Gold Nanoparticles Disturb Small Extracellular Vesicle Attributes of Mouse Embryonic Stem Cells

Fang Hao,[†,‡] Tingting Ku,[§] Xiaoxi Yang,[†] Qian S. Liu,[†] Xingchen Zhao,[†] Francesco Faiola,[†,‡] Qunfang Zhou,[*,†,‡,∥] Guibin Jiang[†,‡,∥]

[†]State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

[‡]College of Resources and Environment, University of Chinese Academy of Sciences, Beijing 100049, China

[§]College of Environment and Resource, Research Center of Environment and Health, Shanxi University, Taiyuan, 030006, China

[∥]School of Environment, Hangzhou Institute for Advanced Study, University of Chinese Academy of Sciences, Hangzhou, 310000, China

* Correspondence to:

Dr. Qunfang Zhou, State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China.

E-mail: zhouqf@rcees.ac.cn

Materials and methods

Reagents. The aqueous solutions (1 mg/mL) of three kinds of AuNPs (AuNP-5, AuNP-20, and AuNP-80) were purchased from Nanocomposix Company (USA), and their surfaces were all functionalized with citrate sodium coatings. The stock solution was sonicated and diluted with cell culture medium to prepare the corresponding working solution. All the other chemicals were bought from Sigma-Aldrich, unless specifically stated otherwise.

AuNP characterization. The aqueous solutions of AuNP-5, AuNP-20, and AuNP-80 (50 μg/mL, 2 μL) were dropped onto the carbon film-coated grids and dried at room temperature. The as-prepared samples were observed and photographed using a transmission electronic microscope (TEM, JEOL H7500, Japan) at the accelerating voltage of 200 kV. Localized surface plasmon resonance absorption spectra ranging from 400 nm - 800 nm were obtained for the test AuNPs using quartz cells on a NIR-3600 spectrometer (Shimadzu, Japan). Their hydrodynamic sizes and zeta potentials were analyzed by a Malvern Zetasizer Nano ZS (Malvern, UK), and the measurement of each sample was replicated for three times. Hyperspectra microscopy (Cytoviva Inc., USA) was used to get the enhanced dark-field images of AuNPs under oil immersion objective (63×) for further hyperspectral analysis.

Culture of mouse embryonic stem cells (mESCs). The J1 mESCs (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences) were seeded in 6-well plates pre-coated with 0.1% gelatin (Merck Millipore, USA), and cultured in KSR medium, which was KnockOut DMEM (Gibco, USA) supplemented with 15% serum replacement (Gibco, USA), 1% Glutamax-I, 1% MEM non-essential amino acids, 1% penicillin and streptomycin (Gibco, USA), 5×10^{-5} mol/L β-mercaptoethanol (Solarbio, China), and 1% leukemia inhibitory factor (LIF, 1×10^{7} U/mL, Merck Millipore, Germany) at 37 ℃ and 5% CO_2 for 12 h. Then, the mESCs were cultured in the complete N2B27 medium, according to previously reported protocol for another 48 h.[1] All exposure experiments of AuNPs were started when

N2B27 medium was introduced.

Cell viability assay. The cell viability experiment was firstly performed to screen the non-cytotoxic concentrations of the test AuNPs (i.e. AuNP-5, AuNP-20, and AuNP-80). Briefly, the mESCs were seeded in 0.1% gelatin pre-coated 96-well plates at the density of 10,000 cells per well and cultured in KSR medium. After 24 h, the medium was replaced with fresh N2B27 medium containing a series of concentrations of AuNPs (0, 0.1, 0.5, 1, 5, 10, 25, 50, 75, 100 μg/mL) and cultured for 24 h and 48 h, respectively. After exposure, the cells were incubated with 10 μM resazurin reagent for another 2 h at 37 ℃. The fluorescence at 530 nm/590 nm (λexcitation/λemission) was recorded by a multiplate reader (VARIOSKAN FLASH, Thermo Fisher Scientific, USA). The fluorescence intensities of exposure groups relative to that of the negative control were finally evaluated.

Measurement of reactive oxygen species (ROS) in mESCs. A commercially available kit (Beyotime, China) was used for the measurement of ROS generation in mESCs upon AuNP treatments. Briefly, the mESCs incubated in 96-well plates were firstly loaded with 10 μM non-fluorescent probe (DCFH-DA) in N2B27 medium. Thirty minutes later, 1 μg/mL AuNPs (i.e. AuNP-5, AuNP-20, and AuNP-80) were added and the exposure lasted for 1 h, 6 h and 24 h, respectively. The fluorescence at λexcitation/λemission of 485 nm/530 nm was measured on a multiplate reader (VARIOSKAN FLASH, Thermo Fisher Scientific, USA). The positive control was set by using 1-h exposure of 10 mM H_2O_2, and the negative control was the mESCs without AuNP treatments. The final results were expressed by the fluorescence intensities of exposure groups relative to that of the negative control.

Hyperspectral microscopic observation. The mESCs were seeded on the glass-slip (CITOGLAS, China) pre-coated by 0.1 % gelatin, and cultured in KSR medium for 24 h. The cells were then submitted to subsequent 48-h culture in N2B27 medium with or without 1 μg/mL AuNPs (i.e. AuNP-5, AuNP-20, and AuNP-80). After treatment, the mESCs were washed by PBS for 3 times and fixed in 4% paraformaldehyde (PFA) for 20 min. After 3-time wash, the cell specimens were

sealed on the glass-slip with nail polish. The as-prepared slides were observed and imaged under the enhanced dark field hyperspectral microscope equipped with a spectrograph CCD camera (Cytoviva Inc., USA). The hyperspectral spectrum from 400 nm to 900 nm with the exposure time of 0.1 s was obtained for at least 30 nanoparticles in each sample. The final acquisition and analyses were conducted using ENVI software (version 4.8. Harris Corporation, USA).

