Neuro-Oncology

Neuro-Oncology 18(11), 1498–1507, 2016
doi:10.1093/neuonc/now099
Advance Access date 18 May 2016

Extracellular vesicles derived from human vestibular schwannomas associated with poor hearing damage cochlear cells

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Background. Vestibular schwannoma (VS) is a tumor of the vestibular nerve that transmits balance information from the inner ear to the brain. Sensorineural hearing loss occurs in 95% of patients with these tumors, but the cause of this loss is not well understood. We posit a role of VS-secreted extracellular vesicles (EVs) as a major contributing factor in cochlear nerve damage.

Methods. Using differential centrifugation, we isolated EVs from VS cell line HEI-193 and primary cultured human VS cells from patients with good hearing or poor hearing. The EVs were characterized using a Nanosight device and transmission electron microscopy and by extracting their RNA content. The EVs' effects on cultured murine spiral ganglion cells and organotypic cochlear cultures were studied using a transwell dual-culture system and by direct labeling of EVs with PKH-67 dye. EV-induced changes in cochlear cells were quantified using confocal immunohistochemistry. Transfection of VS cells with a green fluorescent protein–containing plasmid was confirmed with reverse transcription PCR.

Results. Human VS cells, from patients with poor hearing, produced EVs that could damage both cultured murine cochlear sensory cells and neurons. In contrast, EVs derived from VS cells from patients with good hearing did not damage the cultured cochlear cells.

Conclusions. This is the first report on EVs derived from VSs and on the capacity of EVs from VSs from patients with hearing loss to selectively damage cochlear cells, thereby identifying a potential novel mechanism of VS-associated sensorineural hearing loss.

Keywords: cochlea, extracellular vesicles, sensorineural hearing loss, transwell culture, vestibular schwannoma.

Exosomes (including microvesicles and other vesicular structures released by cells, termed *extracellular vesicles* [EVs]) are cell-derived vesicles, measuring 30 to 200 nm, which contain the genetic profile of their cell of origin, including RNA, DNA, microRNA (miRNA), and proteins within a bilipid membrane.[1–3] EVs are produced by both normal and neoplastic cells[4] of virtually every organ origin and have been identified in virtually every biofluid.[5] EVs are thought to be important mediators of intercellular communication by transferring their cargo between cells, both locally and systemically.[6] EVs are emerging as important serum biomarkers of human diseases, including neurodegenerative diseases[7] and neoplasms[4] of the central nervous system, being able to accurately predict diseases 1–10 years before they become clinically manifest.[7] Moreover, EV-based therapeutics are being developed and have already entered the clinical arena.[8]

EVs produced by vestibular schwannomas (VSs) have not been previously reported. Yet VS, a tumor of the vestibular nerve that transmits balance information from the inner ear to the brain, is the fourth most common adult intracranial neoplasm.[9] VSs occur sporadically as unilateral tumors in 95% of patients or in association with hereditary neurofibromatosis

Received 20 October 2015; accepted 13 April 2016

type 2 (NF2) syndrome, whose hallmark is bilateral VSs. VSs arise from myelin-producing Schwann cells of vestibular nerves due to somatic loss of function of merlin in NF2[10] and to other, as yet undefined, causes. Sensorineural hearing loss (SNHL) occurs in 95% of patients with these tumors,[11] but the cause of this hearing loss is not well understood. The hearing loss from VS growth results in part from tumor compression of the adjacent cochlear nerve that serves as a sensory conduction pathway. However, clinical observations suggest that there may be other explanations for the SNHL: (i) deafness in VS patients can occur suddenly, without change in tumor size[12]; (ii) large VSs may not cause hearing loss, while small ones may[13,14]; and (iii) the size of sporadic VSs within the internal auditory canal does not correlate with the extent of ipsilateral hearing loss[14,15] (albeit there is a correlation between VS size and hearing loss in NF2).[16,17] Histopathological examination of untreated VSs demonstrates ipsilateral cochlear damage, including loss of sensory inner hair cells (IHCs) (75% of specimens), outer hair cells (OHCs) (88%) and spiral ganglion neurons (SGNs) (85%).[13,14] This damage of cells in the cochlea, which is bathed by fluids in proximity to the VS, raises the hypothesis that tumor-induced deafness is related to tumor-secreted materials that bathe the cochlea. We have explored whether VS-derived EVs are mediators of this selective cochlear damage, as we have previously shown that genetic expression profiles of VSs associated with poor hearing (PH) differ from those of VSs associated with good hearing (GH).[18]

Materials and Methods

Human VS Cell Culture

A human NF2 VS-derived cell line immortalized with human papilloma virus E6-7 genes[19] was acquired from House Ear Institute (HEI-193). Primary VS cultures were derived from patients with sporadic VSs after obtaining informed consent. Experimental investigations of human and animal subjects were carried out according to the protocols approved by the Ethics and Human Studies Committee at the Massachusetts Eye and Ear and Massachusetts General Hospital (#196424-22, #717176, #15-003), while honoring the Declaration of Helsinki. Patients' ages were noted at the time of diagnosis. Two hearing metrics were used: (i) pure tone average, defined as the average of the lowest threshold (in dB) for 2 tones among 0.5, 1, and 2 kHz; and (ii) word recognition, defined as the percentage of spoken words from a standardized list a patient can comprehend in quiet. Primary VS cells were cultured as we previously described.[20]

Murine Spiral Ganglion Cell Culture

Spiral ganglion cell (SGC) culture was derived from CBA/CaJ mice purchased from the Jackson Laboratory. We used the standard culturing protocol in our laboratory,[21] with some modifications. Briefly, postnatal day P3-P5 cochleae were isolated and SGCs within the modiolus were dissected. The tissue was incubated in Hank's Balanced Salt Solution (Gibco), trypsin (2.5 mg/mL; Gibco), and collagenase IV (0.5 mg/mL; Sigma-Aldrich) at 37°C for 25 min. Enzymatic digestion was stopped by addition of 10% fetal bovine serum (FBS) (Sigma-Aldrich). After centrifugation of suspended cells at 1000 *g* for 3 min at room temperature, the pellet was resuspended in culture medium, Dulbecco's modified Eagle's medium/F12 (Invitrogen) supplemented with 10% FBS, 1% ampicillin solution (Gibco), 1% GlutaMAX (Invitrogen), 5% sterile horse serum (NHS, Gibco), neurotrophin-3 16 ng/mL (Promega), brain-derived neurotrophic factor 8 ng/mL (Promega), and 2% B-27 supplement (Gibco). The cells were cultured for 24 h in 24-well culture plates (CytoOne) on glass slides treated with poly-L-lysine and laminin (BioCoat BD) before experimental treatment described below. A good dissection was confirmed with high purity of SGNs and Schwann cells as noted morphologically in the culture.

