



MECOM promotes supporting cell proliferation and differentiation in cochlea

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ABSTRACT

Permanent damage to hair cells (HCs) is the leading cause of sensory deafness. Supporting cells (SCs) are essential in the restoration of hearing in mammals because they can proliferate and differentiate to HCs. MDS1 and EVI1 complex locus (*MECOM*) is vital in early development and cell differentiation and regulates the TGF- β signaling pathway to adapt to pathophysiological events, such as hematopoietic proliferation, differentiation and cells death. In addition, *MECOM* plays an essential role in neurogenesis and craniofacial development. However, the role of *MECOM* in the development of cochlea and its way to regulate related signaling are not fully understood. To address this problem, this study examined the expression of *MECOM* during the development of cochlea and observed a significant increase of *MECOM* at the key point of auditory epithelial morphogenesis, indicating that *MECOM* may have a vital function in the formation of cochlea and regeneration of HCs. Meanwhile, we tried to explore the possible effect and potential mechanism of *MECOM* in SC proliferation and HC regeneration. Findings from this study indicate that overexpression of *MECOM* markedly increases the proliferation of SCs in the inner ear, and the expression of Smad3 and Cdkn2b related to TGF signaling is significantly down-regulated, corresponding to the overexpression of *MECOM*. Collectively, these data may provide an explanation of the vital function of *MECOM* in SC proliferation and trans-differentiation into HCs, as well as its regulation. The interaction between *MECOM*, Wnt, Notch and the TGF- β signaling may provide a feasible approach to induce the regeneration of HCs.

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1. Introduction

The organ of Corti is the core component of the auditory system, composed of hair cells (HCs) and supporting cells (SCs). HCs are easily damaged by excessive noise exposure (Guo et al., 2021a), ototoxic drugs (Fu et al., 2021a; He et al., 2017a; Li et al., 2018; Liu et al., 2019a; Zhong et al., 2020; Zhang et al., 2019), aging (He et al., 2021; Zhou et al., 2020), genetic factors (Cheng et al., 2021; Fu et al., 2021b; Lv et al., 2021; Qian et al., 2020; Zhang et al., 2021a) and

infections (He et al., 2020; Zhang et al., 2021b), all of which can cause permanent hearing impairment due to the loss of HCs (Dror and Avraham, 2010). Recent studies have shown that hearing impairment significantly affects the physical and mental health of patients (Stevenson et al., 2015; Overgaard et al., 2021). Therefore, exploration aimed at restoration of mammalian auditory HCs has attracted a growing attention and is considered to be the ultimate strategy for hearing recovery. Although HCs cannot regenerate spontaneously in the mammalian cochlea, previous studies have shown that certain signaling pathways can promote HC regeneration (Liu et al., 2021a; Zhang et al., 2017, 2020a; Li et al., 2016). When SCs are exposed to certain conditions, they may become specialized sensory cells (Chen et al., 2021a; Li et al., 2015). Therefore, the proliferation of SCs and their differentiation into HCs are a promising method in the restoration of hearing dysfunction due to damage to HCs. (Cheng et al., 2019; Wu et al., 2016; Zhang et al., 2020b). The effective regulation of genes and signal pathways involved in this process may be vital to reprogramming SCs

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(Zhang et al., 2020c; Ding et al., 2020; Chen et al., 2021b; Waqas et al., 2016a). Regulation of certain genes or signaling pathways may allow SCs in the resting phase to proliferate and differentiate into HCs (Tan et al., 2019; Lu et al., 2017).

