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AAV-regulated *Serpine2* overexpression promotes hair cell regeneration

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Inner ear hair cell (HC) damage is irreversible in mammals, but it has been shown that supporting cells (SCs) have the potential to differentiate into HCs. *Serpine2*, a serine protease inhibitor, encodes protease nexin 1, and this has been suggested to be a factor that promotes HC regeneration. In this study, we overexpressed *Serpine2* in inner ear SCs cultured in two- and three-dimensional systems using the adeno-associated virus-inner ear (AAV-ie) vector, which promoted organoid expansion and HC differentiation. Overexpression of *Serpine2* in the mouse cochlea through the round window membrane (RWM) injection promoted SC proliferation and HC regeneration, and the regenerated HCs were found to be derived from Lgr5$^+$ SCs. Regenerated HCs have electrophysiological properties that are similar to those of native HCs. Notably, *Serpine2* overexpression promoted HC survival and restored hearing of neomycin-damaged mice. In conclusion, our findings indicate that *Serpine2* overexpression promotes HC regeneration and suggests that the utilization of inner ear progenitor cells in combination with AAVs might be a promising therapeutic target for hearing restoration.

INTRODUCTION

Worldwide, sensorineural hearing loss (SNHL) resulting from the damage of cochlear hair cells (HCs) or spiral ganglion neurons accounts for approximately 90% of all hearing loss types. The main causes of SNHL include ototoxic drug exposure, high-decibel noise, and age-related deafness.[1] Adult mammalian auditory HCs cannot spontaneously regenerate, and therefore hearing loss induced by HC damage is permanent.[2] Currently, there are no biological methods or Food and Drug Administration-approved drugs available for the treatment of hearing loss or regeneration of functional HCs in the mammalian cochlea. Therefore, the development of strategies and drugs to treat HC loss and facilitate HC regeneration in the mammalian cochlea is critical.

Ongoing research in the field of regenerative medicine has shown that supporting cells (SCs) in the mammalian cochlea can directly differentiate into HCs or can differentiate into HCs after first undergoing proliferation.[3] Inner ear SCs are thus considered to be progenitor cells for HC regeneration in mammals.[4] However, the number of HCs regenerated in young and adult mice was small and functionally immature, which was not sufficient to restore hearing. Therefore, there is a need for modified strategies to enhance the amount of regenerated HCs and to facilitate their maturation.

Recent studies have demonstrated the critical roles of transcription factors such as *Atoh1*,[5,6] *Pou4f3*,[7] *Gfi1*,[8] *Gata3*,[9] and *p27Kip1*[10] in HC regeneration. Among them, Atoh1 is a key transcription factor during HC development that efficiently *trans*-differentiates SCs into HCs. In addition, Notch and Wnt signaling pathways involved in

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HC development can also facilitate HC regeneration from SCs.[11,12] These studies suggest that it is possible to regenerate HCs. However, the small number of regenerated HCs and their immature function remain a major problem. Recent studies have shown that the co-regulation of multiple transcription factors and signaling pathways is a better strategy to facilitate HC regeneration and maturation. In addition, some genes such as *Pou4f3* and *Gfi1* have been shown to regulate the fate decision of regenerated HCs induced by *Atoh1*, thus promoting the maturation of neonatal HCs.[13] *Ikzf2* overexpression promotes outer hair cell (OHC) differentiation,[14] while *Tbx2* overexpression promotes inner hair cell (IHC) differentiation.[15] Because functional HC regeneration requires the coordinated regulation of multiple genes, the identification of effective genes for regenerating HCs is a significant focus for gene therapy for hearing restoration.

SERPINs are a superfamily of proteins that encode protease inhibitors, and most of these proteins have functional roles in the process of cell differentiation, fibrinolysis, and apoptosis by inactivating serine and cysteine proteases.[16] Homozygous mutations of *SerpinB6*, which encodes an intracellular protease inhibitor, have been linked to post-linguistic autosomal recessive non-syndromic hearing loss in humans, and deficiency of *SerpinB6* leads to SNHL in mice.[17] *Serpine2*, which has been the most studied member of the superfamily of SERPINs in recent years,[16] encodes the protease nexin-1, which has broad anti-protease activity against serine proteases thus affecting fibrinolysis.[18,19] Nexin-1 is expressed in a wide range of cell types, binds to the glycosaminoglycans on the surfaces of these cells, and inhibits the signaling function of thrombin.[20] Moreover, aberrant expression of *Serpine2* contributes to tumorigenesis and invasion in a variety of cancers, including breast cancer,[21] pancreatic cancer,[22] and hepatocellular carcinoma.[23] We previously separated $Lgr5^+$ SCs (which are thought to be progenitor cells of inner ear HCs) of the apex and base, $Lgr5^+/Lgr5^-$ SCs, and $Lgr5^+$ SCs of neomycin-treated and non-treated cochleae using fluorescence-activated cell sorting (FACS), and found that *Serpine2* had significant differences in expression in the different cell types of the three groups, suggesting that *Serpine2* might play a positive role in regulating HC regeneration.

To efficiently regulate *Serpine2* expression in SCs, we used AAV-ie, which has a transduction efficiency of more than 80% in various SC types, such as Deiters' cells, pillar cells, inner border cells, and inner phalangeal cells in mice. Additionally, AAV-ie can effectively mediate the up-regulation of Atoh1, which induces regeneration of HCs *in vivo*.[24] Due to the high safety of AAV-ie, it has a very promising potential for clinical application in inner ear HC regeneration. Using AAV-ie, we achieved efficient expression of exogenous *Serpine2* in organoids derived from cochlear progenitor cells and in the mouse cochlea. The *in vitro* organoid culture experiments demonstrated that AAV-*Serpine2* could effectively promote cochlear organoid formation and the differentiation of HCs in the organoids. We also found that overexpression of *Serpine2* using AAV-ie promoted the SC proliferation and *trans*-differentiation into HCs *in vivo*. The regenerated HCs have electrophysiological properties that are similar to those of native HCs. Notably, *Serpine2* overexpression promoted HC survival and restored hearing of neomycin-damaged mice. Our findings suggest that the use of AAVs to transduce SCs can regenerate HCs, thus providing a wider range of therapeutic approaches for reversing SNHL.

RESULTS

Serpine2 overexpression promoted the proliferation of inner ear progenitor cells in a 2D culture system *in vitro*

SCs in the inner ear sensory epithelium have characteristics of HC progenitors.[25] To probe the effect of *Serpine2* on inner ear progenitor cell proliferation *in vitro*, we cultured single-cell suspensions of inner ear epithelial tissue to obtain inner ear organoids. Control (AAV-mNeonGreen) and AAV-*Serpine2* (AAV-*Serpine2*-mNeonGreen) viruses were added during the organoid culture (Figure 1A). The diameter and number of organoids are usually used to determine the proliferative ability of stem cells in the inner ear,[26,27] and after *Serpine2* overexpression we found that the organoids' diameter was obviously increased, with the control group averaging 81.3 μm and the AAV-*Serpine2* group averaging 107.3 μm (Figures 1B and 1C). Previous research indicated that inner ear stem cells are capable of differentiating into HCs *in vitro*, among which $Lgr5^+$ cells make up the majority of the population.[28] The cochlear basement membranes of neonatal Lgr5-$EGFP^{CreER/+}$ mice were removed and isolated into individual cells. After digestion, $Lgr5^+$ cells were sorted by FACS for two-dimensional (2D) suspension culture (Figure 1D). The results were consistent with the previous results, demonstrating that *Serpine2* overexpression significantly increased the average organoid diameter, from 33.9 μm in the control group to 40.4 μm in the AAV-*Serpine2* group (Figures 1E and 1F). These results suggest that *Serpine2* can promote inner ear progenitor cell proliferation in the 2D culture system *in vitro*.

