Cellular and Molecular Life Sciences (2022) 79:154
https://doi.org/10.1007/s00018-022-04164-x

ORIGINAL ARTICLE

Characterization of the microRNA transcriptomes and proteomics of cochlear tissue-derived small extracellular vesicles from mice of different ages after birth

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Received: 18 August 2021 / Revised: 30 December 2021 / Accepted: 23 January 2022 / Published online: 26 February 2022

Abstract

The cochlea is an important sensory organ for both balance and sound perception, and the formation of the cochlea is a complex developmental process. The development of the mouse cochlea begins on embryonic day (E)9 and continues until postnatal day (P)21 when the hearing system is considered mature. Small extracellular vesicles (sEVs), with a diameter ranging from 30 to 200 nm, have been considered a significant medium for information communication in both physiological and pathological processes. However, there are no studies exploring the role of sEVs in the development of the cochlea. Here, we isolated tissue-derived sEVs from the cochleae of FVB mice at P3, P7, P14, and P21 by ultracentrifugation. These sEVs were first characterized by transmission electron microscopy, nanoparticle tracking analysis, and western blotting. Next, we used small RNA-seq and mass spectrometry to characterize the microRNA transcriptomes and proteomes of cochlear sEVs from mice at different ages. Many microRNAs and proteins were discovered to be related to inner ear development, anatomical structure development, and auditory nervous system development. These results all suggest that sEVs exist in the cochlea and are likely to be essential for the normal development of the auditory system. Our findings provide many sEV microRNA and protein targets for future studies of the roles of cochlear sEVs.

Keywords Small extracellular vesicles · Cochlea · Development · MicroRNAs · Proteins · Proteomics

Introduction

The cochlea in the inner ear is an important auditory signal transduction organ that develops from embryonic day (E)9 through postnatal day (P)21 [1]. The detection of sound

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waves and transmission of sound information to the brain are both dependent on cochlear hair cell (HCs) [2]. The first cochlear HCs develop at E11, and ultimately three rows of outer hair cells (OHCs), one row of inner hair cells (IHCs), and supporting cells (SCs) beneath the HCs are formed [3]. By P3, the total number of HCs peaks and will remain basically unchanged, while the morphology of the HCs will change as the HCs mature from P3 to P21 [4, 5].

HC maturation involves many complex developmental processes, such as the formation of hair bundles, synapses, and mechanical transduction channels (METs) [6–8]. Hearing formation requires the establishment of proper innervation, and the afferent nerves of the inner ear gradually form an outer spiral bundle of OHCs from P0 to P3 [9]. In the first 7 days after birth, hair bundles and METs develop gradually, and mature innervation patterns emerge gradually between P14 and P21 [9, 10]. At P7, HCs have mature mechanical transduction abilities, which is the most important aspect of formation of the auditory system [11]. Hearing onset and HC synapses gradually mature at P12–14, during which time mice gradually obtain the initial ability to hear [12]. Adult ranges of cochlear potentials can be initially tested at P11, but adult ranges and sensitivities of cochlear potential do not mature until P14 [13]. Mature cochlear potentials are essential for the formation of hearing. At P21, the morphology and function of the cochlea are mature, and hearing function can be measured by auditory brainstem response. During the process of HC maturation, the characteristics of SCs, especially inner ear progenitors, also change dramatically. SCs have been reported to act as inner ear stem cells and to transdifferentiate into HCs by induction of Wnt signaling or inhibition of Notch signaling in newborn mice [14, 15]. However, the stemness of SCs deteriorates with age, and their capacity to divide is completely lost by P14 [16].

It has been reported that many important transcription factors and signaling pathways are associated with the development of the cochlea, such as Sox2, Atoh1 [17], and the Wnt, Notch, and FGF signaling pathways [18, 19]. In addition, many microRNAs (miRNAs), such as miR182, miR183, and miR124, are also reported to regulate inner ear tissue differentiation and to maintain cell differentiation and proliferation [20, 21]. However, the cochlea's development is a complicated process, and many regulatory processes and the factors that are involved remain to be elucidated.

Small extracellular vesicles (sEVs) have become a research hotspot in recent years and are reported to be involved in intercellular signal transmission during many important pathological and physiological processes [22–24]. sEVs have sizes from 30 to 200 nm and can be generated by various cells [25]. The contents of sEVs include numerous proteins and nucleic acids that are protected by a phospholipid bilayer structure from being digested by extracellular substances, and these materials can be delivered to recipient cells and thus contribute to cellular communication and signal transmission [26, 27]. sEVs participate in cell proliferation and differentiation in both pathological and healthy situations through signaling pathways mediated by miRNAs [28–30], and sEVs are involved in intercellular signal transmission during the development of brain neural circuits and in regulating growth patterns during embryonic development [31, 32].

Although sEVs have been extensively studied in cancer and other diseases, limited studies have been performed on the role of sEVs in the cochlea. This may be because as the mice age the otic vesicle outside the cochlea gradually becomes ossified and becomes rigid, especially after P10, which makes it difficult to obtain the substances inside the cochlea. However, it is known that in the utricle SC-derived exosomes can protect HCs against neomycin-induced ototoxicity [33] and that inner ear stem cell-derived exosomes can reduce ototoxic drug damage by transferring miR-182-5p to HEI-OC1 cells [34, 35]. At present, the research on inner ear-derived sEVs is based on in vitro models, and there is no research on sEVs in intact inner ear tissues.

In this study, we extracted cochlear tissue-derived sEVs from mice at different ages after birth and systematically analyzed and characterized their protein and miRNA contents for the first time. We used transmission electron microscopy (TEM), western blotting, and nanoparticle tracking analysis (NTA) to quantify the characteristics of sEVs and then performed proteomics and small RNA-seq to analyze the differentially expressed proteins and miRNAs and to predict the functions of these proteins and miRNAs. These results are expected to provide important information for the subsequent functional analysis of sEVs in the cochlea.

Materials and methods

Isolation of cochlear tissue-derived sEVs

The cochleae were obtained from P3, P7, P14, and P21 FVB mice. sEVs were isolated from 45 mouse cochleae according to the ultracentrifugation method as previously reported [36, 37]. Briefly, the cochleae were dissected, placed in a centrifuge tube with PBS buffer, and then ground for 1 min at 40 Hz in a grinder (Jingxin, Shanghai, China). The sample was filtered with a pore size of 0.22 μm after differential centrifugation to eliminate cell debris and microvesicles (600×*g* for 10 min, 2000×*g* for 15 min, and 12,000×*g* for 50 min, all at 4 °C). The filtered samples were concentrated to 1–1.5 ml in a 50 ml 100 kDa molecular weight cutoff (MWCO) ultrafiltration centrifuge tube (Millipore) at 3000×*g* for 15 min at 4 °C. The samples were then ultracentrifuged at 110,000×*g* for 2 h at 4 °C to obtain sEVs.

After discarding the supernatant, the sEV pellets were resuspended, washed with PBS once, and ultracentrifuged a second time at 110,000×*g* for 2 h at 4 °C. The sEVs were finally resuspended in 400–500 µl PBS for the following experiments.

Transmission electron microscopy

A total of 10 µl of sEV sample was dropped on an electron microscope copper grid and dried at room temperature and then negatively stained with 1.5% phosphotungstic acid (pH 7.4) at room temperature for 5 min. The images of cochlear sEVs were obtained by TEM (JEM-2100, Hitachi, Tokyo, Japan) with an accelerating voltage of 200 kV.

Nanoparticle tracking analysis

NTA (NS300, Malvern, United Kingdom) was used to identify the size and concentration of sEVs. A total of five 60-s videos were obtained for each sample, and the dispersed light signal of the sEVs was gathered using an optical microscope. According to Brownian motion of particles, the sizes and concentrations of the sEVs were averaged from the five videos.

Immunofluorescent staining

Immunofluorescent staining was performed according to a previous study [38]. In brief, cochleae were decalcified with 0.5 M ethylene diamine tetraacetic acid (EDTA) after being fixed in 4% (v/v) paraformaldehyde. The cochleae were then blocked and incubated with primary antibodies. Fluorescence-conjugated secondary antibodies were then added and bound to primary antibodies. A Zeiss LSM 700 confocal microscope was used to capture fluorescent images of the cochleae. The primary antibodies included anti-myosin7a (Proteus Bioscience, #25-6790, 1:1000 dilution), anti-Sox2 (R&D systems AF2018-SP, 1:1,000 dilution), anti-CD63 (ab217345, 1:1,000 dilution), and anti-CD9 (ab92726, 1:1500 dilution). Alexa Fluor 647 donkey anti-goat IgG (Invitrogen, A-21447, 1:400 dilution), Alexa Fluor 555 donkey anti-rabbit IgG (Invitrogen, A-31572, 1:400 dilution), and Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen, A-21202, 1:400 dilution) were used as secondary antibodies.

Western blotting

Small extracellular vesicles were freeze-dried and then lysed in 200 µl RIPA lysis buffer (Beyotime) with 1 × protease cocktail (Roche) for 30 min at 4 °C. The protein quality was assessed using a BCA kit (Beyotime). The samples were boiled for 15 min at 95 °C in 5 × sodium dodecyl sulfate (SDS) loading buffer. SDS polyacrylamide gel electrophoresis was utilized to isolate the sEV proteins, which were then transferred onto a polyvinylidene difluoride membrane at 275 mA for 90 min. The membrane was blocked with 5% BSA (5% (v/v) bovine serum albumin in 0.1% (v/v) Tween-20 in phosphate buffered solution (PBS)) for 1 h at room temperature and then incubated with primary antibody overnight at 4 °C. The second day, the membrane was incubated with HRP-conjugated secondary antibody (Abclonal, 1:2,000 dilution). SuperSignal West Pico Plus chemiluminescent substrate (Thermo Scientific) was employed for visualizing the target bands on a Tanon-5200 automatic chemical imaging system. The primary antibodies were anti-CD63 (ab217345, 1:1000 dilution), anti-CD9 (ab92726, 1:1500 dilution), anti-Tsg101 (ab125011, 1:2000 dilution), anti-mouse EEA1 (Santa Cruz Biotechnology, 1:100 dilution), anti-rabbit Rab7 (Cell Signaling Technology, 1:1,000 dilution), anti-GAPDH (Kangchen, KC-5G4, 1:2000 dilution), anti-Synapsin-1 (Cell Signaling Technology 5297T, 1:1000), and anti-VGLUT3 (Synaptic Systems 135203, 1:1000).

