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Review Article

Hair cell regeneration from inner ear progenitors in the mammalian cochlea

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Abstract: Cochlear hair cells (HCs) are the mechanoreceptors of the auditory system, and because these cells cannot be spontaneously regenerated in adult mammals, hearing loss due to HC damage is permanent. However, cochleae of neonatal mice harbor some progenitor cells that retain limited ability to give rise to new HCs *in vivo*. Here we review the regulatory factors, signaling pathways, and epigenetic factors that have been reported to play roles in HC regeneration in the neonatal mammalian cochlea.

Keywords: Cochlea, inner ear progenitor, hair cell regeneration, transcription factor, signaling pathway

Introduction

Sensorineural hearing loss, one of the most common health problems around the world, is mainly caused by cochlear hair cell (HC) damage or loss [1]. In non-mammalian vertebrates, such as birds and fish, HCs can be spontaneously regenerated from supporting cells (SCs) after damage [2-4]. However, HCs in the adult mammalian cochlea cannot be spontaneously regenerated, and only neonatal cochlear HCs have a limited capacity for regeneration [5, 6]. Damaged mammalian vestibular organs can also generate new HCs from SCs in limited numbers [7-9]. It has been reported that progenitor cells can be isolated from the auditory and vestibular organs of the inner ear and can form spheres and self-renew *in vitro* [10-13]. HCs are regenerated through two mechanisms. In mitotic regeneration, inner ear progenitors re-enter the cell cycle, divide mitotically, and then differentiate into new HCs. In direct trans-differentiation, inner ear progenitors switch cell fate and directly differentiate into new HCs [14-16]. We will focus in this review on the mechanisms through which transcription factors, regulatory factors and signaling pathways regulate HC regeneration.

Inner ear progenitors in the neonatal cochlea

In recent years, researchers have found that the SCs of the cochlea have certain ability for proliferation and differentiation, and as described above, these cells can first divide and then differentiate into HCs or they can trans-differentiate directly into HCs [10, 17]. White et al. isolated P27+ transgenic neonatal mouse cochlear SCs and tested the ability of the cell cycle re-entry and HC regeneration [10]. The presence of both BrdU+ and BrdU- regenerated HCs indicated that SCs can generate new HCs through both direct differentiation and mitotic pathways [10, 18].

Leucine-rich repeat-containing G-protein coupled receptor 5 (*Lgr5*), a Wnt signaling downstream target gene, has been reported to be a progenitor/stem cell marker in many other tissues [19, 20]. Chai et al. and Shi et al. both reported that cochlear Lgr5+ cells, a subset of SCs including inner pillar cells, inner border cells, third-row Deiters' cells, and the lateral

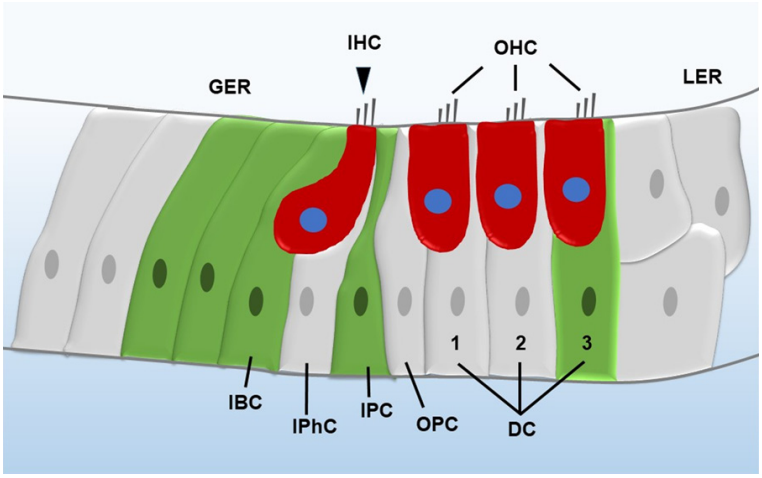

Figure 1. Illustration of the mammalian cochlea. The red cells are HCs, and the green cells are Lgr5+ progenitors. IHC, inner hair cell; OHC, outer hair cell; GER, greater epithelial ridge; LER, lesser epithelial ridge; DC, Deiters' cell; OPC, outer pillar cell; IPC, inner pillar cell; IPhC, inner phalangeal cell; IBC, inner border cell.