Serpine2 overexpression promoted the proliferation of inner ear progenitor cells in a 3D culture system *in vitro*

We further used Matrigel to simulate the three-dimensional (3D) environment to probe the effect of *Serpine2* up-regulation on organoids *in vivo*.[27,29–31] It has been shown that the ability of mouse cochlear epithelial cells to form organoids containing HCs decreases dramatically during the first week of life.[27] Therefore, we researched the influence of forced *Serpine2* expression on cochlear organoid proliferation in P1 and P4 mice, respectively. The same numbers of control and AAV-*Serpine2* virus particles were added the next day. On day 10, the number and diameter of organoids were significantly enhanced after *Serpine2* up-regulation compared with the control group. The control group had an average of 113 organoids and an average diameter of 39.5 μm, whereas the AAV-*Serpine2* group had an average of 444 organoids and an average diameter of 46.7 μm (Figures 2A–2D). EdU was added 1 h before the end of culture on the 10th day to label proliferating cells. Immunofluorescence staining for EdU (Figure 2E) showed that the percentage of EdU^+ organoids and the number of EdU^+ cells per organoid increased after *Serpine2* overexpression compared with controls. Specifically, the control group showed an

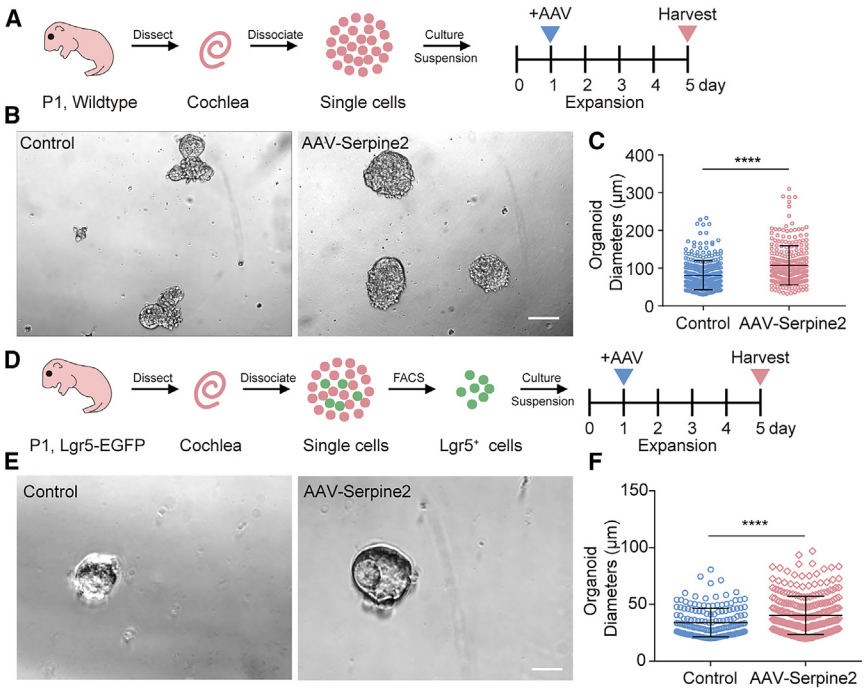

Figure 1. *Serpine2* overexpression promoted cochlear progenitor cell proliferation in two-dimensional cultures *in vitro*

(A) Experimental design. (B) Bright-field images of cochlear organoids in the expansion assay with the overexpression of AAV-mNeonGreen (control) and AAV-*Serpine2*. Scale bar, 100 μm. (C) The diameter of the organoids calculated from (B). (D–F) Similar analyses of Lgr5$^+$ cochlear progenitors by FACS as (A–C). Scale bar, 50 μm.

average of 65.3% EdU$^+$ organoids and an average of two EdU$^+$ cells per organoid, while the AAV-*Serpine2* group exhibited an average of 94.3% EdU$^+$ organoids and an average of seven EdU$^+$ cells per organoid (Figures 2F and 2G). Similarly, we researched the effect of *Serpine2* overexpression on progenitor cell proliferation in older neonatal mice (P4) (Figure 2H). Following *Serpine2* overexpression, the diameter of the organoids increased obviously from an average of 63.5 μm in the control group to 69 μm in the AAV-*Serpine2* group. However, the number of organoids showed no significant change, with averages of 125 and 112 for the control and AAV-*Serpine2* groups, respectively (Figures 2I and 2J). This observation suggested that the ability to form organoids is greatly reduced with increased age. Similarly, EdU immunofluorescence staining indicated that the percentage of EdU$^+$ cells in each organoid increased from an average of 11.1% in the control group to 25.2% in the AAV-*Serpine2* group (Figures 2K and 2L). These results suggest that *Serpine2* can promote inner ear progenitor cell proliferation in the 3D culture system *in vitro*.

We next investigated the mechanism that promotes the proliferative ability of cochlear organoids after *Serpine2* overexpression under 3D culture conditions. We performed qPCR on RNA extracted from virus-transduced organoids and found that *Serpine2* was overexpressed by about 5-fold (Figure 3A), and we analyzed the RNA by transcriptome sequencing (Figures 3B–3G). Pearson correlation indicated that the control and AAV-*Serpine2*-transduced organoids had high similarity (Figure 3B), and a Venn diagram showed that 1,653 and 1,633 genes were enriched in the control and *Serpine2* groups, respectively, and that 20,910 genes were expressed in the two groups (Figure 3C). The gene expression changes induced by *Serpine2* overexpression are shown in the volcano plot and heatmap (| log2FoldChange| > 1.0, p value <0.05) (Figures 3D and 3G). After *Serpine2* overexpression, 708 genes were down-regulated (blue) and 442 genes were up-regulated (red) compared with the control group (Figure 3D). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses showed that the SCs entered into the cell cycle and began to proliferate in response to *Serpine2* overexpression (Figures 3E and 3F). Differentially expressed gene (DEG) analysis indicated that genes related to the Wnt signaling pathway (*Sfrp2*), the cell cycle (*Aurka*, *Birc5*, *Ccnb2*, *Ccna2*, *Chek1*, *Mcm4*, *Mki67*, and *Rbl1*), transcription factors (*Nr2f2*), and cochlear function (*Lin28b* and *Otof*) were up-regulated (Figures 3G and 3H), suggesting that overexpression of *Serpine2* activated the Wnt signaling pathway, which facilitated SC division and primed them for differentiation.

Serpine2 overexpression promoted cochlear progenitor cell differentiation into HCs *in vitro*

First, we investigated the effect of *Serpine2* on the ability of inner ear organoids to differentiate into HCs in 2D cultures (Figure 4A). Myo7a is a marker for HCs, and staining for Myo7a indicated that the total number of HCs was significantly increased after *Serpine2* overexpression, with the control group averaging 228 and the AAV-*Serpine2* group averaging 437 (Figures 4B and 4C). Next, we analyzed the ability of P1 organoids to produce HCs in 3D cultures (Figure 4D). Organoids were transduced by control and AAV-*Serpine2* viruses, respectively, and Myo7a signal was observed in the organoids (Figure 4E). The counts and percentages of Myo7a$^+$ organoids (control: average 23; AAV-*Serpine2*: average 43 and control: average 1%; AAV-*Serpine2*: average 4%, respectively) and the numbers of Myo7a$^+$ cells per organoid (control: average 11; AAV-*Serpine2*: average 22) were significantly higher with *Serpine2* overexpression when the same number of cells was inoculated (Figures 4F–4H). Therefore, *Serpine2* overexpression can facilitate the differentiation of inner ear progenitors into HCs *in vitro*.

Serpine2 overexpression promoted HC regeneration *in vivo*

Inner ear SCs serve as HC precursor cells, which can differentiate into HCs in response to the regulation of different signaling pathways

