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Abstract

Objectives: Isolate, purify, and characterize extracellular vesicles (EVs) obtained from auditory HEI-OC1 cells, and evaluate their suitability for intracochlear transport and delivery of pharmacological drugs and/or pro-resolution mediators of acute inflammatory processes.

Methods: HEI-OC1 EVs were isolated and purified using the exoEasy Maxi Kit, and their size was evaluated by nanoparticle tracking techniques. Bottom-up proteomics of the EVs, either freshly obtained or stored for up to 4 months at −20°C, was performed by LC-ESI-MS/MS. LC-ESI-MS/MS-MRM was used to measure the loading of dexamethasone inside EVs following co-incubation at room temperature for 1 hour with and without 5 minutes sonication.

Results: Routinely, we were able to obtain purified fractions of $>2 \times 10^9$ EVs/mL, with diameters varying between 50 and 800 nm. Bottom-up proteomics showed that among the most abundant EVs proteins, 19.2% were cytoplasmic, 17.2% were membrane localized, 12.3% were cytosolic, and 14.6% were nucleolar. No significant differences between fresh and stored EVs were detected. Importantly, co-incubation of HEI-OC1 EVs (1×10^8 EVs/mL) with dexamethasone (10 mM) resulted in the incorporation of 10.1 ± 1.9 nM dexamethasone per milliliter of EVs suspension.

Conclusions: Altogether, the results suggest that EVs from HEI-OC1 cells could be advantageously used as biological nanocarriers for the delivery of specific molecules and pharmacological drugs into the inner ear.

Keywords

extracellular vesicles, HEI-OC1 cells, proteomics, drug nanocarriers, intracochlear drug delivery

Introduction

Drug-delivery techniques for the treatment of sensorineural hearing loss (SNHL) are categorized as systemic (SIS), intratympanic (IT), and intracochlear (IC), each of them with their particular advantages and disadvantages,[1,2] For example, although SIS is simpler, only a small amount of drug crosses the blood labyrinth barrier (BLB) to reach the inner ear,[3,4] and high doses of medication must be administered to achieve the appropriate concentration in the inner ear. In addition, SIS administration of drugs like corticosteroids has numerous negative side effects that might worsen patient prognosis. IT administration of pharmacological drugs to the middle ear has been heavily used over the past decades for the treatment of sudden SNHL and Meniere's disease.[5-10] IT administration, however, has the limitation that the drug must remain in the middle ear cavity and in contact with the round window membrane (RWM) or the annular ligament of the oval window for enough time to permeate into the cochlea.[6,11] Unfortunately, drugs that enter the middle ear cavity are quickly discharged to the Eustachian tube through mucociliary flow.[12,13] The required increase in frequency of drug administration to compensate this loss not only increases the inconvenience and the cost of therapy but also the incidence of tympanic membrane perforations[14] and undesired infections acquired during the procedure.[5] Finally, active IC drug delivery systems allow

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for better dosing control, higher drug concentrations, and reduced base-to-apex concentration gradients[15-18] but usually require surgical procedures with all their associated risks.[19,20] The recently emerged new avenue for the delivery of drugs in a sustained and controllable manner by using nanoparticles as drug carriers, however, has increased the preference for the IC approach as the drug-delivery technique of choice for the treatment of SNHL.

One major reason for the use of nanoparticles as carriers is that many therapeutic agents are proteins and nucleic acids that have short in vivo half-lives, posing significant challenges to successful therapeutic outcomes. This limitation may be overcome by encapsulating the agent into a nanocarrier able to deliver it, fully active, directly into the target cell. Unlike other nanocarriers, such as liposomes or polymeric nanoparticles, extracellular vesicles (EVs) are a natural endogenous delivery system that transport molecules while avoiding phagocytosis and degradation by macrophages. Other advantages include the fact that EVs continuously circulate for extended periods of time within the body while delivering cargoes directly into the cytoplasm of target cells.[21-24] They are not only stable, but by virtue of avoiding the endosomal pathway, the transfection efficiency for molecules such as siRNA is enhanced.[25]

EVs are small (30-1000 nm) membrane vesicles of endosomal origin that are important for intracellular communication because of their ability to transfer many different molecules to other cells, thereby simultaneously influencing a number of their physiological functions.[21-24] EVs are often broadly categorized into exosomes (30-150 nm) and ectosomes (aka microvesicles; 100-1000 nm), a categorization that not only refers to their different sizes but also to their specific origin. While exosomes are derived from the endosomal system through fusion of multivesicular bodies with the plasma membrane, ectosomes are formed through the direct outward budding of the plasma membrane.[21] However, distinguishing between exosomes and ectosomes is technically challenging since some ectosomes share with exosomes several major characteristics such as size, density, and membrane markers, which impose difficulties for their efficient separation and characterization.[26,27] With these limitations in mind, we use the general term *EVs* for both exosomes and ectosomes.

