Research Progress on Flat Epithelium of the Inner Ear

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Summary

Sensorineural hearing loss and vertigo, resulting from lesions in the sensory epithelium of the inner ear, have a high incidence worldwide. The sensory epithelium of the inner ear may exhibit extreme degeneration and is transformed to flat epithelium (FE) in humans and mice with profound sensorineural hearing loss and/or vertigo. Various factors, including ototoxic drugs, noise exposure, aging, and genetic defects, can induce FE. Both hair cells and supporting cells are severely damaged in FE, and the normal cytoarchitecture of the sensory epithelium is replaced by a monolayer of very thin, flat cells of irregular contour. The pathophysiologic mechanism of FE is unclear but involves robust cell division. The cellular origin of flat cells in FE is heterogeneous; they may be transformed from supporting cells that have lost some features of supporting cells (dedifferentiation) or may have migrated from the flanking region. The epithelial-mesenchymal transition may play an important role in this process. The treatment of FE is challenging given the severe degeneration and loss of both hair cells and supporting cells. Cochlear implant or vestibular prosthesis implantation, gene therapy, and stem cell therapy show promise for the treatment of FE, although many challenges remain to be overcome.

Key words: Wounds and injuries; flat epithelium; cochlear; vestibular; hair cell; supporting cell

Introduction

The sensory epithelia of the mammalian inner ear in the cochlea, utricle, saccule, and crista ampullaris are important for hearing and balance perception. Each of these sensory end-organs consists of mechanotransducing hair cells (HCs), surrounding supporting cells (SCs), and neural endings that innervate to HCs. Different insults result in varying degrees of damage to the sensory epithelium of the inner ear. In most cases, HCs are damaged but SCs remain unaffected and expand to fill the space formerly occupied by the HCs (Leonova and Raphael 1997, Wang *et al.* 2010). In other cases, both HCs and SCs are damaged, resulting in extreme degeneration of the sensory epithelium, which is replaced by a layer of flat cells of irregular contour. This pathologic change occurs in the cochlear and vestibular end-organs of animal models and is referred to as flat epithelium (FE) (Jahan *et al.* 2018, Raphael *et al.* 2007, Wang *et al.* 2017). FE has also been found

in temporal bone specimens of patients with severe deafness or intractable Meniere's disease (McCall *et al.* 2009, Nadol and Eddington 2006, Teufert *et al.* 2006), indicating that FE is an important pathological change in patients with diseases of the inner ear. However, the pathological features and pathogenesis of FE are unclear and inducing HC regeneration in FE to recover hearing or vestibular function is problematic. Herein we review the etiology, characteristics, mechanisms, and intervention strategies for FE of the mammalian inner ear.

1. Etiology

1.1 Genetic factors

Histopathological studies of human temporal bone show hereditary factors result in various degrees of change in the organ of Corti (Bommakanti *et al.* 2019). Severe degeneration of the organ of Corti occurs in patients with nonsyndromic or syndromic profound hearing loss. In patients with *DFNA5* mutation, the organ of Corti is reduced to an FE in the basal and middle turns of the cochlea, which is accompanied by severe atrophy of the cells of the stria vascularis and spiral ganglion (Nadol *et al.* 2015). Mutations in *GJB2*, which encodes connexin 26 (Cx26), are the most common cause of nonsyndromic hereditary deafness. In a mouse model of *GJB2* mutation, HCs and differentiated SCs degenerate in the cochlea, resulting in FE (Sun *et al.* 2009, Takada *et al.* 2014). Although there is no evidence that *GJB2* mutations lead to inner ear FE in human, agenesis of HCs has been observed in human temporal bones with *GJB2* mutations (Jun *et al.* 2000). FE is also found in patients with syndromic hereditary deafness conditions, such as Usher syndrome. Of the three types of Usher syndrome, type 1 presents the most serious hearing loss and vestibular dysfunction, and patients with this type exhibit severe degeneration of the organ of Corti with total loss of HCs and SCs (Nadol and Eddington 2006, Wagenaar *et al.* 2000). The *Pcdh15* mutation is associated with Usher syndrome type 1, and the sensory epithelium of the cochlea is replaced by FE in *Pcdh15* mutation mice (Pawlowski *et al.* 2006). Atonal homolog1 (*Atoh1*) is a crucial basic helix-loop-helix transcription factor for HC development and differentiation. Several studies have reported that *Atoh1* knockout mice exhibit complete loss of differentiated HCs, and the organ of Corti and vestibular sensory