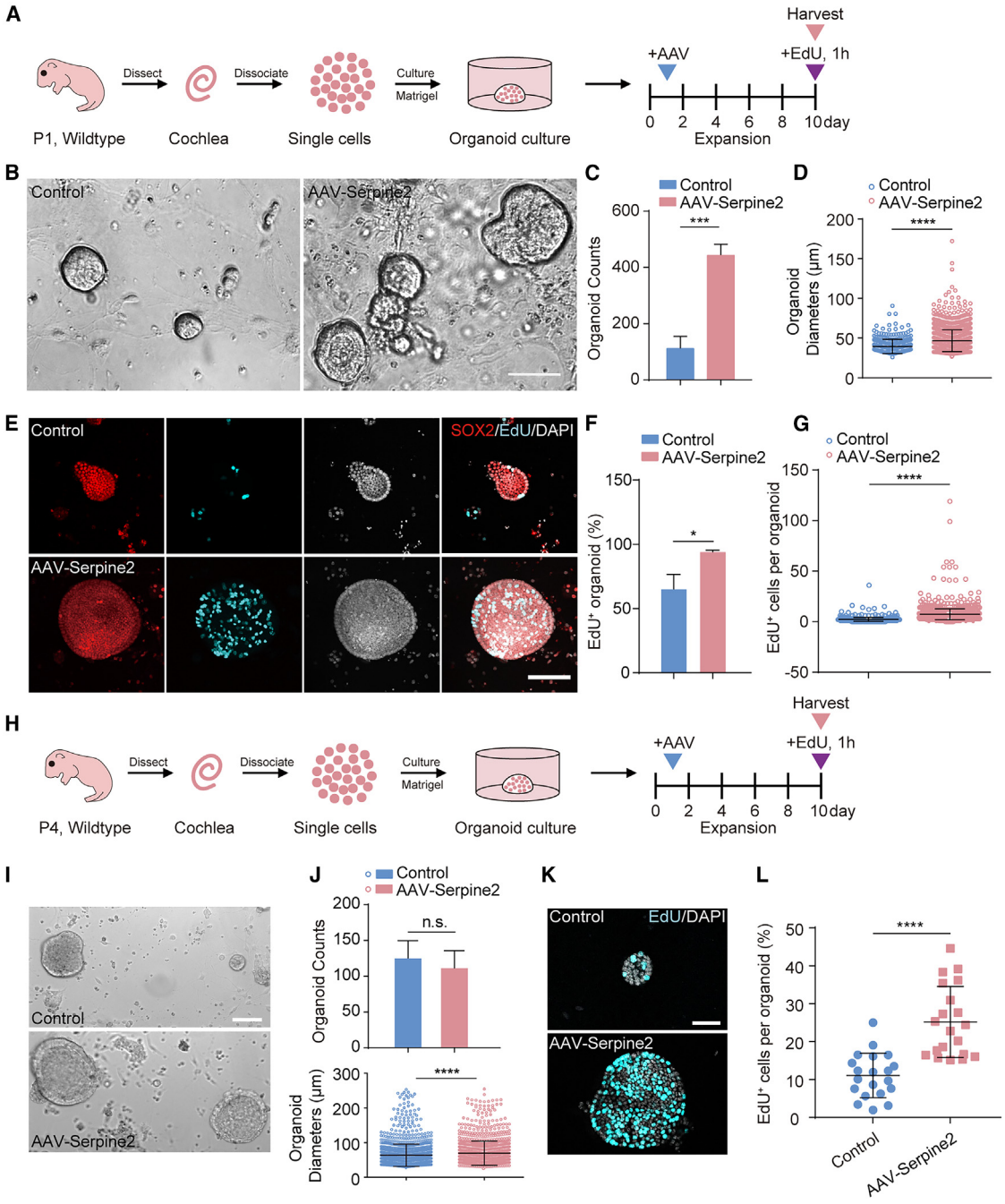

Figure 2. *Serpine2* overexpression promoted cochlear progenitor cell proliferation in three-dimensional cultures *in vitro*

(A) Experimental design. A single EdU incubation was performed for 1 h on day 10. (B) Bright-field images of cochlear organoids in the expansion assay with the overexpression of control and AAV-*Serpine2*. Scale bars, 50 μm. (C and D) The number (C) and diameter (D) of the organoids observed in (B). (E) Immunofluorescence images of the organoids in (B). Sox2 (red) marks SCs. EdU (cyan) marks proliferating cells. Nuclei are marked with DAPI (gray). Scale bar, 100 μm. (F and G) The percentage of EdU$^+$ organoids and the number of EdU$^+$ cells per organoid in (B). (H–J) Similar analyses of cochlear progenitors from P4 mice in the expansion assay as (A–G). Scale bar, 100 μm. (K) Immunofluorescence images of the organoids in (I). Scale bar, 50 μm. (L) The proportion of EdU$^+$ cells per organoid in (K).

including Wnt, Notch, and Atoh1.[5,11,28] The *in vitro* experiments described above showed that *Serpine2* overexpression can facilitate inner ear progenitor proliferation and differentiation into HCs. Therefore, we further investigated whether *Serpine2* promotes HC reprogramming in mice. Control and AAV-*Serpine2* viruses were injected into P1 wild-type neonatal mice through the RWM. On days

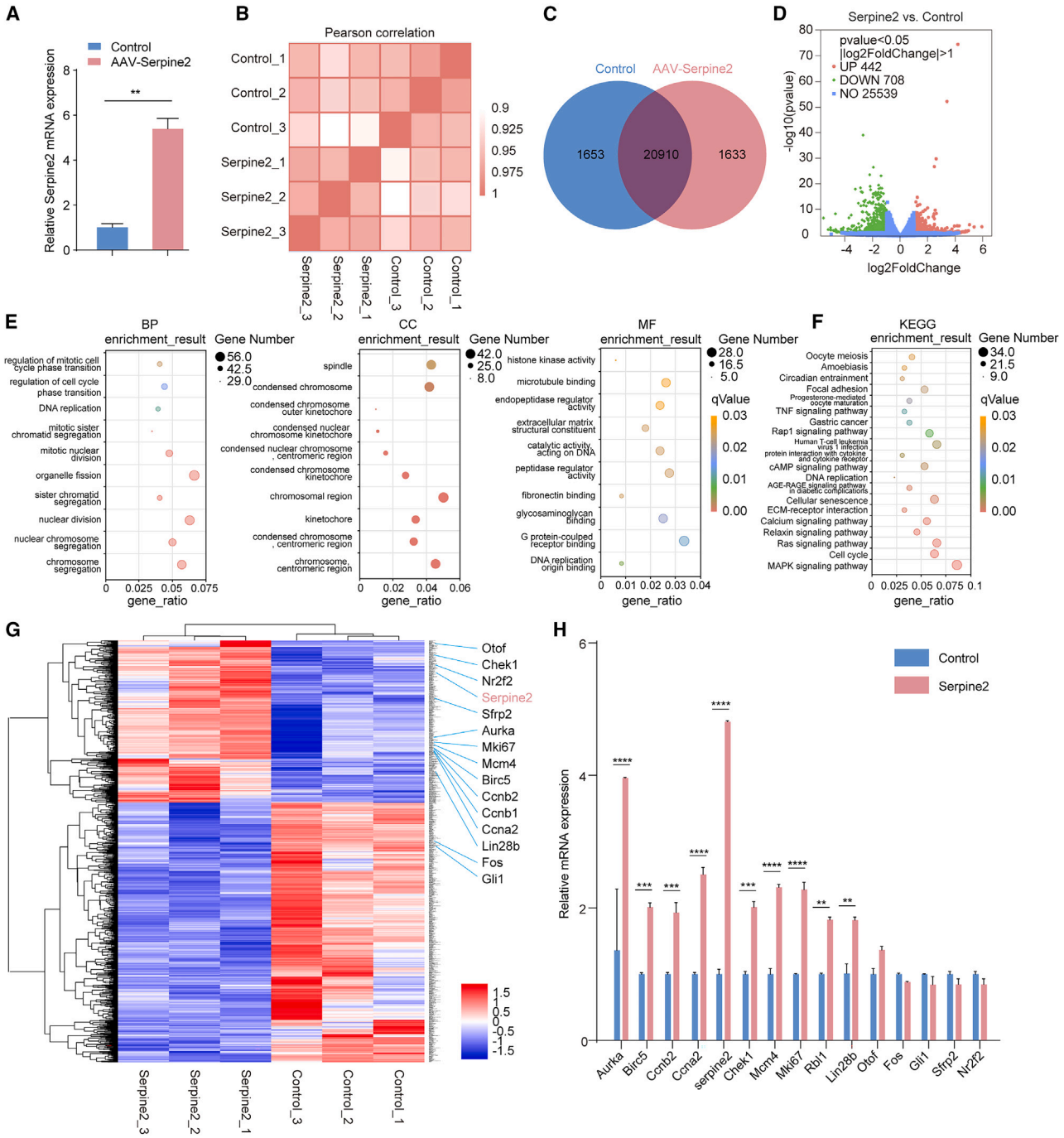

Figure 3. *Serpine2* overexpression promoted cochlear progenitor cell proliferation through the Wnt signaling pathway

(A–G) RNA sequencing data from cochlear organoids injected with control and AAV-*Serpine2*, respectively. (B) Correlation analysis between control and AAV-*Serpine2*-transduced organoids. (C) Venn diagram analysis of differential gene expression. (D) The volcano map shows the overall profile of the DEGs. Up- and down-regulated genes are indicated by red and green dots, respectively, and genes with no statistically significant difference in expression are indicated by blue dots. The screening standards for DEGs were |log2FoldChangel > 1.0, *p* value <0.05. (E) Significantly enriched GO terms of DEGs in *Serpine2*-overexpressing organoids. (F) KEGG analysis between control and AAV-*Serpine2*-transduced organoids. (G) Heatmap of the DEGs. (H) The DEGs identified in (G) were verified by qPCR.