Murine Cochlear Explant Culture

Organotypic cochlear cultures (also known as cochlear explants) were prepared using a similar protocol as described for the culture of SGCs, with several modifications.[22,23] We focused on the middle part of cochlear turns because it consistently gave rise to robust, undamaged explants. Cochlear explants, consisting of the sensory epithelium with IHCs and OHCs, and the attached SGNs, were cultured on glass coverslips previously treated with Cell-Tak (BD Biosciences) to facilitate tissue adhesion. Cochlear explants were incubated at 37°C with 5% CO_2 for 24 h in Dulbecco's modified Eagle's medium, 1% ampicillin solution (Gibco), and 1% N2 supplement (Invitrogen), until experimental treatment. The dissections were initially confirmed by microscopically inspecting each specimen for the presence of morphologically neuron-like cells after culturing and by checking the organization and health of control cochlear explants receiving no treatment after the experiment was finished.

Cochlear explants were subjected to 3 different treatments: (i) 6 μL phosphate buffered saline (PBS) with EVs added to the culture medium, as described below; same volume of PBS served as a negative control; (ii) a dual-culture plate with 2 different concentrations of HEI-193 cells added to the top chamber (1×10^6 and 4×10^6 cells), with a membrane permeable to particles <1 μm in size (#353104, Fisher Scientific), which allowed cell-secreted microvesicles to cross the membrane; (iii) direct addition of EVs isolated from primary human VS tumors from patients with GH or PH. All cultures were incubated for 48 h.

Isolation of Extracellular Vesicles

Approximately 4×10^6 NF2 HEI-193 cells were cultured in 15-cm culture dishes as previously described.[20] After 24 h, the culture plate was washed with PBS. A culture medium was added as outlined above but supplemented with 5% EV-depleted FBS, purified by high-speed centrifugation to deplete EVs from the FBS. After 48 h, conditioned media were collected and centrifuged for 10 min at 300 *g*, then 10 min at 2000 *g*. The supernatant was filtered through a 0.8-μm filter (Millipore). EVs were pelleted by centrifugation at 100 000 *g* for 80 min in a Type 70 Ti Rotor (Beckman Coulter).

The primary human VS cells were cultured for a week. The culture medium was then replaced with 5% EV-depleted FBS for 48 h. The supernatant from each culture plate was collected

to purify and store EVs as described above. When EVs were applied to cochlear explants, the antibiotic penicillin/streptomycin was replaced with ampicillin in the culture medium, since ampicillin is not ototoxic.

Characterization of Extracellular Vesicles

The size distribution and concentration of EVs were assessed with the Nanosight LM20 machine (Malvern Instruments), while EVs' morphology and size were evaluated with transmission electron microscopy. The pellet rich in EVs was suspended in 50 μL PBS (filtered twice) at 4°C and fixed in 2% paraformaldehyde. A grid coated with carbon film was positioned on a suspension drop with EVs. The grids were placed directly on top of a drop of 2% uranyl acetate. The grids were examined with a Technai-12 transmission G2 Spirit Biotwin electron microscope (FEI).

RNA Extraction

For RNA extraction, 1×10^6 cells and EVs from 50 mL of media were used. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), and small RNA was extracted using the miRNeasy Mini Kit (Qiagen) and the RNeasy MinElute Cleanup Kit (Qiagen), while adhering to the manufacturer's protocols. The quantitative and qualitative analysis of the extracted RNA was performed using the NanoDrop 2000 spectrophotometer (Thermo Scientific) and Agilent 2100 Bioanalyzer through an RNA 6000 Pico Kit (Agilent).

Reverse Transcription PCR

Reverse transcription (RT)-PCR was performed using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen), as detailed in the Supplementary materials.

Labeling of Extracellular Vesicles with PKH-67 dye

Plasma membrane and EVs derived from HEI-193 cells were labeled with PKH-67 dye (Sigma-Aldrich), following the manufacturer's protocol and as described by Ekstrom et al.[24] As the EVs have remnants of the cell membrane, the dye PKH-67 was present in secreted vesicles. EVs were suspended in 200 μL PBS after ultracentrifugation and labeled with PKH-67. To eliminate excess dye, the solution was washed 3 times using Vivaspin 2 tubes (Sartorius Stedim Biotech) for 3 min at 4000 *g* at room temperature. The solution was diluted with PBS and ultracentrifuged at 100 000 *g* for 90 min at 4°C. The pellet was diluted with 200 μL PBS; 20 μL of this new solution was added to the culture of SGCs. The number of cells internalizing labeled vesicles was quantified 4 h and 24 h later under light microscopy.

HEI-193 cells, referred to as NF2 cells, stained with PKH-67 were cultured in a dual-culture plate. SGCs, including neurons and Schwann cells, were cultured in the lower portion. The top chamber, with a membrane permeable to particles smaller than 1 μm, had 4×10^6 HEI-193 cells stained with PKH-67. After passing through the permeable membrane, the PKH-67–labeled EVs were internalized by cells in the lower chamber. The frequency of internalization of the labeled vesicles by SGCs was measured at 24 h and 48 h under light microscopy.

Transduction with pCT-CD63-GFP Plasmid

A plasmid containing green fluorescent protein (GFP) was used to evaluate whether the genetic material (RNA) present in EVs could be expressed by the receptor cells. HEI-193 cells were transfected with pCT-CD63-GFP plasmid (Cyto-Tracers, System Biosciences) using Lipofectamine 2000 Reagent (Invitrogen). The transduction efficiency was observed under an inverted microscope to identify living cells (Zeiss). Transfected cells were selected with puromycin 1 mg/mL (Santa Cruz Biotechnology). The EVs from these cells were isolated and RNA was extracted and reverse transcribed into cDNA by RT-PCR to assess the presence of GFP mRNA (168 bp amplicon).

Immunostaining of Cochlear Cells

Hair cells were labeled with the polyclonal rabbit primary antibody against myosin VIIA (myo7A) (Proteus Biosciences). SGNs and their nerve fibers were labeled with a monoclonal mouse primary antibody against class β-tubulin III (Tuj1) (BioLegend). Additional details are provided in the Supplementary materials. The Poly Caspase Assay Kit (Green, Neuromics) was used to stain apoptotic cells.