EVs contain proteins and lipids as well as miRNA capable of regulating an assortment of genes[22,23] (public online databases that catalogue EV-associated components, such as Vesiclepedia,[28] EVpedia,[29] and ExoCarta,[30] are readily available). This unique package of information provides both protection and the option of simultaneous delivery of multiple molecular messengers even to sites remote to the vesicular origin.[31-33] More importantly, EVs can be loaded with specific molecules and/or pharmacological agents using simple procedures,[34-36] stored for extended periods,[37-40] and then used without any processing prior to administration to deliver their tailored cargo through many different routes, including oral administration and intranasal spray.[41-44] This flexibility confers power to alter multiple signaling pathways simultaneously, producing results that extend beyond the initial exposure and making this approach more advantageous than those used for traditional pharmaceuticals.

EVs are secreted by most, if not all, types of cells both in vivo and in vitro, and can be commonly recovered from fluids such as saliva, blood, urine, and breast milk,[45] as well as cell culture media.[31-33] It is known that EVs are involved in many biological processes, including the maturation of erythrocytes, the elimination of unnecessary proteins and RNA, antigen presentation in immune responses, coagulation, inflammation, and angiogenesis.[45] These processes are facilitated through the EVs composition, which is based on that of their parental cell. Thus, we can speculate that EVs from auditory cells should be particularly fitted for the function of nanocarriers in the IC drug delivery approach since they would naturally be expressing molecules associated with the function and protection of the hearing organ. To explore this hypothesis, we decided to investigate whether murine auditory HEI-OC1 cells generate EVs in amounts sufficient for animal and human studies and whether they are able to incorporate pharmacological drugs in order to be used as nanocarriers for IC delivery. In addition, we are reporting here a preliminary proteomic characterization of these EVs.

Materials and Methods

HEI-OC1 cells

Immortomouse-derived HEI-OC1 cells were grown in plastic cell culture dishes with DMEM (Gibco, Dublin, Ireland) supplemented with 10% FBS (HyClone, Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 33°C and 5% CO2 as previously described.[46] Upon reaching approximately 80% confluence, cells were thoroughly washed with PBS, a new cell culture media consisting of DMEM supplemented with 10% exosome-depleted FBS (Cat. No. A2720801, Gibco-Thermo Fisher Scientific) was added, and culture continued. After 24 hours, the cell culture media was removed and processed for EVs isolation and characterization.

EVs isolation and characterization

High-purity EVs fractions were obtained from the cell culture media using the commercially available isolation kit exoEasy (Cat. No. 76064, Qiagen, Germantown, Maryland, USA) following the procedure suggested by the manufacturer. Concentration and size of EVs were measured with the nanoparticle tracking device NanoSight NS300 (Malvern Instruments, Worcestershire, UK), and correlated

