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Arrestin domain-containing protein 1-mediated microvesicles (ARMMs) protect against cadmium-induced neurotoxicity

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ABSTRACT

Exposure to environmental heavy metals such as cadmium (Cd) is often linked to neurotoxicity but the underlying mechanisms remain poorly understood. Here we show that Arrestin domain-containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs)–an important class of extracellular vesicles (EVs) whose biogenesis occurs at the plasma membrane–protect against Cd-induced neurotoxicity. Cd increased the production of EVs, including ARMMs, in a human neural progenitor cell line, ReNcell CX (ReN) cells. ReN cells that lack ARMMs production as a result of CRISPR-mediated *ARRDC1* knockout were more susceptible to Cd toxicity as evidenced by increased LDH production as well as elevated level of oxidative stress markers. Importantly, adding ARMMs back to the *ARRDC1*-knockout ReN cells significantly reduced Cd-induced toxicity. Consistent with this finding, proteomics data showed that anti-oxidative stress proteins are enriched in ARMMs secreted from ReN cells. Together our study reveals a novel protective role of ARMMs in Cd neurotoxicity and suggests that ARMMs may be used therapeutically to reduce neurotoxicity caused by exposure to Cd and potentially other metal toxicants.

1. Introduction

Cadmium (Cd) is a heavy metal that is widely distributed in the environment as a result of various human activities such as mining, smelting, and industrial processes. It is a non-essential element that can accumulate in soil, water, and plants.[1,2] While Cd is classified as a group-1 human carcinogen by International Agency for Research on Cancer (IARC),[3] the metal can also easily cross the blood–brain barrier and accumulate in the brain to affect the central nervous system and cause neurotoxicity.[4,5] Studies of Cd neurotoxicity had mainly focused on induction of oxidative stress[6] and perturbation of calcium homeostasis,[7] which affect neurotransmission.[5] However, it remains unclear whether Cd disrupts other aspects of neural cell function and how such disruption contributes to its neurotoxicity.

Extracellular vesicles (EVs) are small membrane-bound structures that are released by all cells, including neural cells, into the extracellular milieu. EVs play crucial roles in intercellular communication via the shuttling of bioactive cargoes such as proteins, lipids, and nucleic acids, to recipient cells.[8] For instance, cancer cell-derived EVs promoted angiogenesis and coagulation to support tumor progression by transporting RNA and proteins[9,10]; EVs from neurons were shown to promote induction of neural stem cells through cyclin D1.[11] More recently, EVs have been investigated in toxicological studies and much interest has focus on the profile and composition changes caused by environmental pollutants such as polycyclic aromatic hydrocarbons,[12] particular matter,[13] and heavy metals.[14] While these studies identified EVs as potential biomarkers for environmental toxicity, whether the changes in EVs functionally contribute to the respective environmental toxicity is not known. Moreover, EVs were often analyzed in bulk without sufficient consideration of the EV heterogeneity.

Unlike classical exosomes, arrestin domain-containing protein 1 (*ARRDC1*)-mediated microvesicles (ARMMs) represent a different class of EVs that bud directly from the plasma membrane, where the *ARRDC1* protein recruits the ESCRT-I complex protein TSG101 to the cell surface to initiate outward membrane budding.[15,16] *ARRDC1* is not only necessary but also sufficient to drive EVs budding, allowing controlled production of ARMMs, which can be further engineered as a versatile platform for packaging and delivering macromolecules into diseased cells.[17] ARMMs have been shown to mediate intercellular signaling of NOTCH receptors.[16] ARMMs are likely secreted by most tissues as *ARRDC1* is ubiquitously expressed including in the brain. However, little is known about ARMMs secreted by neural cells and their potential role in environmental chemical-induced neurotoxicity.

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In this study, we examined the effect of Cd exposure on the production of EVs, including ARMMs. We took advantage of the essential role *ARRDC1* in ARMMs budding and investigated the functional role of ARMMs in Cd-induced neurotoxicity. CRISPR/Cas9 method was applied to knockout *ARRDC1* in human immortalized neural progenitor cells. We showed that *ARRDC1*-knockout ReN cells were more susceptible to Cd toxicity. Importantly, restoring ARMMs in ReN cells significantly reduced Cd-induced toxicity. Proteomics data showed that anti-oxidative stress proteins are enriched in ARMMs secreted from ReN cells. Together, our study revealed the protein composition of ARMMs form neural progenitor cells and identified ARMMs as a novel mechanism underlying neurotoxicity induced by Cd exposure.

2. Materials and methods

2.1. Cell culture and chemical reagents

ReNcell CX human neural progenitor cell line (SCC007, Millipore Sigma) derived from the cortical region of human fetal brain tissue was cultured in laminin-coated plates using ReNcell NSC maintenance media supplied with EGF and FGF according to supplier's instructions. Cadmium chloride (CAS#10108-64-2, 99.99%) Lead chloride (CAS#7758-95-4, 98%), Sodium (meta) arsenite (CAS#7784-46-5, ≥90%), Manganese chloride (CAS#7773-01-5, ≥99%), Nickel chloride (CAS#7718-54-9, 98%), and Potassium dichromate (CAS#7778-50-9, 99.5%) were purchased from Millipore Sigma and stock solutions at 100 mM were prepared in distilled water. Human fibroblast growth factor (FGF, catalog # PHG0264) and epidermal growth factor (EGF, catalog # PHG0311) recombinant proteins were purchased from Thermo Fisher Scientific.

2.2. Establishment of ARRDC1 CRISPR knockout ReNcell CX cells

A lentiviral CRISPR/Cas9 system was applied to knockout *ARRDC1* in ReN cells using single guide RNAs targeting the exon 1 (GCACCGCTGCCGTTCCGAGG) and 6 (CGACGTACGGACCATTGCGG), respectively. Guides were cloned into lentiCRISPRv2 vector containing hSpCas9 cassette (Addgene) as previously described.[18] Lentivirus were produced in HEK293T cells by co-transfecting vector and packaging plasmids pVSVg and psPAX2. Wild type ReN cells were transduced with lentivirus in the presence of polybrene (8 μg/mL) for 24 h. Cells with stable viral integration were selected for 7 days using puromycin at 1 μg/mL. The knockout efficiency was confirmed by Western blot for *ARRDC1*.

2.3. Extracellular vesicle isolation and purification

ReN cells were seeded at 2×10^6 cells per 10 cm plate and 24 h later were treated with Cd (at 0.5 or 1 μM) or control vehicle (PBS). After 48 h treatment, EVs released by ReN cells was harvested using centrifugation method. In brief, cell culture supernatant was collected and subjected to centrifugation (3220 g) at 4 °C for 10 min. Supernatant was filtered using 0.2 μm filters (Acrodisc) and followed by ultracentrifugation using the SW32Ti rotor in a XE90 centrifuge (Beckman) at 160,000 g for 2 h at 4 °C. The medium was then aspirated, and the pellets enriched with EVs were resuspended in filtered PBS for quantification. The purity and yield of EVs were measured using NanoSight NS300 instrument (Malvern). Samples containing EVs were diluted with filtered-PBS and recorded for 60 s with 5 repeats.

2.4. Cytotoxicity assay

LDH release assay was performed as an indicator of cytotoxicity using CytoTox 96®non-Radioactive cytotoxicity assay kit according to the manufactures instruction (Promega). In brief, cells were seeded in 6-well plates at a density of 2×10^5 cells/well and incubated overnight at 37 °C. The next day, cells were exposed to metals with targeted concentrations in fresh medium. After 48-h exposure, 50 μL of medium from each treatment was collected for the LDH assay, where the CytoTox96®reagent was added and followed with 30-min incubation at room temperature. Stop solution was added after incubation and the absorbance signals were measured at 490 nm on a plate reader.