Elemental analysis of Au in mESCs. The mESCs seeded in 6-well plates at the density of 1×10^5 cells per well were exposed to 1 μg/mL AuNPs for 48 h, following the similar protocol described above. After exposure, the cells were harvested and counted for the cell number (Countess II, Invitrogen, USA). An aliquot of the sample was submitted to cell lysis and protein concentration measurement using BCA kit (Thermo Fisher, USA). Another aliquot of cell sample (3×10^6 cells per group) was submitted to aqua regia digestion using previously-reported protocol,[2] and the as-prepared sample diluted with 3% HNO_3 was quantitatively analyzed for Au contents using inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 8800, USA). The final result of Au concentration was adjusted by protein concentration of each sample. The negative control of the mESCs without chemical treatment was set in parallel, and the negligible level of Au in this group confirmed no cross contamination during exposure experiments. The particle number of AuNPs per cell was estimated using Au concentration in each sample adjusted by cell number and the conversion coefficients of mass-to-particle for AuNPs, which were calculated by the following formula:

$$Conversion\ coefficient = \frac{Mass\ concentration\ (ng/mL)}{Particle\ concentration\ (particles/mL)}.$$

Wherein, the mass concentrations of AuNP-5, AuNP-20, and AuNP-80 were 1 mg/mL, and the particle concentrations were 8.50×10^{14}, 1.20×10^{13}, and 2.40×10^{11} particle/mL, respectively, according to the vendor's manuals. The conversion coefficients of mass to particle were thus calculated to be 1.18×10^{-9}, 8.33×10^{-8}, 4.17×10^{-6} ng/particle for AuNP-5, AuNP-20 and AuNP-80, respectively.

Alkaline phosphatase (AP) activity assay. The mESCs seeded in 6-well plates were performed AuNP-exposure experiments (1 μg/mL AuNPs, 48 h), following the similar protocol described above, or cultured for 48 h without any exposure (negative control). The cells were then fixed in citrate-acetone-formaldehyde buffer (citrate: acetone: formaldehyde = 5: 13: 2, v/v) for 30 seconds, followed by washing, alkaline dye staining and counterstaining with hematoxylin, according to the manual instruction of a commercially-available AP activity kit (Sigma, USA).[3] The pictures were imaged under an inverted microscope (Olympus IX73, Japan). The AP activity in each group was quantitatively evaluated by the grey values of stained cell clones with background subtraction using ImageJ (NIH, USA), and expressed as relative AP activity of the negative control.

Transcriptional levels of pluripotency biomarkers. After 1 μg/mL AuNP treatments or naïve culture for 48 h, both the negative control and the exposed mESCs were harvested for RNA extraction with TRIZOL reagent (Invitrogen, USA) according to the vendor's instruction. After purification and quantitation, the mRNA sample (2 μg) was reversely-transcribed to cDNA using the one-step cDNA synthesis kit (Biorad, USA), and finally submitted to real time-polymerase chain reaction (RT-PCR) on a Roche 480 system (UK) with a SYBR green kit (Biorad, USA). The target genes included Nanog, Oct-4 and Sox-2, and GAPDH was used as the house-keeping gene. The sequences of forward and reverse primers in Table S1 were designed according to previously-reported protocol.[1] The relative mRNA level was normalized by the Ct value of GAPDH using $2^{-\Delta\Delta Ct}$ method.[4]

Protein expressions of pluripotency biomarkers. With the similar exposure protocol described above, the mESCs from different groups were lysed with ice-cold RIPA solution (Solarbio, China) containing 1× protease and phosphatase inhibitor (Cell signaling technology, USA). After centrifugation (15,000 g, 20 min), the protein concentration of each sample was determined by BCA kit (Thermo, USA). Suitable amount of protein samples from different treatments were submitted to Western blot assay. The primary antibodies included goat anti-Oct-4 (Abcam, ab27985), rabbit

anti-Nanog (Abcam, ab80892) and rabbit anti-GAPDH (Abcam, ab9485) and their dilution ratio was 1:1000. The corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from ZSGB-bio (China), and they were used at the ratios of 1:3000 to 1:5000. The target protein bands were developed onto X-ray films with ECL kit (Pierce, USA). The quantitative analysis was performed by measuring the grey densities of protein bands using ImageJ (NIH, USA), and the results were expressed as the relative values of the negative control.

Isolation of small extracellular vesicles (sEVs). The mESCs seeded in 6-well plates were processed for 48-h incubation in N2B27 medium with or without 1 μg/mL AuNPs (i.e. AuNP-5, AuNP-20 and AuNP-80). The protocol for sEV preparation from mESCs was referenced according to the previous study.[5] Briefly, after the removal of floating cells (400 g, 15 min), the culture medium was sequentially centrifuged under a series of conditions (i.e. 2000 g for 20 min, 10,000 g for 30 min) to remove the pellets of apoptotic body (AB) and microvesicle (MV) fractions, respectively. The supernatant was then concentrated using ultrafiltration (100 kDa, Merck Millipore, USA), filtrated using the 0.22-μm sieve and finally centrifuged twice at 100,000 g for 90 min using an Optima L-100K ultracentrifuge equipped with 70 Ti rotor (Beckman Coulter, USA). After transferring away the supernatant, the removal of AuNPs from sEVs was performed by centrifugation of the residue suspension under 12,500 g for 20 min, and the supernatant samples were sEVs, including sEV-ctrl, sEV-5, sEV-20 and sEV-80, respectively, which contained undetectable Au levels measured by ICP-MS. The above processes were carried out at 4 ℃, and the average protein concentrations of the prepared sEV suspensions were about 600 μg/mL after sample lysis and protein quantification using BCA kit (Thermo, USA).

Characterization of sEVs. The sEV samples fixed in 2% PFA were transferred onto carbon membrane-coated copper grids, and air-dried for 20 min. The samples were washed with PBS, and further fixed with 1% glutaraldehyde for 5 min followed by negatively staining with 1% uranylacetate for 1 min.[5] Images were obtained on a

TEM (JEOL H7500, Japan) at an accelerating voltage of 80 kV.

Analysis of sEV protein biomarkers. The isolated sEVs were lysed for protein quantification, and submitted to Western blot following the similar protocol mentioned above. The primary antibodies included rabbit anti-CD63 (Abcam, 1:1000), rabbit anti-HSP70 (Cell signaling, 1:1000), rabbit anti-Flotillin-1 (Cell signaling, 1:1000), and rabbit anti-Calreticulin (Cell signaling, 1:1000). As the protein biomarker of calreticulin was not expressed in sEVs, it was tested herein to confirm no contamination from other cell components in sEV samples.

Immunostaining analysis of sEVs. Total 5 μL of sEV suspensions (600 μg protein/mL) were incubated with PE-CD63 antibody (1:100, Biolegend, CA) at 37 ℃ for 40 min in darkness.[6, 7] Then the sEVs were centrifuged at 100,000 g for 90 min**.** The pellets of sEVs were re-suspended in PBS, and dropped onto the gelatin pre-coated slides, and finally sealed with cover slips by nail polish. The as-prepared samples were imaged by a Leica TCS-SP5 confocal microscopy (Germany).

