Characterization of the microRNA Transcriptomes and Proteomics of Cochlea Tissue-derived Small Extracellular Vesicles From Different Age of Mice After Birth

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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 nm to 200 nm, have been considered as a significant medium for information communication in both the processing of physiological and pathological. 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 proteomics of cochlear sEVs from mice at different ages. Many microRNAs and proteins were discovered to be related with inner ear development, anatomical structure development, and the 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.

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)2 [1]. The detection of sound 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 seven 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]. HC synapses begin to mature at P14, which is when mice begin to gain hearing ability [12]. 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 transdifferentiated into

HCs by induction of Wnt signaling or inhibition of Notch signaling in newborn mice [13, 14]. However, the stemness of SCs deteriorates with age, and their capacity to divide is completely lost by P14 [15].

It has been reported that many important transcription factors and signaling pathways are associated with the development of the cochlea, such as Sox2, Atoh1 [16], and the Wnt, Notch, and FGF signaling pathways [17, 18]. 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 [19, 20]. 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 latest years, and are reported to be involved in intercellular signal transmission during many important pathological and physiological processes [21-23]. sEVs have sizes between 30 nm to 200 nm and can be generated by various cells [24]. 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 [25, 26]. sEVs participate in cell proliferation and differentiation in both pathological and healthy situations through signaling pathways mediated by miRNAs [27-29], and sEVs are involved in intercellular signal transmission during the development of brain neural circuits and in regulating growth patterns during embryonic development [30, 31].

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 [32] and that inner ear stem cell-derived exosomes can reduce ototoxic drug damage by transferring miR-182-5p to HEI-OC1 cells [33, 34]. 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 [35, 36]. Briefly, the cochleae were dissected, placed in a centrifuge tube with PBS buffer, and then ground for 1 minute at 40 Hz in a grinder (Jingxin, Shanghai, China). The sample was filtered via filter with an aperture size 0.22 μm after differential centrifugation to eliminate cell debris and microvesicles ($600 \times g$ for 10 minutes, $2,000 \times g$ for 15 minutes, and $12,000 \times g$ for 50 minutes, all at 4°C). The filtered samples were concentrated to 1–1.5 ml in a 50 ml 100 kDa MWCO ultrafiltration centrifuge tube (Millipore) at $3,000 \times g$ for 15 minutes at 4°C . The samples were then ultracentrifuged at $110,000 \times g$ for 2 hours 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 $110,000 \times g$ for 2 hours at 4°C . The sEVs were finally resuspended in 400–500 μl PBS for the following experiments.

Transmission electron microscopy

For visualizing cochlear sEVs by TEM (Hitachi, Tokyo, Japan), 10 μl of sEV sample was negatively stained with 1.5% phosphotungstic acid on an electron microscope copper grid for 2–5 min.

Nanoparticle tracking analysis

NTA (NS300, Malvern, United Kingdom) was employed to identify the size and concentration of sEVs. A total of five 60-second 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 5 videos.

Immunofluorescent staining

Immunofluorescent staining was performed according to a previous study [37]. In brief, cochleae were decalcified with 0.5 M EDTA after being fixed in 4% (v/v) paraformaldehyde. As followed, cochleae blocked and incubated with primary antibodies. After that, fluorescence-conjugated secondary antibodies were added and bound to primary antibodies. A Zeiss LSM 700 confocal microscope was employed to capture fluorescent photos 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) as secondary antibodies.

Western blotting

sEVs were freeze-dried and then lysed in 200 μl RIPA lysis buffer (Beyotime) with $1\times$ 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 $5\times$ sodium dodecyl sulfate (SDS) loading buffer. SDS polyacrylamide gel electrophoresis was utilized to isolate the sEV proteins, and then transferred onto a polyvinylidene

difluoride membrane at 275mA for 90 minutes. The membrane was blocked with 5% BSA [5% (v/v) bovine serum albumin in 0.1% (v/v) Tween-20 in PBS] for 1h 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 visualized target bands on a Tanon-5200 automatic chemical imaging system. The primary antibodies were anti-CD63 (ab217345, 1:1,000 dilution), anti-CD9 (ab92726, 1:1500 dilution), anti-Tsg101 (ab125011, 1:2,000 dilution), anti-mouse EEA1 (Santa Cruz Biotechnology, 1:100 dilution), and anti-rabbit Rab7 (Cell Signaling, 1:1,000 dilution), and anti-GAPDH (Kangchen, KC-5G4, 1:2,000 dilution).

