are tightly coupled. In the vestibular system, differentiation typically begins near the center of each prosensory patch, and expands out over an extended period of time. For example, the first hair cells appear in the future striolar region of the mouse utricle at embryonic day 11 (Raft et al 2007), but over half of the total hair cells are generated after birth, with small numbers of hair cells still being generated from mitotic progenitors between postnatal days 12-14 (Burns et al 2012b, Kirkegaard & Nyengaard 2005). In the case of the chicken hearing organ, the basilar papilla, the first hair cells are born in the middle of the superior side of the cochlea beginning at embryonic day 6, spreading both inferiorly and to both the base and apex over the next three days (Katayama & Corwin 1989).

The mammalian organ of Corti has a strikingly different arrangement of hair cells and supporting cells compared to all other vertebrate sensory patches. Instead of a quasi-hexagonal arrangement, where each hair cell is surrounded by between 4-8 supporting cells depending on its position in the sensory epithelium (Goodyear & Richardson 1997), hair cells and supporting cells are arranged in uniform rows and invariant proportions along the length of the cochlear duct (Kelley 2006). This serially repeating pattern is generated by a highly unusual pattern of cell cycle exit and differentiation. In the mouse, the prosensory domain of the future organ of Corti begins to exit the cell cycle in the apical tip of the cochlea at embryonic day 12 (Lee et al 2006, Matei et al 2005, Ruben 1967), and a wave of cell cycle exit then proceeds along the prosensory domain from apex to base over the next 48-60 hours, with some cells in the most basal region still incorporating mitotic labels at E14.5-E15.0 (Lee et al 2006). Starting at about E13.5, cells in the mid-basal region of the cochlea begin to differentiate into hair cells by expressing the transcription factor Atoh1 (Chen et al 2002), and this region of differentiating cells spreads down to the apex over the next 3-4 days. Thus, the first cells to exit the cell cycle in the apex of the cochlear duct are the last ones to terminally differentiate into hair cells five days later, while the last cells to exit the cell cycle in the mid-basal region are some of the first to differentiate into hair cells (Figure 1). This dramatic temporal and spatial uncoupling of cell cycle exit and differentiation has no parallel in any other vertebrate tissue.When maturation is complete, numerous morphological, physiological and molecular properties of the cochlear duct and its resident cells vary systematically along this longitudinal axis and are responsible for the gradient of selectivity to sounds of different frequencies (Figure 1).

The mechanisms that propagate the apical-basal gradient of cell cycle exit and the midbasal-apical gradient of differentiation in the mammalian cochlea are poorly understood. The cyclin-dependent kinase inhibitor $p27^{kip1}$ is up-regulated in the cochlea in an apical-basal gradient concomitant with the gradient of cell cycle exit (Figure 1; Chen & Segil 1999, Lee et al 2006), and is necessary for the correct sequence of cell cycle exit and generation of appropriate numbers of hair cells and supporting cells (Chen & Segil 1999, Kanzaki et al 2006, Lowenheim et al 1999). Its expression is regulated at both the transcriptional and protein levels (Lee et al 2006), but no signals have been identified that directly regulate its expression in the cochlea. The wave of hair cell differentiation does not appear to require contact with the underlying mesenchyme, as it can proceed normally when the embryonic cochlear epithelium is maintained alone in organ culture (Montcouquiol & Kelley 2003). Moreover, there is no absolute requirement for a fully intact cochlear epithelium to propagate differentiation signals in a planar fashion, as the correct basal-apical sequence of differentiation can be recapitulated even when the cochlear epithelium is cut into a series of pieces along its length and cultured separately (Montcouquiol & Kelley 2003). Genetic

experiments suggest a role for the transcription factors Neurog1 and Neurod1 in these gradients. The cochlear prosensory region of *Neurog1* mutant mice show a premature exit from the cell cycle of about 24-36 hours earlier than normal, and hair cell differentiation, assayed by expression of Atoh1, commences in the apical, not the basal region of the *Neurog1* mutant cochlea (Matei et al 2005). This disordered pattern of hair cell differentiation is also seen in *Neurod1* mutant mice (Figure 1; Jahan et al 2010). It is important to note that neither Neurod1 nor Neurog1 are expressed in the cochlea at detectable levels during this period of differentiation, suggesting they play an indirect role in triggering the timing of prosensory domain cell cycle exit and differentiation. Both transcription factors are necessary for the differentiation of the spiral ganglion, as both *Neurod1* and *Neurog1* mutant mice have spiral ganglia that are either greatly reduced or completely absent. This suggests the developing spiral ganglion may regulate the tempo of differentiation of cochlear hair cells.

In addition to the basal-apical gradient of differentiation in the cochlea, hair cell differentiation also proceeds in a neural-abneural direction at any given position along the length of the cochlear duct, with inner hair cells differentiating before outer hair cells, and pillar cells separating the two differentiating populations. Although birds do not display the same dramatic discontinuity and separation between inner and outer hair cells, they do show a neural-abneural gradient in hair cell length between “tall” and “short” hair cells (Gleich et al 2004). At present it is not clear whether the temporal separation between inner and outer hair cell differentiation in the organ of Corti reflects distinct sets of inductive signals, or a common inducing signal that is interpreted at different times or by different cellular contexts in the inner and outer hair cell regions. For example, recent evidence suggests that a gradient of BMP signaling normally spreads from the abneural to the neural side of the cochlear duct, and that perturbation of this gradient can change the numbers of both inner and outer hair cells (Groves & Fekete 2012, Hwang et al 2010, Ohyama et al 2010, Puligilla et al 2007). However, a number of mouse mutants display specific defects in the differentiation of outer versus inner hair cells. For example, *Emx2* mutant mice develop inner hair cells but not outer hair cells or Deiters’ cells (Holley et al 2010) and *Jag1* mutant mice develop multiple rows of inner hair cells but also lack outer hair cells and Deiters’ cells along parts of the cochlea (Brooker et al 2006, Kiernan et al 2006). *FGF20* mutant mice also lack outer hair cells and Deiters’ cells, a phenotype that seems to be caused by a failure of the prosensory domain to differentiate correctly (Huh et al 2012). These data suggest that regions containing inner versus outer hair cells may differentiate as distinct developmental units.