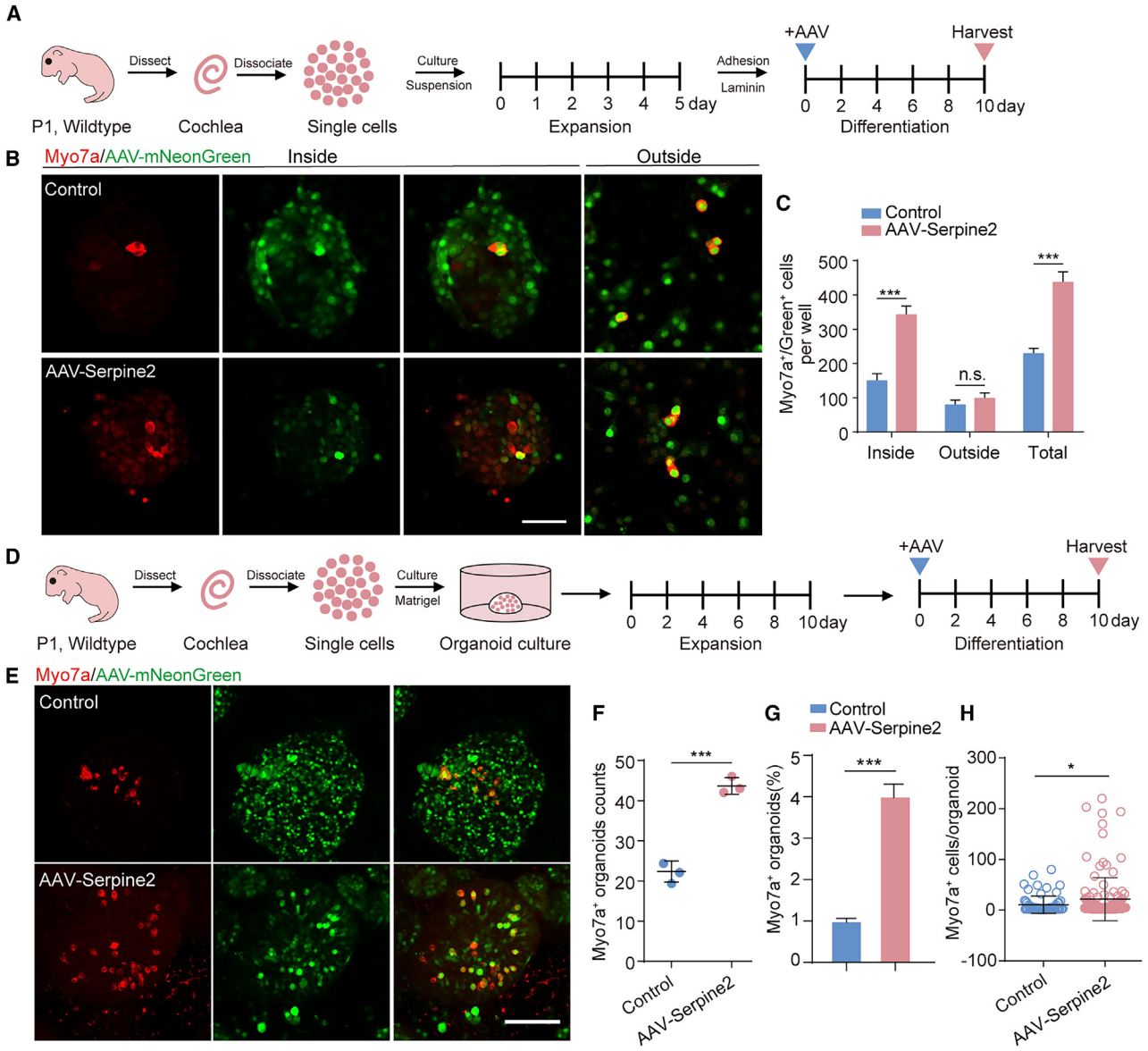

Figure 4. *Serpine2* overexpression promoted cochlear progenitor differentiation into HCs in two- and three-dimensional cultures *in vitro*

(A) Experimental design (two-dimensional differentiation assay). (B) Immunofluorescence staining of differentiated organoids inside and outside of the colony in control and AAV-*Serpine2* overexpression groups. Myo7a (red) marks HCs. AAV-Green (green) marks cells transduced by virus. Scale bar, 50 μm. (C) The numbers of Myo7a$^+$/Green$^+$ cells inside or outside the organoid per well in (B). (D) Experimental design (three-dimensional differentiation assay). (E) Immunofluorescence staining of differentiated organoids in the control and AAV-*Serpine2* overexpression groups. Myo7a (red) marks HCs. AAV-Green (green) marks cells transduced by virus. Scale bar, 100 μm. (F–H) The number of Myo7a$^+$ organoids, the percentage of Myo7a$^+$ organoids, and the amount of Myo7a$^+$ cells in every well in (E).

2–4, the EdU solution was injected subcutaneously, and the cochleae were collected at P7 or P14 (Figure 5A). Immunofluorescence staining indicated that the number of Sox2$^+$/EdU$^+$ cells in cochlear epithelial tissues after *Serpine2* up-regulation was obviously higher than control at P7, with the control group averaging 0 and the AAV-*Serpine2* group averaging 9 (Figures 5B and 5C), indicating that *Serpine2* overexpression could promote SC proliferation *in vivo*. Furthermore, the occurrence of ectopic HCs was observed in the vicinity of the native HC region (Figure 5E). Equal amounts of control and AAV-*Serpine2* viruses (8.1×10^{10} GCs) were injected into P1 wild-type mice cochlea. Immunofluorescence was observed at P7, and the results showed ectopic HCs near IHCs in both the *Serpine2* overexpression group and the control group, but the total number was small and no difference, with the control group averaging 3 and the AAV-*Serpine2* group averaging 5 (Figures 5E and 5F). We suspected that a low number of virus particles resulted in the low expression of

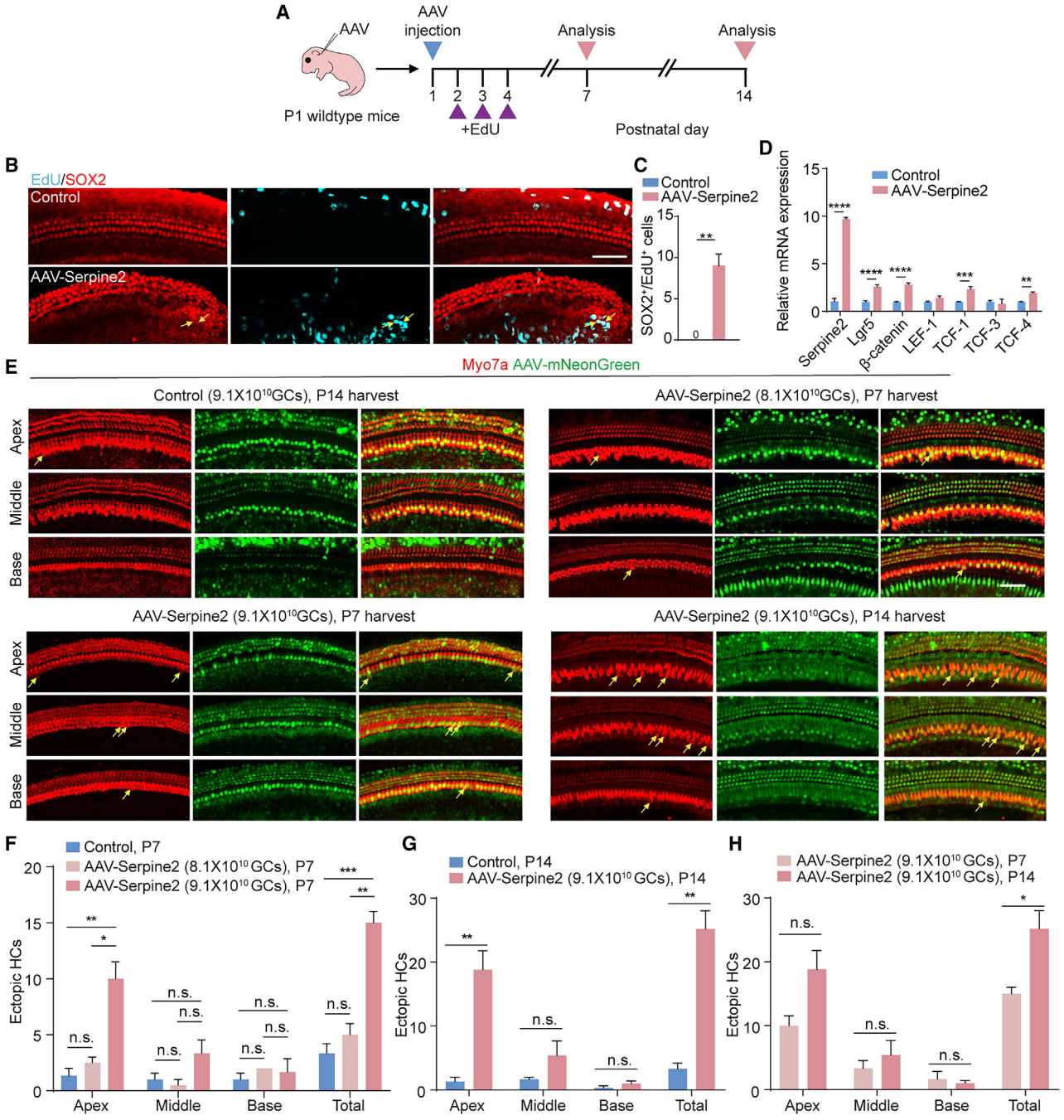

Figure 5. *Serpine2* overexpression promoted cochlear progenitor cell proliferation and HC regeneration *in vivo*

