RNA-Binding Proteins and Inner Ear Hair Cell

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RNA-binding proteins (RBPs) regulate gene expression at the post-transcriptional level. They play major roles in the tissue- and stage-specific expression of protein isoforms as well as in the maintenance of protein homeostasis. The inner ear is a bi-functional organ, with the cochlea and the vestibular system required for hearing and for maintaining balance, respectively. It is relatively well documented that transcription factors and signaling pathways are critically involved in the formation of inner ear structures and in the development of hair cells. Accumulating evidence highlights emerging functions of RBPs in the post-transcriptional regulation of inner ear development and hair cell function.

Keywords: inner ear ; cochlear hair cells ; RNA-binding proteins ; post-transcriptional regulation

1. LIN28 in Cochlear Hair Cell Development and Regeneration

LIN28 (LIN28A and LIN28B) proteins are highly conserved small cytoplasmic RNA-binding proteins (RBPs) that function as pluripotency factors, regulating the transition from self-renewal to a differentiated cell fate [1]. Consistent with this activity, functional analyses in mice suggest that Lin28B plays an important role in hair cell development and regeneration. In the cochlea of mouse embryos, it is highly expressed in prosensory cells and down-regulated at the onset of hair cell differentiation. Prolonged expression of Lin28B delays prosensory cell cycle exit and prevents hair cell differentiation, suggesting that it functions to increase hair cell production [2]. Interestingly, Lin28B inhibits the processing of mature *let7* miRNA, which functions to induce cell cycle exit in progenitor cells [2]. Therefore, the antagonistic actions of Lin28B and *let7* miRNA coordinate the timing of prosensory cell cycle withdrawal for hair cell differentiation. In neonatal murine cochlear organoids and explants, Lin28B antagonizes the activity of *let7* miRNA and increases Akt-mTORC1 signaling to promote hair cell regeneration from immature supporting cells by inducing their de-differentiation and proliferation as well as by directly converting them into hair cells [3]. Thus, Lin28B functions in hair cell regeneration through mitotic and non-mitotic mechanisms, which are dependent on mitotic division or trans-differentiation of supporting cells into hair cells, respectively. The precise mechanism by which Lin28B and *let7* miRNA regulate mTORC1 activity in cochlear epithelial cells awaits further investigation. It is possible that Lin28B directly promotes mRNA translation of mTOR pathway genes or relieves *let7*-mediated repression of their translation [4]. In addition, Lin28B functions to enhance the regenerative competence of maturing supporting cells in the cochlea through cooperation with Follistatin, which inhibits Lin28B-induced TGF-ß signaling that can trigger proliferative quiescence [5]. This suggests that coactivation of Lin28B and Follistatin may represent an endogenous mechanism mediating reprogramming of supporting cells for hair cell regeneration.

Lin28A is required for hair cell regeneration in the mammalian cochlea, and may function in redundant processes with Lin28B [3][5]. Studies using a zebrafish model further illustrate an important role of Lin28A in the recovery of progenitor cells. It has been shown that severe injury with loss of both progenitors and hair cells induces robust transient upregulation of Lin28ab (a zebrafish ortholog of human LIN28A) in regenerating neuromasts through activation of Yap, which directly binds to the *lin28ab* promoter to initiate its transcription in hair cell precursors [6]. Furthermore, Lin28ab inhibits the function of *let7* miRNA to activate the Wnt/ß-catenin pathway for progenitor proliferation and hair cell regeneration [6]. Therefore, studies using different vertebrate models have demonstrated a role for Lin28 paralogs in promoting prosensory cell proliferation and in initiating hair cell regeneration. However, it is unclear how they post-transcriptionally regulate target gene expression in prosensory cells and supporting cells during hair cell development and regeneration. Lin28 and *let-7* miRNA are mutually antagonistic, repressing the expression of each other, and they function as a regulatory pair in stem cell plasticity, somatic cell reprogramming, and tissue regeneration [1]. Thus, further investigation is necessary in order to better understand how Lin28 and *let-7* miRNA interact to modulate gene expression for hair cell regeneration.

2. RBM24 Regulates mRNA Stability and Pre-mRNA Splicing in Hair Cells

RNA binding motif protein 24 (RBM24) contains a highly conserved RRM (RNA recognition motif) at the N-terminus that binds to GU-rich sequences in target transcripts [7]. It displays restricted tissue-specific expression patterns in all vertebrate species [8]. In addition to striated muscles, Rbm24 is strongly expressed in head sensory organs, including the otic vesicle, lens, and olfactory vesicle [9][10]. In the inner ear of neonatal mice, Rbm24 expression is detected in a subset of hair cells and is directly regulated by the transcription factor Atoh1 [11]. This suggests that Rbm24 may be a transcriptional target and function downstream of Atoh1 in hair cell differentiation. Immunofluorescence staining shows that Rbm24 protein highly accumulates in the cytoplasm, with weak localization in the nucleus of inner ear hair cells. It co-localizes with Myo7A in mechanosensory cells of the auditory and vestibular systems, suggesting that it may play a role in sensory hair cell differentiation and function [9].