Epithelial-to-mesenchymal transition (EMT) participates in organ development and cell regeneration (Jolly et al., 2018; Sheng, 2021), and mesenchyme stem cells have a constructive role in cochlear morphogenesis and sensory HC regeneration (Kanzaki et al., 2020; Maharajan et al., 2021). The periotic mesenchyme significantly affects the morphogenesis of sensory epithelium, and similarly affects the formation of HCs (Huang et al., 2020; Ebeid and Huh, 2020). Certain genes and signal pathways in the periotic mesenchyme are necessary conditions for the development of the cochlea (Chen et al., 2017; He et al., 2017b; Cheng et al., 2017). Our previous studies have shown that cochlear sensory cells mainly proliferate from their embryonic day (E) 12 to E13, while primarily differentiate on days E14 to E15 (Shaofeng et al., 2005), which is consistent with the findings from similar studies. (Wilkerson et al., 2019). Moreover, before the fate of cochlear HCs is determined, the periotic mesenchyme adjacent to the sensory epithelium has a more critical role in inducing the morphological development and directed differentiation of sensory epithelium (Huang et al., 2020). Furthermore, the expression level of *MDS1–EVI1* complex locus (*MECOM*) is increased significantly on day E12.5 in the inner ear (Yali, 2016), which indicates that *MECOM* plays a role in the development of the cochlea.

MECOM can encode *EVI1* and *MDS1–EVI1* proteins (hereafter referred to as *PRDM3*) (Pinheiro et al., 2012). *MECOM* is essential for early development, proliferation, hematopoiesis and cell differentiation (Goyama et al., 2008). Studies have shown that *PRDM3* has a significant role in neurogenesis and craniofacial development. Moreover, deletion of *MECOM* causes craniofacial defects and neuronal differentiation deficiency (Shull et al., 2020; Leszczynski et al., 2020a). The function and promoter activity of *PRDM3* (*MDS1–EVI1*) in the hematopoietic system has been described in detail (Christodoulou et al., 2020). Meanwhile, *MECOM* is a known oncogene that can regulate differentiation, proliferation and apoptosis of tumorigenesis. (Rommer et al., 2013; Konantz et al., 2013; Glass et al., 2014). Abnormally high expression of *MECOM* has potent oncogenic properties. Amplification and overexpression of *MECOM* have been discovered in ovarian cancer (Wang et al., 2017), renal cell carcinoma (Palomero et al., 2020) and pancreatic cancer (Ye et al., 2020). Increased *MECOM* expression in acute and chronic myeloid leukemia is related to poor patient survival (Goyama and Kurokawa, 2009). However, expression of *MECOM* in the inner ear and its role in the regeneration of HCs is yet to be understood.

Signaling pathways play an essential part in the proliferation and differentiation of cochlea. *PRDM* proteins have a vital role in controlling cell proliferation, differentiation, development and consequently neoplastic transformation through the regulation of related signaling pathways (Fog et al., 2012; Leszczynski et al., 2020b). *MECOM* belongs to the *PRDM* protein family and also plays a vital role in the development and regulation of stem cell proliferation (Goyama et al., 2008; Kataoka et al., 2011; Yuasa et al., 2005). A previous study has shown that *MECOM* can repress TGF- β

signaling through interaction with the intracellular mediator *smad3* of TGF- β signaling to antagonize the growth arrest effect of the TGF- β signaling pathway, thereby regulating some pathophysiological events, such as cell proliferation, differentiation and apoptosis. In addition, *MECOM* can also interact with a variety of proteins that regulate gene expression and signal transduction to participate in the signaling pathway, such as the Notch and TGF- β signaling pathway (Hohenauer and Moore, 2012; van Donkelaar et al., 2018). This research is expected to discover the expression of *MECOM* during the development of the cochlea and its function and mechanism during the regeneration of HCs.

2. Materials and methods

2.1. Neonatal mouse cochlear sensory epithelium culture

The animals were provided by Fudan Medical University (Shanghai, China). Anesthetized postnatal day 2 (P2) C57BL/6J mice were sacrificed, and the cochlea sensory epithelium was separated from the spiral ganglia. The DMEM/F12 medium (Invitrogen) was used, supplemented with B27 and N2 solutions (Invitrogen). The organ of Corti was transferred onto 10 mm coverslips coated with CellTak (Biosciences, Franklin Lakes, NJ, USA) and filled with 100 μ l nutrient solution. After 12 h, neomycin (1 mM) was added to the nutrient solution for another 12 h. Subsequently, the culture medium containing Ad-*MECOM* (1:200) was added for 3 days to test transfection efficiency. Finally, to assess SC proliferation and HC regeneration with *MECOM* overexpression, Ad-*MECOM* was added to the medium for 5 days. 5-Bromo-2-deoxyuridine (BrdU) was added for labeling during the entire culture period. The medium was replaced every 24 h.