A.2: Atoh1 – the key initiator of hair cell development

The first transcription factor to be expressed in differentiating hair cell progenitors is the basic helix-loop-helix transcription factor Atoh1. *Atoh1* is expressed prior to all other known hair cell genes, and is later down-regulated in maturing hair cells before they begin to participate in hearing and balance (Mulvaney & Dabdoub 2012). *Atoh1* mutant mice lack hair cells in all sensory regions of the ear (Ben-Arie et al 1996), with this deficiency being due to hair cell progenitors failing to differentiate and then dying (Chen et al 2002, Pan et al 2011). This suggests Atoh1 is required to initiate the hair cell differentiation program, but is not required for the function of mature hair cells. Atoh1 is also sufficient to generate hair cells when ectopically expressed in some locations in the developing inner ear (Izumikawa et al 2005, Kawamoto et al 2003, Kelly et al 2012, Liu et al 2012b, Zheng & Gao 2000).

Despite the central role played by Atoh1 in the first steps of hair cell differentiation, surprisingly little is known about the factors that regulate its expression in hair cell progenitors. Manipulation of the BMP, FGF, Shh and Wnt signaling pathways can all alter the numbers of hair cells in inner ear sensory organs (Driver et al 2008, Hayashi et al 2008, Huh et al 2012, Kamaid et al 2010, Li et al 2005, Pirvola et al 2002, Pujades et al 2006,

Stevens et al 2003), although it is far less clear to what extent these effects are due to the direct regulation of *Atoh1* transcription, to indirect transcriptional regulation by induction of other factors that can themselves regulate *Atoh1*, or post-translational modification of the Atoh1 protein (Mulvaney & Dabdoub 2012). Far more attention has focused on regions of the *Atoh1* locus that regulate its transcription, particularly on two enhancer elements located 3.4kb downstream of the *Atoh1* coding region (Figure 2; Helms et al 2000). These enhancers are capable of driving reporter gene expression in the nervous system and in hair cells of transgenic mice (Lumpkin et al 2003), and are strongly conserved in mammals (Helms et al 2000). Atoh1 positively autoregulates its own expression by binding to an E-box motif in this enhancer complex (Helms et al 2000). This autoregulation is necessary for the continued expression of *Atoh1* in hair cells, as *Atoh1* mutant mice cannot maintain expression in hair cells of a GFP reporter transgene containing the autoregulatory enhancer (Raft et al 2007). However, it is not known whether other regulatory sequences in this enhancer initiate expression of *Atoh1* before Atoh1 itself binds to its own enhancer, or whether other enhancers located elsewhere in the *Atoh1* locus are responsible for the first burst of *Atoh1* transcription.

The autoregulatory enhancer region of *Atoh1* contains candidate binding sites for a menagerie of transcriptional activators and repressors, some of which have been shown to bind directly to the enhancer by biochemical assays (Figure 2; Akazawa et al 1995, Briggs et al 2008, D'Angelo et al 2010, Ebert et al 2003, Mutoh et al 2006, Shi et al 2010). Some of these transcription factors can also activate or repress *Atoh1* expression *in vivo* or *in vitro*, although it is not always clear whether these factors work by directly binding the *Atoh1* enhancer or are acting indirectly or through other, as-yet unidentified elements in the *Atoh1* locus. Recent studies have demonstrated that Six1 and its transcriptional co-activator Eya1, which are expressed in the prosensory domain of the cochlea, can bind directly to the *Atoh1* autoregulatory enhancer (Ahmed et al 2012). These two factors are sufficient to induce *Atoh1* expression when electroporated into the greater epithelial ridge adjacent to the organ of Corti (Ahmed et al 2012). This activation of *Atoh1* can be potentiated by the transcription factor *Sox2*, which is strongly expressed in all prosensory domains of the inner ear and is necessary for differentiation of each prosensory domain (Kiernan et al 2005). Sox2 has also been reported to be sufficient to activate *Atoh1* and induce ectopic hair formation in the chick otocyst (Neves et al 2012). *Sox2* is rapidly down-regulated in hair cells as they differentiate, and this down-regulation appears to be required for further maturation of hair cells, since sustained expression of *Sox2* in Atoh1-expressing cells blocks the induction of later hair cell markers such as Myosin 7a (Ahmed et al 2012, Puligilla et al 2010).

A.3: The transcriptional control of hair cell differentiation by Atoh1

Despite the generally accepted importance of *Atoh1* in hair cell development, we know very little about the molecular basis by which *Atoh1* regulates hair cell differentiation. It is conceivable that Atoh1 acts as a "delegator" – sitting atop a large hierarchy of regulatory transcription factors, each devoted to controlling a different aspect of hair cell differentiation. At the other extreme, Atoh1 might act as a "micro-manager", by directly regulating many different aspects of hair cell development and maturation. Moreover, it is not clear whether Atoh1 simply regulates generic aspects of cell identity shared by hair cells in all inner ear sensory organs, or whether it can regulate genes that distinguish different classes of hair cells, such as inner hair cells versus outer hair cells in the cochlea, or type I versus type II vestibular hair cells.

Current efforts to identify direct targets of transcription factors typically involve chromatin immunoprecipitation of the transcription factor bound to DNA in the cells of interest, followed by high throughput sequencing of the DNA (ChIP-sequencing). The application of these approaches to finding Atoh1 targets in hair cells have been confounded by the

relatively small number of hair cells in mammalian sensory organs, and by the large number of other cell types in the cochlea. However, ChIP-sequencing has recently been used to identify Atoh1 targets in cerebellar granule cells, which can be obtained in large numbers and which represent about 50% of the total cells in a neonatal mouse cerebellum (Klisch et al 2011). This work supports the idea that Atoh1 is more of a micro-manager than a delegator – it not only regulates genes associated with transcription, but also cell division, chromosomal organization, metabolism, cell migration and cell adhesion (Klisch et al 2011). Clearly many of these genes are unlikely to be expressed in post-mitotic and non-migratory hair cells, and indeed, a recent comparison of the ChIP-sequencing data from the cerebellum with gene expression data sets from cerebellar granule neurons, dorsal spinal interneurons and hair cells have identified only three genes – *Rab15, Selm* and *Atoh1* itself – that are candidates to be direct targets of Atoh1 in all three cell types (Lai et al 2011). However, the ChIP-sequencing results from the cerebellum have also led to the identification of a consensus Atoh1 binding motif (G/A,C/A,CA,G/T,C/A,TG,G/T,C/T) that is longer and more specific than the generic E-box sequence (CANNTG) bound by many bHLH transcription factors (Figure 2; Klisch et al 2011). This *Atoh1* E-box associated motif, or AtEAM, is present in the majority of sequences bound by Atoh1 in the cerebellum, and thus may be of use in predicting candidate Atoh1 targets in hair cells. The significance of the specificity of the Atoh1-binding AtEAM site was recently demonstrated by replacing the *Atoh1* coding sequence with that of the closely related bHLH factor *Neurog1*, which is expressed in precursors of inner ear neurons, but not hair cells. Mice homozygous for this replacement allele have a severely disrupted organ of Corti containing very few hair cells (Jahan et al 2012). The surviving hair cells were extremely immature and lacked significant stereocilia, although this phenotype was not as severe as that seen in *Atoh1* null mice. This suggests that bHLH transcription factors that are closely related to *Atoh1* are unable to correctly activate sufficient Atoh1 target genes to promote correct hair cell differentiation and survival, even when expressed in the correct cells at the correct time.