(A) Experimental design. (B) EdU immunostaining of AAV-transduced cochlear epithelia. $EdU^+/Sox2^+$ pillar cells are marked by yellow arrows. EdU (cyan) marks proliferating cells. Sox2 (red) marks SCs. AAV dose: 9.1×10^{10} GCs per cochlea. Scale bar, 50 μm. (C) The amount of $EdU^+/Sox2^+$ cells in (B). (D) qPCR results of relative mRNA expression of *Lgr5*, *β-catenin*, *LEF-1*, *TCF-1,* and *TCF-4* in P14 cochleae after *Serpine2* overexpression. (E) Immunofluorescence images of cochleae from P7 or P14 mice injected with control and AAV-*Serpine2* with different viral titers (8.1×10^{10} GCs and 9.1×10^{10} GCs per cochlea) at P1. Ectopic HCs are marked by yellow arrows. Scale bars, 50 μm. (F–H) The numbers of ectopic HCs in P7 and P14 cochleae in (E).

exogenous *Serpine2*, and thus it could not effectively induce HC reprogramming. Therefore, we increased the quantities of AAV-*Serpine2* virus particles (9.1×10^{10} GCs) and again performed immunofluorescence observation at P7. The amount of ectopic HCs in the middle (Control: average 1; AAV-*Serpine2*: average 3) and basal (Control: average 1; AAV-*Serpine2*: average 2) IHC area increased

after *Serpine2* overexpression, and in the apical turn (Control: average 1; AAV-*Serpine2*: average 10), it was significantly greater than control (Figures 5E and 5F). The total amount of ectopic HCs in the cochlea was also obviously higher at P14 than at P7, from an average of 15 in the P7 group to an average of 25 in P14 group (Figures 5E–5H). Therefore, *Serpine2* overexpression can promote cochlear SC division and HC regeneration *in vivo*. Lgr5 is a cell membrane receptor for Wnt signaling and is considered a marker for inner ear stem cells.[32,33] The TCF/LEF transcription factor is the downstream target of the canonical Wnt/β-catenin signaling pathway.[34,35] Therefore, we performed qPCR on cochlear samples from P14 mice injected with control and AAV-*Serpine2* viruses, and found that *Serpine2* overexpression increased the expression of *Lgr5*, *β-catenin*, *LEF-1*, *TCF-1,* and *TCF-4* in the mouse cochlea (Figure 5D). Therefore, *Serpine2* overexpression might promote inner ear HC regeneration through Wnt signaling pathway activation *in vivo*.

Furthermore, AAV-*Serpine2* was injected into the cochlea of P21 wild-type adult mice, and immunofluorescence staining was performed on the cochleae at P30 (Figures S1A and S1B). The results demonstrated no significant difference in *Serpine2* overexpression compared with the control group, with approximately five ectopic HCs observed (Figure S1C). This lack of difference is likely attributed to the low transduction efficiency of AAV-ie in adult mice, with no current serotype exhibiting high transduction efficiency in adult cochlea SCs. In addition, the long-term safety and efficacy of AAV-ie-mediated *Serpine2* overexpression were investigated (Figure S2A). AAV-*Serpine2* was injected into the cochlea of P1 wild-type neonatal mice, followed by auditory brainstem response (ABR) testing at P60. Results revealed no significant difference in ABR thresholds between the injected and uninjected ears (Figure S2B). Subsequent immunofluorescence staining of the cochleae demonstrated that following *Serpine2* overexpression, the number of ectopic HCs ranged from 0 to 2, showing no significant difference from the control group (Figures S2C and S2D). The relative mRNA expression of immune-related inflammatory factors in the cochlea was also assessed after *Serpine2* overexpression, with no significant differences observed compared with the control group (Figure S2E) and the *Serpine2* mRNA expression in brain, liver, and kidney was not abnormal after *Serpine2* overexpression (Figure S2F). These findings suggest that our method is safe for long-term use; however, there was no effect on the long-term efficacy in newborn mice.

$Lgr5^+$ cells are inner ear progenitors and are the source of HC regeneration,[28,29,36] and the injection of tamoxifen in the Cre expression system will cause all subsequent HCs that are derived from $Lgr5^+$ SCs to show tdTomato red fluorescence.[28,37] Thus, we could trace the HCs that were derived from SCs after *Serpine2* overexpression using Lgr5-$EGFP^{CreER/+}$/Rosa26-$tdTomato^{loxp/+}$ mice. After tamoxifen injection at P1, the virus was injected and the cochleae were harvested for immunofluorescence staining at P7 (Figure 6A). tdTomato-positive HCs were detected in both control and *Serpine2*-overexpressing cochleae (Figure 6B), and the number of $Myo7a^+$/$tdTomato^+$ OHCs and IHCs increased significantly after *Serpine2* overexpression (control: average 179; AAV-*Serpine2*: average 325 and control: average 31; AAV-*Serpine2*: average 57, respectively) (Figures 6B and 6C). These results show that *Serpine2* overexpression can facilitate the *trans*-differentiation of inner ear SCs into HCs and that the regenerated HCs are derived from $Lgr5^+$ SCs.

Serpine2 overexpression promoted functional maturation of regenerated HCs *in vivo*

To estimate the maturity of the regenerated cochlear HCs, electrophysiological recordings were conducted using a whole-cell patch-clamp to compare the functionality of ectopic HCs to native HCs. We recorded the voltage potentials of native HCs and ectopic HCs from AAV-*Serpine2* mice at P7 and found that the native HCs generated more action potentials in comparison with ectopic HCs (Figure 7A). Next, we examined voltage-gated K current (IK) in native HCs and ectopic HCs from AAV-*Serpine2* mice and observed the presence of fast-inactivating K^+ channels mediating currents (IK^a) was detected in ectopic HCs (Figure 7B). However, the inward rectifying K current (IK_{in}) was diminished in ectopic HCs, a feature of immature IHCs. Meanwhile, the resting membrane potentials (RMPs) of ectopic HCs and natural HCs were quite similar, with ectopic HCs showing a potential of -29.417 ± 1.633 mV (mean ± SEM, $p = 0.0818$) and native HCs at -35.78 ± 2.857 mV (Figure 7C). Of note, statistics results demonstrated that the rapid component (Figure 7D) associated with the low component (Figure 7E) of averaged potassium currents were decreased more gradually by current injections than that of the native HCs. In summary, these results indicate that ectopic HCs produced by overexpression of *Serpine2* could in part gain functions that mimic the native HCs, underlying AAV-mediated gene regulation of HC regeneration by SC *trans*-differentiation has a promising prospect for clinical treatment of hearing loss.

Serpine2 overexpression promoted HC survival after neomycin injury *in vivo*

Neomycin, an aminoglycoside antibiotic, is known for its ototoxicity.[38] We investigated the effects of AAV-*Serpine2* on a neomycin-damaged mouse model (Figure 8A). AAV-*Serpine2* was administered via injection into the cochlea of P1 wild-type mice, followed by daily neomycin injections (200 mg/kg) from P8 to P14. After 7 days, ABR testing was conducted. Results revealed that, compared with the uninjected ear, ABR thresholds in the injected ear were significantly lower at low frequencies, with the greatest recovery achieving 35 dB at 8 kHz (Figure 8B). Additionally, immunofluorescence analysis indicated substantial HC survival in the cochlea, with an average of 62 OHCs per 100 μm in the uninjected group and an average of 76 OHCs per 100 μm in the injected group (Figures 8C and 8D). These findings suggest that overexpression of *Serpine2* enhances HC survival and facilitates hearing recovery in neomycin-damaged mice.