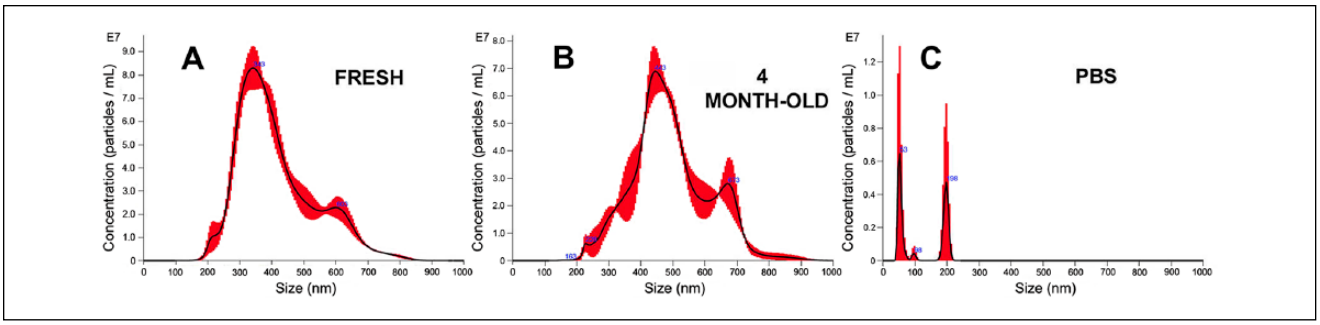

Figure 1. Example of NanoSight measurements. Fresh and 4-month-old (stored at −20°C) extracellular vesicles (EVs) samples were diluted 1:5 in PBS. (A and B) EVs values were automatically corrected with (C) PBS data. Every curve corresponds to a single sample evaluated by triplicate, with 749 frames/replication. Particle concentrations: Panel A: 3.72 × 108 ± 1.96 × 107 particles/mL; Panel B: – 3.18 × 108 ± 8.56 × 106 particles/mL; Panel C: unreliable data.

with protein concentration as measured by Micro BCA Protein Assay Kit (Pierce Biotechnology, Thermo Fisher Scientific).

Proteomic studies

Bottom-up proteomics was performed using well-established protocols. Tryptic peptide samples were desalted using a modified version of Rappsilber's protocol[47] and fractionated via high pH reverse phase chromatography (Poroshell 120, Agilent, Santa Clara, California, USA). The fractions were then injected onto a reverse phase nanobore HPLC column (AcuTech Scientific, San Diego, California, USA) and analyzed with in-line nanospray ionization connected to a hybrid quadrupole-Orbitrap mass spectrometer (QE-Plus, Thermo Fisher Scientific) operating in the positive ion data-dependent collisionally activated dissociation mode (nLC-MS/MS).[48] The data was analyzed in Proteome Discoverer 2.2, which provides measurements of abundance for the identified peptides, and mined using mouse protein databases with a series of bioinformatics methods, including gene ontology (GO) classification and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis.[49,50]

Loading of EVs

To investigate the incorporation of pharmacological drugs, EVs from HEI-OC1 cells (1×10^8 EVs/mL) were co-incubated with dexamethasone (Catalog No. D4902, Sigma-Aldrich, St. Louis, Missouri, USA) at a final concentration of 10 mM for 1 hour at 25°C with and without sonication for 5 minutes. Next, drug-loaded EVs were subjected to a second EVs purification step with exoEasy columns to remove the unloaded drug. For determining the amount of dexamethasone incorporated by the EVs, dried samples were treated with the Amplifex Keto reagent (Sciex, Concord, Canada; 20 µL of diluent and 20 µL of reagent, 60°C, 90 minutes), and after drying, the samples were redissolved in water (50 µL). Aliquots of the solution (20 µL) were analyzed by LC/MS/MS with multiple reaction detection of derivatized dexamethasone (M^+ 507.4 → m/z 448.3) using previously optimized conditions for chromatography and production of the molecular and fragment ion; known amounts of dexamethasone (0, 2.5, 25, and 250 fmol) were treated and analyzed in the same way. Peak intensities from the standards were then used to construct a standard curve, and the quantity of dexamethasone in each sample was determined by interpolation.

Results

Isolation, counting, and sizing of EVs

Although EVs' size and concentration varied among samples, typical values were in the ranges 50 to 800 nm and 2×10^9 to 4×10^9 EVs/mL, respectively (Figure 1). The presence of larger particles (>500 nm) was not a surprise since 0.8 µm filters were used in the process of sample preparation. In contrast, the sharp decrease in the number of EVs with diameters below ~180 nm was completely unexpected. To investigate this issue, we are currently performing sequential microfiltration procedures, dividing the original sample into fractions containing EVs of specific size. In the present study, however, we are only reporting data corresponding to the original sample of HEI-OC1 EVs.

We performed similar measurements with freshly isolated EVs and samples stored for 4 months at −20°C, with results that suggest minimal effects of storage on EVs morphology and integrity (Figure 1). For example, in addition to an increase in variability between replicate measurements in the sample showed in Figure 1, the shift to the right in the size-distribution curves (mode Panel A = 358.1 ± 17.2 nm; mode Panel B = 456.9 ± 14.1 nm) and the slight decrease in EVs concentration (EVs concentration: Panel A = $3.72 \times 10^8 \pm 1.96 \times 10^7$ particles/mL, Panel B

$= 3.18 \times 10^8 \pm 8.56 \times 10^6$ particles/mL) suggest the possible clustering of small vesicles into bigger aggregates.