2.2. Frozen sections

Eight-week-old female and male mice were housed in a cage at a ratio of 2:1, i.e. 6 female and 3 male mice. Timing was defined as Embryonic day (E) 0.5 when a vaginal plug was detected. Embryos ranging from day E9.5 to E18.5 were separated from pregnant mice. Subsequently, the embryos were fixed with 4% PFA for 0.5 h at 37 °C, then immersed in 15% sucrose and transferred to 30% sucrose. Finally, the embryos were embedded in the OCT and serially sectioned (10 μ m thick) with an OTF Cryostat (CM3050, Leica Microsystems, Germany) for Immunohistochemistry.

2.3. RT-PCR assay

GoTaq® qPCR Master Mix (Promega, USA) was used to perform Quantitative RT-PCR on an Applied Biosystem 7500 detection system (Applied Biosystems, USA). We used the $\Delta\Delta$ CT method with housekeeping gene *GAPDH* as a control to analyze gene expression. The specific primers were as follows: *MECOM* forward: AAGTAATGAGTGTGCCTATGGC; reverse: AGTTGACTCTCGAAGCTCAAAC.

2.4. Western blot assay

RIPA buffer was used to obtain the desired protein extract, and

Table 1
Primary and secondary antibodies used in immunohistochemistry.

Primary antibodies	Secondary antibodies
Polyclonal anti-Mecom (1:400; Santa Cruz Biotechnology)	Anti-mouse antibody conjugated to Alexa Fluor 488
Monoclonal anti-BrdU (1:400; ABD Serotech)	Anti-rabbit antibody conjugated to Alexa Fluor 488
Polyclonal anti-myosin VIIA (1:400; Proteus Biosciences)	Anti-goat antibody conjugated to Alexa Fluor 647
Polyclonal anti-SRY-box2 (1:400; Santa Cruz Biotechnology)	Anti-mouse antibody conjugated to Alexa Fluor 594