2.5. Confocal imaging and quantification of reactive oxygen species production

Cells were cultured in 8-chamber slides (glass bottom, Thermo Fisher Scientific) at a density of 3×10^4 cells/well overnight. Cells were exposed to 1 μM of Cd with fresh medium the next day and incubated for 48 h. Cellular reactive oxygen species assay kit (ab113851, Abcam) containing 2′,7′-dichlorofluorescin diacetate (H_2 DCFDA) was used to stain the cells for 45 min under dark at 37 °C, followed by washing with supplied buffer. Fluorescent images were then acquired by ZEISS LSM 880 confocal microscope. For the quantification, cells were cultured in 96-well plates at a density of 5×10^3 cells/well followed by Cd treatments the next day for 48-h. Staining solution that contains H_2 DCFDA (Abcam) was added at the end of incubation for 45 min at 37 °C under dark. The plates were immediately read on a fluorescence plate reader at $E_x/E_m = 485/535$ nm. Images were processed using ImageJ.

2.6. Quantitative real-time PCR (qRT-PCR)

Expression of genes related to antioxidative stress including *SOD1* (gene encoding superoxide dismutase) and *CAT* (gene encoding catalase) as well as the housekeeping gene *GAPDH* was measured by qRT-PCR following the protocols as previously described.[19] In brief, total RNA was extracted using RNeasy kit (Qiagen) and reverse-transcribed to cDNA using random hexamers and Superscript III Kit (Invitrogen). qPCR was performed using SYBR green PCR Master Mix (Thermo Fisher Scientific) in QuantStudio 6 Flex instrument (Thermo Fisher Scientific). The [delta][delta]Ct method was used to compare relative amounts of transcripts. The primers for each gene were shown as follow, *SOD1* (Forward: 5′-GACTGACTGAAGGCCTGCAT-3′, Reverse: 5′-ATCGGCCACACCATCTTTGT-3′), *CAT* (Forward: 5′-GACCACTGGAGCTGGTAACC-3′, Reverse: 5′-CCCGATTCTCCAGCAACAGT-3′), *GAPDH* (Forward: 5′-GAGAAGGCT-GGGGCTCATTT-3′, Reverse: 5′-AGTGATGGCATGGACTGTGG-3′).

2.7. Immunoblot analysis and antibodies

Cells were lysed in M-PER mammalian protein extraction reagent (Thermo Fisher Scientific) supplemented with a protease inhibitor cocktail (Roche). Cell lysates or vesicles resuspended in lithium dodecyl sulfate sampling buffer (Novex) were resolved on a 4%–12% NuPAGE gel (Novex) and transferred onto a PVDF membrane (Bio-Rad). Blots were probed with primary antibodies in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk overnight, followed by HRP-conjugated anti-rabbit antibody (Cell Signaling, 7074S, 1:2000) or anti-mouse antibodies (Cell Signaling, 7076S, 1:2000). Primary antibodies used include anti-*ARRDC1* rabbit polyclonal antibody (in house as described in Ref. 15, 1:2000), anti-CD9 rabbit monoclonal antibody (Cell Signaling, D8O1A, 1:1000), and anti-vinculin rabbit monoclonal antibody (Abcam, AB129002, 1:1000).

2.8. Sample preparation and proteomics by LC-MS/MS

Total proteins from ReN cells were extracted using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific) and the

concentrations were determined using bovine serum albumin standards and 660 nm protein assay reagent (Thermo Fisher Scientific) following the instructions. Proteins were further digested overnight at 37 °C using the FASP approach with trypsin (Promega, Madison, WI)[20] and subjected to mass spectrometry (LC-MS/MS) analysis. Proteins from EVs were prepared in Coomassie blue-stained SDS-PAGE gel and cut into pieces for trypsin digestion. Protein samples were analyzed by the Taplin Biological Mass Spectrometry Facility at the Harvard Medical School following the previous methods.[16] The peptides were reconstituted in 5–10 μl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). They were then loaded onto a reversed-phase analytical column (100 μm × 30 cm) packed with 2.6 μm C18 spherical silica beads after pre-equilibration. Peptides were separated by applying a flow rate of 300 nL/min and a gradient from 5% solvent B (95% acetonitrile, 0.1% formic acid) to 38% solvent B for 60 min. The MS Scan range was 350 to 1250 m/z, with a resolution of 60,000, and for MS/MS, the scan range was set to 2000 m/z. The eluted peptides were ionized by electrospray and analyzed using an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). A tandem mass spectrum of specific fragment ions for each peptide was produced, and peptide sequences were identified by matching UniProt Human database (release 2017_06_20, 80010 entries) using the Sequest software program (Thermo Fisher Scientific, Waltham, MA). The maximum number of missed cleavages was 2, and the mass tolerance values for precursor and fragment ions were 50 ppm and 1 Dalton, respectively. All databases included reversed sequences of peptides, and both the maximum peptide and protein false discovery rates (FDR) were limited to 1%. Protein quantification was based on the intensity of at least two unique peptides to quantify the different protein profiling in the EVs. Total peptide number normalization was performed to ensure that each sample had the same distribution. Finally, a screening of differentially expressed proteins was performed using a cut-off of 1.5-fold change and $p < 0.05$.

2.9. Bioinformatic analysis for proteomic datasets

ShinyGO 0.77 was used for gene ontology (GO) analysis of differential phosphoproteins, including their biological process, and molecular function.[21]

2.10. Statistical analysis

Statistical analysis was performed with GraphPad Prism (version 9.0, La Jolla, CA). Data was analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA. P-values less than 0.05 were considered statistically different. Data were presented as the mean ± standard deviation.

3. Results

3.1. Effects of cadmium on extracellular vesicles (EVs) production

The immortalized human progenitor cell line ReNcell CX cells (ReN cells) have been used extensively as the cell model in neurotoxicology studies.[22–24] We first treated ReN cells with different heavy metals and used the NanoSight Nanoparticle tracking analysis (NTA) to assess extracellular vesicles (EVs) production. As shown in Supplementary Figure S1, among the tested heavy metals, cadmium (Cd) significantly increased EVs production in ReN cells. The effects of Cd on ReN cells EV production are concentration-dependent (Fig. 1A). We further examined the effect of Cd on EVs by comparing EVs from control and Cd-treated ReN cell culture medium. Proteins contained in EVs from control and Cd-treated ReN cells were resolved on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (Fig. 1B) and subjected to mass spectrometry (LC-MS/MS) and proteomic analysis.

Based on normalized peptide numbers, we identified a total of 848 proteins increased (>1.5-fold) in EVs from the Cd-treated cells and among them 392 proteins were only present in Cd-treated group (Fig. 1C and Table S1). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the Cd-induced EV proteins (Fig. 1D) showed that a cluster of proteins are implicated in neurodegenerative diseases such as Alzheimer disease, Parkinson's disease, Huntington disease, and amyotrophic lateral sclerosis. Furthermore, pathways related to vesicle-mediated transportation and protein localization are also enriched (Fig. 1E). One of the enriched proteins increased in Cd-treated ReN cells EVs is ARRDC1 (Table S1). We directly compared the expressions of ARRDC1 in both ReN cell lysates and EVs by western blotting. Consistent with the proteomic data, the western blot result showed that with Cd treatments, *ARRDC1* expression remained similar in the cell lysate but increased in EVs (Fig. 1F&G).