epithelium are replaced by FE in the postnatal stage (Liu *et al.* 2016, Pan *et al.* 2012, Pan *et al.* 2011).

1.2 Aminoglycoside antibiotics

Aminoglycoside antibiotics are important for treating life-threatening bacterial infections, such as tuberculosis, endocarditis, and those of the respiratory and urinary tracts (Jiang *et al.* 2017). However, the ototoxicity of aminoglycoside antibiotics can significantly damage HCs and/or SCs, resulting in absence of the organ of Corti in humans (Kusunoki *et al.* 2004b). In animal models, FE can be induced by administration of high doses of aminoglycosides. In the cochlea of guinea pig, the organ of Corti is replaced by FE 4 days after application of neomycin (Kim and Raphael 2007), suggesting its rapid degeneration. Additionally, cochlear FE occurs when it is lesioned by aminoglycoside plus diuretics in cats and mice (Coco *et al.* 2007, Taylor *et al.* 2012). In the vestibular sensory epithelium, a high dose of streptomycin induces FE in the utricular sensory epithelium (He *et al.* 2020, Wang *et al.* 2017).

1.3 Noise

In the basilar papilla of chicken, exposure to noise (1500 Hz, 120 dB, 24 h or 900 Hz, 120 dB, 48 h) results in moderate damage, which is characterized by loss of HCs but survival and expansion of SCs. When the noise intensity is elevated to 123 dB, both HCs and SCs are damaged, and the basilar papilla is replaced by FE (Cotanche *et al.* 1995). The cochlear sensory epithelium of guinea pig transforms into FE with no signs of differentiated HCs and SCs after two months of noise exposure to gunfire (Yang *et al.* 2012). Similarly, in chinchilla and mice, severe noise exposure leads to degeneration of the cochlear sensory epithelium, which is replaced by FE (Roberto and Zito 1988, Willott JF *et al.* 1994).

1.4 Other factors

Kusunoki *et al*. reported a significant correlation between loss of HCs and aging in the cochlea of temporal bone of aging humans, and the organ of Corti completely degenerated in some regions of the cochlea (Kusunoki *et al.* 2004a). Smittkamp *et al*. found that aging birds sustained total cochlear

damage, and large regions were replaced by hyaline cells (Smittkamp *et al.* 2003). Therefore, aging is a significant factor leading to FE (Yamoah *et al.* 2020). Additionally, inner ear infection may induce FE. Teufert *et al*. found total loss of the organ of Corti in patients with labyrinthitis-induced deafness (Teufert *et al.* 2006). Moreover, a monolayer of epithelial cells is present in the vestibular end-organs of some patients with intractable Meniere's disease (McCall *et al.* 2009).

2. Characteristics of FE of the inner ear

2.1 Morphological characteristics

HCs and differentiated SCs disappear in FE, which consists of a thin layer of epithelial cells of variable size (Kim and Raphael 2007) with surface microvilli (Wang *et al.* 2017). The width of FE cells ranges from less than 20 μm to greater than 40 μm, and the cell height is similar to that of its nucleus (Kim and Raphael 2007). Flat cells typically contain fewer organelles and larger nuclei than normal HCs and SCs. The tissue structure of FE may exhibit polarization and a radial morphology (Taylor *et al.* 2012).