greater epithelial ridge (**Figure 1**), are the inner ear progenitors in the neonatal mouse cochlea [21, 22]. These Lgr5+ progenitors have been shown to regenerate HCs in the neonatal cochlea both *in vivo* and *in vitro*, and Wnt signaling induction either by Wnt agonists or in β-catenin overexpression transgenic mice promotes the proliferation of Lgr5+ progenitors and HC regeneration [21, 23].

In another study, Jan et al. used reporter mice for *Axin2* gene, which is a downstream negative feedback gene of the Wnt signaling pathway [24], and showed in both *in vitro* cell culture and *in vivo* animal experiments that Axin2+ tympanic border cells have similar characteristics as cochlear progenitors. These cells can proliferate into cell colonies and can be differentiated into SCs and HCs. Moreover, the ability of these Axin2+ cells to proliferate and differentiate can be induced by Wnt agonists and suppressed by Wnt inhibitors, similar with Lgr5+ progenitors. Therefore, it is suggested that Axin2+ cells might also be a potential source of progenitors for treating hearing disorders.

Recently, two other genes have been reported to be novel inner ear progenitor markers. The first is *Lgr6*, which is also a Wnt-signaling downstream target gene. Lgr6+ cells, which only include inner pillar cells in the neonatal mouse cochlea, are a subpopulation of Lgr5+ progenitors, and Lgr6+ cells can generate Myosin7a+ HCs *in vitro* in a similar manner as Lgr5+ progenitors [25]. The same number of isolated Lgr6+ cells generates significantly more Myosin7a+ HCs compared to Lgr5+ progenitors, while Lgr5+ progenitors form more cell spheres than Lgr6+ cells *in vitro* [26], which suggests that Lgr6+ cells have greater ability for differentiation and lesser ability for proliferation compared to Lgr5+ progenitors. Another reported inner ear progenitor marker is *Frizzled9*, which is a Wnt receptor gene. Frizzled9 is expressed in inner phalangeal cells, inner border cells, and third-row Deiters' cells in neonatal cochlea, and Frizzled9+ cells could regenerate HCs both *in vivo* and *in vitro*. Moreover, Frizzled9+ cells have a similar capacity for proliferation, differentiation, and HC generation as Lgr5+ progenitors [27].

In summary, the discovery of inner ear progenitors has provided a new approach for cell transplantation therapy. As mentioned above, there are two mechanisms for HC regeneration. One is trans-differentiation in which the inner ear progenitors switch cell fate to become HCs, and the other is mitotic regeneration in which inner ear progenitors proliferate and then differentiate into new HCs. Many transcription factors and signaling pathways are reported to be involved in the development of the inner ear, and several factors have been shown to be involved in HC regeneration in the neonatal mouse cochlea, including Atoh1, $p27^{Kip1}$, pRb, Foxg1, and the Wnt, Notch, Hedgehog, and Ephrin signaling pathways (**Figure 2**).