Consistent with a multifaceted post-transcriptional regulator, RBM24 plays an important role in alternative splicing. It has been shown that deletion of Rbm24 in mice affects the inclusion of the inner ear-specific exon 68 in *Cadherin 23* (*Cdh23*) mRNA, leading to hearing loss and defective motor coordination [12][13]. Cdh23 and Cdh15 are important components of the tip links that interconnect the mechanosensory stereocilia and the kinocilium in the hair bundle for mechanotransduction [14]. Mutations of the *CDH23* gene are responsible for human Usher syndrome 1D (OMIM#601067) and non-syndromic autosomal recessive deafness DFNB12 (OMIM#601386) [15][16][17][18]. Because the inner ear-specific exon 68 of *CDH23* gene encodes amino acids involved in interaction with other proteins in hair cells [19], dysfunction of RBM24 may cause a chain of events that impairs hair cell development and function. In mice, it has been shown that RBM24 promotes muscle-specific alternative splicing by preventing the suppression of exon inclusion mediated by splicing repressors PTBP1 (polypyrimidine tract-binding protein 1), also known as hnRNP I (heterogeneous nuclear ribonucleoprotein I), and hnRNP A1/A2 [20]. Rbm24 likely regulates the inclusion of exon 68 in *Cdh23* mRNA through a similar mechanism because PTBP1 seems to repress inclusion of this exon [12]. Further supporting its functional importance in inner ear development, conditional knockout of Rbm24 in mice has been shown to affect the survival of outer hair cells in the cochlea [21]. Therefore, Rbm24 plays critical roles in the post-transcriptional regulation of hair cell morphogenesis and function. However, although Rbm24 protein is predominantly localized to the cytoplasm of differentiated hair cells in neonatal mice, it is unclear how it is distributed in prenatal sensory hair cells or how it regulates post-transcriptional events during the early stages of inner ear development. Indeed, Rbm24 is expressed in the otic vesicle during early development, at least in E10.5 mouse embryos [8]. Dynamic subcellular trafficking and post-transcriptional regulatory functions of Rbm24 have been demonstrated during muscle cell differentiation and regeneration in mice [22]. Thus, it is of interest to examine whether a similar situation exists during the process of hair cell differentiation in the early embryo. It is worth understanding how Rbm24 acts downstream of transcription factors, such as Atoh1, to relay their activity for hair cell development and regeneration.

3. SFSWAP Functions in Growth and Patterning of Inner Ear Sensory Organs

The splicing factor SWAP (SFSWAP) is a mammalian homolog of *Drosophila* suppressor-of-white-apricot that displays RNA-binding activity and regulates alternative splicing of *CD45* and *Fibronectin* pre-mRNAs in COS cells [23]. In mice, Sfswap is widely expressed in the developing inner ear, then becomes more restricted in the cochlea and the spiral ganglion at birth [24]. Loss of Sfswap function leads to defective inner ear patterning, resulting in reduced numbers of outer hair cells and ectopic inner hair cells in the cochlea as well as smaller cristae and maculae in the vestibular system. This suggests that Sfswap plays an important role in the accurate formation of sensory organs and proper patterning of mechanosensory hair cells. Accordingly, homozygous *Sfswap* mutant mice show mild hearing loss, changed vibratory responses of the organ of Corti, and circling behavior [24][25]. Consistent with its involvement in the expression of Notch pathway genes in the inner ear, *Sfswap* genetically interacts with *Jagged1* in cochlear patterning [24]. However, it is unclear how Sfswap regulates inner ear-specific alternative splicing or whether it is involved in other aspects of the RNA metabolism. Thus, identification of its target RNAs should provide insights into the post-transcriptional mechanisms by which it functions in cochlear patterning. In particular, it is worth examining how it is involved in the post-transcriptional regulation of Notch pathway genes.