2.2 Biological characteristics

2.2.1 Maintenance of intercellular junctions: Intercellular junctions are necessary for homeostasis of the lymphatic fluid of the inner ear. In the normal organ of Corti, the tight junction protein ZO-1 is located between inner hair cells, outer hair cells, and the surrounding SCs in the reticular lamina, and is an important component of the perilymph-endolymph barrier. The gap junction protein Cx26 is present in the basilar membrane and lateral wall of the cochlea and participates in perilymph-endolymph ion transport (Jagger and Forge 2014). ZO-1, Cx26, and Cx30 are present on the surface of FE cells (Kim and Raphael 2007, Taylor *et al.* 2012), indicating that FE maintains the integrity and the ion transport ability of the perilymph-endolymph barrier.

2.2.2 Metabolic activity and mitosis: Protein kinase C (PKC) plays important roles in cell cycle progression, cell differentiation, gene expression, and cytoskeletal remodeling (Isakov 2018). Ladrech *et al.* found that in the normal cochlea of rat, only inner HCs and some types of SCs expressed PKC, while all FE cells strongly expressed PKC, indicating that FE cells have high

metabolic activity (Ladrech *et al.* 2017). The level of mitosis is high at the early stage of flattening (Kim and Raphael 2007). At 4 days after neomycin treatment in the cochlea of guinea pig, flat cells show robust proliferation; however, proliferation is absent at 7 days. The cell-cycle inhibitor $p27^{kip1}$ shows synchronous changes with the extent of mitosis (Kim and Raphael 2007). In moderate lesions, the proliferation rate of SCs is markedly lower than that in FE (Yamasoba and Kondo 2006). *In vitro*, proliferation is initiated by the loss of cell-cell contact, which is important for maintaining epithelial confluence in the inner ear (Meyers and Corwin 2007, Tamiya *et al.* 2010). Therefore, discontinuity of the lesioned epithelium caused by cell death may trigger cell division in FE of the inner ear.

2.2.3 Expression of markers of epithelial and mesenchymal cells: Using scanning electron microscopy, Ladrech *et al.* (Ladrech *et al.* 2017) reported that during FE formation in the inner ear, epithelial cells of the outer spiral sulcus (tectal cells, Hensen cells, Claudius cells, and Boettcher cells) migrated to the medial side to cover the damaged organ of Corti. The expression of epithelial markers, such as E-cadherin and laminin, were decreased. These researchers hypothesized that these epithelial cells underwent the epithelial–mesenchymal transition (EMT) and subsequently acquired certain mesenchymal characteristics. The EMT increases cell differentiation, migration, and apoptosis (Nieto *et al.* 2016). In the nervous system, the EMT is not only involved in organ development and embryo formation but is also closely associated with wound healing, tissue regeneration, and organ fibrosis (Chen *et al.* 2015, Kalcheim 2015, Kuznetsova *et al.* 2014). Moreover, the EMT participates in the development of the inner ear (Johnen *et al.* 2012, Kobayashi *et al.* 2008, Simonneau *et al.* 2003) and the proliferation of inner-ear sensory-epithelium cells of adult vertebrates *in vitro* (Hu and Corwin 2007, Zhang and Hu 2012). Therefore, the loss of cell-cell contact due to severe lesions in the sensory epithelium of the inner ear induces the EMT and cell proliferation, which promote wound healing.

2.2.4 Gene expression profiles: Genes expressed prior to *Atoh1* in undifferentiated sensory precursor cells, such as *BDNF, Sox2,* and *Prox1*, are still expressed in the undifferentiated cochlear sensory epithelium of *Atoh1*-null mice (Dabdoub *et al.* 2008, Fritzsch *et al.* 2010, Fritzsch *et al.* 2005). Pan *et al.* reported that *Fgf10*, a gene expressed in the GER of developing cochlea, and *Bmp4*, a

gene expressed in developing Hensen's and Claudius cells, were expressed in the undifferentiated organ of Corti (Pan *et al.* 2012, Pan *et al.* 2011). Wang *et al.* found that some vestibular FE cells expressed *Sox2* after streptomycin-induced damage (Wang *et al.* 2017). Future studies are still needed to illuminate gene expression profiles of the inner ear FE.