HC regeneration: transcription factors and regulatory factors

Atho1 (also called Math1) is a helix-loop-helix transcription factor that is essential for HC differentiation. The expression of Atoh1 is visible from embryonic day 14.5 in the cochlea. Deletion of the *Atoh1* gene leads to the failure of HC formation, while its overexpression induces ectopic HCs [28, 29]. Atoh1 also plays important roles later during inner ear develop-

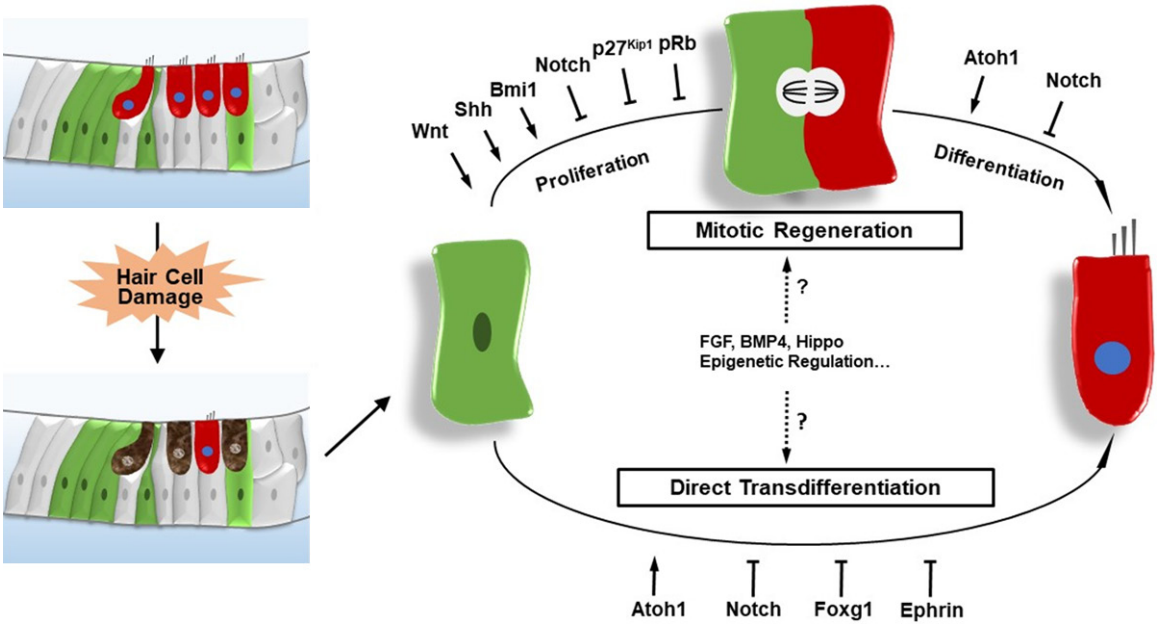

Figure 2. The regulation of HC regeneration in the neonatal mammalian cochlea after HC damage. HCs are regenerated through mitotic regeneration-in which progenitors re-enter the cell cycle, mitotically divide, and then differentiate into new HCs-or through direct trans-differentiation in which progenitors switch cell fate and directly differentiate into new HCs.

ment in HC survival and maturation [30, 31]. In neonatal mice, Atoh1 is also important by promoting HC regeneration, and ectopic activation of Atoh1 induces new HCs generation in young postnatal mice [32, 33]. Moreover, in the young adult deafened guinea pig model, forced expression of Atoh1 induces HC regeneration and decreases the hearing threshold [34]. However, only a subset of these cells is able to give rise to new HCs, and they do so only at early postnatal stages.

Cyclin-dependent kinase inhibitors (CKIs) are divides into two families, the Cip/Kip family and the Ink4 family, which play roles in governing cell cycle transitions and maintaining postmitotic state of numerous cell types [35, 36]. $p19^{Ink4d}$ (Cdkn2d) and $p21^{Cip1}$ (Cdkn1a) have been shown to be required in maintenance of the postmitotic state of HCs [37, 38]. $p27^{Kip1}$ (Cdkn1b), begins to be expressed in prosensory cells during the embryonic development of the mammalian cochlea, and it persists at high levels in SCs of the mature organ of Corti [39, 40]. Deletion of the *p27*^{Kip1} gene in the mouse cochlea results in continuous cell proliferation in the postnatal and adult mouse cochlea and to the appearance of supernumerary HCs and SCs [39, 41]. Deletion of *p27*^{Kip1} in SCs of the neonatal cochlea leads to the proliferation of pillar cells without cell fate conversion [42-44], which suggests that other factors are required to induce the differentiation of SCs into HCs.