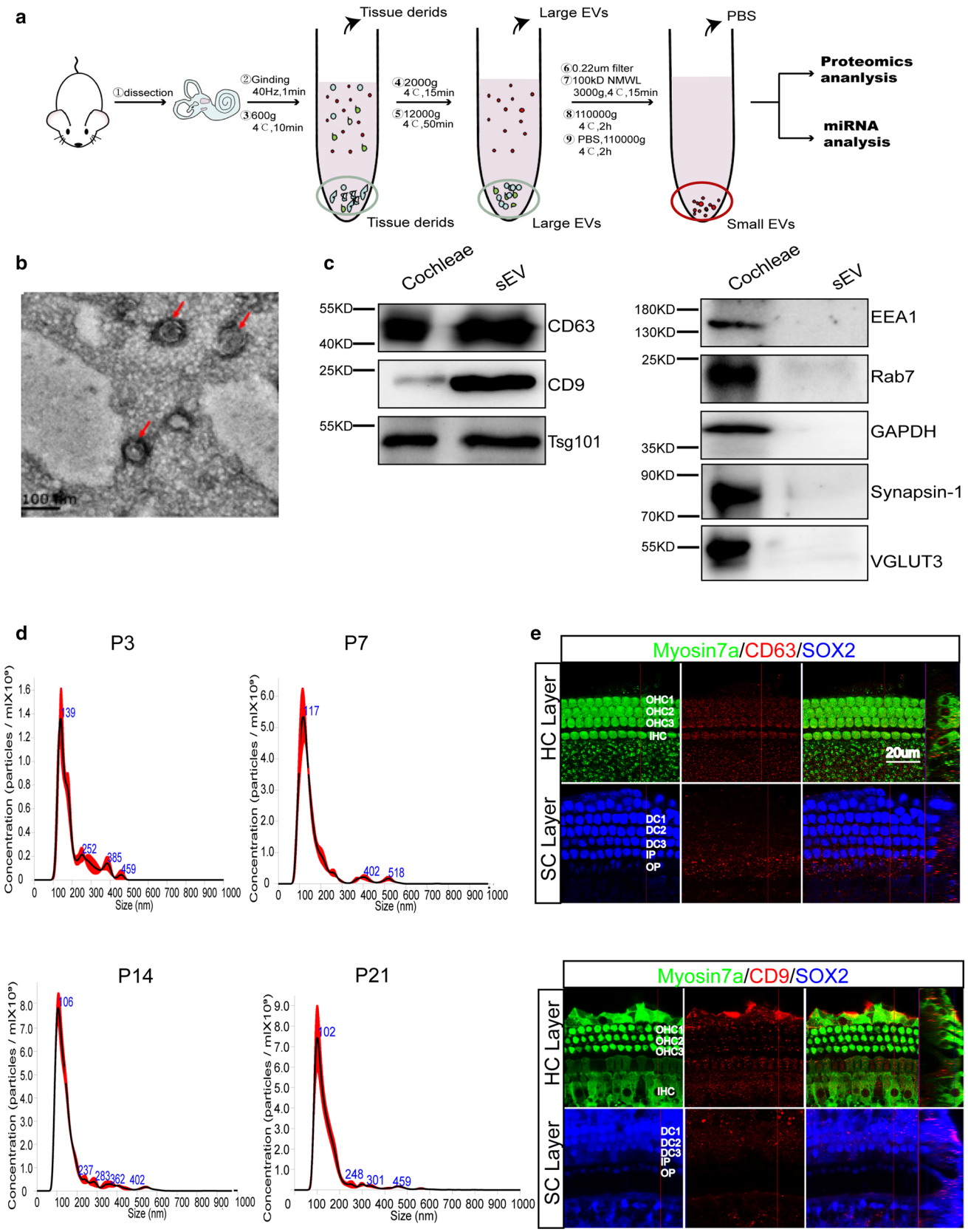
RNA extraction and quantitative real-time PCR

Small extracellular vesicle samples were mixed with 1 ml Trizol (Invitrogen, 15596-026) on ice for 5 min, then centrifuged at 17,970×*g* for 5 min at 4 °C. The sample was mixed with 200 µl chloroform, vortexed to mix well, and then placed on ice for 10 min. After centrifugation at 17,970×*g* for 15 min at 4 °C, the supernatant was mixed with an equal amount of isopropanol, incubated for 10 min, and centrifuged at 17,970×*g* for 10 min at 4 °C. The RNA pellet was washed with 70% ethanol after removing the supernatant then dissolved in 25 µl RNase-free water.

Total RNA from sEVs was reverse transcribed to cDNA using an miRNA first-strand cDNA synthesis kit (Vazyme #MR101) following the manufacturer's directions. Real-time PCR was done using an Applied Biosystems real-time PCR instrument with miRNA Universal SYBR qPCR Master Mix (Vazyme, #MR101-01) to quantify the miRNA expression levels. All primer sequences are listed in the supplemental table. The levels of miRNAs were compared using two-tailed, unpaired Student's *t* tests after being standardized to small nuclear RNA U6.

Small RNA sequencing and analysis

For the small RNA-seq library, a minimum of 2 µg RNA single sample ($n = 3$) was used as the starting material.


a
①dissection
②Ginding 40Hz,1min
③600g 4℃,10min
④2000g 4℃,15min
⑤12000g 4℃,50min
⑥0.22um filter
⑦100kD NMWL 3000g,4℃,15min
⑧110000g 4℃,2h
⑨PBS,110000g 4℃,2h
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P7
Concentration (particles / mlX10⁹)
Size (nm)
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252
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459
117
402
518
P14
P21
106
237
283
362
402
102
248
301
459
e
Myosin7a/CD63/SOX2
Myosin7a/CD9/SOX2
HC Layer
SC Layer
OHC1
OHC2
OHC3
IHC
DC1
DC2
DC3
IP
OP
20um

◂**Fig. 1** Isolation and characterization of cochlear tissue-derived sEVs. **a** The workflow for isolating cochlear tissue-derived sEVs by ultracentrifugation. **b** TEM of cochlear sEVs. Scale bar = 100 nm. **c** Western blotting of cochlear tissue lysate (TL) and sEV samples. CD63, CD9, and Tsg101 were used as sEV markers, and EEA1, Rab7, GAPDH, Synapsin-1, and VGLUT3 from other organelles were used as negative markers. **d** NTA of cochlear sEVs from P3, P7, P14, and P21 mice. **e** Immunofluorescent staining of CD63 and CD9 (red) in the P3 cochlea. Myo7a (green) and Sox2 (blue) were used as HC and SC markers, respectively. OHC, outer hair cell. IHC, inner hair cell. DC, Deiters' cell. IPC, inner pillar cell. OPC, outer pillar cell. IPhC, inner phalangeal cell. Scale bar = 20 μm

Following the manufacturer's protocol, sequencing libraries were created using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA), and miRNA data were evaluated by FASTQC (v 0.11.5). Sequences were aligned to the reference genome derived from MirBase v22.1 (http://www.mirbase.org/) using Bowtie2 (v 2.2.5). The miRNA expression level in each sample was determined by featureCounts (v 2.0.0) and then normalized with the CPM (counts-per-million) algorithm, and differential expression analysis was performed in edgeR (v 3.30.3) using |log2FoldChange| > 2.0 and $p < 0.05$ as the threshold. Short Time-Series Expression Miner (STEM) (v 1.3.13) software was used for expression trend analysis. To avoid too many false positives, only miRNA-targeted genes in the Tarbase v7.0 database [39], which were identified experimentally, were selected.

DIANA-279 miRPath v.3 was used to assess miRNA enrichment pathways [40], and the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were employed to investigate functional annotation and pathway enrichment. The cumulative effects of the specified miRNAs were evaluated using the "genes-Union" algorithm. The Fisher accurate test with a microT threshold of 0.8, false discovery rate (FDR) correction, and a p value threshold of 0.05 was used for enrichment analysis.

Protein digestion

The freeze-dried sEVs were dissolved in buffer consisting of phosphatase inhibitor cocktails, 10 mM TCEP, 40 mM 2-chloroacetamide, 12 mM sodium deoxycholate, 50 mM Tris–HCl, and 12 mM sodium lauroyl sarcosinate (pH 8.5) (Sigma-Aldrich) by boiling for 10 min at 95 °C. After that, the samples were diluted fivefold with 50 mM triethylammonium bicarbonate and digested for 3 h at 37 °C with Lys-C (Wako) at 1:100 (w/w). To further degrade the peptides, the samples were treated overnight at 37 °C with trypsin at a ratio of 1:50 (w/w). To acidify the sample with a concentration of 1% TFA, ethyl acetate solution and 10% trifluoroacetic acid (TFA) were adjusted in a 1:1 ratio according to the aforementioned combination. The sample solution was vortexed before being centrifuged at 15,000×g for 3 min. The organic phase on the top was discarded, and the aqueous phase at the base was harvested and freeze-dried by refrigerated vacuum centrifuge (Laconco CentriVap). The desalting was performed on an 8 mm extraction disk as directed by the manufacturer (3 M Empore 2240-SDB-XC). All samples were stored at – 80 °C.

LC–MS/MS and quantitative data analysis

LC–MS/MS was performed as previously described [41]. Briefly, the peptides were solubilized in 10 μL 0.1% formic acid (FA), and 2 μL of the nanoElute was used for proteomics analysis. All peptides could be separated in a 25 cm internal packed column in the mobile phase with a fluid velocity of 300 nl/min. The timsTOF Pro mass spectrometer (Bruker) was connected to the nanoElute in real time, and the data settings were adjusted to full scan (m/z 100–1700) by the mass spectrometer.

Using the PEAKS Studio X+ program (Bioinformatics Solutions Inc), the raw files were explicitly compared with the UniProt database to obtain clean data. There were no duplicate entries in the identification of proteins and peptides, but special peptides and proteins were found. To examine differential proteins, markers of exosomes, and isolated inner ear proteins in the various samples, the intensities of the peptides were quantified using a label-free approach. The Perseus software was utilized to investigate the differential expression of sEV proteins of the cochlea based on these data. DAVID (https://david.ncifcrf.gov/) was conducted to identify biological process terms from GO and KEGG pathway analyses, and the protein–protein interaction network was obtained by STRING database (http://string-db.org/).

Statistical analysis

All data in this study are shown as the mean ± SD, and all analyses were performed using GraphPad Prism 7 software. When analyzing the different groups, we performed a two-tailed, unpaired Student's t tests to evaluate statistical significance. Statistical significance was defined as a value of $p < 0.05$.