DISCUSSION

Hearing loss affects a large portion of the global population and is an important issue that needs to be addressed. The inability of mammalian inner ear sensory HCs to be regenerated after death or injury makes hearing loss an irreversible process, and the main treatment

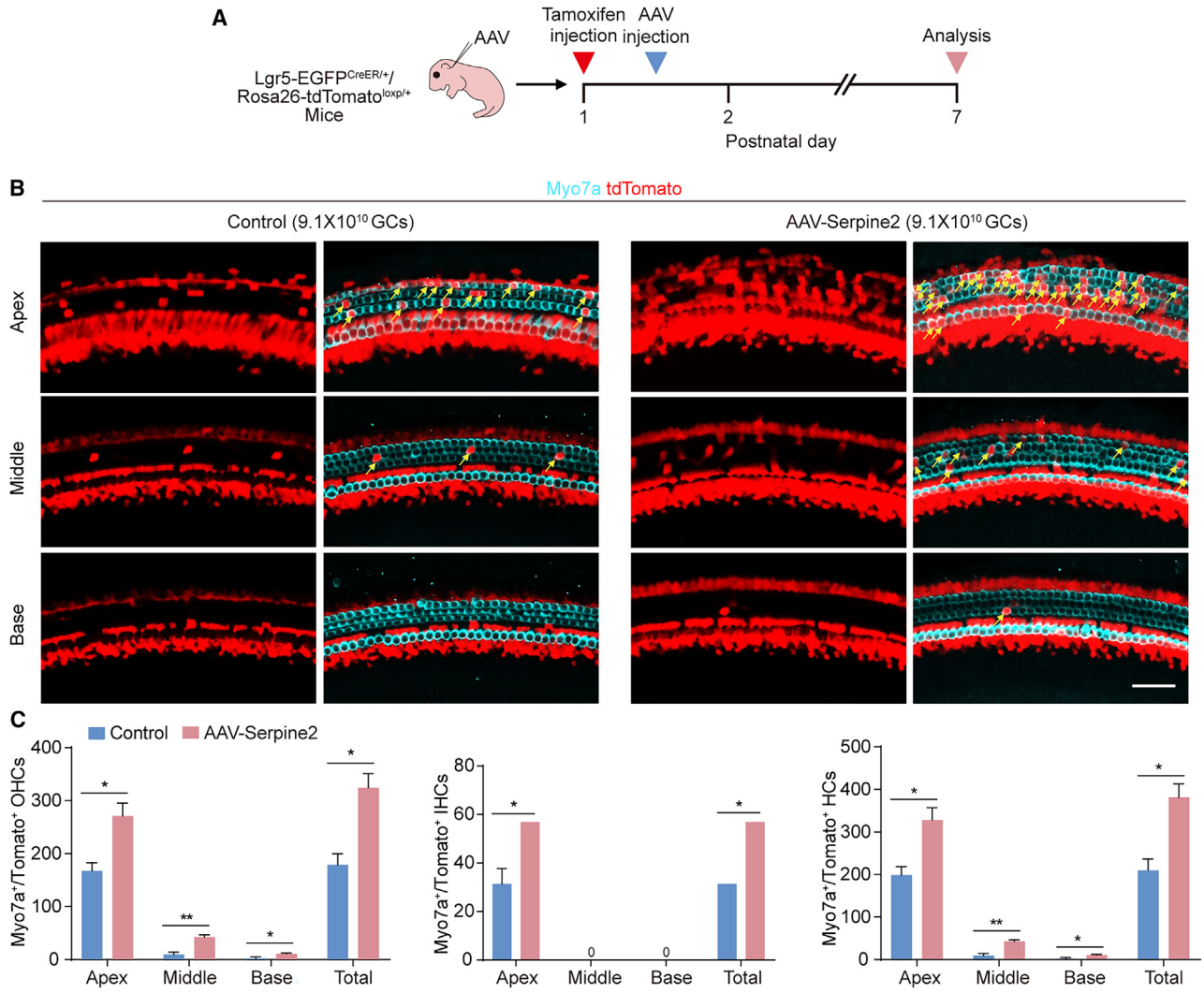

Figure 6. *Serpine2* overexpression promoted Lgr5⁺ progenitor cell *trans*-differentiation into HCs *in vivo*

(A) Experimental design. P1 Lgr5-EGFP$^{CreER/+}$/Rosa26-tdTomato$^{loxp/+}$ mice were injected with tamoxifen intraperitoneally. AAV viruses were injected 12 h later through the RWM, and cochleae were collected at P7. (B) Immunofluorescence images of cochlear epithelia transduced by control and AAV-*Serpine2* viruses. Myo7a⁺/tdTomato⁺ HCs are marked by yellow arrows. Scale bar, 50 μm. (C) The number of Myo7a⁺/tdTomato⁺ OHCs, IHCs, and HCs in every cochlea.

strategies for hearing loss include devices such as cochlear implants and hearing aids. However, these are not universally available and, most importantly, do not address the underlying causes of the problem. Researchers have found that—unlike mammals—birds, fish, and amphibians are able to regenerate inner ear HCs.[39] SCs in birds, fish, and amphibians proliferate rapidly after HC loss, and these epithelial cells can differentiate into new HCs, suggesting that HC loss can be repaired through regenerative replacement mechanisms. Thus, we have begun attempts to develop gene therapies to regulate the expression of certain genes to achieve the regeneration of HCs from inner ear SCs and thus restore auditory function.

Serpine2, an extracellular serine protease inhibitor, is a multifunctional glycoprotein that functions in cellular activities such as vascularization and cellular matrix reconstruction.[40] We pre-screened *Serpine2* as a factor that may affect HC regeneration,[37,41,42] but there have been no reports of *Serpine2* in the field of inner ear HC regeneration. We used AAV-ie, which has a very high transduction efficiency for inner ear SCs,[24] as the vector to deliver the target gene *Serpine2*, and we found that overexpression of *Serpine2* indeed promoted inner ear HC regeneration (Figure 5).

The Notch and Wnt signaling pathways play crucial roles in inner ear SC proliferation and HC regeneration.[11,28] We performed RNA sequencing of the transcriptomes of proliferative cultured inner ear organoids (Figure 3), and GO and KEGG analyses showed that *Serpine2* overexpression supported entry into the cell cycle in a proliferative state. Differential gene expression analysis showed that the cell

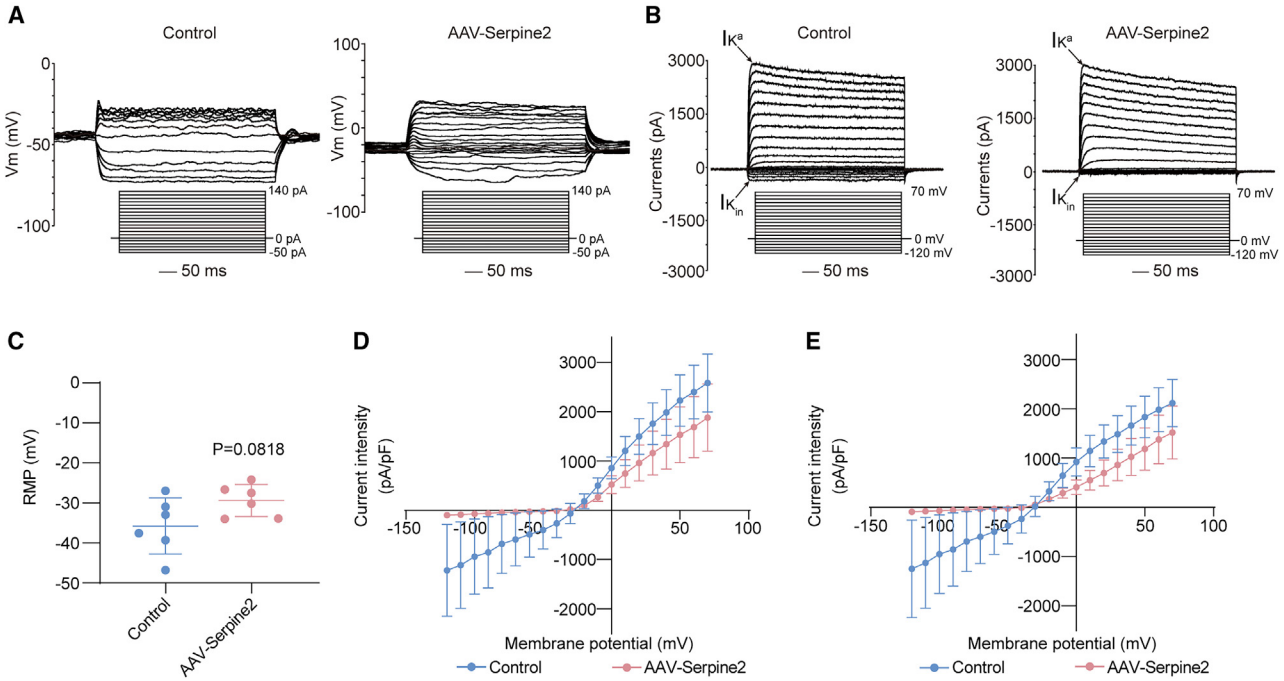

Figure 7. *Serpine2* overexpression promoted functional maturation of regenerated HCs *in vivo*