pRb is a retinoblastoma protein encoded by the retinoblastoma gene *Rb1* and plays important roles in cell cycle exit, differentiation, and survival [45, 46]. And it has been shown that deletion of *Rb1* gene leads to the cell-cycle re-entry of both embryonic and postnatal mammalian HCs [47-49]. In neonatal mice, inactivation of pRb in SCs results in cell cycle re-entry of both pillar and Deiters' cells and an increase in the number of pillar cells. The nuclei of *Rb*$^{-/-}$ mitotic pillar and Deiters' cells were observed to migrate toward the HC layer and these cells divide near the epithelial surface, similar to the SCs in the regenerating avian cochlea. However, there are no newly regenerated HCs, and SC death followed by HC loss occurs [50].

Foxg1 (formerly called BF-1), one of the forkhead box family proteins, is involved in morphogenesis, cell fate determination, and proliferation in many tissues, especially in the brain [51-55]. *Foxg1* knockout mice die in the perinatal period and show shortened cochleae with multiple extra rows of HCs and SCs along

with vestibular defects [56, 57]. It was recently reported that conditional knockdown of *Foxg1* in SCs and progenitors in neonatal mice induces their direct trans-differentiation, but not their proliferation, and subsequently leads to extra HCs [58].

HC regeneration: signaling pathways

During cochlear development, the canonical Wnt/β-catenin signaling pathway regulates cell proliferation, cell fate decision, and HC differentiation, and Wnt signaling activation induces inner ear progenitor proliferation and HC regeneration in both mammalian and non-mammalian vertebrates [59, 60]. The inhibition of Wnt signaling in the embryonic mouse cochlea by small molecule inhibitors or in transgenic mice reduces the proliferation of prosensory cells [61]. Conversely, Wnt signaling activation promotes the prosensory domain formation and increases the number of HCs [62]. As mentioned above, Lgr5 and Lgr6, the Wnt signaling downstream targets, are expressed in embryonic and neonatal inner ear progenitors [22, 25]. And these progenitors can act as inner ear progenitors both *in vivo* and *in vitro* due to their ability of self-renew, proliferation, and differentiation into HCs [21, 23, 63, 64]. In neonatal cochlea, both Wnt agonists treatment and β-catenin overexpression promote the proliferative capacity of Lgr5+ progenitors and subsequent HC formation, whereas Wnt antagonists treatment reduce the proliferation and HC regeneration ability of Lgr5+ progenitors [23, 62, 65]. Wnt activation also causes the Axin2+ tympanic border cells to proliferate and differentiate into HCs and SCs in newborn mice [24]. The combined expression of β-catenin and Atoh1 in Lgr5+ cells increases the HC regeneration capacity of the postnatal cochlea by tenfold, and these newly regenerated HCs can survive until adulthood [66]. However, the combined expression of β-catenin and Atoh1 cannot induce HC regeneration in the adult mammalian cochlea.

Because Notch signaling pathway plays important roles in HC differentiation during inner ear development, many researchers have examined its roles in HC regeneration in postnatal cochlea. In both the zebrafish lateral line and mature avian basilar papilla, inhibition of Notch signaling increases HC regeneration through SC mitotic division and direct trans-differentiation. In contrast, Notch activation maintains SCs in a quiescent state, thereby inhibiting regeneration of HCs [67, 68]. In the mammalian postnatal cochlea, the Notch inhibition by γ-secretase inhibitor upregulates Atoh1 expression and results in the trans-differentiation of adjacent SCs into HCs [69, 70]. Li et al. reported a direct interaction between the Notch and Wnt signaling pathways, that Notch inhibition induces mitotically generated HCs in mammalian cochleae via activating the Wnt pathway [71]. In addition, Notch and Wnt co-regulation promotes SC proliferation and HC regeneration in both the cochlea and utricle in neonatal mice [72, 73]. A particularly exciting finding is that a genetic reprogramming process involving β-catenin activation, Notch1 deletion, and Atoh1 overexpression is established and can promote extensive SC proliferation followed by mitotic HC regeneration [74].