Results

Isolation and characterization of cochlear tissue-derived sEVs

Small extracellular vesicles were isolated from the cochlear tissue of mice at P3, P7, P14, and P21 by ultracentrifugation as described previously [36, 37] (Fig. 1a). Considering that the cochlea is surrounded by the rigid otic vesicle, we dissected the cochleae and ground them in a grinder at 40 Hz as gently as possible so as not to break open the cells. The samples were centrifuged at low speed (600×*g* and 2000×*g*) to remove cell debris and then at high speed (12,000×*g*) to remove large EVs. After passing through a 0.22 μm filter, the samples were concentrated by ultrafiltration with a 100 kDa MWCO ultrafilter. Finally, sEVs were isolated by ultracentrifugation at 110,000×*g*. The RNAs and proteins extracted from sEVs were used for miRNA sequencing and proteomics analysis, respectively. TEM by negative staining indicated the oval shape of sEVs (Fig. 1b), and we characterized the size and number of sEVs from mice of different ages by NTA and confirmed that the diameter of the sEVs was 30–200 nm (Fig. 1d). Typical sEV marker proteins—such as the tetraspanins CD63 and CD9—and the composition of ESCRT-I complex Tsg101 were detected in cochlear tissue-derived sEVs by western blotting (Fig. 1c). Marker proteins for other vesicles, including EEA1 (endosome marker), Rab7 (lysosome marker), GAPDH, and Synapsin-1 and VGLUT3 (synaptic vesicles markers) were used as negative markers of sEVs and were not detected in the sEV samples (Fig. 1c). We also used immunofluorescent staining to confirm the presence of CD63 and CD9 in HCs and SCs (Fig. 1e). We further characterized the morphology and protein markers of sEVs and other vesicle markers in the four age groups by TEM and western blotting (Supplementary Fig. S1), which all showed a cup-like shape and typical sEV

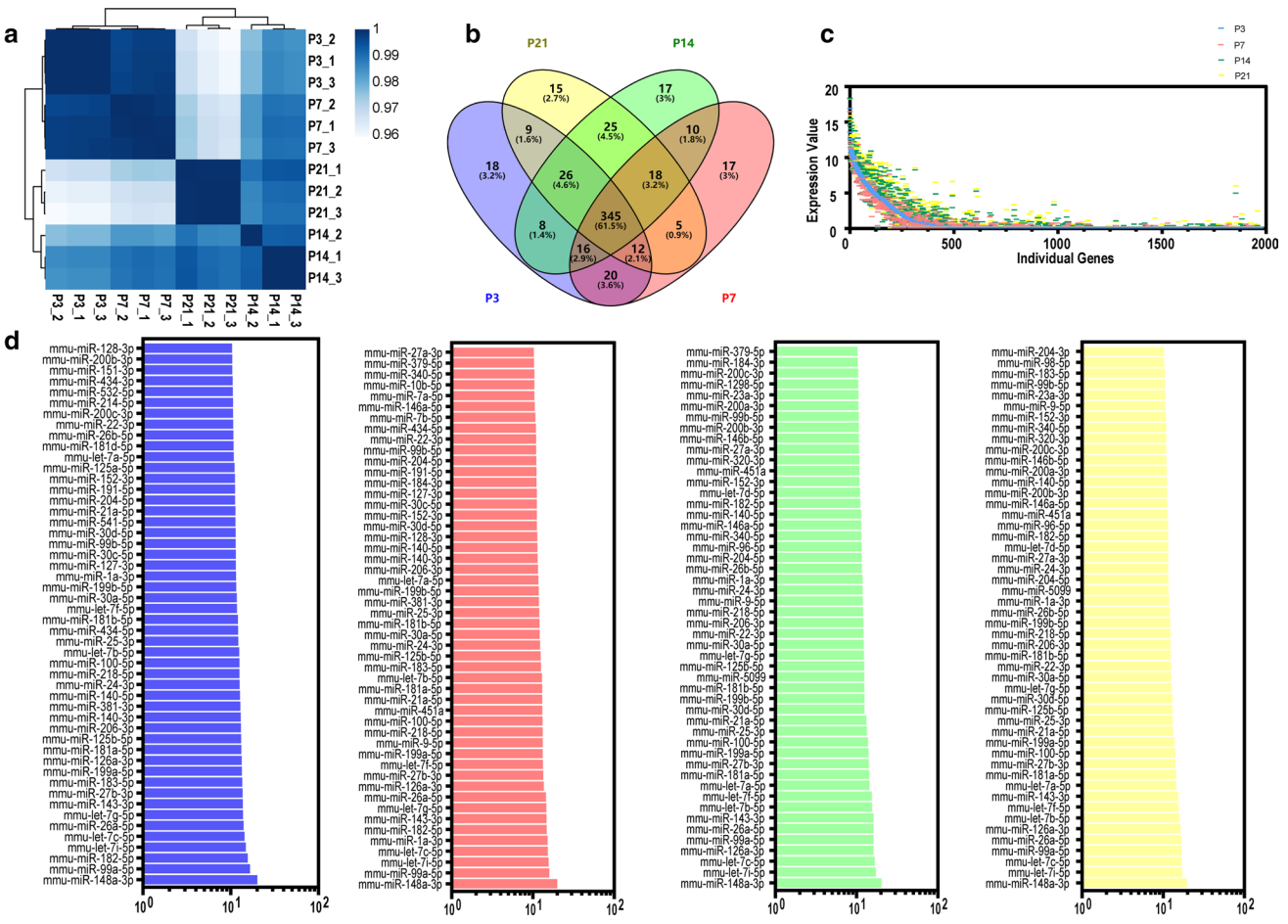


Fig. 2 Transcriptome analysis of cochlear sEV miRNAs from P3, P7, P14, and P21 mice. **a** Cluster analysis of cochlear sEV miRNA sequencing data. **b** The Venn diagram of the miRNA sequencing data. **c** All differentially expressed miRNAs in the four samples. P3 data were used as the control as indicated by the blue line. **d** The top 50 highly expressed cochlear sEV miRNAs from P3 (blue), P7 (red), P14 (green), and P21 (yellow) mice

marker expression. Together, these results suggest that this isolation method of cochlear tissue-derived sEVs is feasible and can yield relatively pure sEVs.

Micro analysis of cochlear tissue-derived sEVs from mice of different ages

Small extracellular vesicles contain a variety of RNAs, especially miRNAs, which play important roles in gene regulation and thus mediate numerous biological processes [42, 43]. Because the role of sEV miRNA in the cochlea is poorly understood, we employed small RNA-seq to evaluate the cochlear tissue-derived sEVs from P3, P7, P14, and P21 mice and to identify differentially expressed miRNA during the development of the cochlea.

The correlations of the samples were tested by hierarchical clustering analysis, and the P3, P7, P14, and P21 groups were well-separated according to their Spearman correlation coefficients (Fig. 2a). We detected a total of 561 miRNAs, including 454, 453, 465, and 455 miRNAs from cochlear tissue-derived sEVs from P3, P7, P14, and P21 mice, respectively (Fig. 2b). Furthermore, there were 18, 17, 17, and 15 miRNAs that were uniquely expressed at P3, P7, P14, and P21, respectively (Fig. 2b). The expression levels of all miRNAs at P3, P7, P14, and P21 are shown in Fig. 2c. We compared the differentially expressed miRNAs between each of the age groups pairwise (Supplementary Fig. S2), and the top 50 most-abundant miRNAs in the four age groups are shown in Fig. 2d.

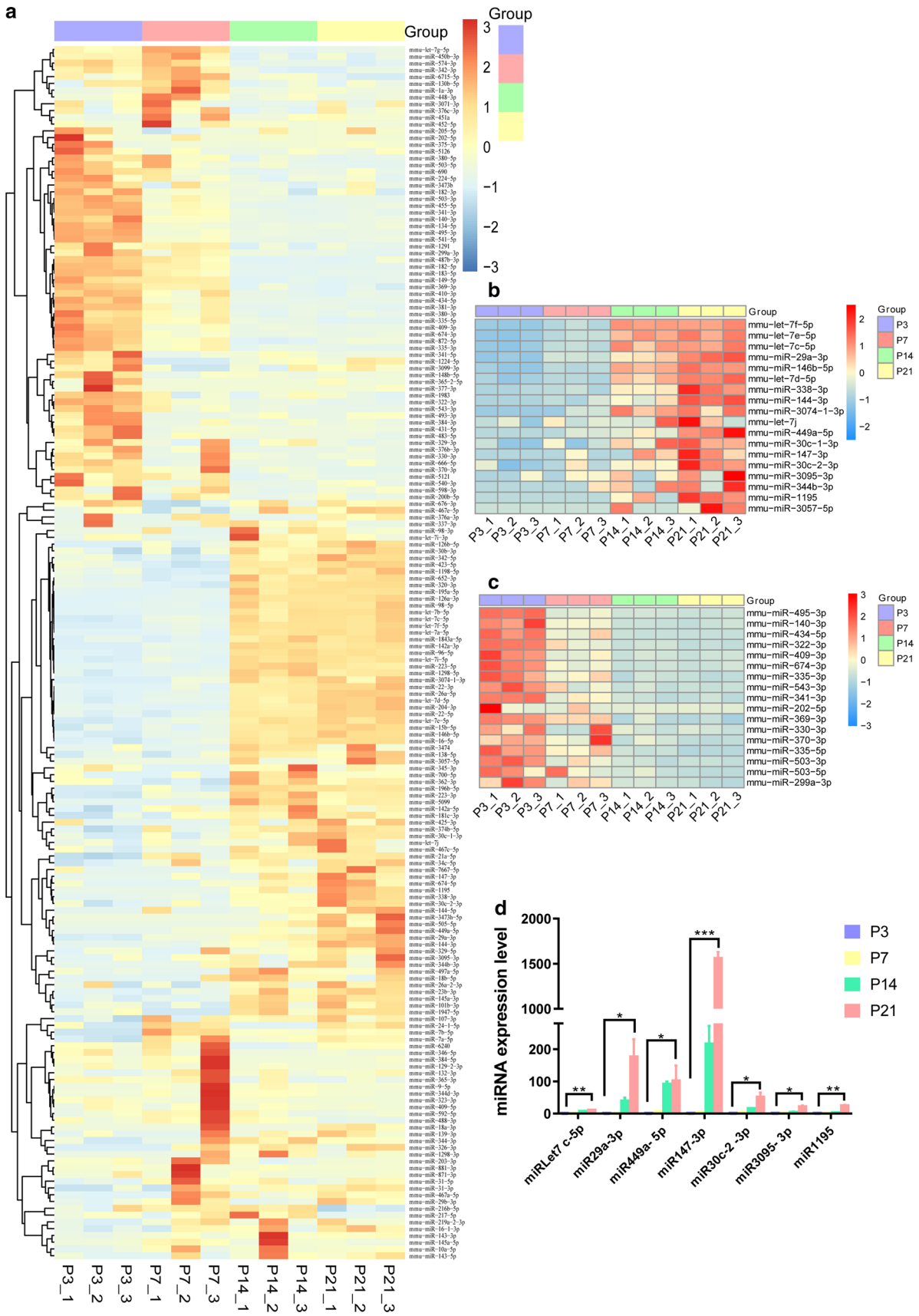
We found 179 miRNAs that were differentially expressed across the four age groups by pairwise comparison, including 57, 33, 29, and 60 miRNAs that were highly expressed at P3, P7, P14, and P21, respectively (Fig. 3a, $p < 0.05$, fold change > 2). Further analysis of the 179 differentially expressed miRNAs showed that 18 of these miRNAs became more prevalent in sEVs with age, while 17 miRNAs decreased with age (Fig. 3b, c). Of the increased miRNAs, miRLet-7f-5p [44], miRLet-7e-5p [45], miRLet-7c-5p [46], miR29a-3p [47], miR146b-5p [48], miRLet-7d-5p [49], miR338-3p [50], miR144-3p [51], miRLet-7j [52], miR449a-5p [53], miR30c-1-3p [54], miR147-3p [55], miR30c-2-3p [56], and miR1195 [57] have been attributed to a range of biological processes including cellular proliferation, cellular differentiation, and cellular signaling and communication. miR3074-1-3p, miR3095-3p, miR344b-3p, and miR3057-5p have no reported biological functions. For the decreased miRNAs, miR495-3p [58], miR140-3p [59], miR434-5p [60], miR322-3p [61], miR409-3p [62], miR674-3p [63], miR335-3p [61], miR543-3p [64], miR341-3p [65], miR202-5p [66], miR369-3p [67], miR330-3p [68], miR370-3p [69], miR335-5p [70], miR503-3p [71], and miR503-5p [72] have been reported to be related to biological processes, and only miR299a-3p has no reported function. We verified the 18 increased miRNAs by qPCR, and Fig. 3d shows that the expression of seven miRNAs (miRLet7c-5p, miR29a-3p, miR449a-5p, miR147-3p, miR30c-2-3p, miR3095-3p, and miR1195) was matched to the results of the bioinformatics analysis. The specific information for these seven miRNAs is listed in Table 1, including gene ID, number of predicted target genes, functional description, and references.