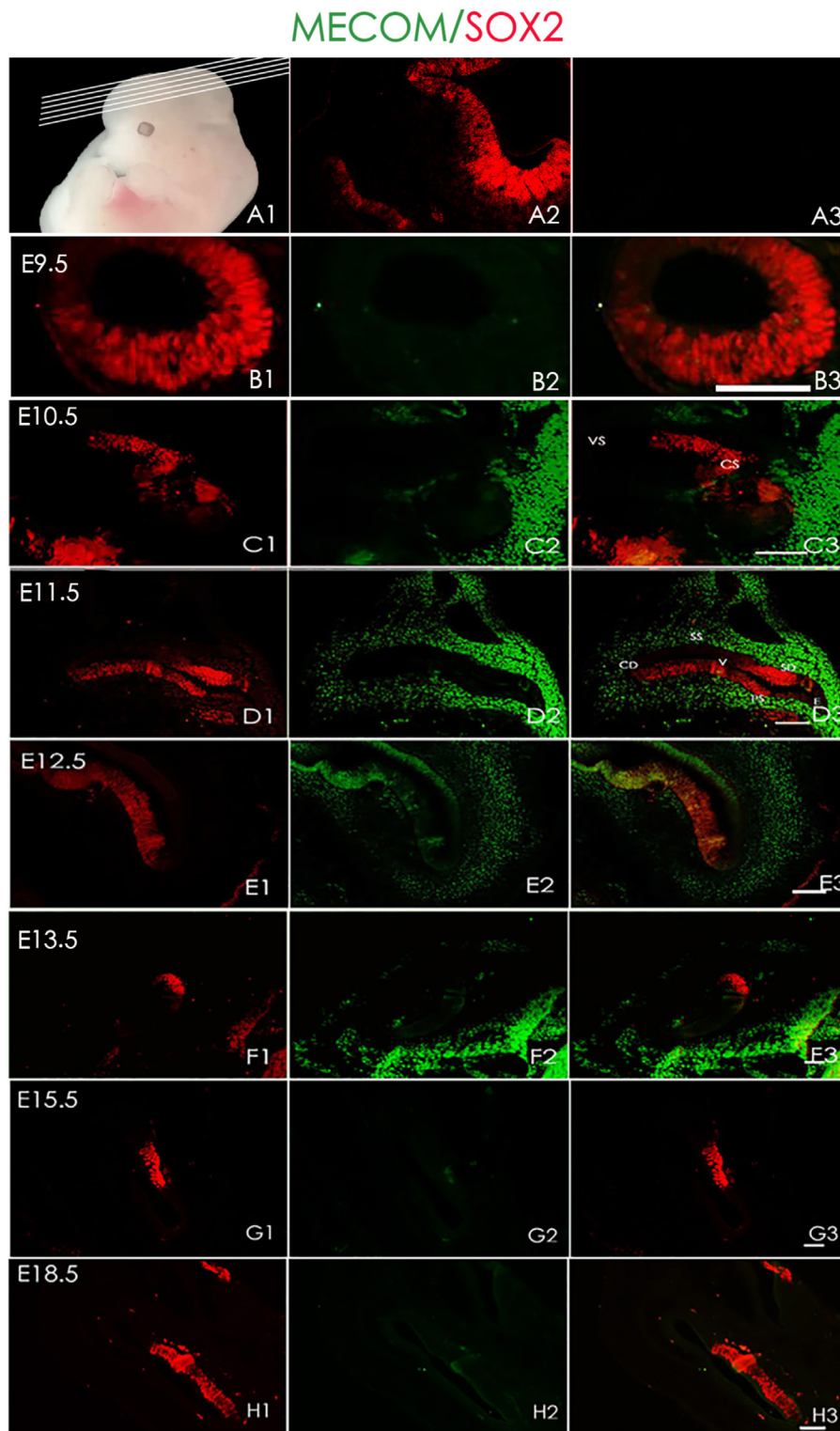


Fig. 1. Patterns of MECOM expression in the development of mouse cochlea. (A1–A3): The direction of frozen sections and negative controls; (B1–B3): No expression of MECOM; (C1–C3): MECOM expression (green) in the dorsal side of the cochlea and the vestibular primordium on day E10.5; (D1–D3): MECOM expression in periotic mesenchyme adjacent to sensory epithelium; (E1–E3): MECOM expression in the periotic mesenchyme and sensory epithelium on day E12.5; (F1–F3): MECOM expression in the inner ear and surrounding mesenchyme after day E13.5; (F1–G3): MECOM expression completely disappeared after day E15.5. Red: Sox2; Green: MECOM. OC: otocyst; VS: vestibular sacs; CS: cochlea capsule; CD: cochlea duct; SS: superior semicircular canal; PS: posterior semicircular canal; E: endolymph; V: vestibule; CL: cochlea; LS: lateral semicircular canal; UT: utricle; SA: saccule. Scale: 50 μ m.