Hedgehog signaling is important for the formation of the dorsoventral axis of the inner ear, and plays important roles in the prosensory domain formation [75], the progenitor proliferation, and HC differentiation during inner ear development [76]. The cell fate of progenitors, whether differentiate into vestibular cells or auditory cells, is depend on the balance between Wnt and Hedgehog signaling [77, 78]. A few studies have reported the roles of Hedgehog signaling in mammalian HC regeneration. Hedgehog signaling induces SC proliferation and HC regeneration in the postnatal rat cochlea after neomycin treatment [79], and Sonic Hedgehog recombinant protein effectively promotes *in vitro* sphere formation, proliferation, and differentiation of Lgr5+ progenitors isolated from the neonatal cochlea. Hedgehog signaling was also proved to induce SC proliferation and HC regeneration in neomycin damaged cochlea by using transgenic R26-SmoM2 mice which constitutively activate Hedgehog signaling in the SCs leads to [80].

Ephrins and their receptors Ephs also play role in HC regeneration. EphA4 receptor is expressed in HCs, while Ephrin-B2 is present in SCs, and this complementary pattern of expression is necessary for the establishment of the compartment boundary between HCs and SCs [81]. Jean Defourny et al. demonstrated that mammalian HCs can be directly generated from SCs

by inhibition of ephrin-B2 signaling. Using either ephrin-B2 conditional knockout mice, shRNA-mediated gene silencing, or soluble inhibitors, they found that downregulation of ephrin-B2 signaling at late embryonic stages after HC production, results in translocation of SC into HC layers and subsequent cell fate switch from SC to HC [81]. Interestingly, throughout inner ear development, Ephrin-B2 and Notch are expressed in similar SC types [82]. Moreover, Ephrin-B2, whose expression is induced by Notch signaling, is reported to be a direct Notch signaling downstream target [83]; therefore, Ephrin-B2 might be required following Notch lateral inhibition in order to segregate the SCs from adjacent HCs.

HC regeneration: epigenetic regulation

Epigenetic factors have recently emerged as important regulators in both inner ear development and in HC regeneration. In the neuromasts of developing zebrafish larva, inhibition of the histone-modifying enzyme lysine-specific demethylase 1 (LSD1) disrupts cell proliferation, induces apoptosis, and reduces the numbers of sensory HCs and SCs [84]. And epigenetic regulation of Atoh1 was reported to guide HC development in the developing mouse cochlea [10]. Inhibition of histone acetyltransferase activity reduces H3K9 acetylation at the Atoh1 locus and therefore prevents *Atoh1* mRNA increase and subsequent HC differentiation. Interestingly, the H3K4me3/H3K27me3 bivalent chromatin structure, observed in progenitors, persists at the *Atoh1* locus in perinatal SCs [10], suggesting the important roles of such structures in HC regeneration.

Histone deacetylase (HDAC) inhibitor treatment of HC-damaged chicken utricles reduces proliferation of SCs, but does not affect HC regeneration [63]. Similarly, inhibition of HDAC activity in HC-damaged zebrafish larvae also reduces SC proliferation and subsequent HC regeneration [23]. Bmi1, a Polycomb group protein and a component of the Polycomb repressive complex 1, maintains the proliferative capacity of SCs by sustaining high levels of Wnt signaling in the neonatal mouse cochlea. In neonatal *Bmi1*-deficient cochleae, SCs fail to re-enter the cell cycle in response to HC damage, and the *in vitro* sphere-forming ability of *Bmi1*-deficient cochlear progenitors is also reduced [11].