Functional analysis of differentially expressed miRNAs in cochlear sEVs

The GO and KEGG pathway analyses of the highly expressed miRNAs at P3, P7, and P14 were performed with DIANA-mirPath v.3 (http://snf-515788.vm.okeanos.grnet.gr/) using the target genes in the Tarbase v7.0 database (http://www.microrna.gr/tarbase). These miRNAs in cochlear sEVs at different ages have different biological functions (Fig. 4). Notably, the GO analysis showed that these miRNAs are mainly involved in anatomical structure development, cell differentiation, developmental maturation, growth, cell cycle, and vesicle-mediated transport (Fig. 4a, c, e, g). Figure 4b, d, f, h shows that the highly expressed miRNAs at P3, P7, P14, P21 are involved in the mTOR, PI3K-Akt, TGF-β, Wnt, Hippo, Notch, and cGMP-PKG signaling pathways. These findings suggest that these pathways are likely to be involved in the development of the cochlea and the formation of the auditory system. Among them Wnt, Notch, TGF-β, and Hippo signaling have been implicated in progenitor cell proliferation and differentiation as well as cell plane polarity during inner ear development [73, 74].

Label-free quantitative proteomics analysis of cochlear tissue-derived sEVs from mice of different ages

Considering that proteins in sEVs also play important roles as biomarkers and in multiple biological processes [75, 76], the protein contents of the sEV sample from the cochleae of P3, P7, P14, and P21 mice was analyzed utilizing label-free quantitative proteomics. Each group included three biological replicates, and the samples clustered well with no outliers (Fig. 5a). A total of 5231 proteins were identified, and 2257 of these were present in all four groups (Fig. 5b). sEV marker proteins (Tsg101, CD63, CD9, CD81, and Flotillin-1) were also found among these proteins by mass spectrometry (Table 2). Figure 5c shows the top 50


a
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P3_2
P3_3
P7_1
P7_2
P7_3
P14_1
P14_2
P14_3
P21_1
P21_2
P21_3
b
Group
mmu-let-7f-5p
mmu-let-7e-5p
mmu-let-7c-5p
mmu-miR-29a-3p
mmu-miR-146b-5p
mmu-let-7d-5p
mmu-miR-338-3p
mmu-miR-144-3p
mmu-miR-3074-1-3p
mmu-let-7j
mmu-miR-449a-5p
mmu-miR-30c-1-3p
mmu-miR-147-3p
mmu-miR-30c-2-3p
mmu-miR-3095-3p
mmu-miR-344b-3p
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mmu-miR-674-3p
mmu-miR-335-3p
mmu-miR-543-3p
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mmu-miR-299a-3p
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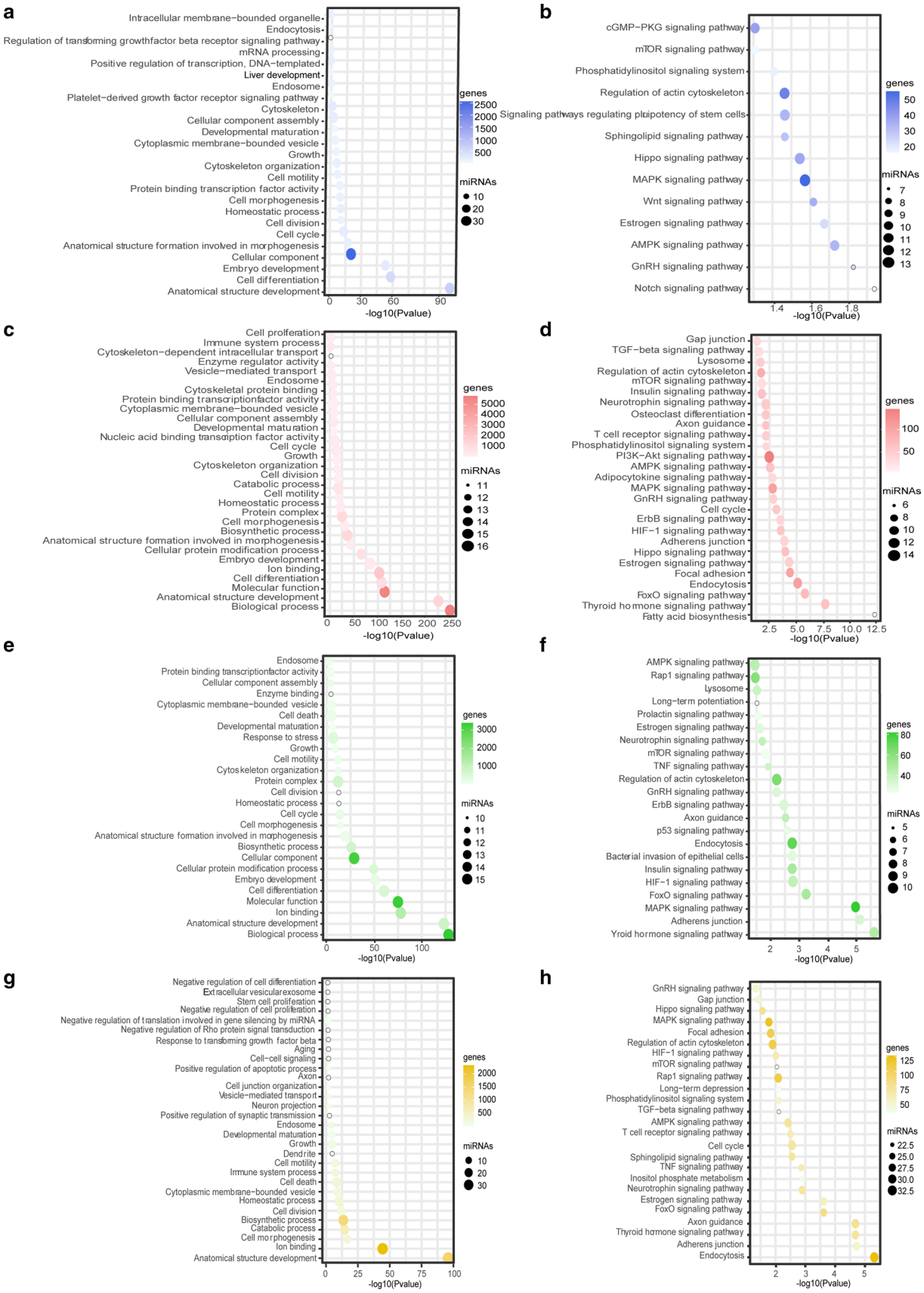
miRLet7 c-5p
miR29a-3p
miR449a- 5p
miR147-3p
miR30c-2 -3p
miR3095- 3p
miR1195
P3
P7
P14
P21

◂**Fig. 3** The differentially expressed cochlear sEV miRNAs from P3, P7, P14, and P21 mice. **a** Heatmap of the differentially expressed miRNAs. **b** Heatmap of the up-regulated miRNAs as mice age. **c** Heatmap of the down-regulated miRNAs as mice age. **d** qPCR verification of some differentially expressed miRNAs. Values lower and higher than the mean are shown by blue and red scales, respectively. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $n=3$

most-abundant proteins in the P3, P7, P14, and P21 sEV samples. In addition, we compared all identified proteins with the Exocarta and Vesiclepedia databases and found that 978 proteins overlapped with Exocarta and 115 proteins overlapped with Vesiclepedia (Supplementary Fig. S3a). Figure S3b shows that 8, 6, 8, and 7 proteins of the top 100 proteins in P3, P7, P14, and P21 cochlea-derived sEVs were reported among the top 100 proteins in the Exocarta and Vesiclepedia databases. These results suggest that many sEV proteins in the Exocarta and Vesiclepedia databases were also found in our sEV samples and that there were sEV proteins in our samples that were not in the EV databases and thus might be newly identified EV proteins in cochlear tissue-derived sEVs.