Particle size and concentration analysis of sEVs. The particle size and particle concentration measurements of sEVs were conducted on a nanoparticle tracking analysis system (NTA, NS300, Malvern, UK) configured with 640 nm laser. Briefly, 10 μL of sEVs (i.e. sEV-ctrl, sEV-5, sEV-20 and sEV-80) were either treated with 0.1% (v/v) Triton-X100 for 10 min at room temperature for background measurement of non-membrane particles,[8] or diluted with PBS to 1 mL,[9] then introduced manually with a syringe. Videos of 30-s duration of each sEV sample was recorded independently for 3 times, and the camera parameters, including camera level of 4, camera gain of 16, and threshold of 4, were set for all experiments. The mobility trajectories of sEVs with different sizes (including 30 nm, 60 nm, 90 nm, 120 nm and 150 nm) were recorded, and the related data analysis was performed using NTA software (version 2.3, Malvern, UK) to obtain the concentrations of particles (C_P) and non-membrane particles (C_{NMP}). To evaluate the correlation between protein levels of sEVs and their particle concentrations, two aliquots of the samples from different groups were submitted to gradient dilution, and performed RIPA lysis for protein

quantitation using BCA kit (Thermo, USA) and NTA measurement, respectively. The linear correlations were simulated for different sEV samples by Origin85 software, and compared with each other. The purities of sEVs from different groups were calculated by the following equation:

$$\text{Purity} = (1 - C_{NMP}/C_P) \times 100\%$$

Quartz crystal microbalance with dissipation (QCM-D) monitoring. sEV rigidity and deposition mass were recorded by a Q-sense E4 system (Biolin Scientific AB, Sweden). The QCM-D measurements were performed as follows. First, a cleaned SiO_2 sensor was pre-coated by poly-lysine solution (PLL, 0.1% w/v, Solabio, China) until both ΔFrequency (Δ*F*) and ΔDissipation (Δ*D*) approached to stable baselines. Next, Tris-HCl buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4)[10] was introduced to remove unbound PLL for another steady baseline. Then, sEVs suspended in Tris-HCl buffer (1:50) were injected into chamber with the continuous record at the third to the eleventh overtones. In all steps, the flow rate was set at 50 μL/min,[11] and the temperature was maintained at 37 ℃. The data were collected and condensed at the fifth overtone by QTools software (Version 3.0.17).

Label-free proteomics analysis. Three batches of sEV-ctrl and sEV-5 samples were lysed in SDT buffer (100 mM Tris-HCl containing 4% w/w sodium dodecyl sulfate and 0.1 M dithiothreitol, pH 7.6), and sonicated on ice for 20 s. The samples were then heated at 60 °C under shaking for 30 min followed by a series of sonication to obtain homogenate. After the protein quantitation, 90 μg protein from each sample was extracted with filter (10 kDa, Millipore). According to previously-reported protocol,[12] the protein lysates were mixed with 200 μL of 8 M urea in ultrafilter tubes, followed by ultracentrifugation-based concentration (14000 g, 40 min) and SDT buffer washing. Then the concentrate was digested overnight with trypsin (1:25, w/w), and the generated peptides were lyophilized, resolved in Milli-Q water and desalted with MILI-SPE C_{18} column (Millipore). The peptides were elicited by 80% acetonitrile. After being lyophilized, the peptide samples were re-suspended in 0.1% formic acid, and submitted to the analysis using high performance liquid

chromatography (HPLC, EASY nLC-1000) coupled with a Q Exactive Orbitrap Mass spectrometer (Thermo Scientific, USA). The HPLC system included a Pepmap 100 column (Thermo Scientific, 100 μm × 2 cm) connected with an EASY column (Thermo Scientific, 10 cm, ID 75 μm, C_{18}-A2). The mobile phase was consisted of de-ionzed water containing 0.1% formic acid (phase A, 16%) and acetonitrile containing 0.1% formic acid (phase B, 84%), and the flow rate was 300 nL/min.[8] Mass spectra with the scanning range of m/z 300-1800 at the resolution of 70,000 were obtained under positive ion mode. Data-dependent scan mode was used by selecting the top 20 abundant ions for fragmentation under the normalized collision energy of 30 eV.

All peptides data were analyzed using the MaxQuant software (v. 1.5.3.17). Carbamidomethylation of cysteines and methionine oxidation were set as fixed modification and variable modification, respectively. Trypsin was selected as the enzyme, with max missed cleavages of two. Peptide and fragment ion tolerances were 6 part per million (ppm) and 20 ppm, respectively. The database incorporated both the forward and reversed sequences to allow the determination of false discovery rate (FDR) of 1% at peptide-spectrum match, peptide, and protein levels. Only proteins in sEV-5 with the abundance higher than 1.5-fold or less than 0.67-fold of that of sEV-ctrl and *p* value less than 0.05 were considered to be differentially-expressed. Protein clustering analysis was performed on R package of complex heatmap. The clustering distance and method were set to Euclidean and average linkage, respectively. Bioinformatics analysis of the results was conducted using the Blast2GO algorithm to obtain the overall GO analysis, including procedures of blast, mapping, annotation and annotation augmentation.

4T1 cell culture and proliferation experiment. 4T1 cells were purchased from Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin and streptomycin (Gibco, USA) at 37 ℃ and 5% CO_2. When sEV exposure experiments were performed, DMEM medium containing 10%

EV-free fetal bovine serum (D-FBS, Vivacell, USA) and 1% penicillin and streptomycin (Gibco, USA) was used for cell incubation. Both FBS and D-FBS (10% in DMEM medium) were submitted to particulate measurement using a Nano ZS system (Malvern, UK) under the similar condition described above, showing the negligible shielding influences from particles in D-FBS (Fig. S11A). The cell viabilities of 4Tl using alamarBlue assay remained the same after 24-h culture in both kinds of culture medium (Fig. S11B), confirming cell viability was not influenced by D-FBS-supplemented culture medium.