4. “Noise/Damage-Related” RBPs in Hearing Loss

There is evidence that RBPs are potentially implicated in noise/damage-induced hearing loss. Quaking (QKI or QK) proteins contain a STAR (signal transduction and activation of RNA) domain and bind to ACUAA motifs in the 3′-UTR to regulate mRNA function [26][27]. The *QKI* gene produces three major protein isoforms through alternative splicing, namely, QKI-5, QKI-6, and QKI-7. In the cochlea of postnatal and adult mice, Qki-6 and Qki-7 isoforms are mostly accumulated in

the cytoplasm of glial cells surrounding spiral ganglion neurons and auditory nerve fibers. It has been shown that auditory nerve degeneration and hearing deficiency in mice caused by noise exposure are associated with a decreased expression of Qki proteins and their numerous possible target genes [28]. Conditional knockout of the *Qki* gene to prevent the expression of all Qki isoforms in cochlear glial cells leads to hearing deficiency due to defective myelination of spiral ganglion neurons and auditory nerve fibers, suggesting that dysfunction of Qki-mediated regulatory process may be an important early target of the noise response [28].

CAPRIN1 (cytoplasmic activation/proliferation-associated protein 1) is a ubiquitously expressed RBP originally identified in lymphocytes and hematopoietic progenitor cells [29]. It contains an arginine-glycine-glycine (RGG) box that functions as an RNA-binding motif for G-rich RNA targets. In the cochlea of postnatal rats, Caprin1 is detected in both hair cells and supporting cells, and it may act as a transcriptional target of Pou4f3 to regulate hair cell survival in response to ototoxic damage [30]. Pou4f3 normally represses the expression of the *Caprin1* gene by directly binding to its regulatory sequence. It has been proposed that ototoxin-induced reduction of Pou4f3 expression in cochlear hair cells could lead to upregulation of the Caprin1 protein, which can form stress granules and may sequester mRNAs of pro-apoptotic genes to inhibit their translation, thereby preventing ototoxin-induced death of sensory hair cells [30][31]. Moreover, inner ear-specific deletion of Caprin1 in mice affects synapse formation between inner hair cells and spiral ganglion neurons, leading to early onset progressive hearing loss. Loss of Caprin1 impairs the ability of the cochlea to recover from noise exposure, suggesting that it is required for the maintenance of the auditory function [32]. Therefore, QKI and CAPRIN1 may be potential therapeutic targets for noise- and ototoxin-induced hearing loss.

5. Post-Transcriptional Inactivation of REST Transcriptional Repressor by SRRM4-Regulated Exon Inclusion

SRRM4 (serine/arginine repetitive matrix 4), also known as nSR100 (neural-specific SR-related protein of 100 kDa), belongs to a large family of proteins harboring serine/arginine (SR)-repeats. It displays RNA-binding activity and regulates a network of brain-specific exons in genes with important function for neural cell differentiation [33]. Bronx waltzer (*bv*) mice show degeneration of cochlear inner hair cells, defective synaptogenesis, deafness, and impaired balance [34][35][36]. Positional cloning maps the *bv* mutation to the *Srrm4*/*nSR100* locus, and transcriptomic analysis indicates that loss of Srrm4 specifically disrupts alternative splicing events in the inner ear [37]. Knockdown of Srrm4 in zebrafish leads to hair cell degeneration in the neuromasts [37]. These observations suggest that Srrm4-regulated alternative splicing plays a conserved role in hair cell development and hearing function.

REST (restrictive element-1 silencing transcription factor), also known as NRSF (neuron-restrictive silencing factor), is a transcriptional repressor that silences the expression of a large number of neural genes in non-neural tissues. However, its function is specifically inactivated in neurons and inner ear hair cells, thereby allowing the expression of neural genes in both cell types [38]. In mouse mechanosensory hair cells, regulated inclusion of the frameshift-causing exon 4 into the *Rest* mRNA is essential for its inactivation, while skipping of this exon allows the expression of a functional Rest protein, causing hair cell degeneration and deafness [39]. Srrm3 and Srrm4 are differentially expressed in inner ear hair cells, and regulate the splicing-dependent inactivation of Rest protein [40]. They likely promote exon incorporation in the *Rest* mRNA in neural cells by directly preventing PTBP1-mediated repression of exon inclusion [41]. In the utricle, Srrm3 expression is dependent on Srrm4-mediated inactivation of Rest function, whereas in cochlear outer hair cells it is independent of Srrm4 due to a transient down-regulation of Rest activity in these cells, as the *Srrm3* gene is itself a target of Rest-mediated transcriptional repression [40]. Therefore, Srrm4 is required for inactivating Rest protein in cochlear inner hair cells and vestibular hair cells, while Srrm3 and Srrm4 function redundantly to inhibit Rest activity in cochlear outer hair cells. This may explain the normal morphology of outer hair cells in the *bv* mutant with loss of Srrm4 function [37][40]. Consistently, combined loss of Srrm3 and Srrm4 in mice causes complete loss of hair cells in the inner ear. Importantly, the DFNA27 mutation (OMIM#612431) that changes the splicing acceptor site of exon 4 in the human *REST* gene prevents SRRM4-dependent alternative splicing of this exon and causes progressive hearing loss [39], further illustrating the requirement of SRRM4-mediated post-transcriptional regulation in REST inactivation for hair cell development and function.