Table 1 Validated miRNA candidates of age-related cochlear tissue-derived sEVs with the numbers of target genes and functional categories

miRNA	Gene ID	Number of target genes	Functional description	Reference
mmu-miRLet7c-5p	387246	558	Cell differentiation, anatomical structure development, ion binding, cell death, growth, developmental maturation, cell cycle, endocytosis, axon guidance, TNF signaling pathway, AMPK signaling pathway, TGF-beta signaling pathway, mTOR signaling pathway, MAPK signaling pathway, MAPK signaling pathway	Chen et al. [45], Morgado et al. [131], Yao et al. [132], Zhang et al. [133] and Chen et al. [134]
mmu-miR29a-3p	387222	451	Cell differentiation, anatomical structure development, ion binding, cell cycle, cell death, anatomical structure formation involved in morphogenesis, cell division, growth, developmental maturation, positive regulation of apoptotic process, axon guidance, TNF signaling pathway, AMPK signaling pathway, TGF-beta signaling pathway, mTOR signaling pathway, MAPK signaling pathway, Hippo signaling pathway	Qu et al. [47], You et al. [135], Volpicelli et al. [136], Wang et al. [137] and Tumaneng et al. [138]
mmu-miR449a-5p	723868	469	Cell differentiation, anatomical structure development, ion binding, cell differentiation, anatomical structure development, ion binding, cell motility, cytoplasmic membrane-bounded vesicle, cell junction, endocytosis, axon guidance, AMPK signaling pathway, TGF-beta signaling pathway, mTOR signaling pathway, MAPK signaling pathway, Hippo signaling pathway	Shu et al. [16], Ni et al. [53], Wu et al. [138] and Bou Kheir et al. [139]
mmu-miR30c-2-3p	723964	209	Anatomical structure development, regulation of epidermal cell differentiation	Tang et al. [56], Hu et al. [140], Hand et al. [141] and Liang et al. [142]
mmu-miR3095-3p	100526502	156	MicroRNA metabolic process, tissue homeostasis, inner cell mass cell differentiation, positive regulation of cAMP-mediated signaling, post-embryonic development, post-embryonic body morphogenesis, cell proliferation in the forebrain, regulation of canonical Wnt signaling pathway, auditory receptor cell fate commitment, positive regulation of transcription of Notch receptor target, regulation of timing of cell differentiation, long-term depression, mTOR signaling pathway	Chiang et al. [143] and Kozomara et al. [144]
mmu-miR147-3p	387165	13	Wnt and MAPK signaling, inflammatory responses	Tang et al. [55] and Liu et al. [108]
mmu-miR1195	100316676	79	Cell differentiation, apoptosis	Zhang et al. [145] and Tagne et al. [57]


a
Intracellular membrane−bounded organelle
Endocytosis
Regulation of transforming growthfactor beta receptor signaling pathway
mRNA processing
Positive regulation of transcription, DNA−templated
Liver development
Endosome
Platelet−derived growth factor receptor signaling pathway
Cytoskeleton
Cellular component assembly
Developmental maturation
Cytoplasmic membrane−bounded vesicle
Growth
Cytoskeleton organization
Cell motility
Protein binding transcription factor activity
Cell morphogenesis
Homeostatic process
Cell division
Cell cycle
Anatomical structure formation involved in morphogenesis
Cellular component
Embryo development
Cell differentiation
Anatomical structure development
0 30 60 90
-log10(Pvalue)
genes
2500
2000
1500
1000
500
miRNAs
10
20
30
b
cGMP−PKG signaling pathway
mTOR signaling pathway
Phosphatidylinositol signaling system
Regulation of actin cytoskeleton
Signaling pathways regulating pluripotency of stem cells
Sphingolipid signaling pathway
Hippo signaling pathway
MAPK signaling pathway
Wnt signaling pathway
Estrogen signaling pathway
AMPK signaling pathway
GnRH signaling pathway
Notch signaling pathway
1.4 1.6 1.8
-log10(Pvalue)
genes
50
40
30
20
miRNAs
7
8
9
10
11
12
13
c
Cell proliferation
Immune system process
Cytoskeleton−dependent intracellular transport
Enzyme regulator activity
Vesicle−mediated transport
Endosome
Cytoskeletal protein binding
Protein binding transcriptionfactor activity
Cytoplasmic membrane−bounded vesicle
Cellular component assembly
Developmental maturation
Nucleic acid binding transcription factor activity
Cell cycle
Growth
Cytoskeleton organization
Cell division
Catabolic process
Cell motility
Homeostatic process
Protein complex
Cell morphogenesis
Biosynthetic process
Anatomical structure formation involved in morphogenesis
Cellular protein modification process
Embryo development
Ion binding
Cell differentiation
Molecular function
Anatomical structure development
Biological process
0 50 100 150 200 250
-log10(Pvalue)
genes
5000
4000
3000
2000
1000
miRNAs
11
12
13
14
15
16
d
Gap junction
TGF−beta signaling pathway
Lysosome
Regulation of actin cytoskeleton
mTOR signaling pathway
Insulin signaling pathway
Neurotrophin signaling pathway
Osteoclast differentiation
Axon guidance
T cell receptor signaling pathway
Phosphatidylinositol signaling system
PI3K−Akt signaling pathway
AMPK signaling pathway
Adipocytokine signaling pathway
MAPK signaling pathway
GnRH signaling pathway
Cell cycle
ErbB signaling pathway
HIF−1 signaling pathway
Adherens junction
Hippo signaling pathway
Estrogen signaling pathway
Focal adhesion
Endocytosis
FoxO signaling pathway
Thyroid hormone signaling pathway
Fatty acid biosynthesis
2.5 5.0 7.5 10.0 12.5
-log10(Pvalue)
genes
100
50
miRNAs
6
8
10
12
14
e
Endosome
Protein binding transcriptionfactor activity
Cellular component assembly
Enzyme binding
Cytoplasmic membrane−bounded vesicle
Cell death
Developmental maturation
Response to stress
Growth
Cell motility
Cytoskeleton organization
Protein complex
Cell division
Homeostatic process
Cell cycle
Cell morphogenesis
Anatomical structure formation involved in morphogenesis
Biosynthetic process
Cellular component
Cellular protein modification process
Embryo development
Cell differentiation
Molecular function
Ion binding
Anatomical structure development
Biological process
0 50 100
-log10(Pvalue)
genes
3000
2000
1000
miRNAs
10
11
12
13
14
15
f
AMPK signaling pathway
Rap1 signaling pathway
Lysosome
Long−term potentiation
Prolactin signaling pathway
Estrogen signaling pathway
Neurotrophin signaling pathway
mTOR signaling pathway
TNF signaling pathway
Regulation of actin cytoskeleton
GnRH signaling pathway
ErbB signaling pathway
Axon guidance
p53 signaling pathway
Endocytosis
Bacterial invasion of epithelial cells
Insulin signaling pathway
HIF−1 signaling pathway
FoxO signaling pathway
MAPK signaling pathway
Adherens junction
Yroid hormone signaling pathway
2 3 4 5
-log10(Pvalue)
genes
80
60
40
miRNAs
5
6
7
8
9
10
g
Negative regulation of cell differentiation
Extracellular vesicular exosome
Stem cell proliferation
Negative regulation of cell proliferation
Negative regulation of translation involved in gene silencing by miRNA
Negative regulation of Rho protein signal transduction
Response to transforming growth factor beta
Aging
Cell−cell signaling
Positive regulation of apoptotic process
Axon
Cell junction organization
Vesicle−mediated transport
Neuron projection
Positive regulation of synaptic transmission
Endosome
Developmental maturation
Growth
Dendrite
Cell motility
Immune system process
Cell death
Cytoplasmic membrane−bounded vesicle
Homeostatic process
Cell division
Biosynthetic process
Catabolic process
Cell morphogenesis
Ion binding
Anatomical structure development
0 25 50 75 100
-log10(Pvalue)
genes
2000
1500
1000
500
miRNAs
10
20
30
h
GnRH signaling pathway
Gap junction
Hippo signaling pathway
MAPK signaling pathway
Focal adhesion
Regulation of actin cytoskeleton
HIF−1 signaling pathway
mTOR signaling pathway
Rap1 signaling pathway
Long−term depression
Phosphatidylinositol signaling system
TGF−beta signaling pathway
AMPK signaling pathway
T cell receptor signaling pathway
Cell cycle
Sphingolipid signaling pathway
TNF signaling pathway
Inositol phosphate metabolism
Neurotrophin signaling pathway
Estrogen signaling pathway
FoxO signaling pathway
Axon guidance
Thyroid hormone signaling pathway
Adherens junction
Endocytosis
2 3 4 5
-log10(Pvalue)
genes
125
100
75
50
miRNAs
22.5
25.0
27.5
30.0
32.5

◂**Fig. 4** The GO and KEGG pathway analysis of differentially expressed cochlear sEV miRNAs from P3, P7, P14, and P21 mice. GO analysis of miRNAs of P3 (**a**), P7 (**c**), P14 (**e**), and P21 (**g**) mouse cochlear sEVs. KEGG enrichment pathways analysis of miRNAs of P3 (**b**), P7 (**d**), P14 (**f**), and P21 (**h**) mouse cochlear sEVs. The size of the bubble indicates the number of miRNAs, and the intensity of the color shows the number of genes targeted by the miRNA in all figures. P3 (blue), P7 (red), P14 (green), P21 (yellow)

We performed quantitative analysis of cochlear sEV proteins and found many differentially expressed proteins between the different age groups (Supplementary Fig. S4), suggesting that the expression of many sEV proteins changes with the development of the cochlea. We found 3,120 proteins that are differentially expressed across the four age