As for the effects of sEVs on 4T1 cell proliferation, the cells were seeded on 96-well plate at the density of 1×10^4 cells per well and cultured in 10% FBS-supplemented DMEM medium (i.e. complete medium) for 24 h. Then the medium was replaced by 10% D-FBS-supplemented DMEM medium (i.e. EV free medium) containing different concentrations of sEVs (30 μg/mL, 50 μg/mL, and 100 μg/mL) derived from different groups of mESCs (e.g. sEV-ctrl, sEV-5, sEV-20 and sEV-80). The exposure lasted for 24 h, and the final cell proliferation was analyzed, according to the protocol used in mESC viability experiments.

Flow cytometry analysis for cell apoptosis. The cell apoptosis was measured by a dead cell apoptosis kit with Annexin V-FITC and PI (Invitrogen, USA). In brief, 4T1 cells cultured in 24-well plates at the density of 2×10^5 cells per well were exposed to sEV-ctrl and sEV-5 for 24 h. The negative control without any treatment was set in parallel. After exposure, the cells were harvested and washed with cold PBS. Then, the cell suspensions were incubated with Annexin V-FITC and PI solutions (100 μg/mL) for 15 min. The stained cells were finally analyzed on a flow cytometry (Novocyte, ACEA, USA) at the wavelengths (λexcitation/λemission) of 488 nm/519 nm and 561 nm/615 nm, respectively.

Cellular uptake of sEVs in 4T1 cells based on morphological observation. The samples of **s**EV-ctrl and sEV-5 (30 μg/mL) were labeled with 1 μM 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbovyanineperchlorate (DiD, Thermo Fish, USA), a kind of lipophilic carbocyanine fluorescent dyes by 1-h incubation at 37 ℃, according to

the previously-reported protocol.[13] The mixture was diluted (1:1000) with fresh D-FBS medium and processed ultrafiltration centrifugation (10 kDa, Millipore, 14000 g, 30 min) to remove excess probes for the following cell exposure tests.

4T1 cells were seeded onto the glass chamber slides (Lab-Tek, Thermo Fisher, USA) at the density of 2×10^4 cells per well. After 24-h incubation in D-FBS medium, the cells were labeled by 5 μM 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO, Thermo Fisher, USA) at 37 ℃ for 15 min, then washed by D-FBS medium for 3 times. The DiD-labeled sEVs were added to the cell cultures and incubated for 6 h. The cells were finally fixed in 4 % PFA for 20 min, and mounted with VECTASHIELD anti-fade mounting medium containing DAPI (H-1200, Vector Laboratories, CA).[14] The fluorescent images were obtained using a Leica SP5 confocal microscopy (Germany) with excitation lasers of 405 nm (DAPI), 488 nm (DiO), and 633 nm (DiD), and the corresponding emission signals were collected at 420-470 nm, 500-600 nm and 700-800 nm, respectively.

Flow cytometry analysis of sEV uptake by 4T1 cells. 4T1 cells were cultured in 12-well plates at the density of 1×10^5 cells per well for 24 h and DiD-labeled sEV-ctrl and sEV-5 samples (30 μg/mL) were added and incubated for 6 h. The negative cell control without sEVs was designed in parallel. After wash with cold PBS, the cells were re-suspended at a density of 2×10^5 cells/mL and submitted to flow cytometry analysis (BD, LSR II, USA). Forward scatter (FSC) and side scatter (SSC) signals were collected using 488 nm laser, while DiD fluorescence channel (695/40) were assessed with 633 nm laser. All threshold gates were set based on control cells, and temperature was maintained at 4℃.

Wound healing assays. 4T1 cells were seeded into 6-well plates with a culture-insert in each well (ibidi GmbH, Germany). The cell density was 5.6×10^5 cells/insert, and the culture was performed in the complete medium for 24 h. The insert was then removed quickly, leaving a cell-free gap with the width of 500 μm, and each well was gently washed by PBS twice to remove the floating cells. The cells were exposed to FBS-free medium containing 30 μg/mL sEVs (i.e. sEV-ctrl, sEV-5, sEV-20 and sEV-

80) for 12 h, respectively. The negative cell control without sEVs was set in parallel. The migration of cells in the gap area was pictured by Olympus microscopy (IX73, Japan). The migration index (M) was calculated as follows:

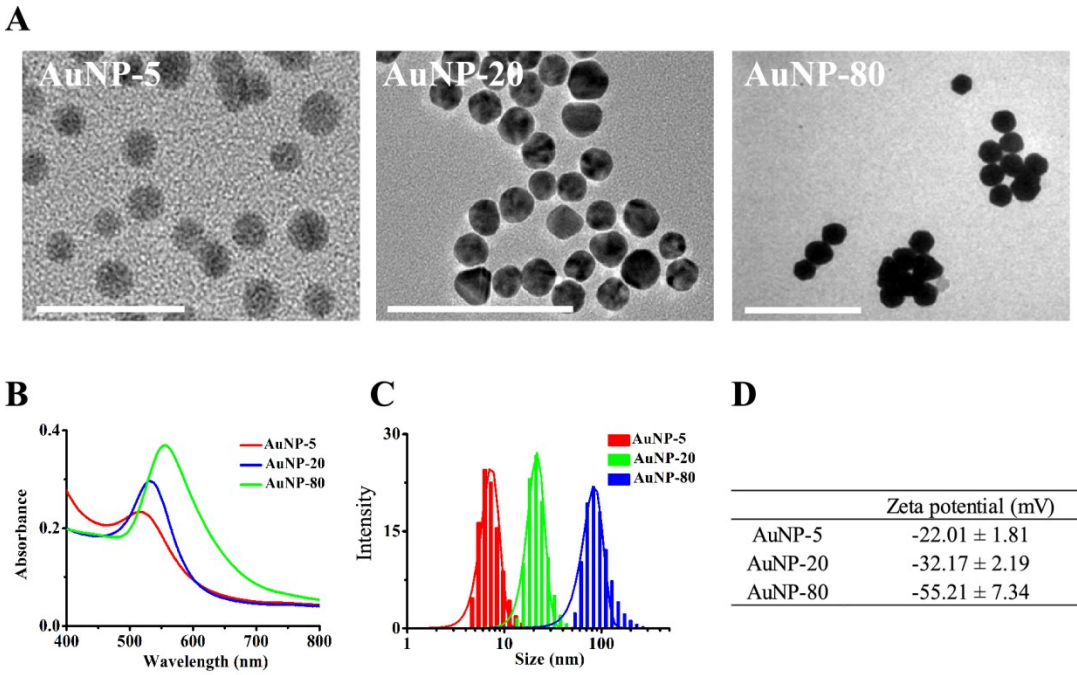
$$M = 1 - \frac{A_{12}}{A_0}$$

Where A_0 is the initial wound area and A_{12} represents the remaining wound area after 12-h treatment. All data calculation was acquired using ImageJ (NIH, USA). The M values were calculated and statistically compared for different sEV treatments.