B: Modulation of hair cell gene expression through microRNAs (miRNAs)

MicroRNAs (miRNAs) are widely appreciated for their role in post-transcriptional repression of gene expression (Hobert 2007, Hobert 2008, Kloosterman & Plasterk 2006, Takacs & Giraldez 2010). miRNAs are single-stranded RNAs, usually 21-23 nucleotides in length, that are generated from long RNA polymerase II transcripts and then processed through a series of double-stranded-specific ribonuclease III enzymes such as Drosha and Dicer (Esquela-Kerscher & Slack 2006, Kosik 2006, Winter et al 2009). Mature miRNAs ultimately bind to target messenger RNA (mRNA) transcripts, which either destabilizes the targets or lowers their translation efficiency (Filipowicz et al 2008, Guo et al 2010, Lewis & Steel 2010, Liu et al 2008). The resulting repression in protein levels rarely exceeds 50%, at least for the small number of miRNAs that have been tested to date (Baek et al 2008, Bartel 2009, Selbach et al 2008). Thus, miRNAs are modulators of protein expression levels, rather than “off” switches. Several comprehensive reviews have recently highlighted the literature related to inner ear miRNAs (Friedman et al 2009, Rudnicki & Avraham 2012, Soukup 2009, Weston & Soukup 2009). In this section, we narrow the focus to the expression and manipulation of miRNAs and their targets in hair cells and hearing. ..

B.1: Manipulating miRNA levels in developing hair cells

To evaluate the role of miRNAs in hair cell development and maintenance, the miRNA processing enzyme, Dicer, has been conditionally deleted in the mouse inner ear. Several different Cre driver lines have been used to eliminate a floxed Dicer allele at different developmental time points. Foxg1-Cre and Pax2-Cre lines excise Dicer at the otocyst stages (Soukup et al 2009), Atoh1-Cre recombines the locus about the time the hair cells are born (Weston et al 2011), and Pou4f3-Cre shortly thereafter (Friedman et al 2009). These

conditional mutants each show defects in hair cell development, with the severity dependent upon the stage of Dicer excision, and probably also on the amount and duration of residual miRNA expression that persists after Cre onset (see Discussion by Soukup, 2009). In general, however, deletion of Dicer in hair cells leads to disrupted hair bundle morphology and subsequent hair cell death.

A major caveat of this approach is that phenotypes resulting from Dicer knockouts may not necessarily reflect a “worst-case scenario” that reveals the full role played by miRNAs. This is because some miRNAs, as well the transcripts that they repress, may act in direct opposition to others that are co-expressed at the same time and place. As a result, the loss of a single species of miRNA (or miRNA family) may throw the system further out of equilibrium than the loss of two opposing miRNAs, or the blockade of all miRNA processing. Such a scenario is seen in the wing imaginal disc of the fly larvae, where the absence of miR-9 alone is more severe than deletion of Dicer (Bejarano et al 2010).

With this in mind, let us consider the necessity of miRNA processing for proper hair cell development and maturation. The miR-183 family includes 3 members (miR-96, miR-182 and miR-183) that are expressed at high levels in young hair cells, are undetectable in supporting cells, and are also present in the inner ear ganglion neurons (Li et al 2010b, Weston et al 2006). There is a discrepancy in the literature about whether these miRNAs remain confined to hair cells in the postnatal organ of Corti (Sacheli et al 2009, Weston et al 2011). Applying whole mount *in situ* hybridization methods to the cochlear epithelium, Weston and colleagues reported that all 3 miR-183 family members behave similarly and remain restricted to hair cells. Expression is stronger in the base than the apex at P0, but then disappears from the base to establish a gradient from apex (high) to base (low) that persists into adulthood (Figure 1; Sacheli et al 2009, Weston et al 2011).

It is thus tempting to speculate that the molecular basis of anatomical variation and frequency selectivity that is systematically arrayed along the length of the cochlea (shown schematically in Figure 1) might be modulated, and perhaps even enforced, by systematic differences in the levels of the miR-183 family and its targets. If so, then loss of this gene family should lead to weaker longitudinal gene expression gradients. A counterintuitive result is obtained when comparing the expression patterns of the miR-183 family with the phenotype observed when Dicer is deleted selectively in hair cells using the Atoh1-Cre driver line. Here, Dicer deletion using Atoh1-Cre *enhances* transcript gradients (Sacheli et al 2009, Weston et al 2011). This leads to the conclusion that, *in toto*, hair-cell-expressed miRNAs serve *to repress* longitudinal transcript gradients, rather than to *promote* them. Resolution of this paradox must await experiments that selectively delete only the miR-183 family to discover whether or not they share this *en masse* effect of miRNA loss. So far, studies in zebrafish have revealed that the number of hair cells in developing macular sensory organs is affected by levels of miR-183 family members: over-expression of miR-96 or -182 initially yields more hair cells, whereas knockdown of miR-96, -182 and/or -183 generates fewer hair cells (Li et al 2010b). While these studies emphasize the impact of the miR-183 family on hair cell fate specification, they cannot address the question of longitudinal maturation gradients, since zebrafish do not have a cochlea.

B.2: miR-96 mutations underlie inherited hearing loss in mice and humans

Progressive, non-syndromic autosomal semi-dominant or dominant hearing loss has been associated with miR-96 mutations in *Diminuendo* mice and in DFNA50 human families, respectively (Lewis et al 2009, Mencia et al 2009). *Diminuendo* hair cells are born and initiate differentiation, but become stalled at an early postnatal stage and fail to differentiate fully into inner and outer hair cells (Kuhn et al 2011). It is uncertain to what degree this phenotype reflects the loss-of-function of the wild type allele versus a gain-of-function

effect of the novel allele in repressing a new set of target genes. One fact arguing in favor of the former mechanism is that two human families have unique point mutations in the seed region of miR-96 (and thus presumably could interact with unique sets of potential new targets; Mencia et al 2009), while a third family has a mutation in the precursor miRNA transcript that results in poor expression levels of the mature miR-96 (Solda et al 2012). Despite these allele differences among the human DFNA50 families and the *Diminuendo* mice, progressive hearing loss is a common outcome. To address the underlying cause of the hair cell phenotype in *Diminuendo* homozygous mice, cochlear tissue was subjected to gene expression profiling in an effort to identify target transcripts (Lewis et al 2009). As might be expected, genes with 3′UTRs carrying heptamer sequences complementary to the seed region of miR-96 were enriched in the pool of transcripts that were up-regulated in the organ of Corti of *Diminuendo* homozygotes. Likewise, the binding site for the *Diminuendo* allele of miR-96 was enriched in the pool of down-regulated transcripts. However, binding sites from this latter group of mouse genes were not conserved in human and rat orthologs. Ultimately, distinguishing gain-of-function from loss-of-function mechanisms may be revealed by genetic rescue of *Diminuendo* hair cells.