2.2.5 Regeneration capacity: Nonmammalian vertebrates possess the ability to completely regenerate HCs in FE in the inner ear (Girod *et al.* 1989). Avian cochlear sensory epithelium exhibits mature-appearing HCs and SCs, and complete recovery from FE of the inner ear caused by exposure to loud noise (Girod *et al.* 1989). In contrast, neither spontaneous nor *Atoh1*-induced HC regeneration occurs in FE of the mammalian cochlea (Izumikawa *et al.* 2008). In the mammalian vestibular FE, a small number of myosin VIIa-positive/Sox2-positive cells are present, and some exhibit surface immature hair bundles, indicating spontaneous regeneration of HCs (Wang *et al.* 2017). *Atoh1* overexpression with the treatment of suberoylanilide hydroxamic acid (SAHA) promotes myosin VIIa expression in vestibular FE cells, suggesting the potential capacity of HC regeneration in vestibular FE (He *et al.* 2020).

2.3. Innervation

SCs protect unmyelinated fibers and express neurotrophic factors, which play an important role in the survival of spiral ganglion neurons (SGNs) and nerve fibers (Sugawara *et al.* 2005, Zilberstein *et al.* 2012). SGNs and nerve fibers degenerate secondary to the loss of HCs and SCs. Additionally, lesions induced by various factors, including noise, aminoglycosides, and aging, can directly damage nerve innervation in the inner ear (Kujawa and Liberman 2009, Makary *et al.* 2011, Raul *et al.* 2001). Nerve degeneration in FE of the inner ear in several animal species has been reported. Izumikawa *et al.* and Shibata *et al.* found that nerve fibers retracted in cochlear FE at 1 week after injury; the number of SGNs was significantly decreased, and the cell body of neurons shrank compared to the normal state (Izumikawa *et al.* 2008, Shibata and Raphael 2010). In the cochlea of Cx26-null mice, SGNs are almost completely lost, and the organ of Corti degenerates (Sun *et al.* 2009). In contrast, in vestibular FE of mice, nerve fibers and neurons show delayed degeneration after damage to HCs and SCs (Wang *et al.* 2017). In the FE of the human inner ear, neurodegeneration may occur over a

long time after hearing loss. The number of SGNs is reduced in cochlear FE, but they do not completely disappear (Nadol and Eddington 2006, Nadol *et al.* 1989). In human vestibular FE, the morphology of calyces and nerve fibers remain relatively normal several years after the onset of Meniere's disease (McCall *et al.* 2009). Nerve maintenance in human FE provides a therapeutic opportunity for functional recovery.

3. Mechanisms of FE formation in the inner ear

The inner ear of nonmammalian vertebrates undergoes self-repair after severe injury. At the early stage, hyaline or cuboidal cells proximate to the basilar papilla (where HCs and SCs are located), migrate into the damaged sensory epithelium to form FE, and subsequently divide and differentiate into mature HCs and SCs (Cotanche *et al.* 1995, Girod *et al.* 1989). Nevertheless, the mechanism of FE formation in the mammalian inner ear is unclear; two hypotheses have been proposed (Figure 1-2). First, the original SCs (Dieter's cells, pillar cells, phalangeal cells or vestibular SCs) dedifferentiate following damage and subsequently form FE. Some cells in the vestibular FE exhibit characteristics of SCs, *i.e.*, expression of the SC marker Sox2 but not the HC marker myosin VIIa (Wang *et al.* 2017). Lineage tracing studies with *Plp-CreER^{T2}:Rosa26^{tdTomato}* mice and *GLAST-CreER^{T2}:Rosa26^{tdTomato}* mice show that some vestibular FE cells express tdTomato (He *et al.* 2020). These studies suggest that FE cells may originate from SCs. However, the extent of damage to SCs required for FE formation is unclear. FE may be present in only some regions of the organ of Corti (Kim and Raphael 2007); alternatively, a small patch of FE may be interspersed with areas of scar formation (Taylor *et al.* 2012). Second, HCs and SCs die after being damaged, and cells surrounding the sensory epithelium migrate into the area occupied by HCs and SCs. Ladrech *et al.* found that cells on the lateral side of the organ of Corti, such as tectal cells and Hensen cells, migrated inwards and covered the scar structure (Ladrech *et al.* 2017). Taylor *et al.* reported that FE cells shared properties with the surrounding cells, e.g., high expression of Cx26 and Cx30; no expression of Cx43, acetylated tubulin or KCC4; and large gap junctions in the lateral walls (Taylor *et al.* 2012). He *et al.* found that transitional epithelial cells might be a source of vestibular FE (He *et al.*