3.2. ARRDC1-knockout ReN cells and responses to Cd exposure

Given the active role of *ARRDC1* in ARMMs production, our result suggests that Cd likely increased the production of ARMMs by ReN cells. We next examined the role of *ARRDC1* in Cd-induced cytotoxicity to ReN cells. We first used a lentiviral CRISPR/Cas9 system that containing guide RNAs targeting exons 1 or 6 of *ARRDC1* to establish ReN cells ARRDC1 knockouts. *ARRDC1* knockout was confirmed by western blotting, where in both cell lysates and EVs, *ARRDC1* was absent compared to the scramble control (Fig. 2A). Overall EVs production decreased by 30%–40% in *ARRDC1*-knockout cells (Fig. 2C), indicating that ARMMs constitute up to 40% of all EVs produced by ReN cells. We then examined Cd toxicity in the *ARRDC1*-knockout and control cells by measuring the LDH release. As shown in Fig. 2C, higher cytotoxicity as indicated by increased LDH release was observed in *ARRDC1*-knockout ReN cells compared to the scramble control cells with consistency in both knockout cell lines.

We next assessed oxidative stress, one of the main cytotoxic mechanisms, in ReN cell by measuring the production of reactive oxygen species (ROS) and antioxidative responses. Total ROS signals in ReN cells were captured using fluorescent staining solution and quantified. Cd treatment induced significantly higher ROS production compared to the control and, importantly, both *ARRDC1*-knockout cell lines showed stronger ROS staining when treated with Cd compared to the scramble control cell line (Fig. 2D&E). Consistent with this, the expression of oxidative stress genes, including superoxide dismutase (SOD) and catalase (CAT), exhibited similar trend as the oxidative stress results, where more profound responses occurred in *ARRDC1*-knockout ReN cells (Fig. 2F&G). Together, our data showed that ReN cells become more susceptible to Cd exposure in the absence of *ARRDC1* and ARMMs.

3.3. Proteomics analysis of ReN cells under Cd exposure

To further explore the mechanism involved in the different responses of *ARRDC1*-knockout ReN cells to Cd, we performed proteomics on both control and *ARRDC1*-knockout ReN cells with or without Cd treatment. First, by comparing the protein profile between Cd-treated and non-treated groups in scramble control ReN cells (Fig. 3A), we identified a set of proteins that were involved in KEAP1-NRF2 pathway, cellular response to chemical stress, iron uptake and transport (Figure S2). Indeed, many proteins related to the NRF2 pathway were increased upon Cd treatment (Fig. 3C). We then compared proteins from *ARRDC1*-knockout and scramble control ReN cells under Cd exposures. As shown in Fig. 3D, a total of 563 proteins with more than 1.5-fold changes and statistical significance ($P < 0.05$) were identified, a majority of which were significantly expressed higher in scramble-control ReN cells under Cd treatment compared to *ARRDC1*-knockout cells (Fig. 3E). Furthermore, the differently expressed proteins were analyzed to uncover the involved pathways. As depicted in Supplementary Figure S3, pathways including glutathione metabolism, fatty acid degradation, and pyruvate metabolism were enriched in *ARRDC1*-knockout ReN cells treated with Cd. Many proteins in related to glutathione metabolism were reduced in *ARRDC1*-knockout cells compared to the control cells (Fig. 3F), suggesting that the knockout may compromise the ability of the cells to detoxify Cd.

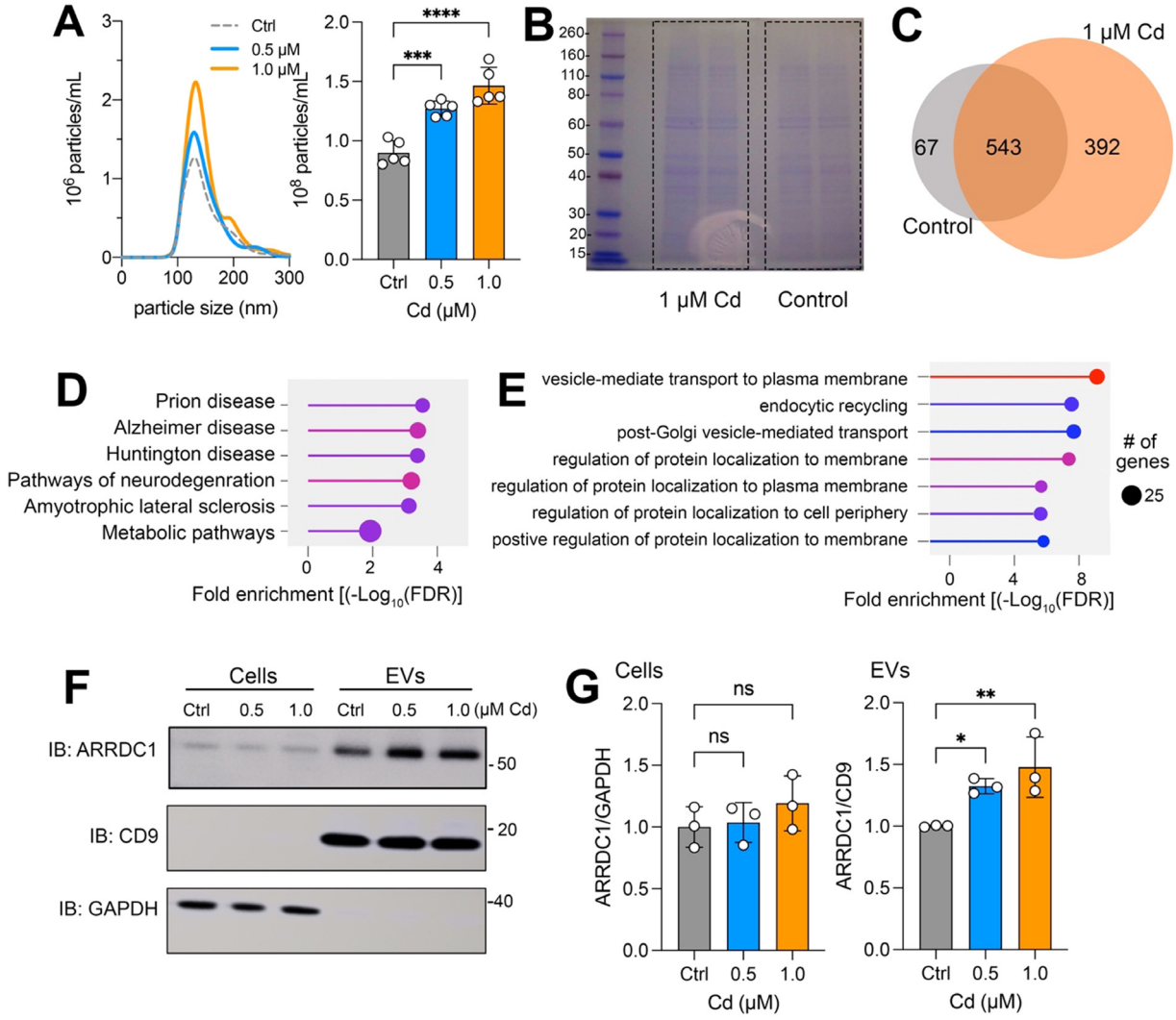


Fig. 1. Effects of Cd on the extracellular vesicles (EVs) release in ReN cells and proteomics changes. (A) EVs profile and quantification results. Each group has three biological replicates and five technical replicates. (B) SDS-Page of proteins in EVs isolated from control and 1 μM-Cd treatment groups and (C) proteins with >1.5-fold change from treatment over the control group. EVs were isolated from three 10-cm plates of cells and combined for proteomic analysis from each group. (D) Pathways related to neurodegenerative diseases from the proteins significantly changed by Cd in ReN cells EVs. (E) Cd affected proteins in EVs that are related to vesicle-mediated transportation and protein localization. (F) Western blot showing the expression of *ARRDC1* in cell lysate and vesicles from ReN cells under Cd treatments and (G) statistical results, each condition has three biological replicates. * P<.