(A) Representative evoked spikes in native HCs and ectopic HCs from AAV-*Serpine2* mice were captured under current-clamp mode, respectively. Step current injection (from −50 to 140 pA, 10 pA per step) elicited spikes and voltage responses that were recorded. (B) Example of K^+ currents of native HCs and ectopic HCs from AAV-*Serpine2* mice recorded in voltage clamp mode, respectively. (C) Dot-plot showing the resting membrane potential (RMP) recorded in native HCs and ectopic HCs corresponding with (B). $n = 6$. (D and E) The I–V plot of averaged fast component (D) and low component (E) of the K^+ currents corresponding to (B). $n = 5$.

cycle-related gene (*Aurka*, *Birc5*, *Ccnb2*, *Ccna2*, *Chek1*, *Mcm4*, *Mki67*, and *Rbl1*) expression was up-regulated, suggesting that the inner ear SC proliferation was promoted by *Serpine2* overexpression. *Sfrp2* activates the Wnt-β-catenin signaling pathway,[43–45] and *Sfrp2* expression was up-regulated in proliferating cultured inner ear SCs after *Serpine2* overexpression, suggesting that the Wnt signaling pathway functions in promoting the inner ear SC proliferation. Meanwhile, *Serpine2* overexpression increased the mRNA expression of *Lgr5*, *β-catenin*, *LEF-1*, *TCF-1*, and *TCF-4*, which were the downstream targets of canonical Wnt/β-catenin signaling pathway, in the mouse cochlea (Figure 5D). Thus, *Serpine2* overexpression might promote HC regeneration through Wnt signaling pathway activation.

Previous studies have shown that *Lin28b* up-regulation increases Akt-mTORC1 activity and causes mature SCs to de-differentiate into progenitor-like cells, which then generate HCs through both mitotic and non-mitotic mechanisms.[27,46] *Otoferlin* functions in the exocytosis of cochlear HCs, and deficiency of *Otoferlin* leads to severe hearing loss.[47,48] The expression of the cochlea-related genes *Lin28b* and *Otoferlin* was up-regulated among the DEGs, again suggesting that *Serpine2* overexpression promotes the differentiation of inner ear SCs into HCs. The cell cycle-related factor *Birc5* is an apoptosis inhibitory protein, and it has been shown that *Birc5* is expressed in the organ of Corti, the lateral wall, and the spiral ganglion of the cochlea and that it has potential cytoprotective effects in the inner ear.[49] We found that *Birc5* was up-regulated in inner ear organoids after *Serpine2* overexpression, suggesting that *Birc5* not only takes part in the proliferation of inner ear SCs, but may also be involved in the formation of inner ear organoids during the cochlea development. Members of the retinoblastoma (pRB) family, including *Rb1*, *Rbl1*, and *Rbl2*, play key roles in the cell cycle by actively interacting with or inhibiting the transcription of important genes involved in proliferation and differentiation. In the inner ear, *Rb1* influences the proliferation, maturation, and survival of HCs, and deficiency of the *Rbl2* gene in mature mice produces excess HCs and SCs.[50] However, there have been no studies on the role of *Rbl1* in the inner ear. We found that *Rbl1* was up-regulated in inner ear organoids after *Serpine2* overexpression, suggesting that *Rbl1* might play a role in cochlear HC regeneration. In addition, other DEGs such as *Aurka*, *Ccnb2*, *Ccna2*, *Chek1*, *Mcm4*, and *Mki67* may be potential targets for HC regeneration.

In conclusion, our findings demonstrate for the first time that *Serpine2* has a promotive effect on HC regeneration through the Wnt signaling pathway (Figure 5), as well as restored hearing of neomycin-damaged mice (Figure 8). And we show that AAV-ie as a tool to deliver *Serpine2* successfully achieved HC regeneration, with long-term safety (Figures 5 and S2). Our findings suggest the use of AAVs to deliver therapeutic genes to inner ear progenitor cells in order to reprogram them to differentiate into functional

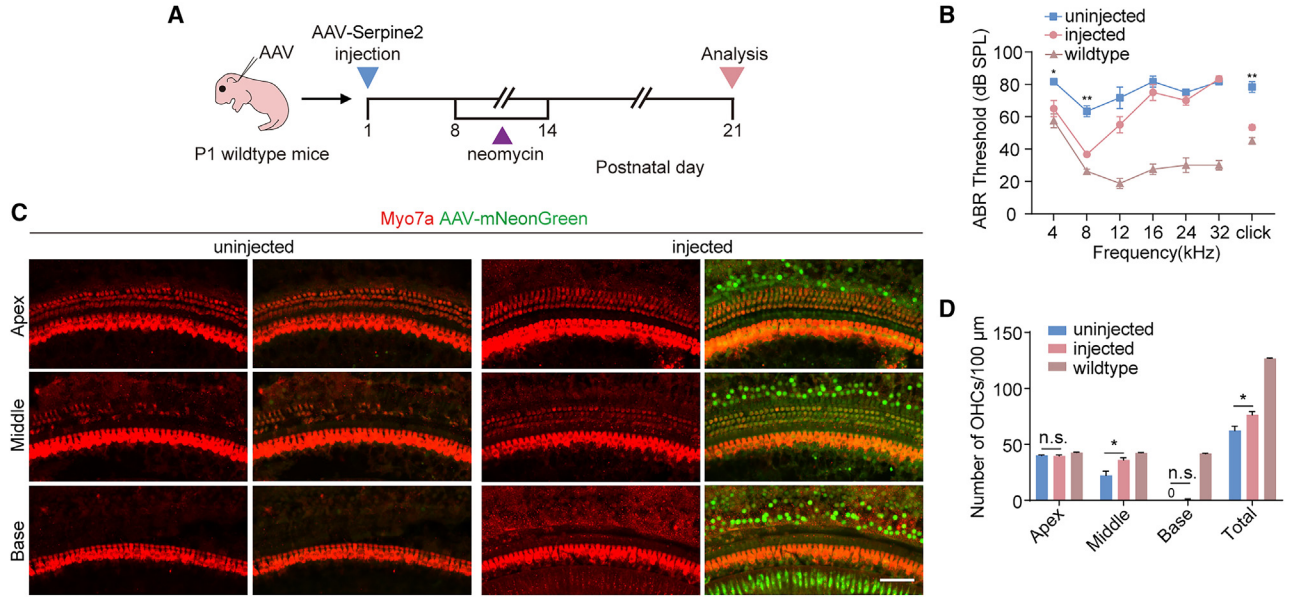

Figure 8. *Serpine2* overexpression promoted HC survival after neomycin injury *in vivo*

(A) Experimental design. (B) ABR thresholds of P21 wild-type mice and mice injected with AAV-*Serpine2*. $n = 3$. Asterisks indicate comparison between the injected and uninjected ears. (C) Immunofluorescence images of cochleae from P21 mice injected with AAV-*Serpine2* (9.1×10^{10} GCs per cochlea) at P1. Scale bars, 50 μm. (D) The numbers of HCs in cochleae of P21 wild-type mice and mice injected with AAV-*Serpine2*.

HCs and restore hearing. However, AAV-ie exhibits low transduction efficiency in adult mouse cochlea SCs, leading to suboptimal HC regeneration outcomes (Figure S1). At present, there are no AAV vectors that exhibit high transduction efficiency in adult mouse cochlear SCs, and thus, suitable serotypes still need to be identified. Furthermore, the overexpression of *Serpine2* in neonatal mice does not support sustained long-term HC regeneration (Figure S2). Consequently, a combined approach involving the regulation of HC regeneration by *Serpine2* alongside other factors known to promote HC regeneration and maturation, such as Gfi1, Pou4f3, and Atoh1,[51] may resolve this issue.

MATERIALS AND METHODS

Animals

Lgr5-EGFP$^{CreER/+}$ and Rosa26-tdTomato$^{loxP/+}$ mice (The Jackson Laboratory, Stock No. 007914 and No. 008875, respectively) were bred in the mouse room. Tamoxifen (Sigma, lot#wxbc9697V) was dissolved in corn oil and administered via intraperitoneal injection to P1 mice (dose: 0.075 mg/g body weight). EdU (Beyotime, ST067-50mg) was dissolved in PBS and administered via subcutaneous injection to P2-4 mice (dose: 0.05 mg/g body weight). All animal experiments were approved by the Institutional Animal Care and Use Committee of Southeast University.