B.3: Evaluation of putative targets for hair-cell-enriched miRNAs

Although the miR-183 family holds particular interest for understanding hair cell biology, the members of this family are also expressed in developing retina and olfactory epithelium (Wienholds et al 2005, Xu et al 2007). Furthermore, they are mis-regulated in certain tumor tissues and cancer cell lines (Guttilla & White 2009, Li et al 2010c, Lin et al 2010, Liu et al 2012c, Lowery et al 2010, Mihelich et al 2011, Sarver et al 2010, Segura et al 2009, Wang et al 2012a, Yan et al 2012, Yu et al 2010). These discoveries have sparked efforts to identify target transcripts and explore their potential functions in other systems, thereby generating lists of genes that could eventually be evaluated as targets in hair cells. Bioinformatic analysis is useful as a preliminary target screening tool. Target-search algorithms, such as PicTar, TargetScan and Microcosm, favor transcripts containing sequences in their 3′ untranslated regions (3′UTRs) that are complementary to the seed regions of miRNAs, particularly nucleotides 2 through 7 or 8 (Lewis et al 2005, Lewis et al 2003). Other criteria can include the number of putative miRNA binding sites as well as their evolutionary conservation. Each of the common target-prediction programs can generate lists of genes numbering in the hundreds for a single miRNA. Ultimately, though, the translational repression of potential targets requires validation.

In vitro assays can be employed to evaluate protein levels directly (such as with immunostains or Western blots) or to monitor translational activity indirectly using luciferase reporters. By way of offering an example of the latter method, Figure 3 shows results from luciferase reporter assays performed in human embryonic kidney cells (HEK293T cells) for 20 human 3′ UTRs that varied widely in their score values as predicted targets for miR-182. Normalized luciferase activity was reduced by at least 30% in the presence of ds-miR-182 mimic for 14 tested targets. Interestingly, the strength of the scores generated from both TargetScan and PicTar algorithms showed moderately good correlations with the magnitude of the knockdowns in the luciferase assays. An exception was seen for the *myosin1C* (*myo1C*) 3′UTR reporter, which showed relatively greater repression by miR-182 in comparison with the other potential targets (Figure 3C, D).

Combining these results with other assays reported in the literature, we compiled a list of validated targets of miR-96, miR-182 and miR-183 (Table 1). A number of these transcripts are expressed in hair cells, as determined by deep sequencing of purified hair cells (SHIELD; Shared Harvard Inner-Ear Laboratory Database; https://shield.hms.harvard.edu/gene_search.html). For these genes, the miR-183 family likely acts to modulate target protein levels, possibly to initiate or maintain a mature hair cell phenotype. Others have

speculated that while miR-183 may promote hair cell differentiation, miR-96 and miR-182 may instead promote a proliferative state (Hei et al 2011). This idea is based on their association with oncogenesis in other cells types, as well as temporal changes in their expression levels as sphere-forming cochlear progenitors are grown under differentiation conditions for two weeks: miR-183 peaks at 6 days as the other two fall precipitously. However, the percentage of cells differentiating into hair-cell-like cells was not evaluated in these cultures, and is likely to be below 0.3% (Diensthuber et al 2009). A resolution of the potentially conflicting activities of miR-183 family members will require a more comprehensive accounting and verification of their direct targets, as well as *in vivo* validations such as miR-specific knockouts.

C: The genetic mechanisms and constraints of hair cell regeneration

All non-mammalian vertebrates examined to date have the capacity to regenerate their hair cells. This can occur either as part of normal turnover (for example, in the constant replacement of lateral line hair cells in bony fish or in the vestibular organs of birds; (Ma & Raible 2009, Roberson et al 1992), or following loss of hair cells after injury in fish, amphibians, lizards and birds (Avallone et al 2008, Corwin & Cotanche 1988, Ma & Raible 2009, Ryals & Rubel 1988, Straube & Tanaka 2006). Supporting cells are capable of directly trans-differentiating into hair cells following injury – this has been best characterized in birds and begins in the first 24 hours after hair cell loss (Cafaro et al 2007). The potential depletion of supporting cells by this regenerative strategy is offset by a robust proliferation of supporting cells that begins several days after damage, and it is likely that at least some of these cell divisions are asymmetric, generating both hair cells and supporting cells (Stone & Cotanche 2007). Mammals, in contrast, have a very limited capacity for hair cell regeneration (Groves 2010). Although a very small amount of proliferation can be seen in the mature vestibular system of mammals following damage, together with a small number of morphologically immature hair cells (Forge et al 1993, Forge et al 1998, Kawamoto et al 2009), the degree of vestibular hair cell regeneration seen in mammals is a fraction of that seen in other vertebrates. Moreover, there is almost no evidence of proliferation or hair cell recovery following damage to the mature organ of Corti (Roberson & Rubel 1994, Yamasoba & Kondo 2006). Nevertheless, the widespread occurrence of hair cell loss in aging humans has prompted many comparisons between mammals and non-mammalian vertebrates in the hopes of identifying genetic or biochemical pathways that underlie regeneration but which may lie dormant in mammals.

C.1: To what extent is hair cell regeneration a recapitulation of hair cell development?

As discussed in section A, *Atoh1* is both necessary and in some circumstances sufficient for hair cell differentiation. Its role appears to be confined to establishing rather than maintaining hair cell fate, as it is rapidly down-regulated in hair cells as they mature. A small number of *Atoh1*-expressing cells can be observed in sensory organs of non-mammalian vertebrates that exhibit normal hair cell turnover, such as fish lateral line organs (Ma et al 2008) or bird vestibular organs (Cafaro et al 2007), This suggests that *Atoh1* is re-activated in supporting cells during the process of regeneration, an idea borne out by the observation that *Atoh1* is rapidly and broadly up-regulated in fish and bird supporting cells following damage (Cafaro et al 2007, Lewis et al 2012, Ma & Raible 2009, Ma et al 2008). Recent experiments using adenovirally-transduced reporters of *Atoh1* enhancer activity in cultures of the chicken basilar papilla treated with ototoxic antibiotics suggest that only about 50% of supporting cells that re-activate the *Atoh1* enhancer go on to differentiate into hair cells as defined by the expression of Myosin6 (Lewis et al 2012). It is possible that some cells that begin to express *Atoh1* are eventually diverted back to a supporting cell fate by processes such as Notch-mediated lateral inhibition. Supporting this, the Notch ligand *Delta1* is one of the first genes to be re-activated during hair cell regeneration in birds and

fish (Ma et al 2008, Stone & Rubel 1999). Moreover, pharmacological inhibition of Notch signaling in drug-damaged chick basilar papilla cultures increased the proportion of hair cells that derived from cells activating the *Atoh1* enhancer from about 50% to 75% (Lewis et al 2012).