Protein profiling of EVs

A preliminary proteomics profile identified about 850 proteins belonging to HEI-OC1 EVs (Supplemental Table in the online version of the article). Interestingly, 69 out of the top 100 proteins (69%) listed in ExoCarta as those more frequently identified in exosomes were found in this profile, and this value increased to 84% (42/50) for the top 50 proteins. Moreover, if we focus on the first 30 of these 100 top proteins listed in ExoCarta, only 4 were not detected in this preliminary study (26/30, 86.7%; Table 1).

Among the full set of proteins identified in our samples, 19.2% were cytoplasmic, 17.2% localized in membranes, 12.3% in the cytosol, and 14.6% in the nucleus (Figure 2). Investigating their primary function, we found that 31.0% were involved in protein binding, 16.4% showed catalytic activity, 12.6% RNA binding, and 6.6% had a structural role (Figure 3). Likewise, looking at their molecular function, we found that 21.4% were associated primarily with cell metabolism, 21.2% were involved in the regulation of biological processes, and 13.7% with cell organization and defense (Figure 4). Comparison with the proteomic profile of whole HEI-OC1 cells indicated, as expected, that EVs concentrate some proteins while others were below the limit of detection. No significant differences between fresh and stored EVs were detected (results not shown).

Table 1. Twenty-Six out of the 30 (86.7%) Proteins More Frequently Identified in Exosomes Were Also Found Expressed in Extracellular Vesicles From HEI-OC1 Cells.[a]

Score ExoCarta	Gene Symbol	Present in HEI-OC1 EVs?	Abundance Score in HEI-OC1 EVs
1	CD9	No	—
2	HSPA8	Yes	41
3	PDCD6IP	Yes	60
4	GAPDH	Yes	40
5	ACTB	Yes	18
6	ANXA2	Yes	317
7	CD63	No	—
8	SDCBP	Yes	70
9	ENO1	Yes	52
10	HSP90AA1	Yes	13
11	TSG101	No	—
12	PKM	Yes	39
13	LDHA	Yes	194
14	EEF1A1	Yes	44
15	YWHAZ	Yes	47
16	PGK1	Yes	420
17	EEF2	Yes	57
18	ALDOA	Yes	437
19	HSP90AB1	Yes	14
20	ANXA5	No	—
21	FASN	Yes	792
22	YWHAE	Yes	26
23	CLTC	Yes	202
24	CD81	Yes	248
25	ALB	Yes	48
26	VCP	Yes	9
27	TPI1	Yes	188
28	PPIA	Yes	362
29	MSN	Yes	292
30	CFL1	Yes	301

[a]As listed in ExoCarta (http://exocarta.org/Archive/ExoCarta_top100_protein_details_5.txt; Accessed January 29, 2019).

Loading of EVs with pharmacological drugs

Investigating the loading of HEI-OC1 EVs with dexamethasone (10 mM), we found that they incorporated significant amounts of drug by simple co-incubation at room temperature for 1 hour, followed by a second EVs purification step with exoEasy columns to remove the unloaded drug. The measured values were 10.1 ± 1.9 nM dexamethasone per mL of EVs suspension, and loading was not significantly improved by 5 minutes sonication. Similarly, no significant differences in drug loading between fresh and stored EVs were detected (results not shown).

Discussion

We are reporting here the first results of our research on EVs generated by murine auditory HEI-OC1 cells. Our data indicate that this cell line generates EVs in amounts sufficient for future animal and human studies and that they are able to easily incorporate dexamethasone, suggesting that they could be ideal nanocarriers for IC drug delivery. In addition, the preliminary proteomic characterization of these EVs provided data that when compared with those from similar studies of EVs from different cellular origin, support the idea that they express a unique set of proteins, perhaps including some associated with the function and protection of the hearing organ.