Future perspectives

Although HC regeneration can be induced by many factors and signaling pathways in the neonatal mammalian cochlea, HCs cannot be regenerated in the adult mammalian cochlea and current technologies are still quite far from restoring hearing functions in the HC-damaged mammalian cochlea. Thus, further research is needed to find ways to induce HC regeneration in both the neonatal and adult mammalian cochlea.

First, more pathways and important factors, including those that might regulate the proliferation and differentiation of stem cells and progenitors, such as FGF, BMP4, and Hippo signaling pathway, should be explored in the study of HC regeneration. The FGF signaling pathway has been shown to be important in inner ear development and to be related to the otic placode induction and the otic vesicle development [85-87]. Deletion of the FGF receptor 1 (*Fgfr1*) gene in the inner ear results in decrease of the number of proliferative prosensory cells and subsequent decrease of the numbers of HCs and SCs [88, 89]. The roles of the FGF signaling pathway in HC regeneration has been explored in the utricles of chickens and the lateral lines of zebrafish [90-93]. Many reports has shown that BMP4 plays important roles in mammalian and non-mammalian inner ear development [94-100], and it is recently reported that BMP4 can also antagonize HC regeneration in the avian auditory epithelium [101]. The Hippo/Yap signaling pathway plays important roles in development, homeostasis, and regeneration in many tissues and cancer cells [102-106], and it has been reported that Hippo/Yap controls proliferation and differentiation of lung and plays key roles in regeneration and fibrogenesis after kidney injury. In zebrafish lateral line, Yap1 plays important roles in HC differentiation. Knockdown of Yap1 in developing zebrafish affects development of the lateral line system and recapitulates the Prox1a deficiency in mechanosensory cells of neuromast [107]. All of the above factors and signaling pathways can be used as good candidates for further HC regeneration study in the mammalian inner ear. As mentioned above, many epigenetic regulators such as LSD1, histone modifications, and HDAC inhibitors, which have been studied in inner ear development and HC regeneration in

non-mammalian organisms, are also very good candidates for studying HC regeneration in the mammalian inner ear.

Second, the interactions of multiple pathways in cell proliferation and HC differentiation should be explored. As mentioned above, some research has studied the cross talk between two or more signaling pathways and factors [72-74], but these studies are far from regenerating HCs and repairing inner ear damage in adult mammals.

And lastly, the maturation and survival of newly generated HCs and HC regeneration in adult mammals still remains a challenge. Bradley Walters et al. found that combining $p27^{Kip1}$ deletion with ectopic Atoh1 expression surmounts age-related decline of HC regeneration from SCs, leading to conversion of SCs to HCs in mature mouse cochleae and after noise damage [108]. Moreover, co-activation of GATA3 or Pou4f3 and Atoh1 promoted conversion of SCs to HCs in adult mice and activation of Pou4F3 alone also converted mature SCs to HCs *in vivo* [108]. In another recent report, Yilai Shu et al. reported that transient co-activation of cell cycle activator Myc and inner ear progenitor gene Notch1 induces proliferation of diverse adult cochlear sensory epithelial cell types, and enables adult SCs to respond to transcription factor Atoh1 and efficiently trans-differentiate into HC-like cells [109]. Although it is excited to see these two recent reports that HC could now be regenerated from SCs in adult mice by genes and signaling regulation, the regeneration efficiency and the maturation of regenerated HCs remains still a problem. More efforts, such as other genes and signaling co-regulation, apoptosis inhibition and maturation induction of newly regenerated HCs, should be made in the future.

In summary, much effort has been put into exploring the mechanisms of HC regeneration in the mammalian inner ear, and many factors and signaling pathways have been shown to play important roles in the neonatal cochlea. However, these studies are still far from regenerating HCs and repairing HC damage in adult mammals, which is the ultimate research objective in this field.

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Disclosure of conflict of interest

None.

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