3.4. Effects of adding ARMMs to ReN cells under Cd exposure and role of ARMMs

Because *ARRDC1*-knockout ReN cells are more susceptible to Cd exposure, we hypothesize that ARMMs play a protective role in Cd-induced toxicity. To further test this hypothesis, we examined the effect of adding ARMMs back to ReN cells. We isolated the EVs from wild type ReN cells, which were further quantified by nanoparticle tracking analysis and added to the ReN cells with Cd treatment as well as non-treatment controls (Fig. 4A). First, EVs were characterized by western blot which showed the expression of ARRDC1 as well as CD9 and vinculin, markers for vesicles and cell lysate, respectively (Fig. 4B). Different doses of EVs were added back to ReN cell (scramble control and *ARRDC1*-knockout). As shown in Fig. 4C, the amount of ARRDC1 in cells was enhanced as the concentration of added EVs increased, where at the concentration of 2×10^5 EV/cell, ARRDC1 amount was restored back to normal level as shown in scramble-control ReN cells. In the control groups (without Cd treatment), the addition of EVs from wild type ReN cells did not change LDH release. However, in both *ARRDC1*-knockout ReN cells treated with 1 μM of Cd, the LDH release was significantly reduced with the addition of EVs (Fig. 4D), indicating a protective function of EVs in ReN cells in response to Cd. We also tested EVs from *ARRDC1*-knockout ReN cells. In contrast to EVs from wild type ReN cells, the addition of ARRDC1-knockout ReN-derived EVs did not reduce Cd toxicity in scramble control or *ARRDC1*-knockout ReN cells (Fig. 4E). These results together support the hypothesis that ARMMs play protective roles in ReN cells against Cd toxicity.

3.5. Proteomics analysis of ARMMs from ReN cells

To investigate the mechanism underlying ARMMs' protective role in ReN cells, we performed proteomics on EVs from scramble-control and *ARRDC1*-knockout ReN cells (Fig. 5A). Proteins contained in EVs were resolved on SDS-PAGE gel (Fig. 5B) and subjected to mass spectrometry (LC-MS/MS). By comparing the proteins between scramble control and *ARRDC1*-knockout EVs, we identified a total of 348 proteins that decreased more than 1.5 folds in two groups of knockout EVs compared to scramble-control (Table S2). These proteins significantly decreased in the EVs from *ARRDC1*-knockout ReN cells were considered as the ARMMs proteins. As shown in Fig. 5C, consistent with the biogenesis of microvesicles, many proteins in ARMMs are extensively involved in the vesicle-mediated transport to the plasma membrane and endocytic

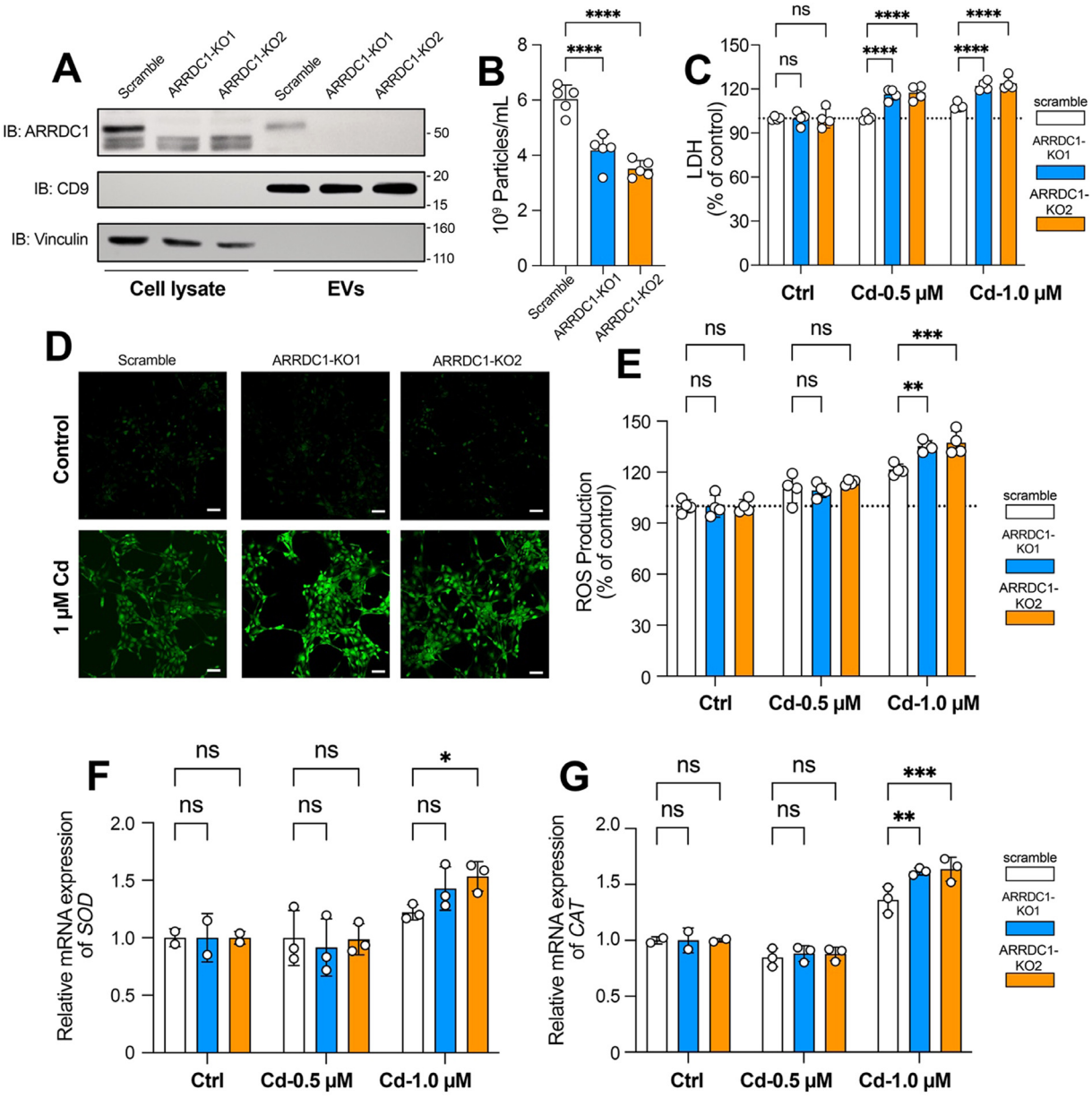


Fig. 2. Characterization of *ARRDC1* knockout in ReN cells and response to Cd exposure. (A) Western blotting to show the successful knockout of *ARRDC1* in ReN cells. (B) EV concentrations isolated from scramble control and *ARRDC1*-knockout ReN cells. Each group has three biological replicates and five technical replicates. (C) Cytotoxicity LDH. (D) representative confocal images of scramble and knockout ReN cells exposed to 1 μM-Cd (scale bar, 100 μm) and (E) Quantification of ROS production. Each treatment has three biological replicates and four technical replicates. (F) *SOD* and (G) *CAT* gene expressions. Each treatment has three replicates.

recycling. These proteins include TSG101 and Vps28—components of the ESCRT-I complex that are required for ARMM budding.[15,16] In addition, several members of the Rab family GTPases (RAB21, RAB4A, RAB8A, 5A, RAB14, RAB7A, RAB11B, RAB1A, RAB35, RAB1C, RAB34, RAB5C, RAB2A, RAB10 and RAB13), which are coordinators of vesicle traffic,[25] are enriched in ReN cell ARMMs. Interestingly, many proteins related to antioxidant and peroxidase activities are also found in ARMMs (Fig. 5D). The levels of these antioxidant proteins decrease significantly in EVs secreted by *ARRDC1*-knockout ReN cells (Fig. 5E). These proteomic results identified the protein components of ARMMs from ReN cells and suggest a model in which the transfer of antioxidant proteins may underly the protective effect of ARMMs in Cd neurotoxicity (Fig. 5F).