2020). Future studies on cellular-lineage tracing using specific Cre mouse lines to fate-map cell types in the inner ear would provide insight into the mechanism of FE formation.

4. Intervention strategies for FE of the inner ear

4.1 Cochlear implantation and innervation protection

Cochlear implantation is effective for profound sensorineural hearing loss (Naples and Ruckenstein 2020). Cochlear implantation bypasses the damaged sensory HCs and directly stimulates the SGNs. Thus, the outcome of cochlear implantation is dependent on the presence of sufficient neurons and nerve fibers in the cochlea. Because innervation of the inner ear is degenerated in FE (Nadol and Eddington 2006, Nadol *et al.* 1989, Wang *et al.* 2017), preservation of innervation following damage is vital for a satisfactory outcome of cochlear and vestibular implants (Perez *et al.* 2017). Following cochlear damage, overexpression of neurotrophins induces considerable regrowth of peripheral auditory fibers in the basilar membrane area and preserves SGNs. Therefore, induced overexpression of neurotrophins has potential for maintaining innervation or inducing nerve regeneration in FE (Budenz *et al.* 2015, Fukui and Raphael 2013, Shibata *et al.* 2011, Shibata *et al.* 2010, Wise *et al.* 2010).

4.2 Gene therapy

Gene therapy has been used in animals with inner ear diseases for more than 20 years (Guo *et al.* 2018, Raphael Y *et al.* 1996, Wang *et al.* 2014). *Atoh1* is an important regulator of the development and differentiation of HCs (Li *et al.* 2016, Richardson and Atkinson 2015, Zhong *et al.* 2019). Overexpression of *Atoh1* promotes the differentiation of SCs into HCs in the developing inner ear and damaged inner ear of mouse (Gao *et al.* 2016, Hicks *et al.* 2020, Liu *et al.* 2012, Sayyid *et al.* 2019). Nevertheless, overexpression of *Atoh1* in cochlear FE fails to induce HC regeneration (Izumikawa *et al.* 2008). In vestibular FE, *Atoh1* overexpression plus SAHA induces vestibular FE to express myosin VIIa; however, these cells were morphologically different from mature HCs (He *et al.* 2020). These studies indicate that flat cells do not possess properties of the original SCs, which poses a great challenge for gene therapy in FE of the inner ear. As flat epithelial cells may have regressed to an early stage of differentiation, regeneration of FE is unlikely to be induced by

exclusively manipulating *Atoh1* (Izumikawa *et al.* 2008, Yamoah *et al.* 2020). A variety of combinatorial genetic approaches have been applied to regeneration of HCs in the inner ear (Srivastava and DeWitt 2016). HC differentiation requires an essential set of genes, including *Atoh1, Pou4f3, Gfi1,* and *miRNA-183* (Jahan *et al.* 2015, Pauley *et al.* 2008, Yamoah *et al.* 2020)*,* and co-expression of *Atoh1* with other factors, such as β-catenin, GATA, and Pou4f3, induces robust HC regeneration in the mouse cochlea (Kuo *et al.* 2015, Ni *et al.* 2016, Walters *et al.* 2017). Transcriptome analyses have identified multiple genes that function during inner-ear development and regeneration, suggesting targets for FE gene therapy (Reh *et al.* 2016, Scheffer *et al.* 2015).