The specimens were imaged with a confocal microscope (Leica SP5), in 0.5-μm steps, according to a published protocol[25] with modifications. The samples were evaluated under magnification of 20×, 63×, and 126×. To capture representative morphology of each specimen, photographs were taken from the central region. In addition, various sectional slices were projected on a single plane using Leica software. The counting of IHCs, OHCs, and neural fibers was performed along 100 μm of cochlear length. The number and area of neuronal somata were quantified in a 1×10^4 μm^2 area using the ImageJ program. Specifically, for all cell types, an appropriately stained cell (eg, Myo7A stained hair cell) that was distinguishable as an individual cell with a discernible nucleus was counted regardless of cell size or degree of damage.

Statistical Analysis

We used Excel 2013 and GraphPad Prism 6 for Windows for statistical analyses. The D'Agostino–Pearson test was used to test normality of distribution. Student's *t*-test was applied as a parametric test. The Mann–Whitney and Kruskal–Wallis tests were applied as nonparametric tests. Dunn's multiple comparison test and Benjamini–Hochberg correction for multiple hypotheses were conducted on generated *P*-values. $P < .05$ was considered significant.

Results

Human Vestibular Schwannoma Cells Secrete Extracellular Vesicles

As EVs released by VS have not been previously reported, we first tried to isolate them from a human VS cell line. We used the HEI-193 cell line, which we refer to as NF2 cells, as this cell line was developed from a VS of a patient affected with NF2.[19] After differential centrifugation of cell-conditioned culture media, numerous small EVs were identified by transmission electron microscopy ($n = 2$; Fig. 1A). The size of the EVs

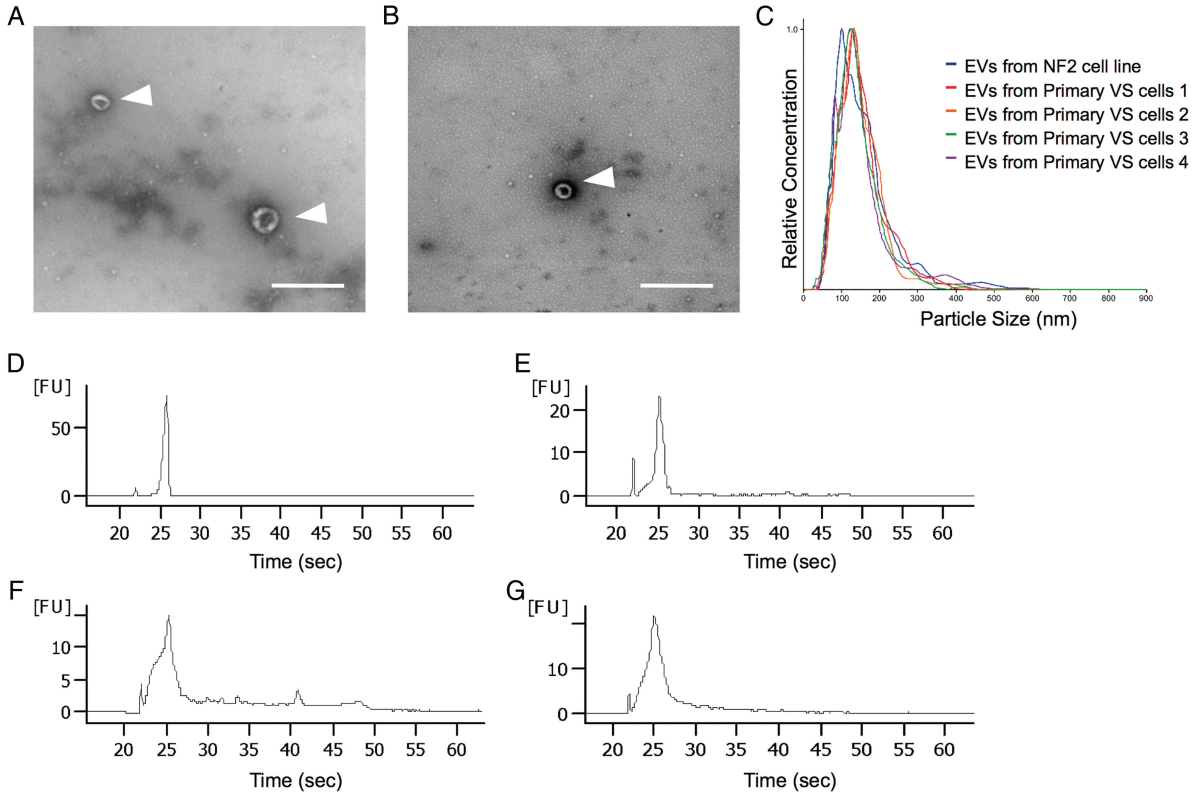

Fig. 1. VSs secrete EVs that contain small RNAs. Electron microscopy images show EVs in conditioned culture media from (A) the NF2 vestibular schwannoma cell line and (B) primary human VS cells. Round particles with defined edges resemble exosomes (marked with white arrowheads). (C) Particle distribution by size in conditioned culture media from the NF2 cell line and primary human VS cells ($n = 4$). The highest concentration is set to 1. Representative images of spectrophotometry of small RNA from cultured (D) HEI-193 cell line and (E) primary human VS cells. Representative images of spectrophotometry of small RNA present in isolated EVs from cultured (F) HEI-193 cell line and (G) primary human VS cells. FU, fluorescence unit. Scale bar: 500 nm (A, B).

ranged from 30 nm to 680 nm, and the most frequent size was 102 nm (Fig. 1C). Since we used EV-depleted media for our cultures, EVs detected were released from the HEI-193 cells. The distribution of particles in EV-depleted media is shown in Supplementary Fig. S1. The shape of the vesicles resembled upwardly facing cups of glass, consistent with previously described EVs.[26] Similarly shaped vesicles were observed in culture media in which primary human VS cells were grown ($n = 2$; Fig. 1B). These primary cells were obtained from indicated surgeries on patients with sporadic, unilateral VSs. The size distribution of EVs was similar between primary VS cells ($n = 4$) and NF2 cells (Fig. 1C).

Vestibular Schwannoma–Derived Extracellular Vesicles Contain RNA

It is known that RNA delivered through EVs can be functional—for example, mRNAs can be translated into proteins, and miRNAs can inhibit protein translation.[27] We found small RNAs in cultured NF2 cells ($n = 10$; Fig. 1D) and cultured primary VS cells ($n = 10$; Fig. 1E). The peak at 25s is consistent with miRNA, but could also reflect other small noncoding RNAs. We further found small RNA species in isolated EVs from NF2 cells ($n = 10$; Fig. 1F) and primary human VS cells ($n = 10$; Fig. 1G).