4. Discussion

In this study, we examined the emerging role of EVs in metal-induced neurotoxicity. We showed that Cd, a ubiquitous environmental metal contaminant, stimulates the production of EVs, including ARMMs, in human neural progenitor cells (ReN cells). The lack of ARMMs renders ReN cells more susceptible to Cd toxicity, and conversely, the addition of ARMMs reduced Cd-induced toxicity. We further showed that anti-oxidative stress proteins are enriched in ARMMs secreted by ReN cells. Together our results demonstrate that ARMMs, an important class of EVs, protect ReN cells against Cd-induced neurotoxicity and further suggest that ARMMs may be used therapeutically to reduce neurotoxicity caused by exposure to Cd and potentially other metal toxicants.

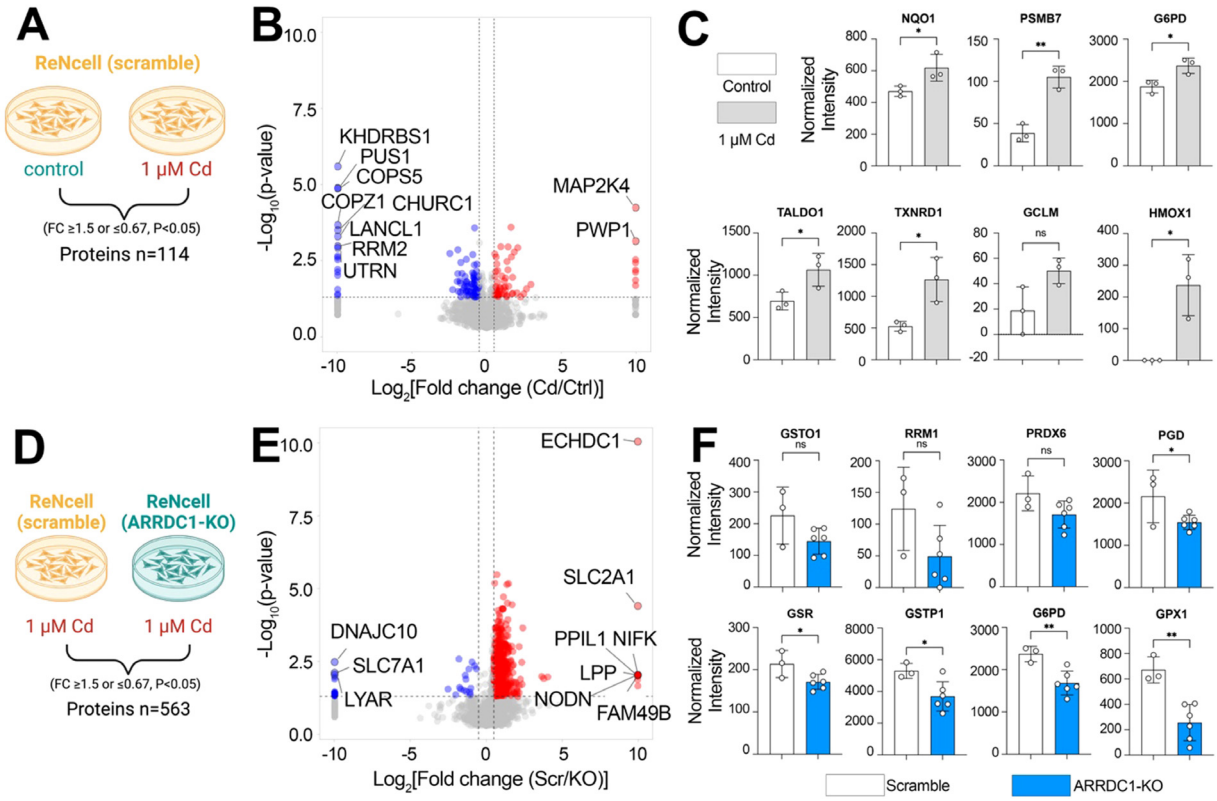


Fig. 3. Proteomics analysis of ReN cells (scramble control and *ARRDC1*-knockout ReN cells) under Cd treatment, each group contained three biological replicates. (A) scheme of the proteomics comparison between Cd-treatment and control in ReN scramble cells. (B) Volcano plot showing the protein changes. (C) Level changes of representative proteins that are related to NRF2-mediate nuclear events. (D) scheme of the proteomics comparison between scramble and *ARRDC1*-Knockout ReN cells under Cd-treatment. (E) Volcano plot showing the protein changes. (F) Level changes of representative proteins that are related to glutathione metabolism.

This study unveiled the protective role of ARMMs and provided a new mechanism that helps explain the neurotoxicity caused by Cd exposure. Previously known mechanisms of Cd toxicity mainly engage in the regulation of genes that are related to stress responses and transcriptional and translational factors, induction of cellular autophagy and apoptosis, and oxidative stress that leads to damage of lipids, proteins, and DNA.[26] For instance, Cd was reported to alter the genes targeted to the p53, mTOR, and ciliary neurotrophic factor-mediated Jak1 and Jak2 tyrosine kinase signaling pathways in human neural cell models.[27–29] Oxidative stress induced by Cd in neural cells features excessive production of reactive oxygen species, and as a response, antioxidant defense systems are activated including enzymes such as superoxide dismutase and catalase.[30] Interestingly, our study determined many of the proteomic components of ARMMs from ReN cells are related to antioxidative responses including glutathione s-transferase (GSTP1, GSTM2), peroxiredoxin (PRDX1, 4, 6), and glutathione peroxidase (GPX3) (Fig. 5D), which have been extensively reported to be involved in antioxidative response in other *in vitro* cell models and *in vivo* animal models under Cd stress.[31–33] Therefore, these data are consistent with previous studies on the role of oxidative stress in Cd neurotoxicity and, more importantly, support a model in which transfer of antioxidant proteins via ARMMs may underlie the protective effects of the vesicles in Cd neurotoxicity (Fig. 5F).

Our study provides causal evidence between EVs and Cd neurotoxicity, thus further expanding the roles of EVs in toxicity studies. It has long been recognized that exposure to heavy metals such as Cd strongly correlates with neurodegenerative diseases via experimental and epidemiological evidence.[28,34,35] EVs were reported as the mediators between manganese exposure and Parkinson's disease, where the EVs promoted the transfer of protein α-synuclein between neurons and microglia and induced inflammation and neuronal cell death.[36] Similarly, in this study, EVs production was stimulated with Cd treatments and proteomic investigation of these EVs revealed a group of proteins that are related to neurodegenerative diseases were identified (Fig. 1D), providing the first evidence from the perspective of EVs to show the correlation between neurodegenerative diseases and Cd exposure. Furthermore, EVs have been reported to be associated with toxicities caused by environmental contaminants. For example, secretion of EVs was stimulated in macrophages exposed to fine atmospheric particulate matter, which can further convey the signals and induce pro-inflammatory phenotype on pulmonary epithelial cells.[37] Polycyclic aromatic hydrocarbons were reported to increase EVs production by both *in vitro* endothelial cells and in urines from rats, and the composition in the EVs were impacted.[12,38] These studies suggest the potential of EVs as the mediators of chemical-induced toxicity and biomarkers for diseases and monitoring exposures. However, attention was mostly paid to the changes of EV production and cargo profile after chemical treatments, which would be rather the effects than the reasons for chemical toxicity and fail to reveal the role of EVs comprehensively and unbiasedly in chemical-induced toxicity. Our study took advantage of the function of *ARRDC1* in EVs production and CRISPR/Cas9-based gene editing tool and compared the Cd toxicity after the EVs production was repressed. Effects of *ARRDC1*-knockout on the proteomic changes in ReN cells in response to Cd showed that several key pathways with significant differences were involved, including the metabolism of glutathione, cysteine and methionine, glycolysis, and protein processing in the endoplasmic reticulum. These results help explain the protective effects of *ARRDC1*, provide more complementary evidence for ARMMs as the biomarkers for neurotoxicity, and ultimately, provide a better understanding of the biological functions of EVs in hazardous effects caused by environmental pollutants.