Why use HEI-OC1 cells

Mesenchymal stem cells (MSCs) are known as one of the most prolific producers of EVs.[51,52] In addition, MSCs derived from adipose tissue possess a natural high capacity to modulate both innate and adaptive immune responses,[53-55] promoting immune tolerance and facilitating inflammatory resolution in a paracrine manner through their EVs.[56-63] Because of that, MSC EVs are probably the most frequently used model for studies such as those reported here. However,

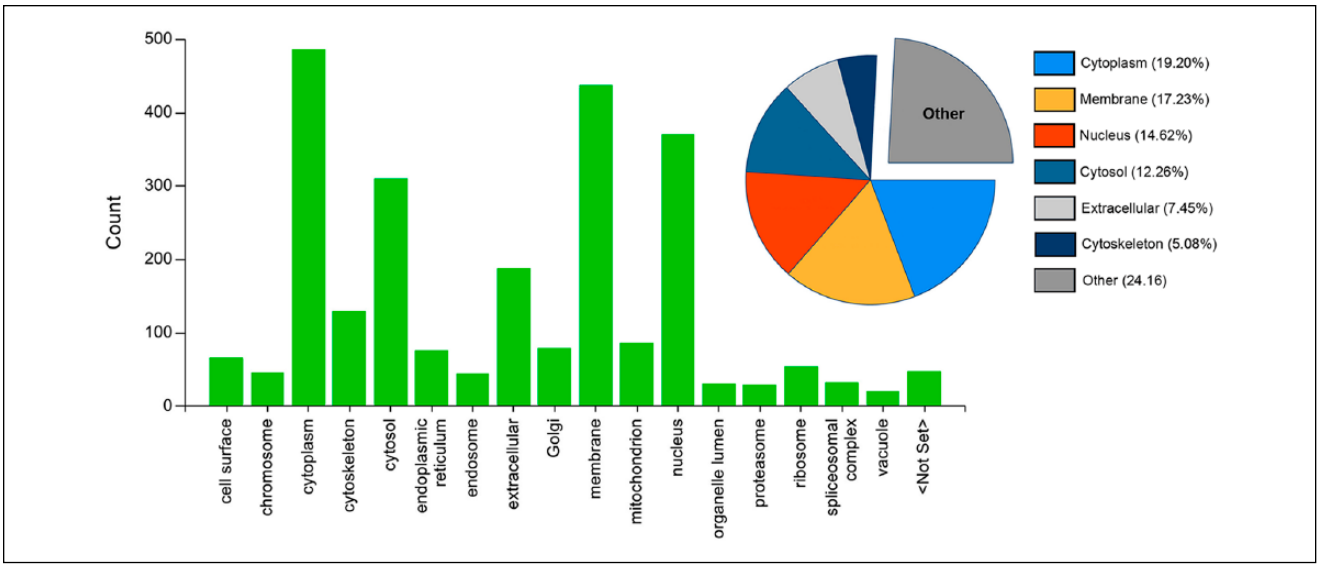

Figure 2. Proteomic analysis. Characterization of proteins from HEI-OC1 EVs by cellular localization.

it is currently accepted that EVs' therapeutic value may be increased by their molecular composition, which is based on the parental cell from which they are derived. Thus, EVs from auditory cells could be more suited for the function of nanocarriers in the IC drug delivery approach than MSCs, since they would naturally contain molecules associated with the function and protection of the hearing organ.

HEI-OC1 is a murine auditory cell line generated in our laboratory about 15 years ago.[46] Currently, with more than 230 published research papers and reviews discussing results obtained with them, they are one of the most frequently used auditory cell line in hearing research. In fact, HEI-OC1 cells have been used in studies with glucocorticoid-loaded nanocarriers to palliate cisplatin ototoxicity, and the results showed a good correlation between HEI-OC1 cells and live mice.[64] Thus, since HEI-OC1 *are* auditory cells with an ample literature supporting their usefulness for hearing research, they could be a good candidate for generating EVs to be used as IC drug delivery nanocarriers.

HEI-OC1 cells as EVs producers

Although EVs' concentration varied among samples, our results indicate that typical values were above 2×10^9 EVs/mL. Ongoing experiments, however, suggest that this number could be significantly increased by collecting the cell culture media after 36 to 48 hours rather than the 24-hour period used in the experiments reported here. In addition, EVs' concentration may be further enlarged by replacing common ultrafiltration procedures by tangential flow filtration techniques. We estimate that samples containing around 1×10^{11} EVs/mL could be routinely obtained with these methodological improvements. Thus, we are confident that HEI-OC1 cells are able to produce EVs on a clinically applicable scale.