Spiral Ganglion Cells Can Internalize Tumor-Derived Extracellular Vesicles

To determine whether cochlear cells could internalize EVs secreted by VS cells (donor cells), PKH-67 dye that fluorescently labels cell membranes was used in 2 different experiments. First, EVs (5×10^{11} particles/μL) from 4×10^{6} NF2 cells were stained with PKH-67 and added directly to the culture of dissociated murine SGCs (recipient cells) (Fig. 2A–D). The advantage of this monolayer of SGCs consisting of SGNs and Schwann cells is easy visualization of the internalized EVs by the recipient cells. Our focus on SGCs is motivated by the histopathological findings of a major loss of these cells in human temporal bones affected by VS.[13] Second, donor NF2 cells were stained with PKH-67 and placed in the top chamber of a dual-culture dish with cultures separated by a transmembrane filter and with a monolayer of murine SGCs in the bottom well (recipient cells) (Fig. 2E–G). As EVs form by budding of the bilipid cell membrane from the NF2 cells, they acquire PKH-67–stained membrane from their donor cells. These EVs cross the culture dish's permeable synthetic membrane, which allows particles sized <1 μm to pass through and reach SGCs in the bottom chamber of the transwell plate. Using these 2 approaches of labeling EVs, we found that both pre-PKH-67–labeled and newly forming PKH-67–labeled EVs could be internalized by SGCs

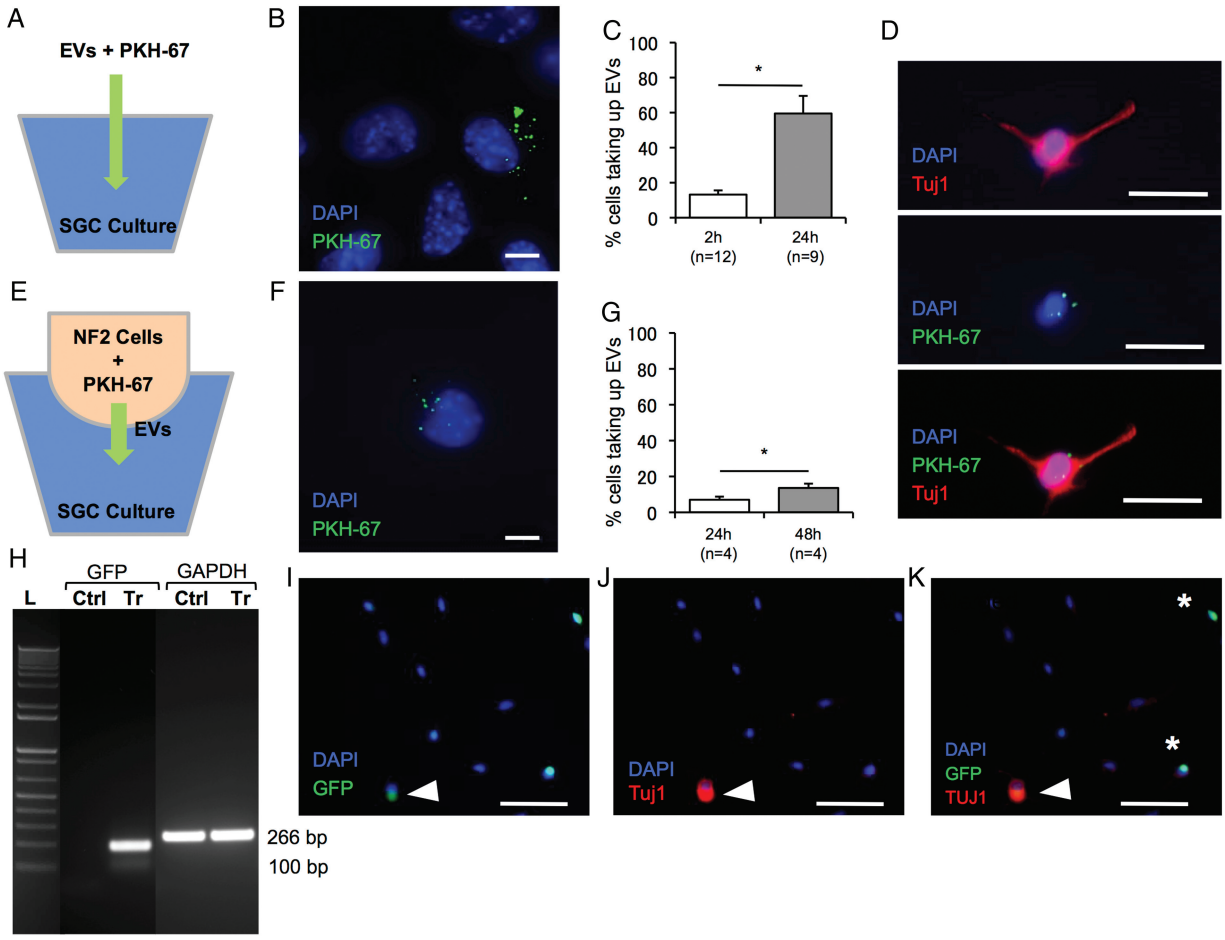

Fig. 2. EVs can transfer RNA to cochlear cells in culture, including SGCs. To study the internalization of the vesicles, (A–D) PKH-67-labeled EVs were added directly into SGC cultures or (E–G) PKH-67-labeled NF2 donor cells were cultured in a transwell plate, allowing PKH-67-labeled secreted EVs to pass through the membrane in the top chamber and reach recipient SGCs cultured in the bottom chamber. (B, F) The PKH-67-labeled EVs were identified as green cytoplasmic spots and (C, G) quantified over time (mean ± SEM, $*P < .05$). (D) A representative SGN with internalized vesicles. (H) EVs isolated from NF2 cells (lane 1) did not have the GFP mRNA, but EVs from NF2 cells transfected with pCT-CD63-GFP plasmid (lane 2) contained the GFP mRNA. L: DNA ladder. GAPDH (glyceraldehyde 3-phosphate dehydrogenase): housekeeping control mRNA for the samples in lanes 1 and 2. (I-K) Adding EVs from cells transfected with the GFP expression plasmid directly to cultured SGCs resulted in the cells expressing the GFP protein, as illustrated on a representative SGN (arrowhead). Scale bar: 2 μm (B, D, F) or 5 μm (I, J, K). DAPI, 4′,6′-diamidino-2-phenylindole.