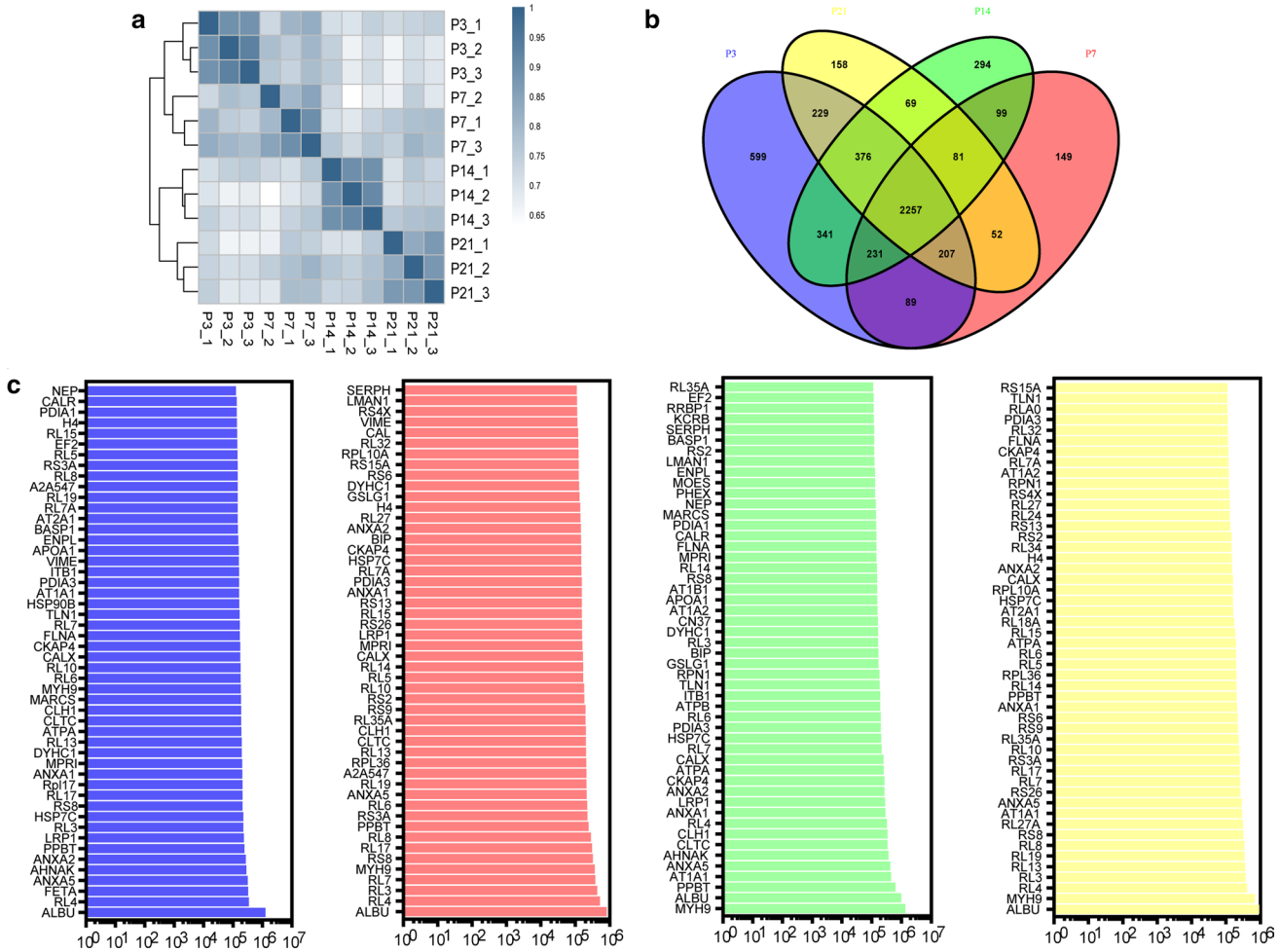
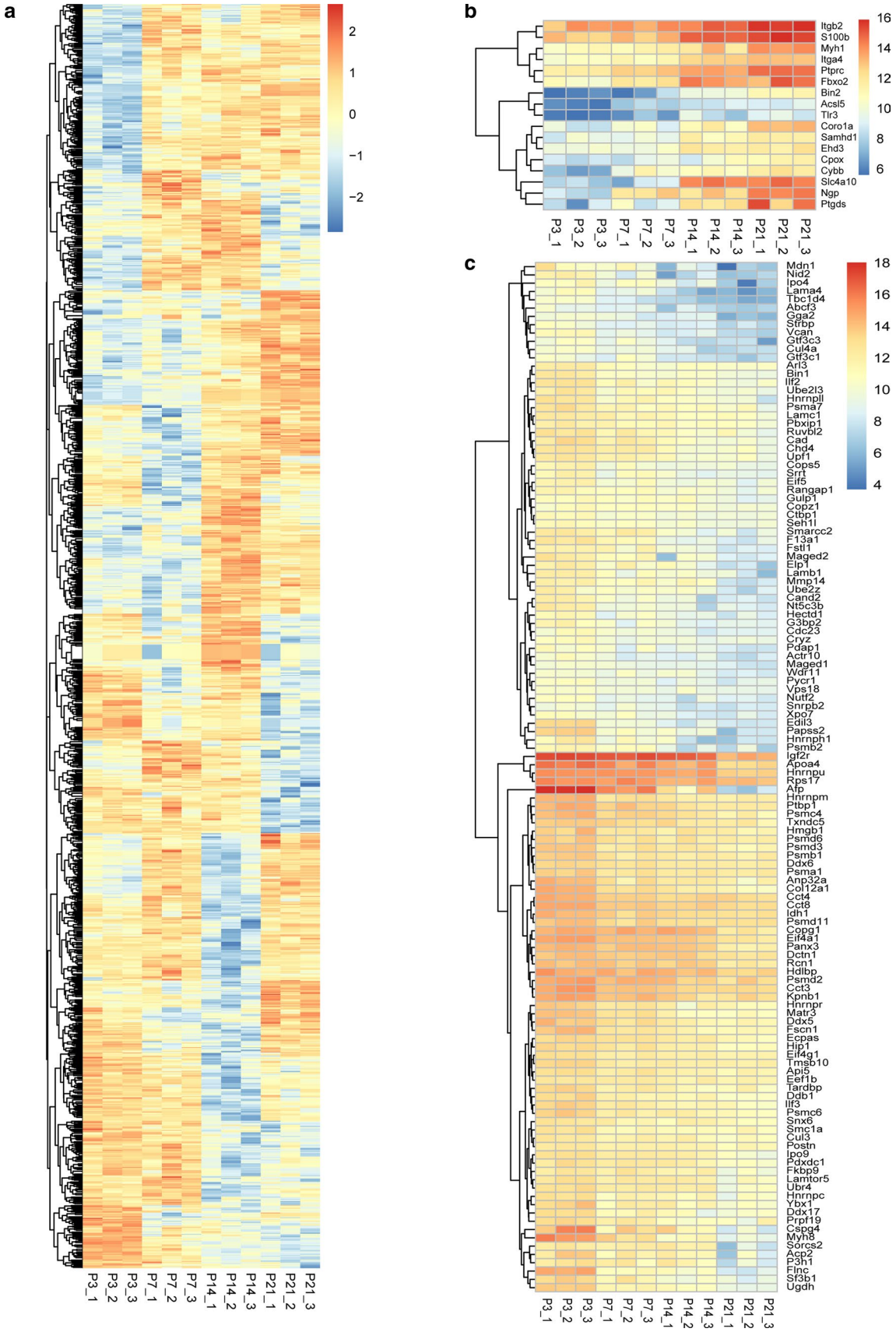


Fig. 5 Proteomics analysis of cochlear sEV proteins from P3, P7, P14, and P21 mice. **a** Cluster analysis of cochlear sEV proteomics data. **b** The Venn diagram of cochlear sEV proteomics data. **c** The top 50 most highly expressed proteins of cochlear sEVs from P3 (blue), P7 (red), P14 (green), and P21 (yellow) mice

Table 2 Mass spectrometry analysis identified typical sEV proteins

Name	Accession	− 10lgP	Intensity			
			P3	P7	P14	P21
TSG101	Q61187	214.54	4.11E+03	1.57E+03	2.75E+03	1.79E+03
CD9	P40240	293.38	4.24E+04	1.99E+04	5.30E+04	3.44E+04
CD63	P41731	195.28	7.86E+03	2.46E+03	1.52E+04	4.64E+03
CD81	P35762	248.37	1.46E+04	5.28E+03	2.05E+04	5.33E+03
Flotillin-1	O08917	376.55	1.03E+04	3.66E+03	1.31E+04	4.46E+03


a
2
1
0
−1
−2
P3_1
P3_2
P3_3
P7_1
P7_2
P7_3
P14_1
P14_2
P14_3
P21_1
P21_2
P21_3
b
Itgb2
S100b
Myh1
Itga4
Ptprc
Fbxo2
Bin2
Acsl5
Tlr3
Coro1a
Samhd1
Ehd3
Cpox
Cybb
Slc4a10
Ngp
Ptgds
16
14
12
10
8
6
c
18
16
14
12
10
8
6
4
Mdn1
Nid2
Ipo4
Lama4
Tbc1d4
Abcf3
Gga2
Strbp
Vcan
Gtf3c3
Cul4a
Gtf3c1
Arl3
Bin1
Ilf2
Ube2l3
Hnrnpll
Psma7
Lamc1
Pbxip1
Ruvbl2
Cad
Chd4
Upf1
Cops5
Srrt
Eif5
Rangap1
Gulp1
Copz1
Ctbp1
Seh1l
Smarcc2
F13a1
Fstl1
Maged2
Elp1
Lamb1
Mmp14
Ube2z
Cand2
Nt5c3b
Hectd1
G3bp2
Cdc23
Cryz
Pdap1
Actr10
Maged1
Wdr11
Pycr1
Vps18
Nutf2
Snrpb2
Xpo7
Edil3
Papss2
Hnrnph1
Psmb2
Igf2r
Apoa4
Hnrnpu
Rps17
Afp
Hnrnpm
Ptbp1
Psmc4
Txndc5
Hmgb1
Psmd6
Psmd3
Psmb1
Ddx6
Psma1
Anp32a
Col12a1
Cct4
Cct8
Idh1
Psmd11
Copg1
Eif4a1
Panx3
Dctn1
Rcn1
Hdlbp
Psmd2
Cct3
Kpnb1
Hnrnpr
Matr3
Ddx5
Fscn1
Ecpas
Hip1
Eif4g1
Tmsb10
Api5
Eef1b
Tardbp
Ddb1
Ilf3
Psmc6
Snx6
Smc1a
Cul3
Postn
Ipo9
Pdxdc1
Fkbp9
Lamtor5
Ubr4
Hnrnpc
Ybx1
Ddx17
Prpf19
Cspg4
Myh8
Sorcs2
Acp2
P3h1
Flnc
Sf3b1
Ugdh
P3_1
P3_2
P3_3
P7_1
P7_2
P7_3
P14_1
P14_2
P14_3
P21_1
P21_2
P21_3

◂**Fig. 6** The differentially expressed cochlear sEV proteins from P3, P7, P14, and P21 mice. **a** Heatmap of the differentially expressed cochlear sEV proteins. **b** Heatmap of the up-regulated proteins as mice age. **c** Heatmap of the down-regulated proteins as mice age. Values lower and higher than the mean are shown by blue and red scales, respectively

groups (Fig. 6a), and among them the expression level of 17 proteins increased with age (Fig. 6b), while the expression level of 124 proteins decreased with age (Fig. 6c). These results suggest that the expression patterns of proteins in the cochlear tissue-derived sEVs are correlated with age and may play significant roles in the formation of the inner ear system.

Functional analysis of differentially expressed cochlear sEV proteins

We performed GO analysis to identify the biological processes, molecular functions, and cell membrane components of the differentially expressed proteins (Fig. 7a–c). For the enriched biological processes, GO annotations indicated that these proteins are involved in cell, development, ion, neuron, signal communication, and vesicle processes. We mapped the top 20 molecular functions and cell membrane components of these proteins, and this showed that these proteins are mostly involved in ion binding, catalytic activity, protein binding, and RNA binding. In addition, the cellular components analysis revealed that these proteins are mostly found in the cytoplasm, the endomembrane system, and the plasma membrane.

We next conducted KEGG pathway analysis of the differentially expressed proteins, which showed that these proteins are mostly involved in the neurotrophin, AMPK, mTOR, PI3K-Akt, and cGMP-PKG signaling pathways and in endocytosis (Fig. 7d). According to GO, KEGG analysis and previous reports, we screened out 20 proteins from 141 age-related proteins (17 increased and 124 decreased with age as we showed above) that are reported to play roles in the inner ear and/or to be involved in the processes of development, cell differentiation, and proliferation. Table 3 lists these 20 top candidate proteins, including their UniProt accession numbers, functional descriptions, and references, which should be studied in the future to elucidate their roles in cochlear sEVs. These results suggest that cochlear sEVs may act as mediators in intercellular communications. Finally, to analyze the interactions between differentially expressed sEV proteins, we created a STRING protein interaction network (Fig. 7e).