RNA extraction and quantitative real-time PCR

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

Total RNA from sEV was reverse-transcribed to cDNA using a miRNA 1st strand cDNA synthesis kit (Vazyme #MR101) following manufacturer's directions. Real-time PCR was done using an Applied Biosystems real-time RCR instrument by miRNA Universal SYBR qPCR Master Mix (Vazyme, #MR101-01) to quantify the miRNA expression levels. All primers sequences are listed in Table of supplement. The levels of miRNAs were compared utilizing 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 building material. Following the manufacturer's protocol, sequencing libraries were created employing NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA), and miRNA data was 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 $|\log_2\text{FoldChange}| > 2.0$ and $p < 0.05$ as the threshold. Short Time-Series Expression Miner (STEM) (v 1.3.13) software was used for expression trend analysis. In order to avoid too many false positives, only miRNA-targeted genes in the Tarbase v7.0 database,[38] which were identified experimentally, were selected.

DIANA-279 miRPath v.3 was used to assess miRNA enrichment pathways [39], and the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) 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 minutes at 95°C. After that, the samples were diluted 5-fold with 50 mM triethylammonium bicarbonate and digested for 3 hours at 37°C with Lys-C (Wako) at 1:100 (w/w). To further degrade the peptides, the samples were treated overnight in a 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 to the aforesaid combination. The sample solution was vortexed before being centrifugation at $15,000 \times g$ for 3 minutes. The organic phase on the top was discarded, and the aqueous phase at the base was harvested and frozen dried by refrigerated vacuum centrifuge (Laconco CentriVap). The desalting experiment was developed on an 8 mm extraction disk as directly by manufacturer (3M Empore 2240-SDB-XC). All samples were stored at -80°C .

LC-MS/MS and quantitative data analysis

LC-MS/MS experiment method refer to previous study [40]. Briefly, the peptides were solubilized in 10 μL 0.1% formic acid (FA), then taken 2 μL into the nanoelute for proteomics analysis. All peptides can be separated in a 25 cm internal packed column in the mobile phase with a fluid velocity of 300nl/min. The timsTOF Pro mass spectrometer (Bruker) is connected to Nanoeluate in real time, and the data settings are adjusted to full scan (m/z 100 to 1,700) 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 exosome, and isolated inner ear proteins in various samples, the intensities of 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 analysis, and the protein-protein interaction network obtained by STRING database (<http://string-db.org/>).

Statistical analysis

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

Table1 Mass spectrometry analysis identified typical sEV proteins

Name	Accession	Intensity				
		-10lgP	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

Results

Isolation and characterization of cochlear tissue-derived sEVs

sEVs were isolated from the cochlear tissue of mice at P3, P7, P14, and P21 by ultracentrifugation as described previously [35, 41] (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 ($600 \times g$ and $2,000 \times g$) to remove cell debris and then at high speed ($12,000 \times g$) to remove large extracellular vesicles. After passing through a $0.22 \mu\text{m}$ filter, the samples are concentrated by ultrafiltration with a 100 kDa MWCO ultrafilter. Finally, sEVs were isolated by ultracentrifugation at $110,000 \times g$. 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), and GAPDH, 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). Together, these results suggest that this isolation method of cochlear tissue-derived sEVs is feasible and can yield relatively pure sEVs.

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

sEVs contain a variety of RNAs, especially miRNAs, that 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 exosomes from P3, P7, P14, and P21 mice to discover 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 coefficient (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 (Fig. S1), 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. The miRNAs 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 that 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 7 miRNAs (miRlet7c-5p, miR29a-3p, miR449a-5p, miR147-3p, miR30c-2-3p, miR3095-3p, and miR1195) was matched to the results of bioinformatics analysis.

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, and g). Fig. 4(b, d, f, and h) show 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 likely actively 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 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 5,231 proteins were identified, and 2,257 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 1). Fig. 5c shows the top 50 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 (Fig. S2a). Fig. S2b 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.

We performed quantitative analysis of cochlear sEV proteins, which showed that there were many differentially expressed proteins between the different age groups (Fig. S3), 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 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). Most of these proteins are reported to be involved in neurodevelopmental process [77], cilia formation [78], ion homeostasis [79], cell proliferation and differentiation [80], and signaling [81]. 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). These results suggest that cochlear sEVs may act as mediators in intercellular communications. Finally, in order to analyze the interactions between differentially expressed sEV proteins, we created a STRING protein interaction network (Fig. 7e).

Discussion

sEVs 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 [32, 82]. 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 in order to extract EVs from fat, brain, and tumor tissues [35, 83-86], while other studies have ground the tissues as a necessary step for extracting EVs [85, 87-89]. 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 [90]. Considering the above factors, we improved the extraction method based on the scheme of Crewe *et al.* [35]. We used low-frequency grinding of the cochlear tissue to avoid breaking open the cells, and we increased the centrifugal force ($12,000 \times g$) 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 and EEA1 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 [91]. Among them, miRNAs are reported to have a role in numerous of biological processes including organ development and maturation and cell communication [92, 93]. 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 via triggering the Wnt/ β -catenin signaling pathway [36]. 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 miRNA, 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, 94], and upregulation of miR30c-2-3p suppresses gastric cancer and the proliferation of renal cell carcinomas [56, 95]. 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, 96, 97]. In addition, upregulation of miR29a-3p rescues bronchopulmonary dysplasia and has a negative regulatory effect on the Smad, NFkB, and canonical Wnt signaling pathways [98-100]. 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) [100]. These 7 miRNAs, whose expression increases as mice age, 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.