(Fig. 2B and F, respectively). The fraction of cells taking up EVs increased from 2 h to 24 h in culture and was significantly higher for pre-PKH-67–labeled (Fig. 2C) than newly forming PKH-67–labeled EVs ($P = .001$; Fig. 2G), probably due to the higher ratio of EVs to cells in the former. SGCs internalizing EVs were identified as neurons based on immunostaining for the Tuj1 neuronal marker (Fig. 2D). The PKH-67–labeled EV-depleted media are shown in Supplementary Fig. S1A.

Extracellular Vesicles Can Transfer Tumor-Derived RNA to Cochlear Cells In vitro

Having established that SGCs can internalize tumor-derived EVs, we next tested whether SGCs could express the EVs' genetic cargo. NF2 cells were transfected with a pCT-CD63-GFP plasmid containing an expression cassette for GFP. RNA was extracted from EVs secreted by NF2 cells and reverse transcribed into cDNA. EVs from control NF2 cells that were not transfected with the plasmid lacked the GFP mRNA (Fig. 2H, lane 1). The EVs from GFP transfected cells did contain GFP mRNA (Fig. 2H, lane 2); when these EVs were applied to cultured SGCs, GFP labeling was observed under fluorescence microscopy. GFP expression was noted in different cell types (Fig. 2I), including Tuj1-labeled neurons (Fig. 2J, arrowhead) and non-neuronal cells (Fig. 2K, asterisks).

Extracellular Vesicles from NF2 Cells Cause Damage in Cultured Cochlear Explants

Having demonstrated that dissociated SGNs can internalize NF2 cell-secreted EVs and express their genetic content, we tested whether NF2 cell-secreted EVs could also affect cochlear cells in a multilayered organotypic murine cochlear culture, referred to as a cochlear explant. Cochlear explants allow examination and quantification of hair cells and SGNs, the 2 cochlear cell types most commonly damaged in patients affected

by VSs.[13] Six microliters of EVs isolated from NF2 cell-conditioned media at a concentration of 2.5×10^{10} particles/μL were applied to cochlear explants from postnatal day 3–5 mice for 48 h; explants treated with an equal volume of PBS served as controls. Following incubation, cochlear explants were stained with anti-Myo7A antibodies to label IHC and OHC (Fig. 3A) and/or anti-Tuj1 antibodies to label neurites (Fig. 3B) and cell bodies of SGNs (Fig. 3C) and then examined using confocal microscopy. Compared with untreated explants (Fig. 3A–C, left panels), the explants treated with NF2 cell-derived EVs (Fig. 3A–C, right panels) demonstrated degenerative changes, especially at the level of neurites and SGN somata. These changes were quantified by counting the number of IHCs per 100 μm (Fig. 3D), OHCs per 100 μm (Fig. 3E), neurites per 100 μm (Fig. 3F), SGNs per 1×10^4 μm^2 (Fig. 3G), and the area of SGN somata (Fig. 3H). Control cochlear explants from different animals ($n = 7–8$) were compared with EV-treated cochlear explants from different animals ($n = 7–8$). Although the EV treatment did not affect the number of IHCs (Fig. 3D) or OHCs (Fig 3E), it significantly reduced the number of nerve fibers (Fig. 3F, $P < .05$) and neurons ($P < .05$; Fig. 3G) and the area of neuronal somata ($P < .00001$; Fig. 3H).

Cochlear Damage Caused by Extracellular Vesicles from NF2 Cells Can Be Rescued by Heparin

We further used a dual-culture system, with different concentrations of NF2 cells in the top chamber as donor cells and murine cochlear explants in the bottom chamber of a transwell plate as recipient cells, to demonstrate the dose-dependent damaging effect of NF2 cell-secreted EVs on cochlear cells (Fig. 4). Cochlear hair cells (Fig. 4A), neurites (Fig. 4B), and SGNs (Fig. 4C) were analyzed when the top chamber contained either no cells, which served as a control ($n = 11$), 1×10^6 NF2 cells ($n = 3$), 4×10^6 NF2 cells ($n = 3$), or 4×10^6 NF2 cells plus 200 μg/mL heparin ($n = 3$). Heparin is known to promote aggregation of EVs and hinder their uptake by receptor cells.[28] After 48 h in culture, the more densely plated NF2 cells (4×10^6) caused a significant loss of IHCs ($P < .04$) and OHCs ($P < .02$) compared with the control group (Fig. 4A, E, and F). There was a trend for hair cell preservation with heparin treatment (Fig. 4A), but it did not meet a significance level of <.05 (Fig. 4E and F). The hair cell loss was due to other toxic factors in the supernatant of NF2 culture media, such as secreted proteins, as hair cell loss was much less apparent when only purified EVs were applied (Supplementary Fig. S2). When analyzing neurites, the group with 4×10^6 NF2 cells tended to have fewer neurites—the trend that could be prevented with heparin cotreatment (Fig. 4B), albeit not significantly (Fig. 4G). The size of SGNs was significantly reduced in the groups with 1×10^6 NF2 cells and 4×10^6 NF2 cells compared with the control group (Fig. 4C, I, and J–M). There was a trend for prevention of SGN loss (Fig. 4H) and a statistically significant ($P < .05$) prevention of neuronal shrinkage with heparin cotreatment (Fig. 4C, I, and J–M).