Assay for protein biomarkers regulating 4T1 cell migration. 4T1 cells were seeded in 24-well plates at a density of 2×10^5 cells per well, and cultured for 24 h. The exposure was subsequently performed by stimulating the cells with 50 μg/mL sEVs (i.e. sEV-ctrl, sEV-5) for 6 h. The negative control without any treatment was set in parallel. After wash with ice-cold PBS, the cells were lysed in RIPA solution and quantitatively measured for protein concentration. The as-prepared samples were submitted to Western blot assay following the protocol described above. The primary antibodies included rabbit anti-Erk 1/2 (Cell signaling, 1:1000), rabbit anti-phosphoErk 1/2 (Cell signaling, 1:1000), rabbit anti-cofilin (Cell signaling, 1:1000), and rabbit anti-GAPDH (Cell signaling, 1:1000).

Statistical analysis. All experiments were independently carried out at least for three times, and the final results were represented as mean values ± standard deviations (SDs). The graphs were plotted using GraphPad Prism 7. One-way analysis of variance (ANOVA) with the Bonferroni multi-group comparison test was used for the difference analysis of different groups, and the *p* value less than 0.05 (*) or 0.01 (**) was considered to be significantly different.

Supplementary Figures



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	Zeta potential (mV)
AuNP-5	-22.01 ± 1.81
AuNP-20	-32.17 ± 2.19
AuNP-80	-55.21 ± 7.34

Fig. S1 Characterization of the test AuNPs (*n* = 3)**.** (A) TEM graphs of AuNP-5, AuNP-20, and AuNP-80. Scale bars represent 20 nm for AuNP-5, 100 nm for AuNP-20 and 500 nm for AuNP-80, respectively. (B) Localized surface plasmon resonance absorption spectra of the test AuNPs. (C) Hydrodynamic sizes of the AuNPs. (D) Zeta potentials of the AuNPs.

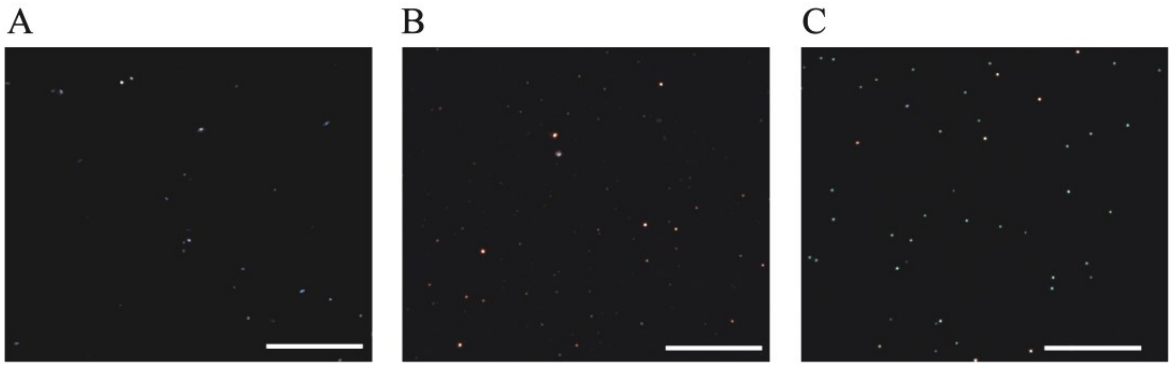


Fig. S2 Enhanced dark-field images for the test AuNPs. (A) AuNP-5. (B) AuNP-20. (C) AuNP-80. Scale bars represent 25 μm.

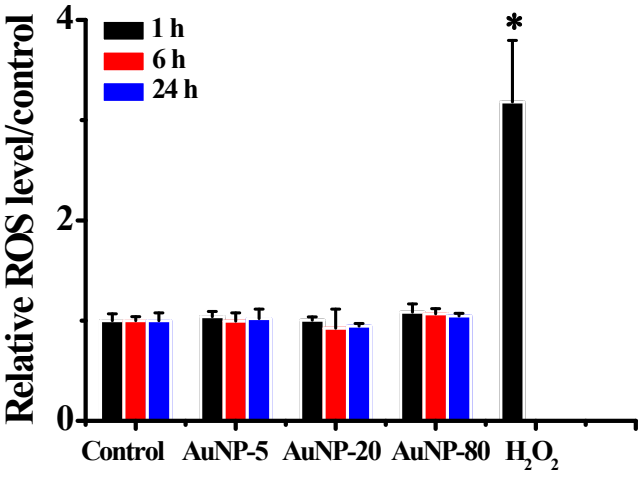


Fig. S3 ROS generation in mESCs from different groups (n = 3). *p < 0.05 versus the control.