Discussion

Small extracellular vesicles are important mediators in cellular communication and signal transmission, and they also can be used as naturally occurring carriers for drugs and biomarkers in clinical trials. At present, most researchers extract sEVs from in vitro culture systems, and previous research on inner ear sEVs has also relied on in vitro culture systems [33, 77]. However, the in vitro culture environment cannot truly replicate the in vivo environment, and sEVs derived from inner ear tissues can more accurately depict sEV functions in the inner ear. Therefore, we extracted sEVs from cochlear tissue for the first time and studied the miRNA transcriptomes and proteomics of the cochlear tissue-derived sEVs. We found that typical sEVs could be isolated from the cochlea by ultracentrifugation, and we identified 561 miRNAs and 5,231 proteins in cochlear tissue-derived sEVs that are engaged in multiple biological functions, including cellular communication, development, and vesicle production.

The cochlea is surrounded by the otic vesicle that gradually ossifies and becomes rigid as the mouse ages, especially after P10, and this makes it difficult to dissect the basilar membrane for extracting cochlear tissue-derived sEVs. Some recent studies have used enzyme digestion for the purpose of maintaining the integrity of the cells as much as possible to extract EVs from fat, brain, and tumor tissues [36, 78–81], while other studies have ground the tissues as a necessary step for extracting EVs [80, 82–84]. Crescitelli et al. showed that the digestive enzymes in the existing tissue extraction methods are ineffective for bone tissue, and the methods for this type of tissue need further optimization [85]. Considering the above factors, we improved the extraction method based on the scheme of Crewe et al. [36]. We used low-frequency grinding of the cochlear tissue to avoid breaking open the cells, and we increased the centrifugal force (12,000×*g*) for removing large vesicles and for isolating sEVs. TEM and NTA showed that the cochlear tissue-derived sEVs we extracted had typical sEV shapes and sizes. The western blotting also showed that the typical sEV markers—CD63, CD9, and Tsg101—could be detected in the sEV samples, while contaminating proteins Rab7, EEA1, Synapsin-1 and VGLUT3 from other vesicles and the intracellular protein GAPDH were not detected, which further confirmed the integrity and relative purity of the sEVs extracted by our method.

One of the important contents of sEVs is nucleic acids, which include miRNAs, lncRNAs, tRNAs, mtDNAs, and

ssDNA [86]. Among them, miRNAs are reported to have a role in numerous biological processes including organ development and maturation and cell communication [87, 88]. In addition, miR-318 from mesenchymal stem cell-derived sEVs promotes chondrogenesis by suppressing TAOK1 [43], and miR135a derived from epithelial exosomes accelerates the mesenchymal production of dentin matrix proteins by triggering the Wnt/β-catenin signaling pathway [37]. Therefore, small RNA-seq was performed to characterize the miRNAs in cochlear tissue-derived sEVs and to elucidate their possible roles in the cochlea. We identified 561 miRNAs in cochlear sEVs, including 179 differentially expressed miRNAs, and we found that the expression of 18 miRNAs increased and 17 miRNAs decreased as the mice aged. We used qPCR to verify the expression of miRNAs and found that 7 miRNAs (miRLet7c-5p, miR29a-3p, miR449a-5p, miR147-3p, miR30c-2-3p, miR3095-3p, and miR1195) were consistent with the RNA-seq analysis results. Overexpression of miRLet7c-5p can inhibit laryngeal squamous cell carcinoma cell proliferation and can regulate microglial activation during the repair of brain injury [46, 89], and upregulation of miR30c-2-3p suppresses gastric cancer and the proliferation of renal cell carcinomas [56, 90]. miR29a-3p, miR449a-5p, and miR147-3p have been reported to be accumulated in exosomes derived from oral squamous cells, macrophages, and bronchoalveolar lavage fluid [53, 55, 91, 92]. In addition, upregulation of miR29a-3p rescues bronchopulmonary dysplasia and has a negative regulatory effect on the Smad, NFκB, and canonical Wnt signaling pathways [93–95]. Importantly, miR29a-3p directly targets the Wnt-related genes *DVL3* (Dishevelled 3), *CSNK2A2* (casein kinase 2 alpha 2 polypeptide), *FZD3* (Frizzled family receptor 3), and *FZD5* (Frizzled family receptor 5) [95]. These seven miRNAs whose expression increases as mice age are listed in Table 1, and they may be involved in the development of the cochlea after birth and may act as new targets to be further studied in the future to elucidate the detailed mechanisms behind cochlear development.

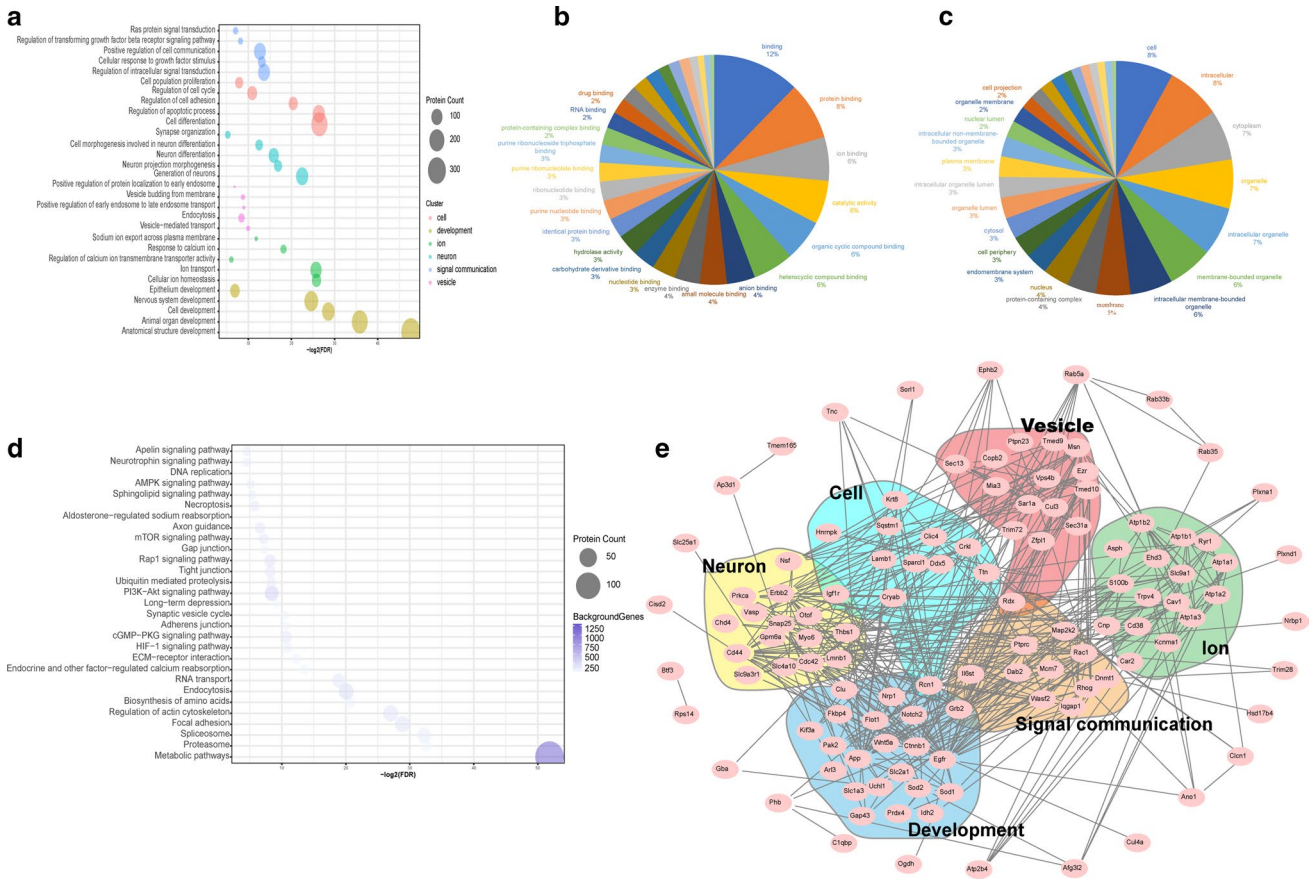


Fig. 7 GO and KEGG pathway analysis of differentially expressed cochlear sEV proteins from P3, P7, P14, and P21 mice. Differentially expressed cochlear sEV proteins were identified by GO analysis as part of biological processes (**a**), molecular components (**b**), and cell membranes (**c**). **d** KEGG pathway analysis showing the significantly enriched pathways of differentially expressed cochlear sEV proteins in the four age groups. The size of the bubble shows the amount of protein, and the intensity of the color indicates the number of genes targeted by the protein in **a** and **d**. **e** The STRING network analysis for cochlear sEV proteins

Table 3 Protein candidates of age-related cochlear tissue-derived sEVs with functional categories