6. Mutations of the ESRP1 Gene in Humans Cause Alternative Splicing Defects and Hearing Loss

ESRP1 (epithelial splicing-regulatory protein 1), previously known as RBM35A, belongs to the hnRNP family of RBPs and regulates alternative splicing of *FGFR2* pre-mRNA in epithelial tissues [42]. Importantly, frameshift and missense mutations of the *ESRP1* gene have been identified in individuals with profound bilateral sensorineural hearing loss (SNHL) by whole-exome sequencing [43]. Analysis of ESRP1-dependent alternative splicing events using patient-derived iPSCs (induced pluripotent stem cells) indicates altered exon inclusions for several mRNAs, including *ENAH*, *NF2, RALGPS2*, and

ARHGEF11 [43], suggesting that mutations of *ESRP1* cause hearing loss by disrupting alternative splicing. Loss of Esrp1 in mice causes defective inner ear morphogenesis and prevents cochlear hair cell differentiation, mostly by disrupting epithelial-specific splicing of *Fgfr2* pre-mRNA in cochlear epithelial cells, leading to inappropriate expression of the mesenchymal Fgfr2-IIIc isoform that displays a different ligand-binding specificity [43]. These data highlight the importance of ESRP1-mediated alternative splicing in inner ear development and link *ESRP1* mutations with SNHL (OMIM#618013).

There are two highly homologous *Esrp* genes in vertebrates, *Esrp1* and *Esrp2* [42]. Although no mutations of *ESRP2* have been associated with SNHL, there is evidence that Esrp1 and Esrp2 display both distinct and redundant functions in regulating the epithelial splicing program during tissue and organ morphogenesis in mice [44]. Moreover, at least in zebrafish and chicks, Esrp2 is expressed in the otic placode and along the tonotopic axis [45][46]; thus, there is a possibility that it may have a function in inner ear development.

7. Possible Role of Musashi1 in the Maintenance of Stem Cell Fate for Hair Cell Regeneration

Musashi1 (Msi1) is a neural RBP with two RRMs and is expressed in neural stem/progenitor cells [47]. In mice, expression of the Msi1 protein is present in all otocyst cells between E12 and E14, and is absent in vestibular hair cells at E14 and in cochlear hair cells at E16 [48]. However, Msi1 can be detected in different supporting cells during postnatal and adult life, including pillar and Deiters cells [48][49][50]. Interestingly, the subcellular localization of Msi1 in supporting cells undergoes cytoplasm to nucleus translocation during the first two weeks after birth, implying a dynamic function in inner ear development [48]. In the utricle of chick embryo, Msi1 is localized to the basal side of supporting cells and is upregulated along with downstream genes of the Notch pathway following aminoglycoside-induced ototoxic damage [51]. During the spontaneous regeneration process of vestibular hair cells in guinea pigs, Msi1 becomes redistributed in the cytoplasm of supporting cells, which undergo asymmetric division to produce hair cells [52]. These observations suggest that nuclear Msi1 may play a role in the maintenance of stem cell fate. However, functional analyses are needed to determine the role of Msi1 in hair cell regeneration. Because Msi1 contributes to self-renewal of neural stem cells by increasing Notch signaling activity through translational repression of *Numb* mRNA [47], it is of interest to examine whether this post-transcriptional mechanism functions to maintain stem cell fate in the inner ear

8. Other RBPs Implicated in Inner Ear Development and Hair Cell Regeneration

Zebrafish possess sensory hair cells in both the ear and in lateral line neuromasts. They are structurally and functionally equivalent to hair cells in the mammalian inner ear, and are subjected to similar molecular regulation [53]. In contrast to mammals, zebrafish can robustly regenerate inner ear and neuromast hair cells after mechanical injury or ototoxin-induced damage [54]. Therefore, the zebrafish model has become particularly attractive for understanding molecular mechanisms underlying the development and regeneration of vertebrate sensory organs. Mutagenesis screening and functional analyses in this model have identified several RBPs potentially involved in ear development and hair cell regeneration. One reverse genetic study has demonstrated the requirement of Rbm24a for hair cell morphogenesis in the inner ear and neuromasts [55]. IGFBP3 (insulin-like growth factor binding protein 3) is expressed in the otic vesicle at early stages of development. Using the morpholino-mediated knockdown approach, it has been shown that IGFBP3 is required for inner ear growth, hair cell differentiation, and semicircular canal formation [56]. In the developing mouse cochlea, the expression of IGFBP3 is restricted to the prosensory domain, suggesting that it may have conserved role in inner ear development [57]. However, the post-transcriptional regulatory function of IGFBP3 needs further investigation.

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