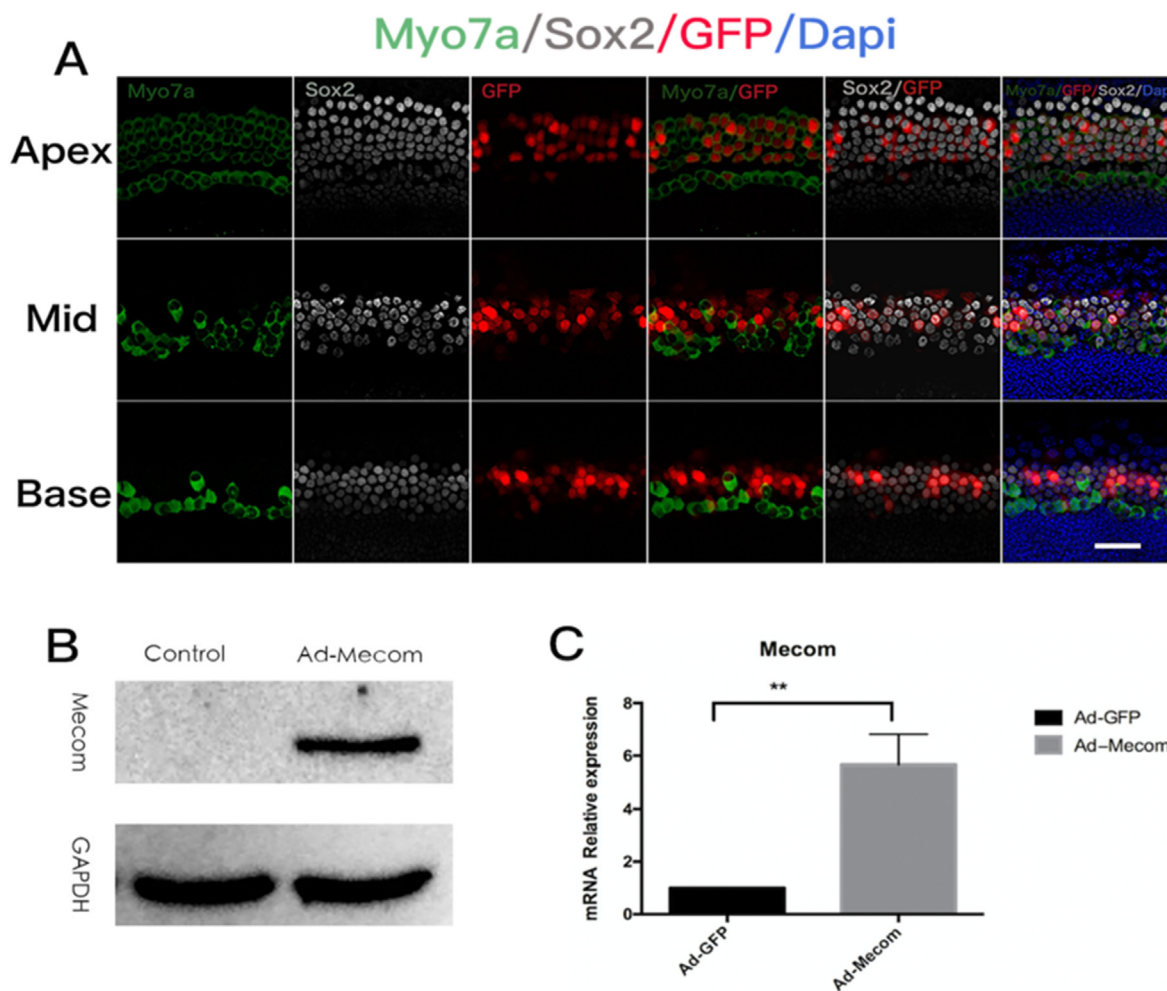


Fig. 2. Adenovirus infection in the sensory epithelium of the cochlea. (A) Adenovirus infection after three days of incubation in the three turns. Efficiencies of SC infection in the three rows: Apex: 7.2 ± 4% (n = 4), 50 ± 3.3% (n = 4) and 42 ± 6.3% (n = 4); Middle turn: 30.7 ± 2.7% (n = 4), 2.1 ± 1.9% (n = 4) and 0%; (B) Western Blot analysis showing significantly increased expression of MECOM protein compared to the control; (C) level of gene expression of *Mecom* after Ad-MECOM incubation. Scale bar = 50 μm. Red: Virus fluorescence; Gray: Sox2; Green: Myo7a. **P < 0.01; *P < 0.05.

concentration was measured using the BCA protein kit. The extract was boiled, separated and transferred to PVDF membranes (Biorad, Shanghai, China). Blocked for 1 h, PVDF was incubated overnight with anti-GAPDH (1:1000; Kangcheng, Shanghai, China), anti-MECOM (1:800; Santa Cruz Biotechnology). Then the blot was treated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) for 1 h, and was finally detected by X-Omat X-ray film.