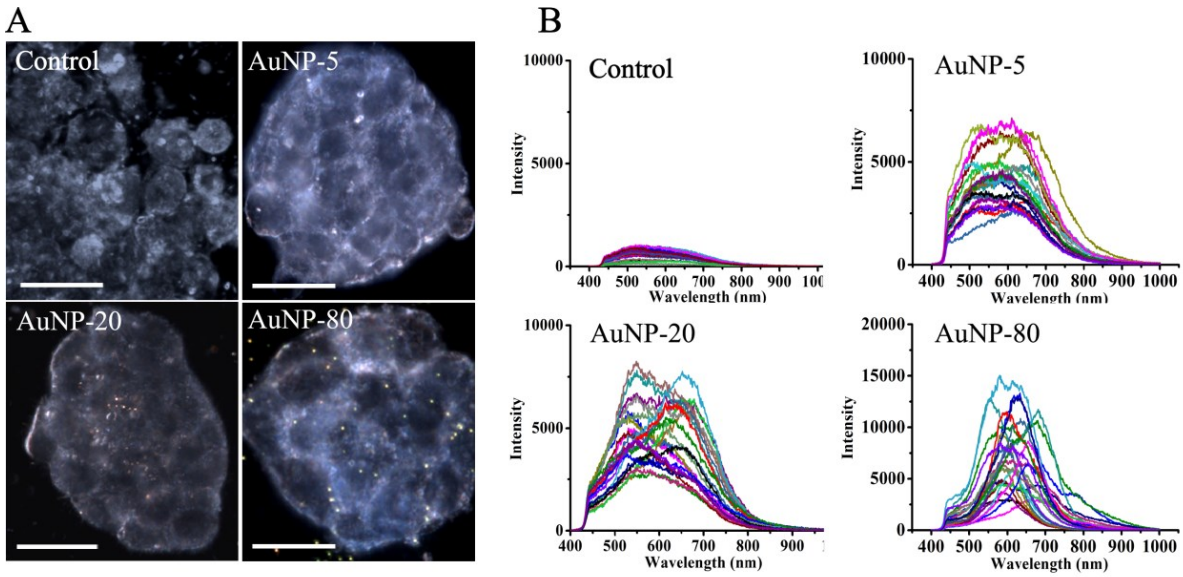
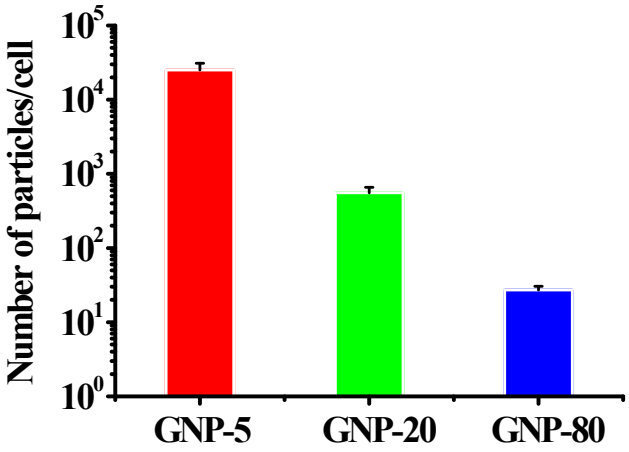


Fig. S4 Cellular uptake of AuNPs by the mESCs. (A) Dark-field images for control and AuNP-treated cells. The scale bars show 25 μm. (B) The normalized hyperspectral scattering signals of AuNPs in the mESCs. The intensities of AuNP exposure groups were higher than 2500, whereas that of the negative control was below 700, which was similar to the previously-reported finding.[15] Meanwhile, the scattering peaks of the adsorption spectrums were widened in AuNP-exposure groups, showing the potential aggregation of AuNPs in cells.[16]



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367 **Fig. S5 The calculated AuNP numbers per cell in different exposure groups.**

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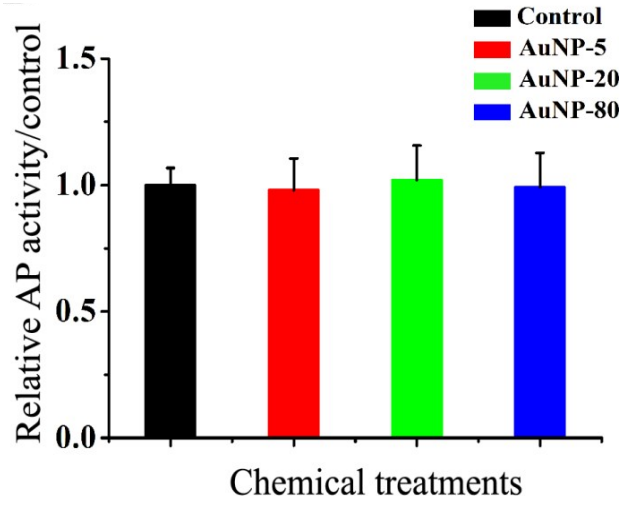

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370 **Fig. S6 Relative AP activities of mESCs with AuNP treatments** (n = 3)

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372 **Table S1. The primer sequences for RT-PCR analysis.**

Gene name	Sequence of the primers (5'-3')	
	Forward	Reverse
GAPDH	CCTGCGACTTCAACAGCAAC	TAGGGCCTCTCTTGCTCAGT
Oct4	TGGATCCTCGAACCTGGCTA	CTCAGGCTGCAAAGTCTCCA
Nanog	GGAGGACTTTCTGCAGCCTT	TGCCCTGACTTTAAGCCCAG
Sox2	AACCGATGCACCGCTACGA	TGCTGCGAGTAGGACATGCTG

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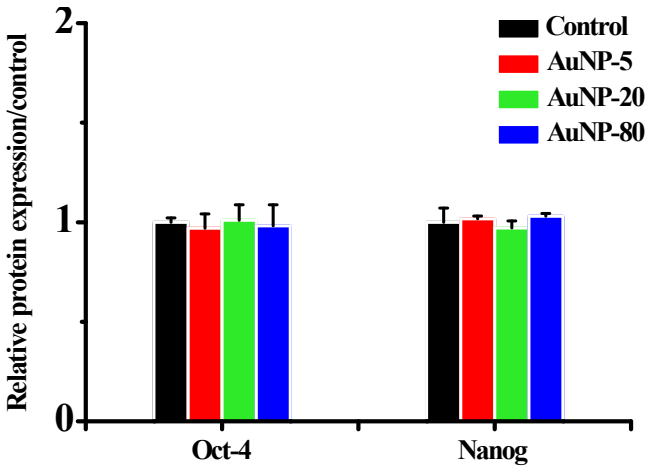

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376 **Fig. S7 Relative protein expressions of pluripotency biomarkers in the mESCs** (*n*
377 = 3).