Although only very limited hair cell regeneration is seen in the vestibular system of mammals, recent work suggests that the potential of mammalian sensory tissue to respond to damage may be greater than previously realized. Transduction of *Atoh1* reporter constructs into cultures of the drug-damaged adult mouse utricle showed that an average of almost 200 cells/utricle activated the *Atoh1* enhancer after 18 days of culture (Lin et al 2011). However, very few (less than 5%) of these cells expressed Atoh1 protein or hair cell markers such as Myosin7a. Similar results have also been observed an *in vivo* ototoxic lesion to the vestibular system (Kawamoto et al 2009). As in birds, the efficiency of hair cell generation in mouse utricular cultures could be significantly increased – by almost 30-fold - by blocking Notch signaling (Lin et al 2011), although the numbers of hair cells generated by the damaged mouse utricle is still far less than those seen in birds even after Notch inhibition.

These results suggest that hair cell regeneration has at least some superficial similarities with hair cell development, in that *Atoh1* is activated rapidly and in a manner that is at least partly subject to regulation by the Notch pathway (Puligilla & Kelley 2009). Moreover, mammalian vestibular tissue is able to initiate at least some of these regenerative steps, albeit at a much lower efficiency than in other vertebrates. One significant difference is that regenerating hair cells are derived from supporting cells rather than prosensory progenitors. At present, the lack of unambiguous markers to distinguish these two cell populations confounds the question of whether supporting cells undergo partial de-differentiation into a prosensory cell state during the course of regeneration, or are able to directly activate *Atoh1* and become hair cells from their mature state.

C.2: Age-dependent obstacles to hair cell regeneration in mammals

A number of studies suggest that young mammals possess at least some of the molecular components to respond to damage by replacing hair cells or triggering proliferation of supporting cells, but that this potential is rapidly lost with age. For example, cochlear hair cells can be replaced in the embryonic organ of Corti after laser ablation, but not in neonatal mice (Kelley et al 1995). Genetic ablation of neonatal mouse utricular hair cells with diphtheria toxin leads to significant mitotic replacement of hair cells, most likely by supporting cells, but this phenomenon is no longer observed in five day old mice (Burns et al 2012a). Supporting cells purified from the neonatal mouse cochlea are capable of re-entering the cell cycle and generating $Atoh1^{+}$ hair cells in culture, but this capacity is lost by two weeks after birth (White et al 2006). Similarly, the ability of cochlear or vestibular sensory tissue to generate proliferative non-adherent sphere cultures also declines with age (Oshima et al 2007).

What are the age-dependent constraints on supporting cell proliferation and hair cell replacement in mammals? Recent work from Corwin and colleagues has established a very strong correlation between the decline in the ability of supporting cells to spread and proliferate in normal or damaged utricular explants with the establishment of thick bands of F-actin and E-cadherin in the apical junctions between supporting cells (Burns et al 2008). In contrast, the cortical actin bands in chicken utricular supporting cells remain thin throughout the life of the animal. In the absence of perturbation studies, it is not clear at present whether the establishment of thick F-actin/E-cadherin-containing bands in mammals directly regulates the capacity of supporting cells to divide, or whether it is simply an indicator of more global changes occurring in supporting cells with age that might also

include their ability to generate hair cells. In this regard, it is interesting to note that supporting cells in the striolar region of the utricle – which has the capacity to activate Atoh1 expression in the adult mouse after damage and inhibition of Notch signaling – contain significantly less E-cadherin and have thinner actin bands than their extra-striolar counterparts (Collado et al 2011).

In addition to changes in cell structure, it is also likely that the mature sensory epithelium of mammals may exhibit changes in responsiveness to cell-cell signaling. In particular, several recent studies suggest the Notch signaling pathway may no longer be instrumental in maintaining the balance between hair cells and supporting cells in older animals. For example, blocking Notch signaling in the undamaged chicken basilar papilla or the undamaged zebrafish larval lateral line has no effect on supporting cell proliferation or hair cell generation (Daudet et al 2009, Ma et al 2008), suggesting that either Notch signaling is not necessary to maintain the mature array of hair cells and supporting cells, or that other factors are able to compensate for the loss of Notch activity. Moreover, although pharmacological or genetic inhibition of Notch signaling can rapidly up-regulate *Atoh1* and promote the expression of hair cell markers such as Myosin6 and Myosin7a in perinatal mouse cochlear or utricular cultures, these effects decline rapidly with age, both *in vitro* and *in vivo* (Doetzlhofer et al 2009, Hori et al 2007, Lin et al 2011).

As described above, some of the age-dependent changes in the ability of mammalian sensory epithelium to regenerate may be due to a failure to re-activate expression of Atoh1 after it is down-regulated in early postnatal life. In an attempt to bypass these age-dependent limitations, a number of studies have used adenoviral or transgenic expression of *Atoh1* to generate new hair cells (e.g. Izumikawa et al., 2005). Two recent studies used transgenic techniques to activate *Atoh1* expression either throughout the inner ear (Kelly et al 2012) or specifically in sub-populations of supporting cells (Liu et al 2012b). In either case, expression of *Atoh1* in supporting cells or in non-sensory cochlear epithelium was able to induce new hair cells, some of which assembled stereociliary bundles and displayed voltage-dependent currents (Kelly et al 2012, Liu et al 2012b). However, in both studies, the ability of *Atoh1* to promote ectopic hair cell formation in all regions of the cochlea declined rapidly and was severely compromised by two weeks of age, the time at which hearing begins in mice (Kelly et al 2012, Liu et al 2012b). This failure was also seen when *Atoh1* was activated in supporting cells of adult mice in which hair cells had been ablated with ototoxic drugs (Liu et al 2012b).