HEK 293T cell culture

HEK 293T cells were thawed and resuscitated in a 37°C water bath. Then the cells were seeded in cell culture dishes (Greiner, #664160). The 10% culture medium contained DMEM (Gibco, #C11995500BT-500mL), 10% fetal bovine serum (FBS) (Vivacell, #C04001-500), and 1% penicillin/streptomycin (Gibco, #15140122). For cell passage, the cells were treated with 0.05% trypsin-EDTA (Gibco, #25200072) when the density was about 90%. When the cell boundary became significantly brighter under the microscope, the trypsin was removed, and the digestion was terminated with 10% culture medium. Next the cells were gently blown off the dish. The samples were collected into centrifuge tubes and were seeded in cell culture dishes at an appropriate proportion for passage.

Organoid culture

2D organoid culture

Single cells were obtained from the cochlear basal membranes of P1 Lgr5-EGFP$^{CreER/+}$ or wild-type neonatal mice by digesting them with 0.25% trypsin-EDTA. Trypsin inhibitor (Worthington, #59S11627) was added to terminate digestion, and the samples were collected. Lgr5^{+} SCs were sorted by FACS. Cells were cultured in a 96-well plate (Corning, #3474) with complete culture medium and infected with control and AAV-*Serpine2* viruses. After 5 days, live cells were photographed. Cells were transferred to four-well dishes coated with laminin for *trans*-differentiation culture with the addition of control and AAV-*Serpine2* viruses (AAV dose: 2×10^{10} GCs per well), and cells were collected after 10 days of differentiation culture. The complete culture medium included DMEM/F12 (Thermo Fisher, #11320033), 1% N2 (Invitrogen, A1370701), 2% B27 (Thermo Fisher, #12587010), epidermal growth factor (EGF) (20 ng/mL; Life, #PHG0311), insulin growth factor (IGF) (50 ng/mL; Sigma, #I8779), β-fibroblast growth factor (β-FGF) (10 ng/mL; #Peprotech, 100-18C), and 0.1% ampicillin (Sangon Biotech, #A610028-0025).

3D organoid culture

Single cells were extracted from the basal membranes of P1 wild-type mice cochleae. The single cells were suspended in DMEM/F12, mixed with 35% Matrigel (Corning, #354230), and placed in 24-well dishes at 30 μL/well. To solidify the Matrigel, the plates were placed in a 37°C incubator for 30 min. Once solidified, the proliferation medium (500 μL/well) was added. The following day, control and AAV-*Serpine2* viruses (AAV dose: 2×10^{10} GCs per well) were introduced. The culture medium for cell proliferation included DMEM/F12, 1% N2, 2% B27, 10 ng/mL β-FGF, 20 ng/mL EGF, 1 mM valproic acid (Sigma-Aldrich, #P4543), 3 μM CHIR99021 (Axon, 1386), 2 μM 616452 (Sigma-Aldrich, #446859-33-2), and 0.1% ampicillin. After 10 days, the differentiation medium was added with the control and AAV-*Serpine2* viruses (AAV dose: 2×10^{10} GCs per well). The differentiation medium included DMEM/F12, 1% N2, 2% B27, 5 μM LY411575 (Sigma-Aldrich, #SML0506), 3 μM CHIR99021, and 0.1% ampicillin.

Virus packaging and purification

In the three-plasmid packaging method, the target gene, viral capsid, and auxiliary plasmid were combined with PEI (Polyscience, #23966) transfection reagent at the appropriate ratios. The combination was added to a culture dish containing HEK 293T cells at a density of approximately 90%, and the cells were harvested after 96 h. Virus purification was performed as previously described.[24] The SYBR (Vazyme, #Q311) method with primers designed from the WPRE region was used to determine the genomic titer of AAVs.

AAV injection through the RWM

Newborn mice were anesthetized in ice. A small incision was cut between the ear and neck of the mice with scissors under a microscope, and the fat and muscle were carefully removed with tweezers to expose the round window of the cochlea. The AAV virus was injected into the mice cochlea through the RWM. The incisions were closed with tissue adhesive (3M Vetbond, #1469SB), and the mice were placed on a heating pad at 37°C to recover.

Immunofluorescence staining

The excess tissue around the cochlea was stripped away under a microscope, and the cochlea was fixed with 4% PFA (Beyotime, #P0099). Then 0.5 mM EDTA (Biosharp, #BL518A) was added for decalcification. The cochlea was cut into three parts (apex, middle, and base) with a scalpel under a microscope and blocked with donkey serum (10%; Solarbio, #017-000-121) for 1 h. Cultured cells were fixed with 4% paraformaldehyde (PFA) and blocked with 10% donkey serum for 1 h, respectively. The samples were incubated with primary antibodies against Myo7a (Proteus Biosciences, #25–6790, 1:1000 dilution) and Sox2 (Santa, #SC-17320, 1:400 dilution) for 12 h, and then they were incubated with the corresponding secondary antibody. DAKO (DAKO, #S3023) was added to prevent fluorescence quenching. Images were obtained and processed with a confocal laser scanning microscope (Zeiss, LSM 700).

Quantitative real-time PCR and RNA sequencing

Total RNA of tissue/organoids was extracted using Trizol reagent (Thermo Fisher, #15596018). The Reverse transcription kit (Thermo Fisher, #K1622) was used to synthesize the cDNA. Quantitative PCR (qPCR) was performed on a CFX96 Real-Time PCR System using the QPCR Mix (Vazyme, #Q311). The primer sequences are listed in Table S1. All libraries for RNA sequencing were analyzed for quality and concentration using Novogene's bioanalyzer. Sequencing data were analyzed using the NovoMagic platform.

ABR testing

ABR tasting was performed using the Tucker-Davis Technologies System III (TDT). Mice were anesthetized and subsequently placed in a soundproof chamber. Electrodes were inserted on the top of the mouse's head and on either side of the ears. These electrodes recorded the brain's electrical activity in response to auditory stimuli, including ABR and click. The tested frequency range covered from low to high frequencies, specifically 4 kHz, 8 kHz, 12 kHz, 16 kHz, 24 kHz, and 32 kHz. The sound pressure level (SPL) was incrementally increased from 0 to 90 dB in steps ranging from 5 to 10 dB.

Electrophysiological recordings

Electrophysiological recording of cochlear tissues is followed as previously described.[51] Briefly, we used the apical turn of cochleae at P7 mice in either males or females and performed the whole-cell patch clamping for recording of native HCs and ectopic HCs induced by AAV-*Serpine2*, respectively. Tissues were embedded and perfused with HEPES-buffered artificial cerebrospinal fluid (ACSF) in an ice-cold and sterile condition and cochlear cell types were imaging by an inverted microscope using a differential interference contrast and a 60 × 1.00NA water-immersion objective. The ACSF is composed of the following gradients: 11.2 mM $NaHCO_3$, 6 mM KCl, 1.3 mM NaH_2PO_4, 11 mM D-glucose, 1.3 mM $MgCl_2$, 1.3 mM $CaCl_2$, and 115 mM NaCl.[52] Ectopic HCs were selected as their disorganized HC bundles (stereocillia) with fluorescence labeling. Whole-cell patch clamps were recorded using an Olympus microscope (BX51WI) and AXON 7500B amplifiers. Patch pipettes (with pipette resistance of 10–15 MΩ) contained the following gradients: 0.1 mM $CaCl_2$, 135 mM KCl, 5 mM HEPES-KOH (325 mOsm, pH 7.35), 2.5 mM Na_2ATP, 5 mM EGTA-KOH, and 3.5 mM $MgCl_2$. During recordings, native HCs and ectopic HCs were maintained at a holding potential of −70 mV. After low-pass filtering recordings at 2 kHz, data analysis was done using Graphpad 9 and Clamfit 10.

Statistical analysis

GraphPad Prism 9 software was used to conduct the statistical analyses, and the data are presented as the mean ± SEM. The p value was calculated by Student's t test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, n.s. refers to no significance. All experiments were carried out three times or more, and all repeated experiments were independent of each other. Two-tailed, unpaired Student's t tests were conducted to determine statistical significance between two groups, and the criteria for statistical significance was $p < 0.05$.

DATA AND CODE AVAILABILITY

All data associated with this study are present in the paper.

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AUTHOR CONTRIBUTIONS

R.C., J.Q., B.T., and L.Z. conceived and designed the experiments. Q.S., F.T., X.W., X.G., and X.C. performed most of the experiments. Y.L., N.L., X.Q., Y.Z., Z.Z., and M.W. performed genotyping and cell culture. R.C., J.Q., L.Z., and Q.S. discussed the data analysis, interpretation, and presentation and wrote the manuscript with contributions from all authors.

DECLARATION OF INTERESTS

J.Q. and F.T. have filed a patent on the use of AAV-ie for gene therapy in the inner ear. The authors declare that they have no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2024.102396.

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