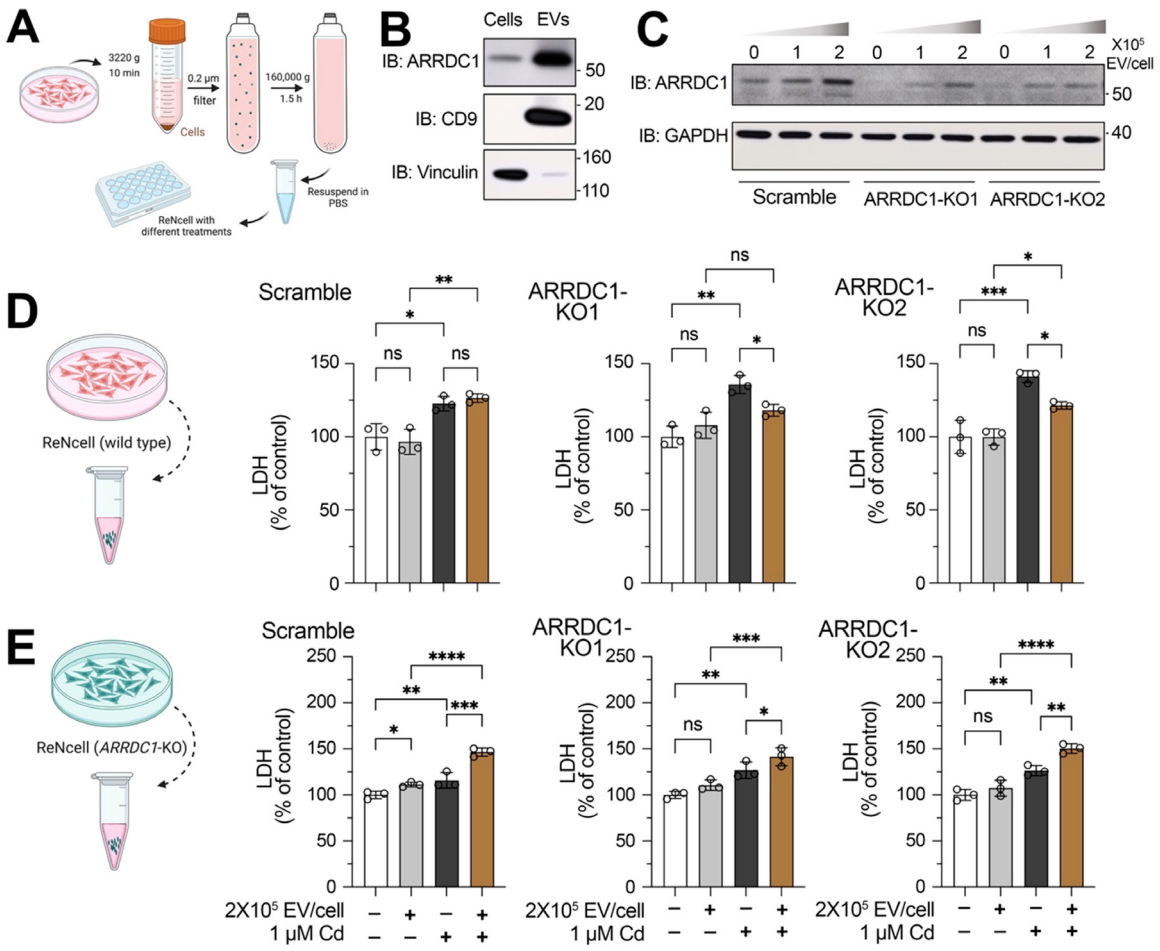


Fig. 4. Effect of adding back EV to ReN cells and response to Cd exposure, each group contained three biological replicates. (A) scheme of EVs isolating and adding back design. (B) western blot showing the *ARRDC1* expression ReN cells EV. (C) Western blotting showing *ARRDC1* expression after adding different dose of EV back to scramble and *ARRDC1*-knockout ReN cells. (D) LDH cytotoxicity results of scramble and *ARRDC1*-knockout ReN cells under Cd treatment and effect of adding wild type EV (2×10^5 EV/cell). (E) LDH cytotoxicity results of scramble and *ARRDC1*-knockout ReN cells under Cd treatment and effect of adding *ARRDC1*-knockout EV (2×10^5 EV/cell).

The mechanism of ARMMs biogenesis as well as the protein composition have been well recognized.[15,16] For instance, a previous study showed that ARMMs isolated from a panel of human cells including HEK293T, HCC1419, A549 and MCF-7 cells contain active NOTCH receptors and mediate a non-canonical intercellular NOTCH signaling.[16] This study characterized ARMMs released from human neural progenitor cell lines and identified proteins involved in vesicle-mediated transportation to plasma membrane (Fig. 5C), which confirmed the consistence of ARMMs biogenesis across different cell types. More importantly, a group of proteins related to the activities of antioxidant and peroxidase were also recognized (Fig. 5D), which indicates a new functional role which ARMMs may play in biological process, especially in response to oxidative stress induced by xenobiotics. This study also found that the production of ARMMs was affected by Cd exposure, indicating that ARMMs can be a potentially important biomarker for chemical exposure. In addition, great efforts have been made to apply ARMMs as a versatile platform for packaging and delivery different biological cargos including RNAs and proteins.[17] The protective role of ARMMs in ReN cells against Cd highlights the potential for engineering ARMMs to target chemical-induced neurotoxicity and neurodegenerative diseases.

Our study has several potential limitations. First, we used the immortalized human neural progenitor cell line as the *in vitro* model due to its representation of an important neural cell type and wide applications in neurotoxicity studies.[39,40] However, this cell line does not model other important, mature, and fully differentiated cell types such as neurons, astrocytes, or microglia in the brain. Second, our study essentially tested the EVs from ReN cells and investigate the role of ARMMs in this neural cell type by focusing on autocrine regulation without examining intercellular communications, which would provide a more comprehensive understanding of the roles of ARMMs in cell-to-cell communications and responses to xenobiotic stress in the brain. In addition, we tested relatively high concentrations of Cd with short exposure time; long-term exposure with lower concentrations could be considered in future studies. Finally, whether the ARMMs role established in cell-based *in vitro* model can be extrapolated to *in vivo* remains to be determined. Together, future studies addressing these limitations are needed to further establish the important role of ARMMs in modulating the neurotoxic effects caused by Cd and other environmental metal contaminants.