There are many possible reasons why the mature mammalian ear becomes refractory to over-expression of Atoh1. In a recent review of Atoh1 function, Mulvaney and Dabdoub (2012) discuss a number of ways in which Atoh1 activity and binding to its E-box DNA targets might be regulated at the post-translational level, and each of these might contribute to the failure of Atoh1 to activate hair cell-specific gene expression in the adult mammal. For example, the C-terminus of Atoh1 has a conserved serine-rich domain that contains potential phosphorylation sites that could regulate its activity (Mulvaney & Dabdoub 2012). Basic helix-loop-helix proteins like Atoh1 require bHLH binding partners such as E47 to correctly bind to their E-box targets. Competition for the partners of Atoh1 by inhibitory Id HLH family members, or by other bHLH proteins, might reduce or abolish the transcriptional activity of Atoh1 in adult tissue. Atoh1 activity can also be modulated in different cell types by forming complexes with different bHLH factors that promote binding to different sets of E-boxes. The expression of such factors in adult supporting cells might direct Atoh1 away from the promoters of hair cell genes and hence block its hair cell promoting activity. Finally, it is possible that other classes of transcription factors co-operate with Atoh1 in young sensory tissue but are no longer expressed in older tissue, thus depriving Atoh1 of transcriptional partners.

A final possibility for why Atoh1 is unable to promote hair cell formation in the mature mammalian inner ear is that direct transcriptional targets of Atoh1 become epigenetically modified in supporting cells with age and are rendered unavailable for transcription, even in the presence of exogenous Atoh1. In such a model, some form of epigenetic reprogramming would be required to render mammalian supporting cells competent to respond to Atoh1 again. Whether this could be achieved by altering the activity of histone-modifying complexes or would require defined reprogramming factors (for example, similar to those required to reprogram fibroblasts into neurons; (Lujan et al 2012, Vierbuchen et al 2010, Yang et al 2011) is not clear. A better understanding of the epigenetic regulation of Atoh1 targets in the inner ear is hampered by a lack of good candidates for genes directly regulated by Atoh1. As discussed above, two recent studies used ChIP-seq, RNA-seq and mining of microarray databases to identify and validate a set of potential direct targets of Atoh1 in cerebellar granule cells and dorsal spinal cord interneurons (Klisch et al 2011, Lai et al 2011). However, of these, only three – *Rab15, Selm* and *Atoh1* itself – have been shown to be expressed in hair cells.

ACRONYMS

ChIP-Seq	Chromatin immunoprecipitation and deep sequencing
GFP	Green Fluorescent Protein
miRNA	MicroRNA

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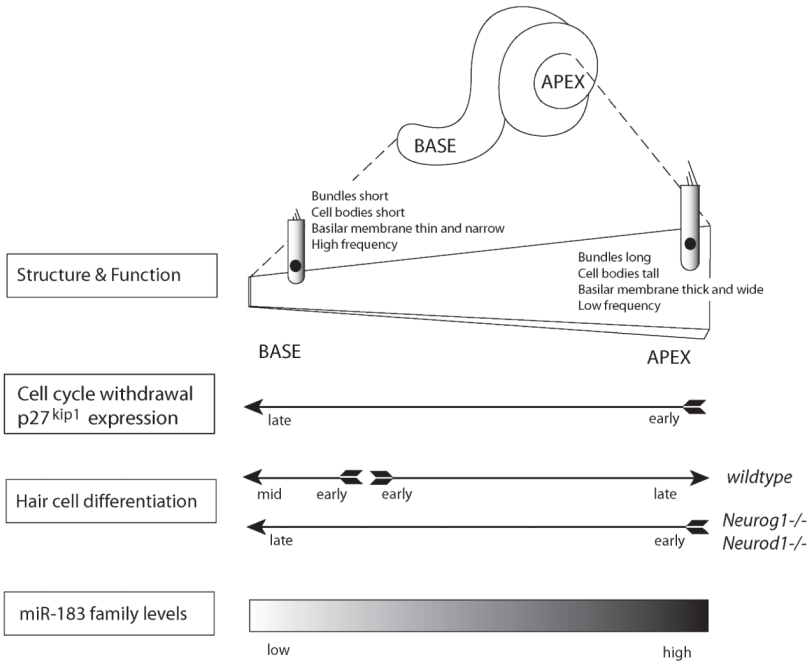

Figure 1.

Longitudinal gradients of the mammalian organ of Corti in normal and mutant mice. The cochlea coils from base to apex and exhibits systematic gradients in the dimensions of its fluid-filled chambers, as well as the width and thickness of the basilar membrane (shown uncoiled). Lying on the basilar membrane is the delicate organ of Corti, with hair cells and supporting cells (not shown) also changing systematically in their physical dimensions (shown schematically with one outer hair cell at the extremes). Arrows depict temporal gradients in cell cycle exit, $p27^{kip1}$ expression and hair cell differentiation in normal and mutant mice. In the mature organ of Corti, the miR-183 family is expressed in a gradient from base (lowest levels) to apex (highest levels).