2.5. Immunohistochemistry

For immunohistochemistry analysis, we fixed the tissue with 4% paraformaldehyde for 0.5 h, which was then incubated with a blocking solution for another 1 h. We used the primary and secondary antibodies in Table 1 and visualized the cell nuclei with 4',6-diamidino-2-phenylindole (1:800, Sigma-Aldrich). A negative control was prepared by omitting the primary antibodies in staining.

2.6. Statistical analysis

All tests were repeated at least four times independently. The SPSS 19.0 software was used to analyze the comparison between

the two groups. P < 0.05 was considered as statistically significant.

3. Results

3.1. MECOM expression pattern in development of cochlea

To investigate the function of MECOM in SC proliferation and trans-differentiation, we first examined the expression of MECOM during cochlear development. We observed changes in the level of MECOM expression from day E9.5 to E18.5. When the expression of Sox2 was visible on the dorsolateral and ventromedial sides of the otic vesicle, MECOM was not detected in the inner ear (Fig. 1 B1–B3). Next, the expression of MECOM was captured on the dorsal side of the cochlea and vestibule on day E10.5 when Sox2 was observed in the dorsolateral side of the otic vesicle and cochlea capsule (Fig. 1 C1–C3). MECOM was mainly located in the periotic mesenchyme between day E10.5 and E13.5 (Fig. 1 C1–E3). On day E12.5, MECOM was expressed in both periotic mesenchyme and sensory epithelium where Sox2 was also expressed (Fig. 1 E1–E3). Furthermore, from day E13.5 on, the fluorescence signal of MECOM in the cochlea and surrounding mesenchyme gradually weakened and finally disappeared on day E15.5 (Fig. 1 F1–F3, G1–G3). The dynamic spatiotemporal distribution patterns of MECOM suggested