5. Conclusion

In summary, we established an *in vitro* role for ARMMs in Cd-mediated neurotoxicity. Our study implicates a novel mechanistic link between EV-mediated cellular communication and the effect of heavy metal exposures on neurotoxicity. Further studies on ARMMs' role in neurotoxicity may ultimately contribute to the development of preventative and therapeutic strategies for neurotoxicity induced by environmental chemicals.

CRediT authorship contribution statement

Zunwei Chen: Conceived the project, Designed the experiments, Performed most of the experiments, Wrote the paper with input from

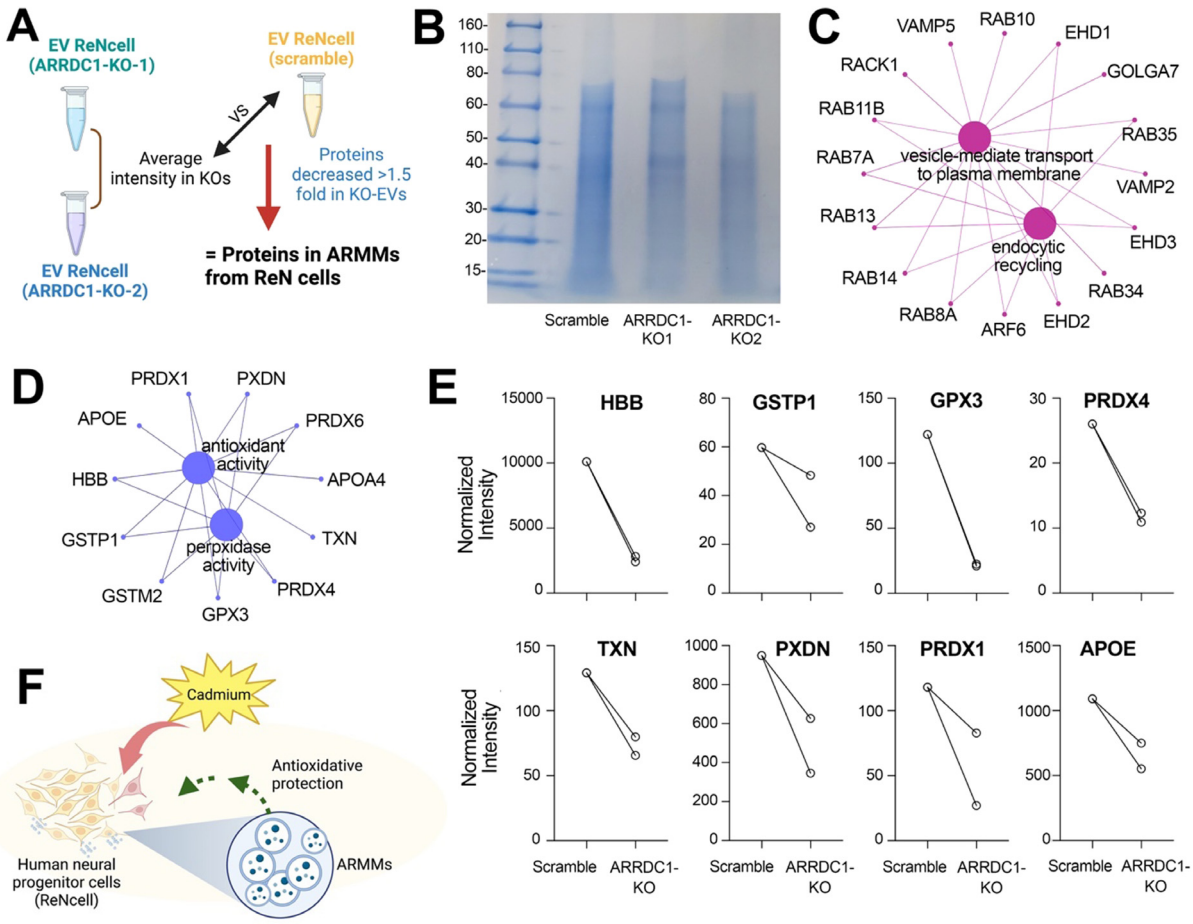


Fig. 5. Proteomics analysis of EVs isolated from scramble and *ARRDC1*-knockout ReN cells. EVs were isolated from three 10-cm plates of cells and combined for proteomic analysis from each group. (A) Comparison strategy of ARMMs proteomics. (B) SDS-PAGE of EVs after purification for proteomics analysis. (C) Functions related to vesicle-mediated transportation and endocytic recycling of ARMMs from ReN cells. (D) Proteins related to antioxidant and peroxidase activity of ARMMs from ReN cells. (E) Different levels of representative proteins related to antioxidant and peroxidase activity of EVs from scramble and *ARRDC1*-Knockout ReN cells. (F) Schematics showing the protective roles ReN cells ARMMs against Cd toxicity.

other co-authors. **Zhi Qiao:** Performed bioinformatics analysis of the proteomics data. **Charlotte R. Wirth:** Made the ARRDC1-knockout ReN cells. **Hae-Ryung Park:** Made the ARRDC1-knockout ReN cells. **Quan Lu:** Conceived the project, Designed the experiments, Supervised the project, Wrote the paper with input from other co-authors.

Declaration of competing interest

Quan Lu is a scientific founder, advisor and shareholder of Vesigen Therapeutics and Evantigen Inc. All other coauthors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.vesic.2023.100027.

References

1. Genchi G, Sinicropi MS, Lauria G, Carocci A, Catalano A. The effects of cadmium toxicity. *Int J Environ Res Public Health*. 2020;17(11):3782.
2. Wang M, Chen Z, Song W, Hong D, Huang L, Li Y. A review on cadmium exposure in the population and intervention strategies against cadmium toxicity. *Bull Environ Contam Toxicol*. 2021;106:65–74.
3. IARC IAfRoC. Arsenic, metals, fibres and dusts. IARC monographs on the evaluation of carcinogenic risks to humans. 2012.
4. Li C-X, Talukder M, Xu Y-R, Zhu S-Y, Zhao Y-X, Li J-L. Cadmium aggravates the blood–brain barrier disruption via inhibition of the wnt7a/β-catenin signaling axis. *Environ Pollut*. 2023;324:121400.
5. Méndez-Armenta M, Rios C. Cadmium neurotoxicity. *Environ Toxicol Pharmacol*. 2007;23(3):350–358.
6. Liu J, Qu W, Kadiiska MB. Role of oxidative stress in cadmium toxicity and carcinogenesis. *Toxicol Appl Pharmacol*. 2009;238(3):209–214.
7. Tonoli E, Verduci I, Gabrielli M, Prada I, Forcaia G, Coveney C, Savoca MP, Boocock DJ, Sancini G, Mazzanti M. Extracellular transglutaminase-2, nude or associated with astrocytic extracellular vesicles, modulates neuronal calcium homeostasis. *Prog Neurobiol*. 2022;216:102313.
8. Maas SL, Breakefield XO, Weaver AM. Extracellular vesicles: Unique intercellular delivery vehicles. *Trends Cell Biol*. 2017;27(3):172–188.
9. Feng Q, Zhang C, Lum D, Druso JE, Blank B, Wilson KF, Welm A, Antonyak MA, Cerione RA. A class of extracellular vesicles from breast cancer cells activates vegf receptors and tumour angiogenesis. *Nature Commun*. 2017;8(1):14450.
10. Skog J, Würdinger T, Rijn SVan, Meijer DH, Gainche L, Jr. WTCurry, Carter BS, Krichevsky AM, Breakefield XO. Glioblastoma microvesicles transport rna and proteins that promote tumour growth and provide diagnostic biomarkers. *Nature Cell Biol*. 2008;10(12):1470–1476.