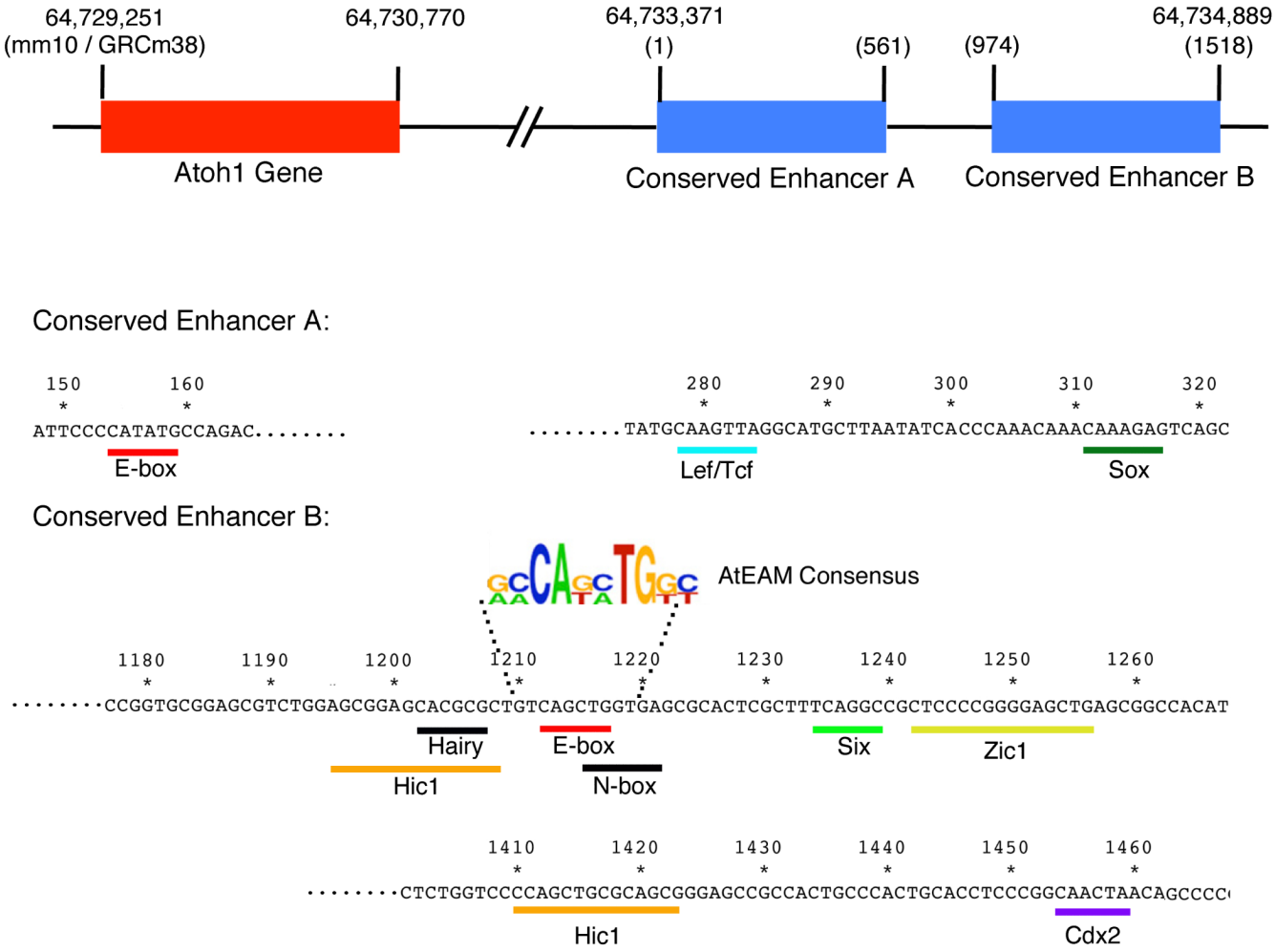

Figure 2.

A diagram of the mouse *Atoh1* locus, showing the position of its 3′ autoregulatory enhancer. Numbers refer to the position on mouse chromosome 6, according to the current build of the mouse genome (mm10/GRCm38). The *Atoh1* autoregulatory enhancer consists of two conserved elements, A and B. Transcription factor binding sites that have been experimentally verified are shown in color on relevant regions of the enhancer sequence. Two candidate binding sites, a hairy-preferred motif and an N-box are shown in black; although these have not been experimentally verified, they have been featured in many previous reviews of this regulatory region. The AtEAM consensus sequence for Atoh1 binding identified by Klisch et al. (2011) is shown aligned to the likely E-box site in Enhancer B.

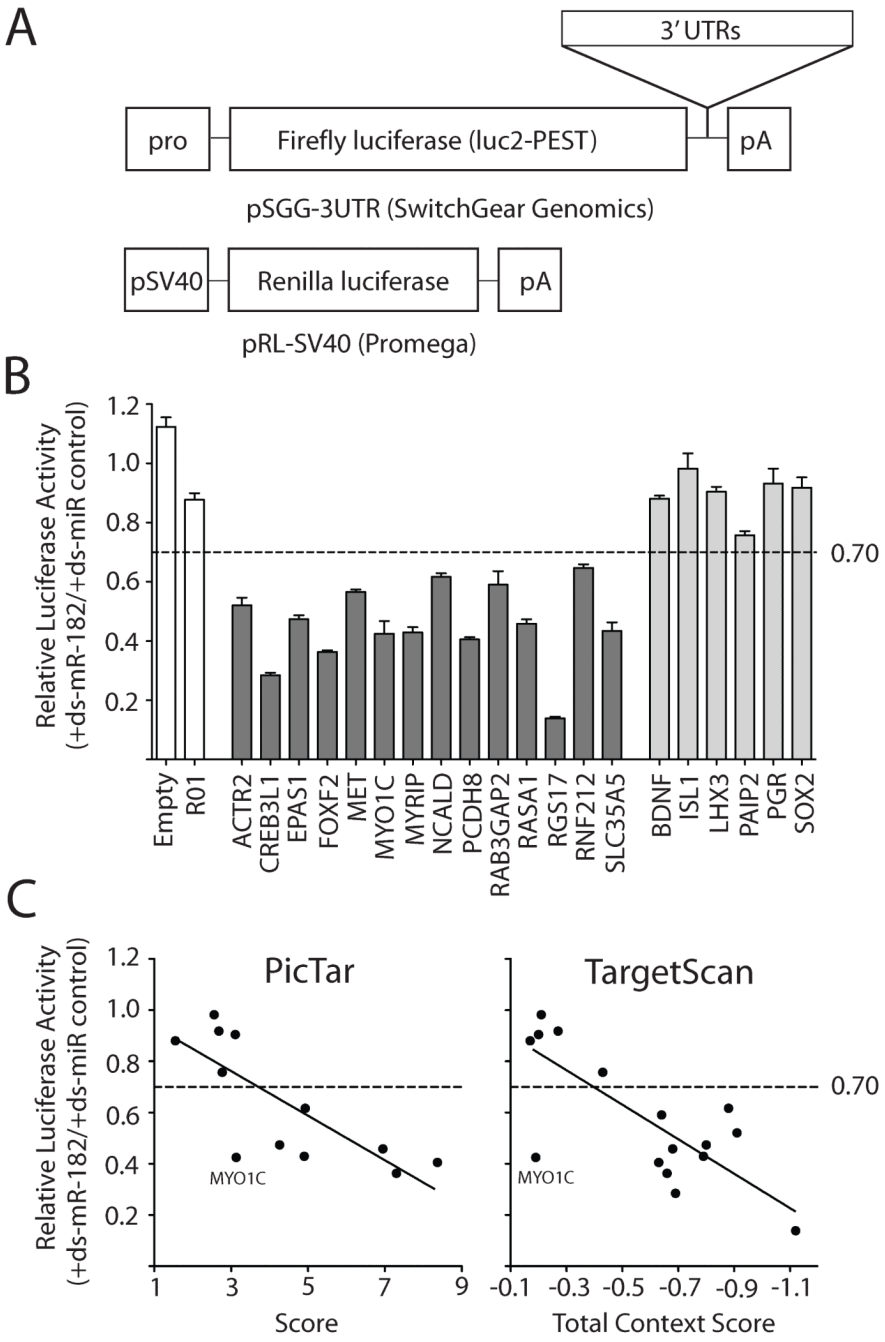

Figure 3.