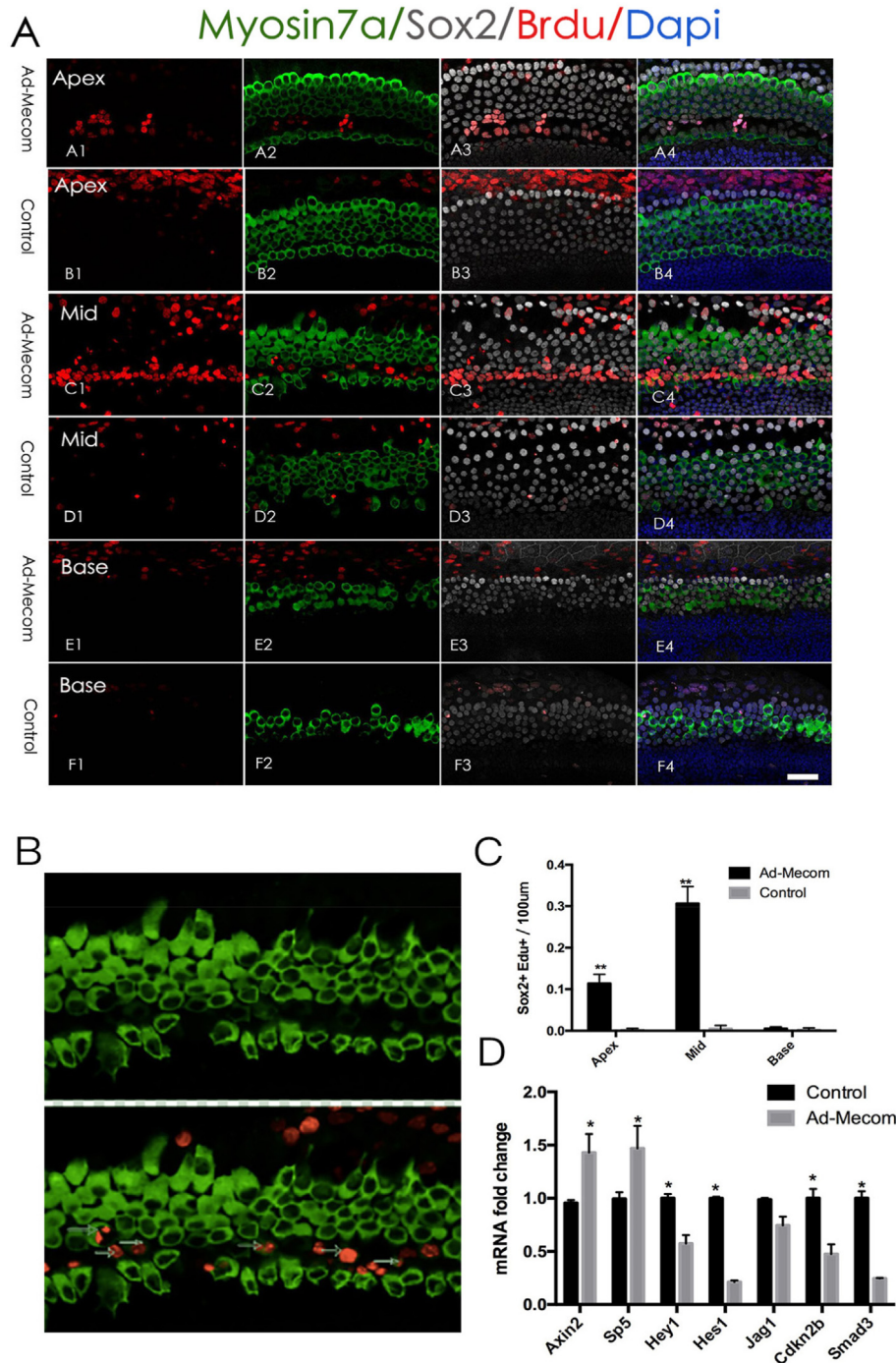


Fig. 3. MECOM promotes the proliferation and differentiation of SCs into HCs. (A–B) Compared with Ad-empty controls, Sox2+/BrdU + double-labeling cells are visible in significant numbers in the apical and middle turns. Myo7a+/BrdU + cells appeared following MECOM overexpression in the middle turn; (C) After Ad-MECOM treatment, the number of Sox2+/BrdU + double labeling cells increased significantly in the apical and middle turns ($P < 0.05$). (D) qPCR showing down-regulated *Hes1* and *Hey1*, *Smad3* and *Cdkn2B* and up-regulated *Sp5* and *Axin2*.

that it might be correlated with the development of HCs, hence its involvement in the proliferation and differentiation of sensory cells and the maturation of HCs.

3.2. Effects of MECOM overexpression on supporting cells

We constructed an adenovirus-MECOM vector. The EGFP/Sox2

and EGFP/Myosin7a double labeling used immunofluorescence staining to show the distribution and efficiency of infection in HCs and SCs (Fig. 2A). The infection efficiency was $7.2 \pm 4\%$, $50 \pm 3.3\%$, and $42 \pm 6.3\%$ in SCs in the three turns, respectively, and $30.7 \pm 2.7\%$, $2.1 \pm 1.9\%$ and 0% in HCs (Fig. 2A). We performed qPCR and Western Blot analysis to confirm MECOM overexpression in the cochlea (Fig. 2B and C).

3.3. Influence of MECOM in SCs proliferation and differentiation and mechanisms

After confirming the overexpression of MECOM, its roles in the proliferation of SCs was investigated by counting Sox2+/BrdU + double labeled cells. The rate of SC proliferation with Ad-MECOM treatment was $11.4 \pm 2.2\%$ ($n = 4$) and $30.7 \pm 4.1\%$ ($n = 4$) in the apical and middle turns, respectively, in contrast to $0.2 \pm 0.4\%$ ($n = 4$) and $0.5 \pm 0.8\%$ ($n = 4$), respectively, in the control group ($P < 0.05$). Compared with the control group, the number of proliferative SCs markedly increased in the apical and middle turns, indicating that SC proliferation was promoted by MECOM overexpression (Fig. 3A and C). However, no significant differences were seen in the basal turn. To examine the role of MECOM in the trans-differentiation of SCs, we counted myosin7a⁺/BrdU⁺ double-labeled cells and found that their number arose in the middle turn, which usually does not appear in the cochlea (Fig. 3B), indicating that MECOM overexpression might induce SC differentiation into HCs. We performed qPCR to investigate the mechanism of MECOM overexpression after HC injury and found increased expression of *Sp5* and *Axin2*, which are downstream genes of Wnt signaling. Conversely, Notch signaling downstream genes, including *Hes1* and *Hey1*, decreased, while the TGF- β signaling-related genes, *Smad3* and *Cdkn2B*, were also down-regulated (Fig. 3D). In summary, the proliferation and differentiation of SCs appeared following overexpression MECOM, which may be due to activation of Wnt and TGF- β signaling and inhibition of Notch signaling.