Protein	Accession	Functional description	Expression trends with age	Reference
Fbxo2	Q80UW2	Biological regulation, regulation of cell population proliferation, regulator for age-related hearing loss	↑	Atkin et al. [99] and Nelson et al. [100]
Tlr3	Q99MB1	Biological regulation, anatomical structure development, immune system process, regulation of apoptotic process, positive regulation of cell communication, animal organ development, signal transduction	↑	Yamada et al. [101], Gollmann-Tepeköylü et al. [102] and Krisher et al. [146]
Slc4a10	Q5DTL9	Ion transport, system development, anatomical structure development, nervous system development, cell differentiation, generation of neurons, sodium ion transmembrane transport, neuron differentiation, multicellular organism growth	↑	Sinning et al. [98], Huebner et al. [103] and Christensen et al. [147]
S100b	P50114	Metabolic process, regulation of cellular component organization, response to stimulus, system development, anatomical structure development, multicellular organism development, regulation of cell death, ion homeostasis, cell differentiation, regulation of apoptotic process, nervous system development, neurogenesis, regulation of intracellular signal transduction, positive regulation of cell communication	↑	Raponi et al. [148], Zeng et al. [149], Nishiyama et al. [150] and Riuzzi et al. [151]
Ddx5	Q61656	Regulation of localization, regulation of transport, positive regulation of biological process, metabolic process, cell proliferation and differentiation	↓	Legrand et al. [152], Dardenne et al. [153] and Arun et al. [154]
Chd4	Q6PDQ2	Cellular metabolic process, transmembrane transport, cellular localization, response to oxidative stress, neuronal differentiation	↓	Xu et al. [111], Tomofuji et al. [155] and Sun et al. [156]
Sorcs2	Q9EPR5	Maintain ciliary morphology, apoptosis, neuroprotection	↓	Forge et al. [118], Leloup et al. [157] and Malik et al. [158]
Idh1	O88844	Cellular metabolic process, multicellular organism development, anatomical structure development, system development, system development	↓	Zhang et al. [54], Kim et al. [121], Itsumi et al. [159] and Rohle et al. [160]
Panx3	Q8CEG0	Establishment of localization, cellular component organization or biogenesis, transport	↓	Abitbol et al. [119, 120] and Ishikawa et al. [161]
Hnrnp	P49312	Cellular localization, metabolic process, regulation of cellular protein localization, mRNA processing, multicellular organism development, anatomical structure development, system development, cellular developmental process, cell differentiation, positive regulation of cell cycle process, cell division, animal organ development	↓	Chen et al. [105] and Ruan et al. [162]
Ptbp1	P17225	Cellular metabolic process, RNA processing, regulation of protein localization, multicellular organism development, anatomical structure development, system development, generation of neurons, nervous system development, regulation of cell development, cell differentiation, regulation of neuron projection development, regulation of neuron differentiation	↓	Mokabber et al. [112], Linares et al. [163], Georgilis et al. [164] and Vuong et al. [165]
Lama4	P97927	Regulation of localization, cell adhesion, multicellular organism development, system development, cellular developmental process, cell differentiation, regulation of multicellular organismal development, regulation of cell migration	↓	Suzuki et al. [49], Kim et al. [116] and Gorfu et al. [166]
Ilf3	Q9Z1X4	Negative regulation of transcription, cell proliferation, cell apoptosis	↓	Jia et al. [107] and Larcher et al. [175]

Table 3 (continued)

Protein	Accession	Functional description	Expression trends with age	Reference
Lamtor5	Q9D1L9	Cellular localization, regulation of metabolic process, regulation of cell death, regulation of apoptotic process, regulation of intracellular signal transduction, positive regulation of cell communication	↓	Zhang et al. [88] and Liu et al. [108]
Psmd2	Q8VDM4	Cellular catabolic process, regulation of biological process, cell proliferation, cell cycle	↓	Li et al. [109]
Ddb1	Q3U1J4	Metabolic process, response to stimulus, organelle organization, regulation of cell death, homeostatic process, regulation of apoptotic process, regulation of cell cycle, signal transduction	↓	Zheng et al. [110], Zhao et al. [126], Yang et al. [167] and Gao et al. [168]
Ruvbl2	Q3TXT7	Establishment of localization, metabolic process, positive regulation of biological process, cellular component assembly, cellular response to stress, regulation of gene expression, regulation of signaling, regulation of cell communication, regulation of signal transduction	↓	Zhang et al. [42] and Hong et al. [114]
Cul4a	Q3TCH7	Metabolic process, regulation of biological process, developmental process, anatomical structure development, animal organ development, regulation of response to stimulus, regulation of cell differentiation, immune system process, regulation of multicellular organismal development, regulation of cell cycle, regulation of cell population proliferation	↓	Liu et al. [115], Pan et al. [169], Kopanja et al. [170] and Waning et al. [171]
P3h1	Q3V1T4	Skeletal development, hearing loss	↓	Fratzl-Zelman et al. [172], Pokidysheva et al. [173] and Vranka et al. [174]

We also performed GO and KEGG analysis on the highly expressed miRNAs. GO analysis showed that these miRNAs are important for growth, development, maturation, anatomical structure development, ion binding, cell differentiation, and cell proliferation, all of which are relevant to cochlear development events. The enriched miRNAs in the sEVs are involved in the Hippo, MAPK, Wnt, Notch, TGF-β, and PI3K-Akt signaling pathways, most of which are essential to the development of the cochlea and in regulating the pluripotency of stem cells. These results showed that miRNAs enriched in cochlear tissue-derived sEVs may be essential for cell communication during inner ear development.

Proteins are another major component of sEVs and play significant roles in cell communication, mediation of immune responses, and proliferation of cancer cells and as markers for disease diagnosis [96, 97]. We performed proteomics analysis of the cochlear tissue-derived sEV proteins and identified 5,231 proteins, including 3,120 differentially expressed proteins, in the four age groups. We also found the sEV marker proteins CD63, CD9, CD81, and Tsg101 in the proteomics data, which again verified the purity of our isolated cochlear sEVs. We identified 1,051 proteins in the cochlear sEVs that overlapped with proteins in the Vesiclepedia and Exocarta databases.

Among the differentially expressed sEV proteins, we found that the expression of 17 proteins increased and the expression 124 proteins decreased as the mice aged. For the 17 increased proteins, Slc4a10, Fbxo2, and S100b are related to the process of neurodevelopment and in regulating the differentiation and excitability of neurons [98, 99], which suggests that these three proteins may be involved in the innervation of the cochlea that is required for hearing function. Fbxo2 is enriched in the inner ear and is a key regulator of age-related hearing loss [100]. Tlr3 is also present in the inner ear and regulates immune responses [101, 102], and Tlr4 acts as a mediator in protecting HCs from damage by exosomes secreted by SCs [33]. This suggests that Tlr3 might also have a protective role on inner ear development. In addition, Slc4a10 is important for maintaining ion homeostasis in the inner ear, and the absence of Slc4a10 can lead to hearing loss [103, 104]. Among the proteins that decrease with age, Hnrnp [105], Ddx5 [106], Ilf3 [107], Lamtor5 [108], Psmd2 [109], Ddb1 [110], and Chd4 [111] are reported to be related to cell proliferation and differentiation. Ptbp1 [112], Chd4 [113], Ruvbl2 [114], Cul4a [115], and Lama4 [116] are required for early developmental processes and neuronal differentiation, and some proteins are also present in the inner ear, such as P3h1 [117], Sorcs2 [118], Panx3 [119, 120], Idh1 [121], and Lamb1 [122]. P3h1 knockout mice showed dysplasia of middle ear bones and hearing impairment [117]. Sorcs2 regulates HC development by maintaining the shape of the cilia [118], while Idh1 is a protein found in the cochlea and may play a role in age-related hearing loss by acting as an antioxidant [121, 123]. Panx3 is a pannexin channel protein and is mainly present in the cochlear bone structure and is essential for the maintenance of cochlear morphology [119, 120], and the expression of Panx3 is regulated during development and reaches its peak at P8 [119]. The GO and KEGG analysis of 17 increased and 124 decreased proteins and previous reports of these proteins, some of which are expressed in the inner ear, showed that they affect the ion balance of the inner ear and mediate inner ear immunity and cilia formation [98, 99, 101, 103, 119]. In addition, some proteins are involved in various developmental processes of the skeletal and nervous systems [111, 115, 117] and regulate cell proliferation, differentiation, apoptosis, and other cellular communication processes [110, 111]. Table 3 lists the 20 top candidate proteins among them. These cochlear sEV proteins may play important roles and may be used as new targets for studying the development of the cochlea in the future.

We conducted GO and KEGG analysis of the differentially expressed proteins. The GO analysis revealed that cochlear tissue-derived sEV proteins play significant roles in various biological processes such as Ras protein signal transduction, cell proliferation, cell differentiation, neuron differentiation, endocytosis, cellular ion homeostasis, nervous system development, and organ development and that these proteins are involved in many molecular functions, including ion binding, protein binding, and RNA binding. The cellular components analysis showed that sEVs can be secreted from the cell, cytoplasm, and endomembrane system. According to the KEGG pathway analysis, these proteins are involved in axon guidance, the synaptic vesicle cycle, the AMPK signaling pathway, the mTOR signaling pathway, the PI3K-Akt signaling pathway, and endocytosis. Synapses on HCs are connected to spiral neurons for transmitting signals to the brain, and this activity is essential for hearing function [8, 124, 125]. In addition, these pathways have also been reported to be critical for the biological functions of the inner ear. Down-regulating the AMPK signaling pathway can reduce noise-induced damage to HCs and can prevent age-related hearing loss [126, 127]. The mTOR signaling pathway is involved in reprograming Myc/NICD to promote HC regeneration [16], and age-related hearing loss and HC damage can be relieved by inhibiting the mTOR signaling pathway [128, 129]. Balancing the AMPK and mTOR signaling pathways can further protect HCs from damage by ototoxic drugs [130]. We also created a STRING protein-interaction network investigating the interactions between differentially expressed sEV proteins, and this showed that sEV proteins involved in vesicles, development, neurons, signal communication, cellular processes, and ion homeostasis have close interactions with each other and with other differentially expressed cochlear sEV proteins. These results indicate that sEVs may be critical for the development of the

cochlear nervous system, as well as for the protection and regeneration of HCs during development.

In summary, we isolated cochlear tissue-derived sEVs from mice of different ages after birth by ultracentrifugation and characterized the miRNA transcriptomes and proteomics of these sEVs to elucidate their possible roles. We found 561 miRNAs and 5,231 proteins in the cochlear sEVs, and among them 179 miRNAs and 3,120 proteins were differentially expressed at different ages. We further analyzed these differentially expressed miRNAs and proteins and found that the expression of many miRNAs and proteins may be relevant to the maturation of HCs, to changes in SC characteristics, to neural development, and to the protection of HCs from P3 to P21. These miRNAs and proteins might be used as new targets for further studying the detailed mechanism of cochlear development after birth.

Based on our results, we speculate that sEVs play a regulatory role in the maturation of HCs, HC regeneration from inner ear stem cells, and neural development in the inner ear after birth, and this should be further confirmed in future studies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00018-022-04164-x.

Acknowledgements Thanks to all colleagues who contributed to this research.

Funding This work was supported by grants from the National Key RD Program of China (nos. 2021YFA1101300, 2020YFA0112503), the Strategic Priority Research Program of the Chinese Academy of Sciences (No. XDA16010303), the National Natural Science Foundation of China (nos. 82171149, 81970892, 82030029, 81970882), the Natural Science Foundation of Jiangsu Province (nos. BK20190062 and BE2019711), the Science and Technology Department of Sichuan Province (no. 2021YFS0371), the Shenzhen Fundamental Research Program (no. JCYJ20190814093401920, JCYJ20210324125608022), Open Research Fund of State Key Laboratory of Genetic Engineering, Fudan University (No. SKLGE-2109), and the Fundamental Research Funds for the Central Universities for the Support Program of Zhishan Youth Scholars of Southeast University (no. 2242021R41136).

Availability of data and material All the data and material analyzed in this research are included in this article and its supplementary files.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval and consent to participate All animal studies followed the authorized guidelines of Southeast University's Animal Care and Use Committee and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The number of animals was kept to a minimum, and all efforts were made to reduce their suffering. Written informed consent was obtained from individuals or their guardians.

Consent for publication Not applicable.

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