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 were 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 [101, 102]. 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 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 [103, 104], 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 for age-related hearing loss [105]. Tlr3 is also presented in the inner ear and regulates immune responses [106, 107], and Tlr4 acts as a mediator in protecting HCs from damage by exosomes secreted

by SCs [32]. This suggests that Tlr3 might also have a protective role on inner ear's development. In addition, Slc4a10 is important for maintaining ion homeostasis of inner ear, and the absence of Slc4a10 can lead to hearing loss [108, 109]. Among the proteins that decrease with age, Hnrnp [110], Ddx5 [111], Ilf3 [112], Lamtor5 [113], Psmd2 [114], Ddb1 [115], Psmd6 [116], and Chd4 [117] are reported to related to cell proliferation and differentiation. Ptp1 [118], Chd4 [119], Ruvbl2 [120], Cul4a [121], and Lama4 [122] are required for early developmental processes and neuronal differentiation, and some proteins also presented in the inner ear, such as P3h1 [123], Sorcs2 [78], Panx3 [124, 125], Idh1 [126], Lamb1 [127]. P3h1 knockout mice showed dysplasia of middle ear bones and hearing impairment [123]. Sorcs2 regulates HC development by maintaining the shape of the cilia [78]. Idh1 is protein found in the cochlea and may play a role in age-related hearing loss act as an antioxidant [126, 128]. Panx3 is a pannexin channel protein and is mainly presented in the cochlear bone structure and is essential for the maintenance of cochlear morphology [124, 125], and the expression of Panx3 is regulated during development and reaches its peak at P8 [124]. According to previous reports, these cochlear sEV proteins may play important roles and may be used as new targets for 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 a significant role in various biology 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. These proteins are invested in axon guidance, the synaptic vesicle cycle, the AMPK signaling pathway, the mTOR signaling pathway, the PI3K-Akt signaling pathway, and endocytosis, according to the KEGG pathway analysis. Synapses on HCs are connected to spiral neurons for transmitting signals to the brain, and this activity is essential for hearing function [8, 129, 130]. 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 the age-related hearing loss [131, 132]. The mTOR signaling pathway is involved in reprogramming Myc/NICD to promote HC regeneration [15], and age-related hearing loss and HC damage can be relieved by inhibiting the mTOR signaling pathway [133, 134]. Balancing the AMPK and mTOR signaling pathways can further protect HCs from damage by ototoxic drugs [135]. 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 sEV 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 microRNA 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 miRNA and proteins and found that the expression of many miRNAs and proteins may be relevant to the maturation of HCs, to changes in SCs 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 during the development of the inner ear after birth, and this should be further confirmed in future studies.

Declarations

Ethics approval and consent to participate

All studies of animal 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 amount of animals was kept to a minimum, and all efforts were made to reduce their suffering.

Consent for publication

Not applicable.

Availability of data and material

All the data analyzed by this research is included in this article and its supplementary files.

Competing interests

The authors declare that they have no conflict of interest.

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Author contributions

RC, SZ, and WAT designed the experiments. PJ, JA, LW, YZ, HX, and MT isolated and characterized the cochlear sEVs. XM performed the miRNA analysis. SH and LM performed the proteomics analysis. PJ and SZ analyzed all data and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 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 sEV samples. CD63, CD9, and TSG101 were used as sEV markers, and EEA1, Rab7, and GAPDH 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 .

Figure 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.

Figure 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. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$.

Figure 4

The GO and KEGG pathway analysis of differentially expressed cochlear sEV miRNAs from P3, P7, P14, and P21 mice. (a, c, e, g) GO analysis of miRNAs of P3 (a), P7 (c), P14 (e), and P21 (g) mouse cochlear sEVs. (b, d, f, h) KEGG enrichment pathways analysis of miRNAs of P3 (b), P7 (d), P14 (f), and P21 (h) mouse cochlear sEVs. The size of the bubble described the number of miRNAs, and the intensity of the

color shows the amount of genes targeted by the miRNA in all figures. P3 (blue), P7 (red), P14 (green), P21 (yellow).

Figure 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.

Figure 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.

Figure 7

GO and KEGG pathway analysis of differentially expressed cochlear sEV proteins from P3, P7, P14, and P21 mice. (a-c) Differentially expressed cochlear sEV proteins are identified by GO analysis that 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 describes the number of genes targeted by the protein in a and d. (e) The STRING network analysis for cochlear sEV proteins.

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