4.3 Stem cell therapy

Stem cells possess self-renewal ability and can be induced to differentiate into many types of cells (Cruciani *et al.* 2019, Travnickova and Bacakova 2018). Inner ear or other stem cells can be induced to differentiate into hair cell-like cells *in vitro* (Longworth-Mills *et al.* 2015, Savary *et al.* 2007, Warnecke *et al.* 2017). However, the following difficulties must be overcome to induce differentiation of stem cells to HCs *in vivo*: 1) exogenous stem cells are unable to adapt to the high potassium ion concentration in endolymph, which causes their death (Lee and Park 2018); 2) tight junctions at the apical end of FE hamper colonization by stem cells; and 3) differentiation into functional HCs may require a series of complex regulatory processes. HeLa cells and human embryonic stem cells survive in the normal auditory epithelium and FE for at least 7 days if the potassium concentration is reduced (Lee *et al.* 2017, Park *et al.* 2014). Further research is needed to prolong the survival and induce stem cell differentiation.

5. Conclusion

FE is a pathological change that occurs after severe damage to the sensory epithelium of the inner ear. FE is present in human temporal bone specimens and mouse inner ear samples with profound hearing loss and/or vestibular disorders induced by ototoxic drugs, noise, and genetic factors. FE has different characteristics than inner ear sensory cells and shows innervation degeneration of variable degrees. Cochlear FE is unable to regenerate in mature mice, but vestibular FE may have limited regeneration ability. The expression levels of molecular markers change during FE formation,

which may provide insight into the characteristics and formation of FE. Further studies should focus on means by which gene regulation and/or stem cell colonization can promote FE regeneration and maintain innervation.

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Figures:

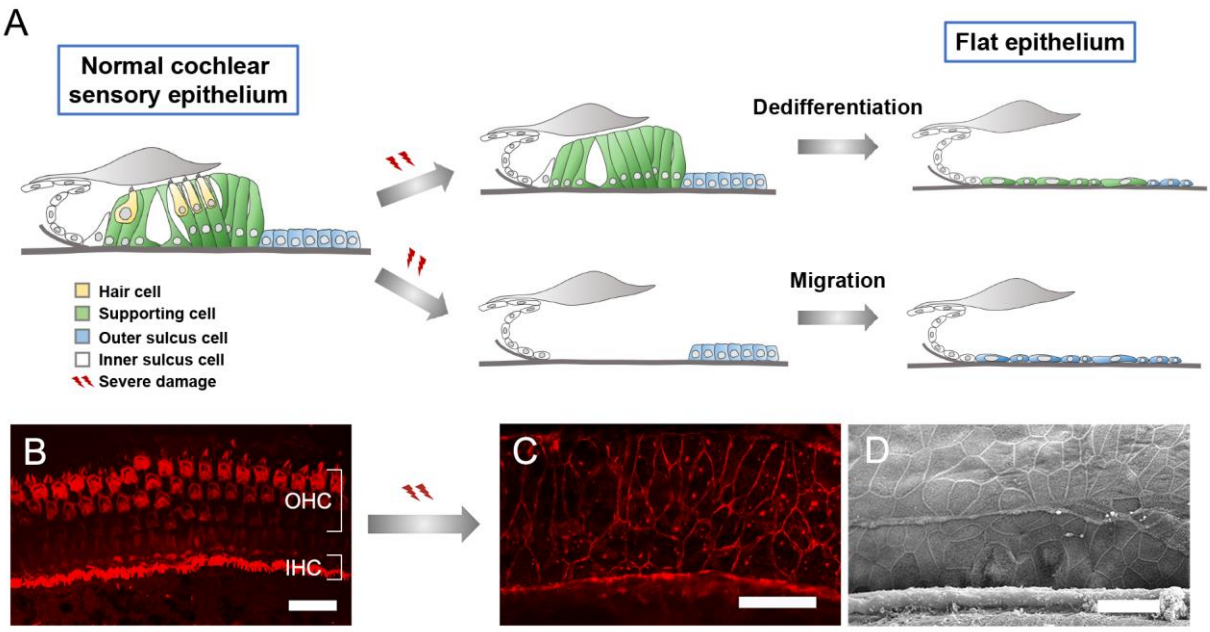

Figure 1. Postulated mechanisms of formation of cochlear flat epithelium (FE) in mammals. (A) Schematic figures of cochlear FE formation. (B) Confocal image showing normal out hair cells (OHCs) and inner hair cells (IHCs) stained by phalloidin. (C) A confocal image of cochlear FE stained by phalloidin shows that normal OHCs and IHCs are lost and the sensory epithelium is replaced by flat cells. (D) A scanning electron microscopic image of cochlear FE. Scale bars represent 20 μm (B, C and D).

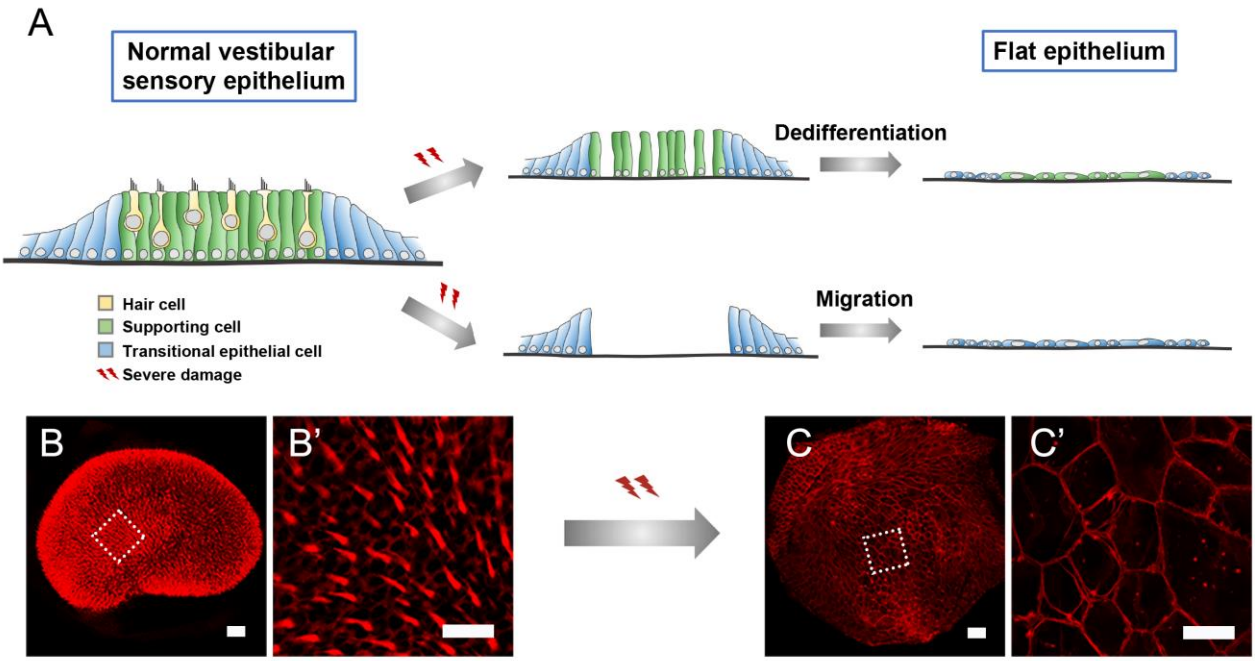

Figure 2. Postulated mechanisms of formation of vestibular flat epithelium (FE) in mammals. (A) Schematic figures of vestibular FE formation. (B-B') Confocal images showing normal vestibular sensory epithelium stained by phalloidin. (B') High-magnification image of the dotted square in (B) shows stereocilia structure. (C-C') Confocal images showing vestibular FE stained by phalloidin. (C') High-magnification image of the dotted square in (C) shows the contour of flat cells. Scale bars represent 50 μm (B and C) or 20 μm (B' and C').