4. Discussion

Deafness is one of the most common human diseases, which seriously affects the health of patients. Clinically, deafness is mainly caused by abnormal inner ear development, the treatment methods of which are relatively limited. Therefore, looking for more genes that cause deafness, exploring their key regulatory mechanisms, and finding and developing specific therapeutic agents for the pathogenic mechanisms are important research directions for clinical interventions in hereditary inner ear malformations and deafness. In the mammalian inner ear, HCs in the organ of Corti are responsible for transducing mechanical sound stimulation into cochlear electric signals (Gao et al., 2019; Liu et al., 2019b; Qi et al., 2019; He et al., 2019), while spiral ganglions relay information from HCs to higher auditory centers in the central nervous system (Guo et al., 2019, 2020, 2021b, 2021c; Hu et al., 2021; Liu et al., 2021b; Wei et al., 2021). Hearing dysfunction is often characterized by the loss of HCs in the cochlea. Unlike non-mammalian species, mammals cannot regenerate HCs spontaneously after injury (Dabdoub and Nishimura, 2017; Cox et al., 2014; Wang et al., 2015; Chai et al., 2012; Waqas et al., 2016b). Thus, inducing SCs to division and trans-differentiation into HCs is a potentially promising method to rescue hearing loss caused by HCs damage (You et al., 2018).

In our study, we firstly observed that MECOM was mainly located in the periotic mesenchyme between days E10.5 and E13.5. Then, we constructed an adenovirus vector to overexpress MECOM in the sensory epithelium. Following overexpression of MECOM, a large number of SCs was seen in the cochlea sensory epithelium at the apical and middle turns. We also found that regenerating HCs appeared in middle turns, which normally does not occur, and was most likely to be the result of differentiation from SCs. These results indicate that MECOM may induce HC regeneration to restore cochlear function. We performed qPCR to investigate the expression changes of Wnt signaling downstream genes, Notch signaling downstream genes and TGF- β signaling-related genes, and found activation of Wnt and TGF- β signaling and inhibition of Notch

signaling in the presence of MECOM overexpression. Overall, our data showed MECOM-promoted proliferation of the SCs with significant changes in transcription levels of TGF β /BMP signaling pathway related genes. These findings indicate that MECOM is likely to coordinate and cooperate with Smad3 and Cdkn2b to regulate TGF- β signaling, thereby inducing the proliferation and differentiation of SCs.

In the field of HC regeneration, research has focused on HC regeneration in vertebrates and its ability to restore auditory function. At the same time, studying molecular mechanisms that control regeneration capabilities provide the hope to find new prospects of treatment for hearing loss. Our research is the first to demonstrate that MECOM overexpression can potentially induce proliferation of SCs and stimulate regeneration of HCs. Therefore, our findings carry important clinical significance for hearing loss caused by HC damage. MECOM may be a potential therapeutic target to induce HC regeneration after HC loss.

The usage of MECOM may become a non-invasive therapy in future clinical applications for appropriate hearing disorder indications and can potentially improve research methods. Utility of MECOM may continue to be a promising research tool in the development of new treatments for hearing impairment caused by HCs damage. However, our study did not address in-depth mechanisms. We believe that MECOM gene knock out at different stages of inner ear development may be used to further study its effects on inner ear development and its mechanisms in detail in future studies, which may open up new fields and lead to new ideas in the research of inner ear development and HC regeneration.

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