Luciferase assays for validating potential targets of miR-182. (A) Plasmids for *in vitro* luciferase assays. Human 3′UTRs were placed downstream of the coding region for destabilized Firefly luciferase (purchased from SwitchGear Genomics) and these test plasmids were then co-transfected with Renilla luciferase plasmids into HEK293T cells, along with double-stranded (ds) miRNA mimics (Thermo Scientific Dharmacon). (B) Relative luciferase activity. At 24 hours post-transfection, luminescence originating from Renilla protein was used to normalize the transfection efficiency in each well of a 96-well plate using the Dual-Glo Luciferase Assay System (Promega). Then, the luminescence from Firefly luciferase in the presence of miRIDIAN double-stranded (ds) miRNA-182 mimic

was compared that obtained in the presence of a miRIDIAN ds-miRNA mimic negative control #1 (5′-UCACAACCUCCUAGAAAGAGUAGA-3′) provided by the manufacturer. Values are shown as means plus standard errors from at least six replicates performed over at least two independent experiments. White bars are negative controls. Dark bars are constructs with luminescence ratios below 0.70 (dashed line); the knockdowns by miR-182 are statistically significant in comparison to a plasmid carrying the R01 3′UTR that has no predicted binding sites for miR-182 (t-test, $p<0.05$). Grey bars are constructs that were not significantly different from the R01. (C) Relative luciferase activity shown as a function of target prediction scores for PicTar and TargetScan. Note that each program outputs a score for only a subset of the 20 tested 3′UTRs. Linear regression analysis of the data gives modest correlation coefficients for TargetScan ($R^2 = 0.5961$) and PicTar ($R^2 = 0.5851$)

TABLE 1
Validated targets of hair-cell-enriched miRNAs based on bioactivity assays

miR-96 Target	Reference
ACVR2B (h)	Solda et al., 2012
ADCY6 (h)	Xu et al., 2007; (%) Jalvy-Delvaille et al., 2012
AQP5 (m, h)	Lewis et al., 2009; Mencia et al., 2009
ARRDC3(m)	Zhu et al., 2011
AVIL (m) #	Lewis et al., 2009
CACNB4 (h)	Solda et al., 2012
CASP2 (m)	Zhu et al., 2011
CELSR2 (m, h)	Lewis et al., 2009; Mencia et al., 2009
COL2A1 (h) *	Mencia et al., 2009
FMNL2 (h) **	Mencia et al., 2009
FN1 (h) %	Jalvy-Delvaille et al., 2012
FOXO1 (h)	Guttilla et al, 2009; (%) Jalvy-Delvaille et al., 2012
FOXO3 (h)	Lin et al., 2010
GPC3 (h) %	Jalvy-Delvaille et al., 2012
HTR1B (h)	Jensen et al., 2009; Jensen et al., 2010
KRAS (h)	Yu et al., 2010
LMX1A (h) **	Mencia et al., 2009
MITF (h)	Xu et al., 2007
MYLK (h) **	Mencia et al., 2009
MYO1B (h) *	Mencia et al., 2009
MYRIP (m, h)	Lewis et al., 2009; Mencia et al., 2009; Solda et al., 2012
NEUROD4 (m)	Zhu et al., 2011
NR3C1 (m)	Riester et al., 2012
ODF2 (m, h)	Lewis et al., 2009; Mencia et al., 2009
PGR (h, rhesus, not m)	Liu et al., 2012 (a)
RAD51 (h) $	Wang et al., 2012 (c)
REV1 (h)	Wang et al., 2012 (c)
RYK (m, h)	Lewis et al., 2009; Mencia et al., 2009
SEMA6D (h) *,#	Mencia et al., 2009
SLC19A2 (h) **	Mencia et al., 2009
SLC39A1 (h) %	Mihelich et al., 2011
SLC39A3 (h) %	Mihelich et al., 2011
SLC39A7 (h) %	Mihelich et al., 2011
SPAST (h) %	Henson et al., 2012
ZIC1 (h) *	Mencia et al., 2009

miR-182 Target	Reference
ACTR2 (h)	Fig. 3
ADCY6 (h)	Xu et al., 2007; (%) Jalvy-Delvaille et al., 2012
ARRDC3 (m)	Zhu et al., 2011
BCL2 (h)	Yan et al., 2012
BRCA1 (h)	Moskwa et al., 2011
CASP2 (m)	Zhu et al., 2011
CCND2 (h)	Yan et al., 2012
CLOCK (h)	Saus et al., 2010
CREB3L1 (h)	Fig. 3
EPAS1 (h)	Fig. 3
FOXF2 (h)	Fig. 3
FOXO1 (h)	Guttilla et al., 2009; (%) not a target in Jalvy-Delvaille et al., 2012
FOXO3 (h)	Segura et al., 2009
MET (h)	Fig. 3
MITF (h)	Xu et al., 2007; Segura et al., 2009; Yan et al., 2012
MTSS1 (h)	Wang et al., 2012 (a)
MYO1C (h)	Fig. 3
MYRIP (h)	Fig. 3
NCALD (h)	Fig. 3
PCDH8 (h)	Fig. 3
RAB3GAP2 (h)	Fig. 3
RASA1 (h)	Fig. 3
RGS17 (h)	Sun et al., 2010; Fig. 3
RNF212 (h)	Fig. 3
SLC30A1 (h) %	Mihelich et al., 2011
SLC30A7 (h) %	Mihelich et al., 2011
SLC35A5 (h)	Fig. 3
SLC39A1 (h) %	Mihelich et al., 2011
SLC39A7 (h) %	Mihelich et al., 2011
SOX2 (h) #	Weston et al., 2011
SPAST (h) %	Henson et al., 2012
TBX1 (m)	Wang et al., 2012 (b)

miR-183 Target	Reference
EGR1 (h)	Sarver et al., 2010
ITGB1 (h)	Li et al., 2010 (a)
KIF2A (h)	Li et al., 2010 (a)
PDCD4 (h)	Li et al., 2010 (c)

BOLDgenes expressed in cochlear hair cells (GFP+) at greater than 50 reads at P0, P4 and/or P7 in Pou4f3-GFP mice; SHIELD database

italicsgenes repressed at least 30% in P0 cochlear hair cells (GFP+) vs. other cochlear cells (GFP−) in Pou4f3-GFP mice; SHIELD database

m, h mouse, human

* tested as a potential target for hs-mR-96(13G>A), but also repressed by wildtype hs-miR-96

** tested as a potential target for hs-mR-96(14C>A), but also repressed by wildtype hs-miR-96

$ miR-96 binding site is present in the coding region, not in the 3′UTR

% not validated by luciferase assay

these may not be valid targets because they were repressed less than 30% in the presence of the miRNA