A surprising result of these preliminary study was the distribution of EVs by size as estimated with the nanotracking technique. As illustrated in Figure 1, there is a prominent peak for particles with diameters of ~340 nm (Figure 1A) and ~445 nm (Figure 1B). Most importantly, in both cases, there is a significant decrease in the concentration of particles with diameters below ~200 nm. This result seems to indicate that HEI-OC1 EVs consist mostly of ectosomes, with a significant lower number of exosomes. However, although currently considered one of the most reliable techniques for counting and sizing exosomes, the nanotracking technique is not exempt of problems.[65,66] Current experiments aimed at optimizing our measurements suggest that Nanosight measurements are strongly dependent on the optical properties of the particles, which vary with their size. This problem is particularly evident in experiments such as those reported here, where the samples include particles in a very wide size range (50-800 nm). Further studies with sample fractions containing EVs of specific size and/or different techniques for vesicles counting and sizing (eg, Tunable Resistive Pulse Sensing) are required for elucidating this issue.

In contrast, our results showing only small differences between fresh obtained EVs and EVs stored for 4 months at −20°C were expected since it was already recognized in the literature that EVs are relatively insensitive to freeze/thaw cycles.[65]

Proteins in HEI-OC1 EVs

As already mentioned, 69% of the 100 proteins listed in ExoCarta as those more frequently identified in exosomes

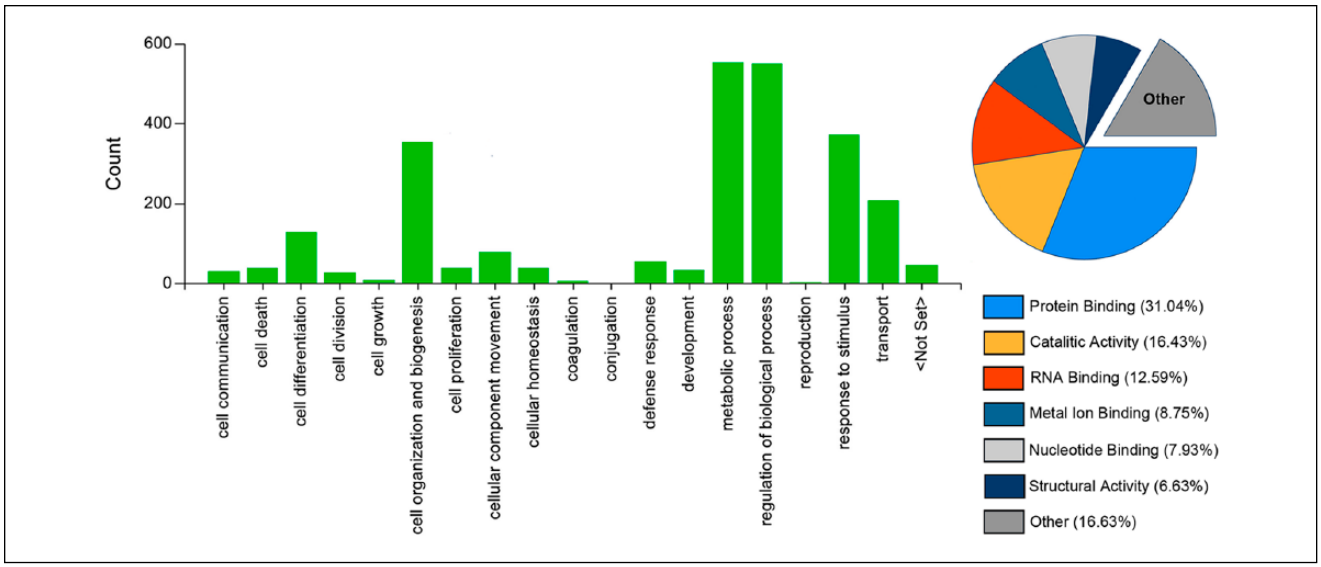

Figure 3. Proteomic analysis. Characterization of proteins from HEI-OC1 EVs by associated biological process.