11. Song L, Tian X, Schekman R. Extracellular vesicles from neurons promote neural induction of stem cells through cyclin d1. *J Cell Biol*. 2021;220(9).
12. Le Goff M, Lagadic-Gossmann D, Latour R, Podechard N, Grova N, Gauffre F, Chevance S, Burel A, Appenzeller BM, Ulmann L. Pahs increase the production of extracellular vesicles both in vitro in endothelial cells and in vivo in urines from rats. *Environ Pollut*. 2019;255:113171.
13. Chen X, Guo J, Huang Y, Liu S, Huang Y, Zhang Z, Zhang F, Lu Z, Li F, Zheng JC. Urban airborne pm2. 5-activated microglia mediate neurotoxicity through glutaminase-containing extracellular vesicles in olfactory bulb. *Environ Pollut*. 2020;264:114716.
14. Dhanraj P, Venter C, Bester MJ, Oberholzer HM. Induction of hepatic portal fibrosis, mitochondria damage, and extracellular vesicle formation in sprague-dawley rats exposed to copper, manganese, and mercury, alone and in combination. *Ultrastruct Pathol*. 2020;44(2):182–192.
15. Nabhan JF, Hu R, Oh RS, Cohen SN, Lu Q. Formation and release of arrestin domain-containing protein 1-mediated microvesicles (armms) at plasma membrane by recruitment of tsg101 protein. *Proc Natl Acad Sci*. 2012;109(11):4146–4151.
16. Wang Q, Lu Q. Plasma membrane-derived extracellular microvesicles mediate non-canonical intercellular notch signaling. *Nature Commun*. 2017;8(1):1–9.
17. Wang Q, Yu J, Kadungure T, Beyene J, Zhang H, Lu Q. Armms as a versatile platform for intracellular delivery of macromolecules. *Nature Commun*. 2018;9(1):960.
18. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for crispr screening. *Nature Methods*. 2014;11(8):783–784.
19. Park H-R, Sun R, Panganiban RA, Christiani DC, Lu Q. Microrna-124 reduces arsenic-induced endoplasmic reticulum stress and neurotoxicity and is linked with neurodevelopment in children. *Sci Rep*. 2020;10(1):1–11.
20. Qiao Z, Zhang Y, Ge M, Liu S, Jiang X, Shang Z, Liu H, Cao C, Xiao H. Cancer cell derived small extracellular vesicles contribute to recipient cell metastasis through promoting hgf/c-met pathway*[s]. *Mol Cell Proteomics*. 2019;18(8):1619–1629.
21. Ge SX, Jung D, Yao R. Shinygo: A graphical gene-set enrichment tool for animals and plants. *Bioinformatics*. 2020;36(8):2628–2629.
22. ALNasser MN, AlSaadi AM, Whitby A, Kim D-H, Mellor IR, Carter WG. Acai berry (euterpe sp.) extracts are neuroprotective against l-glutamate-induced toxicity by limiting mitochondrial dysfunction and cellular redox stress. *Life*. 2023;13(4):1019.
23. Breier JM, Radio NM, Mundy WR, Shafer TJ. Development of a high-throughput screening assay for chemical effects on proliferation and viability of immortalized human neural progenitor cells. *Toxicol Sci*. 2008;105(1):119–133.
24. Chang X, Lu W, Dou T, Wang X, Lou D, Sun X, Zhou Z. Paraquat inhibits cell viability via enhanced oxidative stress and apoptosis in human neural progenitor cells. *Chem Biol Interact*. 2013;206(2):248–255.
25. Stenmark H. Rab gtpases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol*. 2009;10(8):513–525.
26. Đukić-Ćosić D, Baralić K, Javorac D, Djordjevic AB, Bulat Z. An overview of molecular mechanisms in cadmium toxicity. *Curr Opinion Toxicol*. 2020;19:56–62.
27. Chen L, Xu B, Liu L, Luo Y, Zhou H, Chen W, Shen T, Han X, Kontos CD, Huang S. Cadmium induction of reactive oxygen species activates the mtor pathway, leading to neuronal cell death. *Free Rad Biol Med*. 2011;50(5):624–632.
28. Forcella M, Lau P, Oldani M, Melchioretto P, Bogni A, Gribaldo L, Fusi P, Urani C. Neuronal specific and non-specific responses to cadmium possibly involved in neurodegeneration: A toxicogenomics study in a human neuronal cell model. *Neurotoxicology*. 2020;76:162–173.
29. Monroe RK, Halvorsen SW. Cadmium blocks receptor-mediated jak/stat signaling in neurons by oxidative stress. *Free Rad Biol Med*. 2006;41(3):493–502.
30. Lopez E, Arce C, Oset-Gasque M, Canadas S, Gonzalez M. Cadmium induces reactive oxygen species generation and lipid peroxidation in cortical neurons in culture. *Free Rad Biol Med*. 2006;40(6):940–951.
31. Wang L, Gallagher EP. Role of nrf2 antioxidant defense in mitigating cadmium-induced oxidative stress in the olfactory system of zebrafish. *Toxicol Appl Pharmacol*. 2013;266(2):177–186.
32. Wu KC, Liu JJ, Klaassen CD. Nrf2 activation prevents cadmium-induced acute liver injury. *Toxicol Appl Pharmacol*. 2012;263(1):14–20.
33. Zhang L, Yang F, Li Y, Cao H, Huang A, Zhuang Y, Zhang C, Hu G, Mao Y, Luo J. The protection of selenium against cadmium-induced mitophagy via modulating nuclear xenobiotic receptors response and oxidative stress in the liver of rabbits. *Environ Pollut*. 2021;285:117301.
34. Oggiano R, Pisano A, Sabalic A, Farace C, Fenu G, Lintas S, Forte G, Bocca B, Madeddu R. An overview on amyotrophic lateral sclerosis and cadmium. *Neurol Sci*. 2021;42:531–537.
35. Ruczaj A, Brzóska MM. Environmental exposure of the general population to cadmium as a risk factor of the damage to the nervous system: A critical review of current data. *J Appl Toxicol*. 2023;43(1):66–88.
36. Harischandra DS, Rokad D, Neal ML, Ghaisas S, Manne S, Sarkar S, Panicker N, Zenitsky G, Jin H, Lewis M. Manganese promotes the aggregation and prion-like cell-to-cell exosomal transmission of α-synuclein. *Sci Signal*. 2019;12(572):eaau4543.
37. Martin PJ, Héliot A, Tremolet G, Landkocz Y, Dewaele D, Cazier F, Ledoux F, Courcot D. Cellular response and extracellular vesicles characterization of human macrophages exposed to fine atmospheric particulate matter. *Environ Pollut*. 2019;254:112933.
38. van Meteren N, Lagadic-Gossmann D, Chevanne M, Gallais I, Gobart D, Burel A, Bucher S, Grova N, Fromenty B, Appenzeller BM. Polycyclic aromatic hydrocarbons can trigger hepatocyte release of extracellular vesicles by various mechanisms of action depending on their affinity for the aryl hydrocarbon receptor. *Toxicol Sci*. 2019;171(2):443–462.
39. Cheung KAK, Abeysinghe P, Vaswani K, Tucker K, Bassett J, Mitchell PA, Mosaad EM, Logan J, Mitchell MD. Characterisation of rencells cx and vm stimulated with interleukin-1β and lipopolysaccharide. *Neurochem Int*. 2022;156:105326.
40. Zhao X, Yang Z, Liang G, Wu Z, Peng Y, Joseph DJ, Inan S, Wei H. Dual effects of isoflurane on proliferation, differentiation, and survival in human neuroprogenitor cells. *J Am Soc Anesthesiol*. 2013;118(3):537–549.