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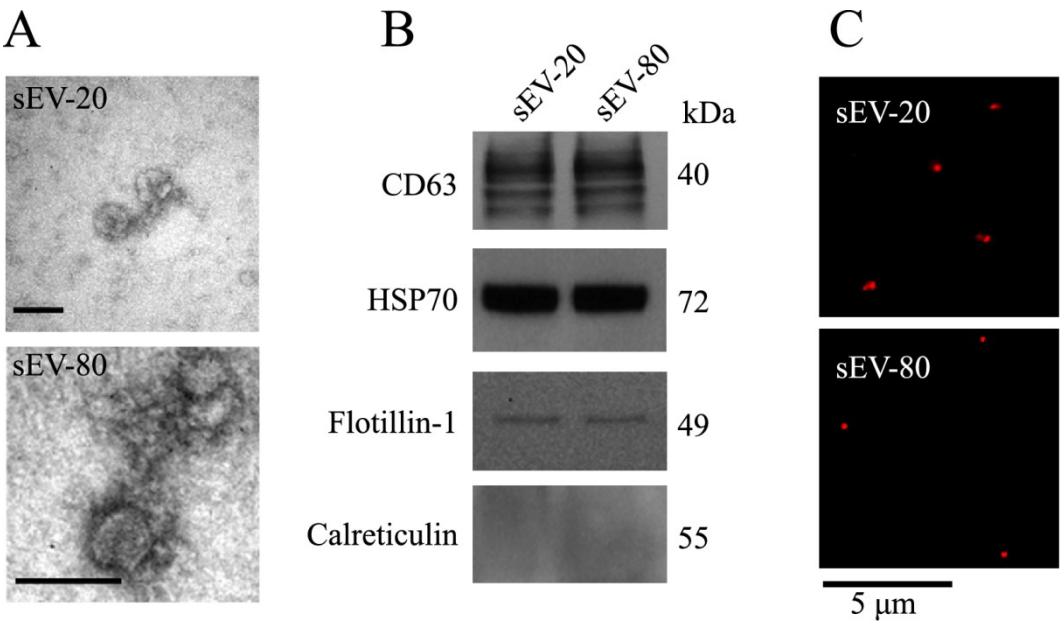

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380 **Fig. S8 Characterization of sEV-20 and sEV-80.** (A) TEM images (Scale bars =
381 100 nm). (B) Western blots for the protein markers. (C) Immunoblotting of CD63 in
382 sEVs (Scale bar = 5 μm).

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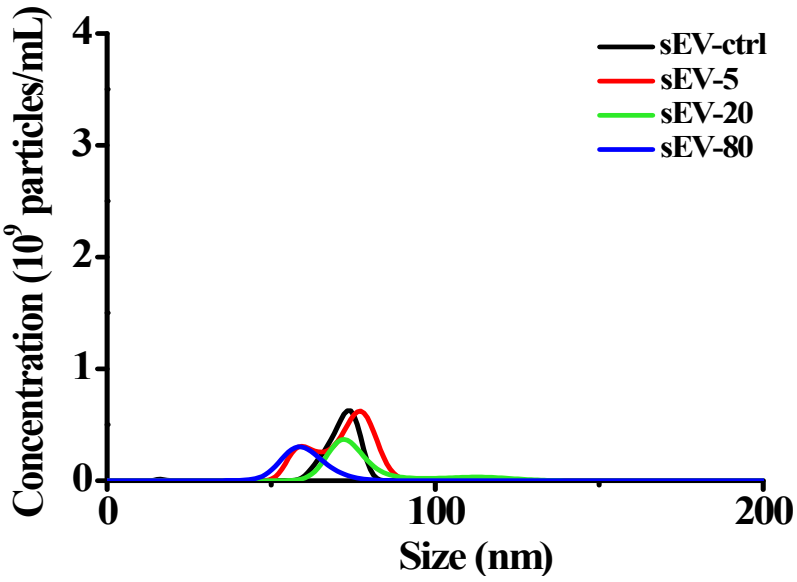

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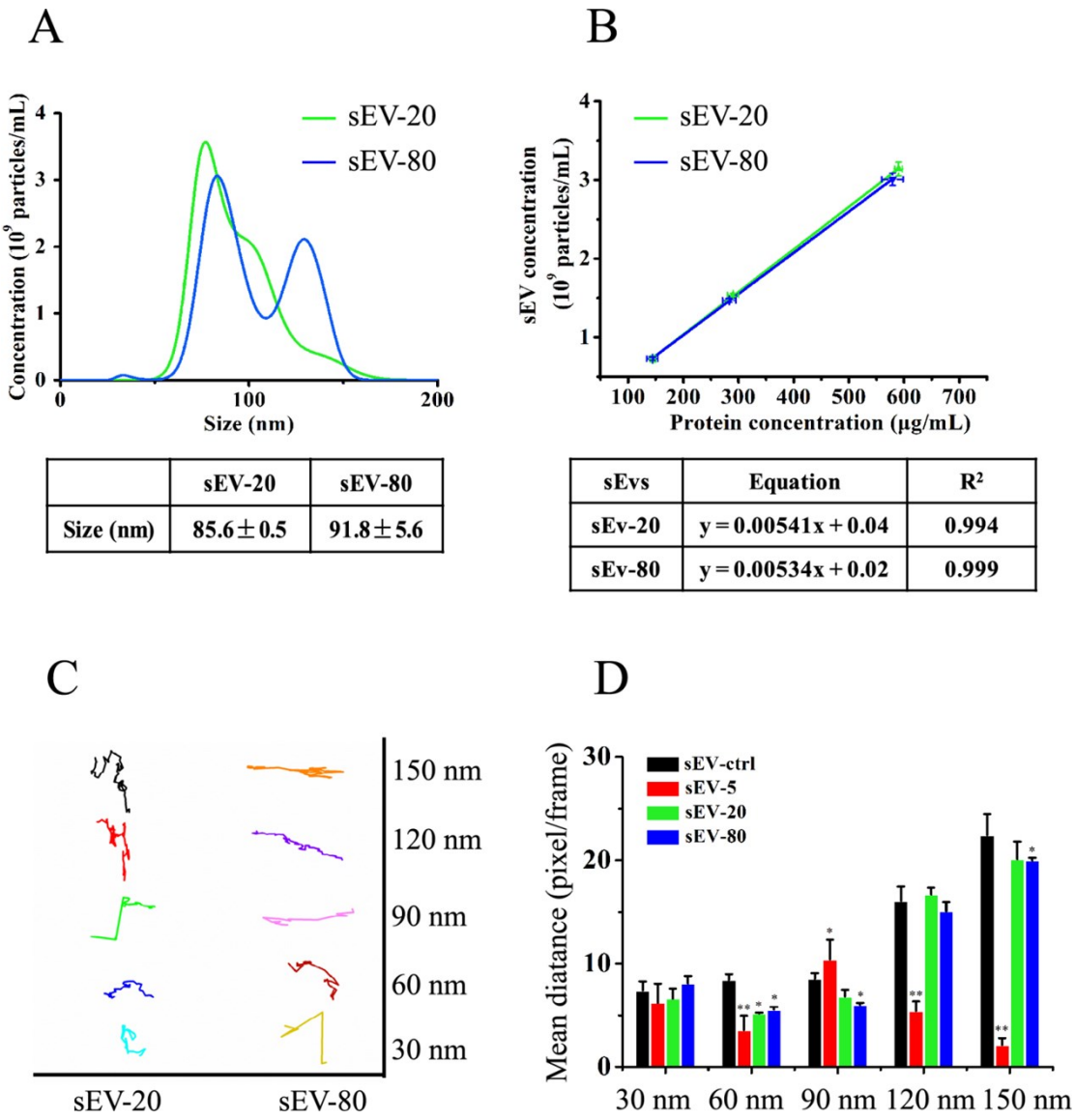
385 **Fig. S9 NTA data for non-membrane particles in sEV samples.** The pretreatment