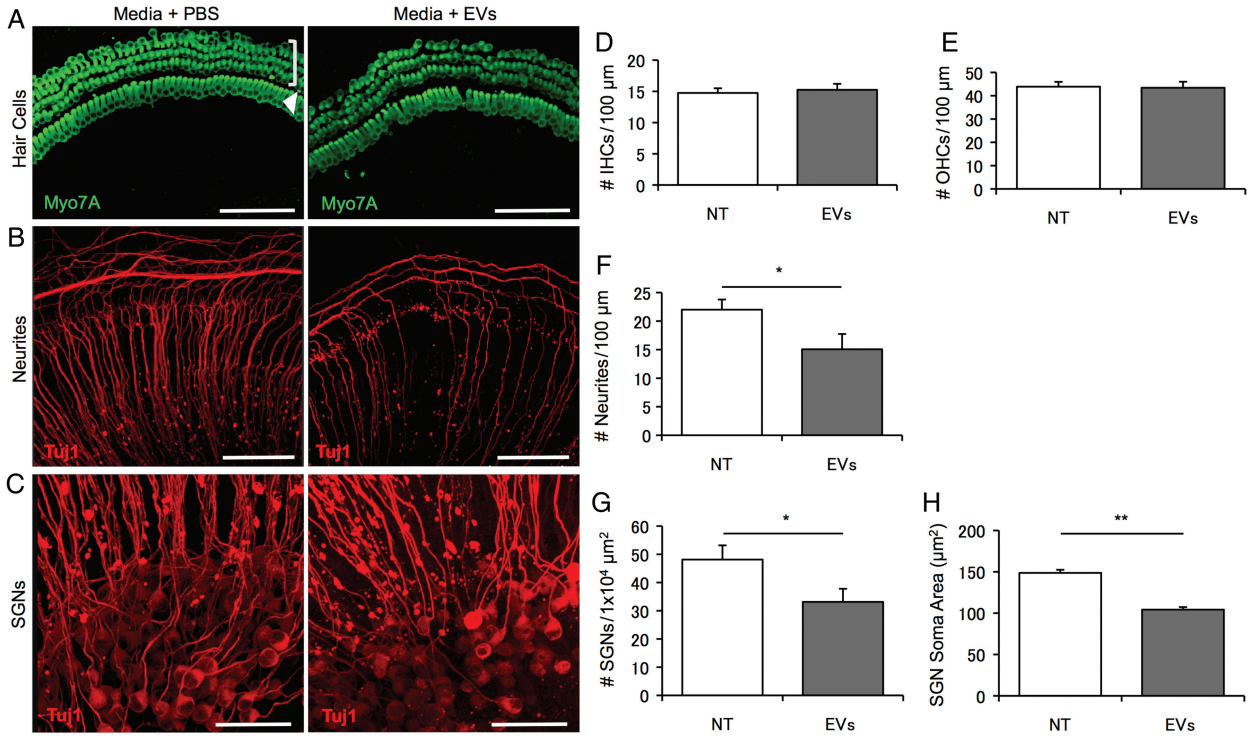

Fig. 3. EVs from NF2 cells cause damage in cultured cochlear explants. Representative images of hair cells: (A) a single row of IHCs (white arrowhead) and 3 rows of OHCs (white bracket), (B) neurites, and (C) SGNs, exposed to control PBS (6 μL) or NF2 cell-derived EVs (6 μL). Number of cells was quantified for (D) IHCs per 100 μm, (E) OHCs per 100 μm, (F) neurites per 100 μm, (G) SGNs per 1×10^4 μm^2, and (H) area of the neuronal somata for the control group (NT, no treatment) and the group treated with EVs (mean ± SEM, $n = 7–8$ explants from different animals for each in the control and EV-treated groups, $^*P < .05$, $^{**}P < .01$). Scale bar: 100 μm (A, B, C).

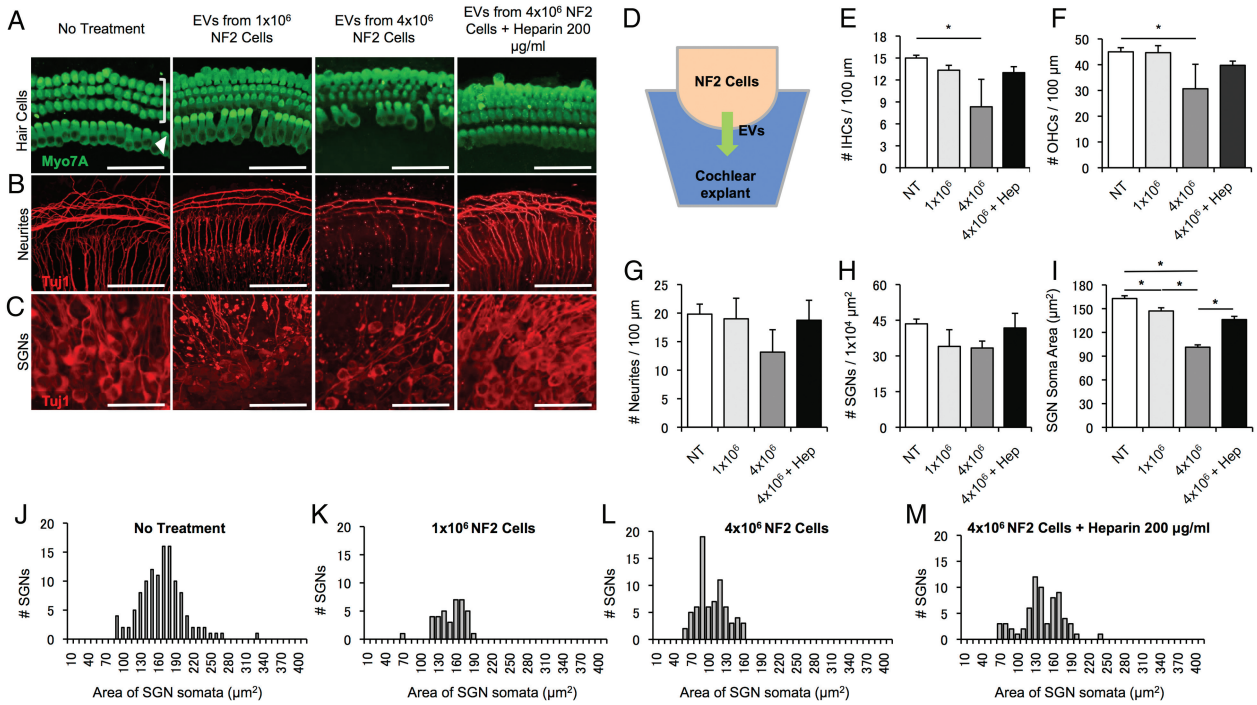

Fig. 4. Cochlear damage caused by EVs from NF2 cells can be rescued by heparin. Representative images of (A) hair cells (a single row of IHCs: white arrowhead and 3 rows of OHCs: white bracket), (B) neurites, and (C) SGNs treated with control media (no treatment), EVs from 1×10^6 NF2 cells, EVs from 4×10^6 NF2 cells, and EVs from 4×10^6 NF2 cells along with heparin in a transwell coculture system schematized in (D). Quantification of the number of IHCs per 100 µm (E), OHCs per 100 µm (F), neurites per 100 µm (G), SGNs per 1×10^4 µm^2 (H), and area of neuronal somata (I) for control media (NT, no treatment, white column), EVs from 1×10^6 NF2 cells (light gray column), EVs from 4×10^6 NF2 cells (dark gray column), and EVs from 4×10^6 NF2 cells along with 200 mg/mL heparin (black column) (mean ± SEM, $n = 11$ explants were used for NT controls and $n = 3$ different explants were used for all other experiments, $^*P < .05$). The distribution of the area of SGN somata in cochlear explants treated with control media (no treatment) (J), EVs from 1×10^6 NF2 cells (K), EVs from 4×10^6 NF2 cells (L), and EVs from 4×10^6 NF2 cells along with 200 mg/mL heparin (M). The data in panels (J)–(M) are summarized in the panel (I). Scale bar: 50 µm (A, C) or 100 µm (B). Hep, heparin.