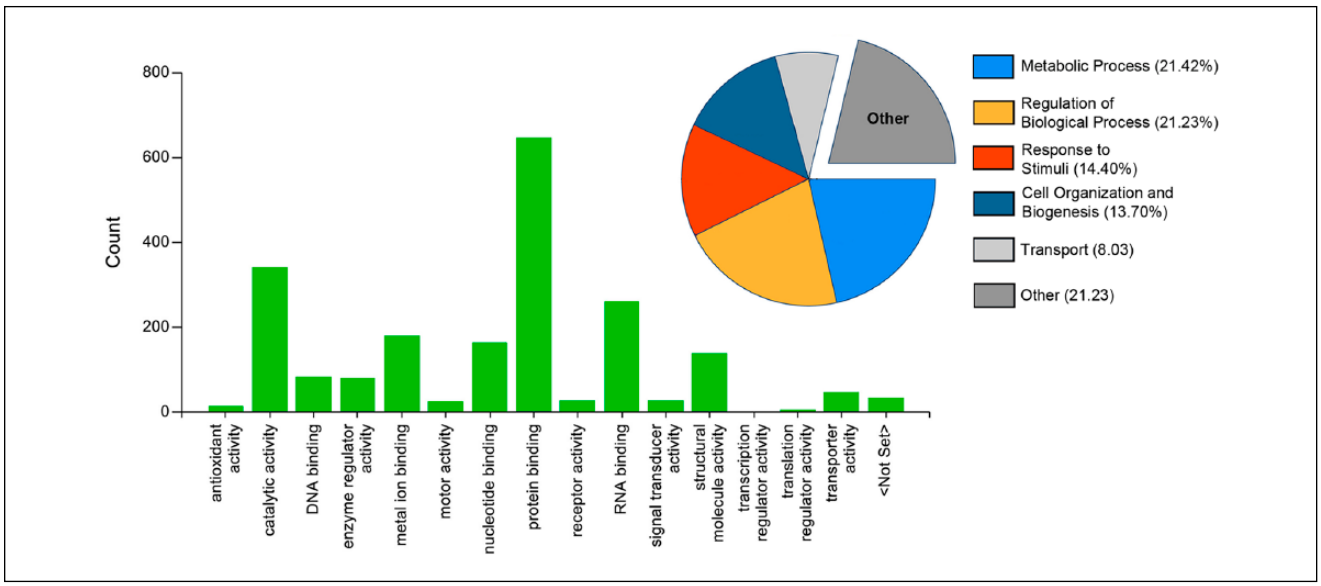

Figure 4. Proteomic analysis. Characterization of proteins from HEI-OC1 EVs by molecular function.

were found in HEI-OC1 cells, and this value increased to 86.7% for the first 30 in the list. Interestingly, from the generally accepted exosomes biomarkers (CD9, CD63, and CD81), only CD81 was definitely detected in our samples (Table 1). Likewise, although the annexins (ANXA) 2, 3, 4, and 6 are relatively abundant in HEI-OC1 EVs, 2 of the most common annexins found in exosomes (ANXA1 and ANXA5) are either expressed at low levels or not expressed at all. Although our study must be further extended, these preliminary results support the idea that EVs from auditory cells would have a particular molecular profile, perhaps associated with the function and protection of the hearing organ.

HEI-OC1 EVs as potential drug nanocarriers

It is already known that EVs can be loaded with specific molecules and/or pharmacological agents using simple procedures.[34-36] For instance, curcumin was efficiently loaded into exosomes after only 5 minutes of incubation at 22°C and was shown to mediate significant anti-inflammatory effects in several disease models,[34,42] and both microRNA (miR-150) and

the antineoplastic chemotherapy drug doxorubicin were loaded into EVs by co-incubation at 37°C for 1 hour and 2 hours, respectively.[39,67] Our results confirm that EVs from HEI-OC1 cells can be loaded with pharmacological compounds (in our case dexamethasone) by simple co-incubation of the vesicles with the compound just as EVs from different cellular origin. Clearly, the incorporation of a given therapeutic agent into HEI-OC1 EVs will be strongly dependent of the particular physicochemical properties of the agent, and parameters such as time and temperature of co-incubation and drug concentration should be optimized in every case. Nevertheless, the present results provide strong support to the idea that HEI-OC1 EVs could be used as IC drug delivery nanocarriers.

Conclusions

Since HEI-OC1 are auditory cells able to produce abundant EVs and these EVs can be quickly isolated in near pure form and loaded with pharmacological drugs by simple co-incubation at room temperature, they appear to be an excellent alternative to MSC for studies involving the inner ear and as nanocarriers in IC drug delivery therapeutic approaches. Further studies, however, are still necessary to fully characterize the EVs generated by HEI-OC1 cells and confirm their value for current and future clinical strategies aimed at protecting the inner ear and preventing noise-, drug-, and age-related hearing loss.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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