Extracellular Vesicles from Vestibular Schwannomas Associated with Poor Hearing Damage Cochlear Cells

Having established that EVs derived from the NF2 cell line can damage cochlear cells in an explant model (Figs 3 and 4), we next tested whether EVs derived from primary human VSs could have a similar effect. Primary VSs can be associated with GH in the ipsilateral ear, defined as word recognition better than 70% out of a possible 100% and pure tone average <30 dB hearing level, or PH. This classification system was developed by the American Academy of Otolaryngology–Head and Neck Surgery.[29] We have previously demonstrated that primary cultured VS cells are representative of their parent tumors.[20] We applied EVs from conditioned media of cells from 3 VSs from patients with GH, using an average concentration ± SEM of $1.9 \times 10^{10} \pm 0.6$ particles/µL, and from 3 VSs from patients with PH in the ipsilateral ear, with an average concentration of $2.8 \times 10^{10} \pm 0.9$ particles/µL. Patient demographics are summarized in Table 1 and Supplementary Fig. S3. Individual audiograms for all patients are shown in Supplementary Fig. S4. The tumors in the GH and PH group were matched with respect to patients' age, sex, and tumor size. Moreover, the size distribution of the EVs and concentration of particles were found to be similar between EVs from GH ($n = 3$) and PH ($n = 3$) VSs ($P > .05$). The EVs from cells cultured from each tumor were applied to 3–4 different cochlear explants, for a total of 10 different explants for the GH VS group and 10 different explants for the PH VS group (Fig. 5); cochlear explants from 10 different animals served as no-treatment controls. Compared with no-treatment controls (Fig. 5A, left panel), explants treated with EVs from VSs associated with good (Fig. 5A, middle panel) or poor hearing (Fig. 5A, right panel) did not lose IHCs (Fig. 5E) or OHCs (Fig. 5F). However, the number of neurites (Fig. 5B and G) and SGNs (Fig. 5C and H) as well as the area of SGN somata (Fig. 5I) were significantly reduced in explants treated with EVs from VSs associated with PH ($P = .04$, $P < .0001$, and $P < .0001$, respectively) compared with the control group, but not in explants treated with EVs from VSs associated with GH. Individual measurements in Fig. 5I are depicted as the distribution of the area of SGN somata in Fig. 5K–M. Using the Poly Caspase kit to mark apoptotic cells with a green stain (Fig. 5D), we found a significantly greater number of apoptotic SGNs in cochlear explants treated with EVs from cultured VSs associated with PH than in control explants ($P = .015$) or in explants treated with EVs from cultured VSs associated with GH ($P = .022$; Fig. 5J). The number of counted SGNs was 149, 123, and 162, respectively, which was not statistically significant among the groups.

Table 1. Patient demographics

Tumor ID	Age	Gender	Side	Tumor Size (mm)	Hearing Level			
					Ipsilateral Ear		Contralateral Ear	
					PTA (dB)	WR (%)	PTA (dB)	WR (%)
VS 1	55	F	Right	22	12	96	8	98
VS 2	46	F	Right	24	12	94	10	100
VS 3	49	F	Left	12	22	84	6	94
VS 4	**45**	**F**	**Left**	**12**	**58**	**20**	**2**	**100**
VS 5	**53**	**F**	**Right**	**29**	**35**	**94**	**18**	**100**
VS 6	**61**	**F**	**Right**	**20**	**40**	**20**	**15**	**100**

Abbreviations: PTA, pure tone average; WR, word recognition. Poor hearing patients in bold font.

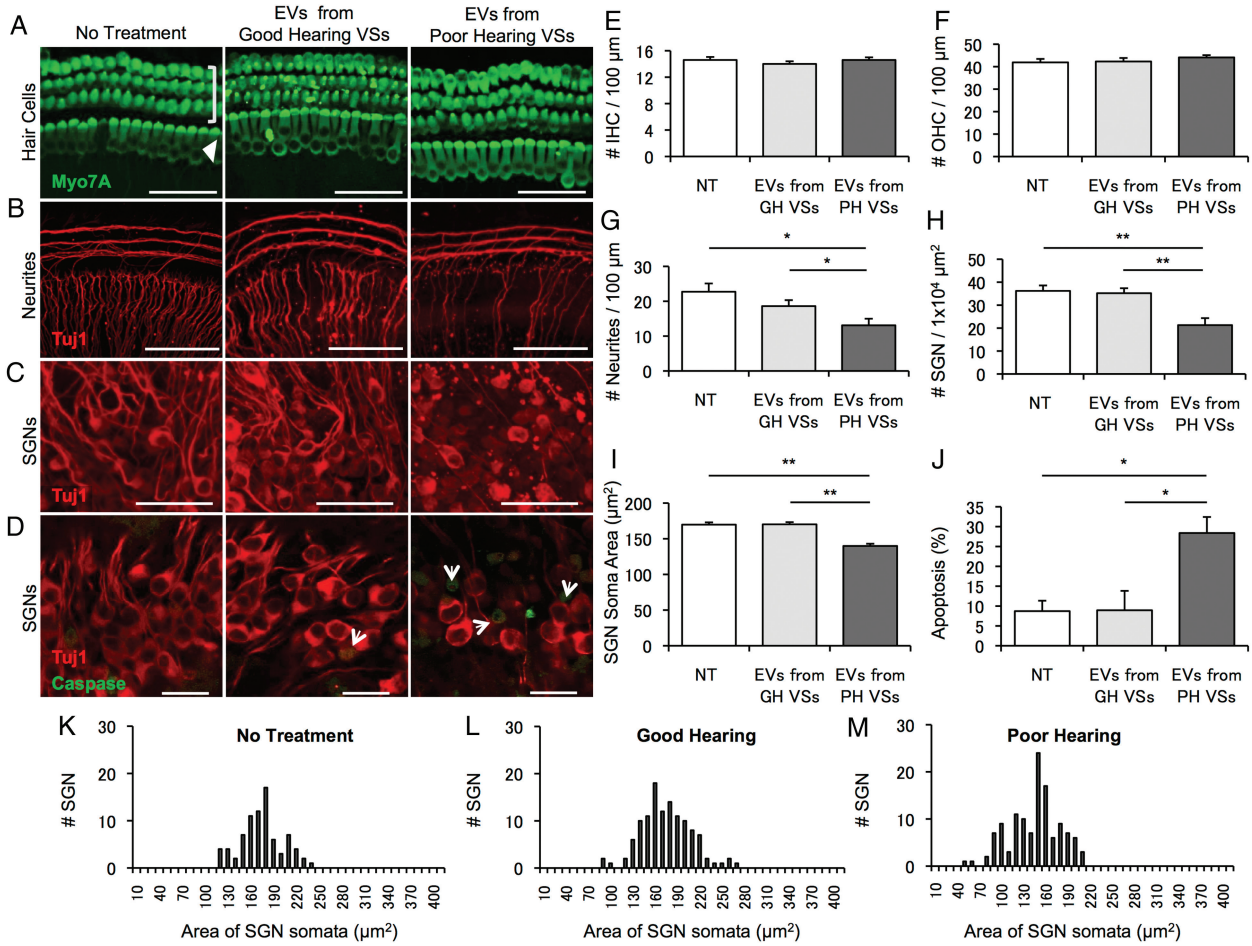

Fig. 5. Application of isolated EVs from VSs associated with PH causes cochlear explant damage. Representative images of (A) hair cells (a single row of IHCs [white arrowhead] and 3 rows of OHCs [white bracket]), (B) neurites, (c) SGNs, and (D) SGNs labeled with polycaspase stain to identify apoptotic cells treated with control media (NT, no treatment; 6 µL of PBS), isolated EVs (6 µL of 2×10^{10} particles/µL) from a VS associated with GH, and extracted EVs (6 µL of 2.9×10^{10} particles/µL) from a VS associated with PH are shown. Quantification of the number of (E) IHCs per 100 µm, (F) OHCs per 100 µm, (G) neurites per 100 µm, (H) SGNs per 1×10^4 µm^2, (I) area of neuronal somata, and (J) apoptosis noted in SGN for control media (NT, no treatment, white column), isolated EVs from a VS associated with GH (light gray column), and isolated EVs from a VS associated with PH (dark gray column) (mean ± SEM, $^*P < .05$, $^{**}P < .01$). $n = 10$ explants from different animals for each of 3 groups (E–I), except for (J) apoptosis assay where $n = 3$ or 4 explants from different animals for the NT, the GH, and the PH VS groups. The distribution of the area of SGN somata in cochlear explants treated with control media (no treatment) (K), isolated EVs from a VS associated with GH (L), and isolated EVs from a VS associated with PH (M). The data in panels (K)–(M) are summarized in panel (I). Scale bar: 25 µm (D), 50 µm (A, C) or 100 µm (B).

Discussion

Our study is the first to isolate EVs produced by cultured primary VS cells and a cultured NF2 VS cell line. In order to elucidate the function of EVs from VSs, we further assessed whether VS-secreted EVs could cause cochlear damage. Using a cochlear explant culture model and a transwell coculture system, we demonstrated that SGNs are most susceptible to this damage; hair cells are also vulnerable, especially to EVs secreted by NF2 VS cells. Importantly, EVs secreted by VSs associated with PH, but not EVs secreted by VSs associated with GH, produced a damaging effect on the SGNs.

VS secretions provide an alternative and complementary mechanism to mechanical compression of the cochlear nerve in mediating VS-induced hearing loss. The neurotoxic and ototoxic components of the VS secretome could reach the adjacent cochlea via the fundus of the internal auditory canal to affect cochlear function. Our focus on VS secretions is motivated by clinical observations that cannot be simply explained by cochlear nerve compression. Specifically, there is no correlation between the tumor extent within the internal auditory canal or radiographic tumor size and audiometric threshold shifts in people with sporadic VSs.[14,15] In addition, VS patients can develop sudden or progressive audiometric threshold shifts despite the lack of VS growth.[15] A role for VS secretions in SNHL is supported by the observations that total levels of protein in cochlear fluids of VS patients are 5–15 times higher than in healthy individuals[30,31] and that the proteome of cochlear fluid is different in VS patients compared with those without the tumor.[32] We have found that VSs associated with GH secrete higher levels of fibroblast growth factor 2 than VSs associated with PH, irrespective of the tumor size,[33] while VS secretion of tumor necrosis factor alpha correlates with poor hearing.[34] Moreover, our cDNA microarray study of VSs stratified by hearing has revealed differences in gene expression profiles between VSs that do and those that do not cause hearing loss.[18] Another cDNA microarray study reported that expression levels of platelet derived growth factor–A gene inversely correlated with SNHL in VS patients.[35] The apparent intrinsic genetic differences in VSs that do versus those that do not cause SNHL are likely to be reflected in VS-derived EVs, since EVs contain the genetic profile of their cell of origin.[36] It is likely that both EVs and free soluble molecules in VS secretions modulate cochlear function. An advantage of EVs is that they can concentrate donor cell–specific cargo.[37] For future clinical application, it will be important to identify the specific factors in EVs responsible for the cochlear damage noted in our study. Identification analyses such as next-generation microRNA sequencing and proteomic analysis of VS-released EVs could provide insight into the molecular pathways leading to hearing loss due to VSs.

Conclusions

Our findings strongly motivate future work to identify the EV-derived cargo mediating cochlear damage, as this could provide insight into much needed prognostic and therapeutic targets for prevention and treatment of hearing loss due to VSs and potentially other causes. Understanding mechanisms of hearing loss due to VS, such as by VS-secreted EVs, can expedite the path to pharmacotherapies against this common, debilitating symptom of VS for which approved nonsurgical therapies do not exist.

Supplementary material

Supplementary material is available at *Neuro-Oncology Journal* online (http://neuro-oncology.oxfordjournals.org/).

Funding

This work was supported by a Department of Defense grant W81XWH-14-1-0091 (to K.M.S.), the Bertarelli Foundation (to K.M.S.), Nancy Sayles Day Foundation (to K.M.S.), the Lauer Tinnitus Research Center (to K.M.S.), a National Institute on Deafness and Other Communication Disorders grant T32DC00038 (to support S.D.) and the NIH Common Fund NCI CA179563 (to X.O.B.).

Acknowledgments

We thank R. van Noorden and H. van Veen for help with transmission electron microscopy.

Conflict of interest statement. None declared.

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