386 of 0.1% Triton-X100 was performed for sEVs to obtain non-membrane particles.[8]

387



	sEV-20	sEV-80
Size (nm)	85.6±0.5	91.8±5.6

sEvs	Equation	R^2
sEv-20	y = 0.00541x + 0.04	0.994
sEv-80	y = 0.00534x + 0.02	0.999

Fig. S10 Characterization of sEV-20 and sEV-80 (n = 3)**.** (A) The hydrodynamic sizes. (B) Linear correlation between particle concentrations and protein contents of sEVs. (C) Typical mobility trajectories of sEVs with different sizes from NTA assay. (D) The mean trajectory distances of sEVs with different sizes. *p < 0.05, and **p < 0.01 versus sEV-ctrl.

Table S2. The purities of sEVs from different groups.

	sEV-ctrl	sEV-5	sEV-20	sEV-80
C_p (particles/mL)	5.5×10^{10}	7.3×10^{10}	8.3×10^{10}	6.6×10^{10}
C_{NMP}(particles/mL)	1.6×10^{9}	3.2×10^{9}	3.8×10^{9}	1.8×10^{9}
Purity	97%	96%	95%	97%

Table S3. The specifically-expressed proteins in sEV-ctrl or sEV-5.

Protein	Protein Name	Gene Name	Coverage/%
B9EJV3	GREB1-like protein	Greb1l	1.6
O09118	Netrin-1	Ntn1	9.6
O09167	60S ribosomal protein L21	Rpl21	11.2
P10711	Transcription elongation factor A protein 1	Tcea1	4
P27048	Small nuclear ribonucleoprotein-associated protein B	Snrpb	10
P62196	26S proteasome regulatory subunit 8	Psmc5	7.4
P62889	60S ribosomal protein L30	Rpl30	13.9
P63325	40S ribosomal protein S10	Rps10	5.5
P97379	Ras GTPase-activating protein-binding protein 2	G3bp2	6
Q08943	FACT complex subunit SSRP1	Ssrp1	2.4
Q8BHW2	Protein OSCP1	Oscp1	7.1
A2ASQ1	Agrin	Agrn	1.6
P23242	Gap junction alpha-1 protein	Gja1	15.2
Q3TYQ9	Aldehyde oxidase 4	Aox4	1.9
Q8QZY6	Tetraspanin-14	Tspan14	10.4
Q9ESU6	Bromodomain-containing protein 4	Brd4	1

Table S4. The differentially-expressed proteins in sEV-5 compared with sEV-ctrl.

Protein	Protein Name	Gene Name	Coverage/%	$FC_{sEV\text{-}5/sEV\text{-}ctrl}$[a]	t test p value
P14115	60S ribosomal protein L27a	Rpl27a	22.3	3.1204	0.0161
P49312	Heterogeneous nuclear ribonucleoprotein A1	Hnrnpa1	13.1	2.8109	0.0055
P27659	60S ribosomal protein L3	Rpl3	7.9	2.0963	0.0137
P10126	Elongation factor 1-alpha 1	Eef1a1	39.6	2.0143	0.0438
Q61937	Nucleophosmin	Npm1	22.9	1.9476	0.0500
P47911	60S ribosomal protein L6	Rpl6	12.2	1.8238	0.0270
Q02248	Catenin beta-1	Ctnnb1	4.5	0.6508	0.0005
Q8VDN2	Sodium/potassium-transporting ATPase subunit alpha-1	Atp1a1	17	0.5981	0.0380
Q9WU78	Programmed cell death 6-interacting protein	Pdcd6ip	31.9	0.5961	0.0470
O35874	Neutral amino acid transporter A	Slc1a4	4.9	0.5558	0.0500
Q80UG2	Plexin-A4	Plxna4	1.1	0.4514	0.0457
P09242	Alkaline phosphatase, tissue-nonspecific isozyme	Alpl	27.1	0.2779	0.0133
P14094	Sodium/potassium-transporting ATPase subunit beta-1	Atp1b1	17.8	0.2349	0.0467

[a] $FC_{sEV\text{-}5/sEV\text{-}ctrl}$ is the fold change of the protein abundance in sEV-5 versus that of sEV-ctrl.

Table S5. GO enrichment analysis.

GO ID	Signaling pathway	Category	Protein
GO:0014704	Intercalated disc	Cell component	P23242,Q02248,P14094,Q8VDN2
GO:0044291	Cell-cell contact zone	Cell component	P14094,P23242,Q02248,Q8VDN2
GO:0061695	Transferase complex	Cell component	P62196,Q9ESU6,P10711
GO:0008023	Transcription elongation factor complex	Cell component	Q9ESU6,Q08943
GO:0016591	RNA polymerase II	Cell component	P10711,P62196
GO:0005667	Transcription factor complex	Cell component	Q02248,Q62318,P10711,P62196
GO:0000049	tRNA binding	Molecular function	P10126,P47911,P63325
GO:0060590	ATPase regulator activity	Molecular function	P14094,A2ASQ1
GO:0019829	Cation-transporting ATPase activity	Molecular function	P14094,Q8VDN2
GO:0022853	Active ion transmembrane transporter activity	Molecular function	P14094,Q8VDN2
GO:0015662	ATPase activity, coupled to transmembrane movement	Molecular function	P14094,Q8VDN2
GO:0042625	ATPase coupled ion transmembrane transporter activity	Molecular function	P14094,Q8VDN2
GO:0008016	Regulation of heart contraction	Biological process	Q8VDN2,P14094,A2ASQ1,P23242
GO:0036376	Sodium ion export	Biological process	A2ASQ1,P14094,Q8VDN2
GO:0140115	Export across plasma membrane	Biological process	P14094,Q8VDN2,A2ASQ1
GO:0060047	Heart contraction	Biological process	Q8VDN2,P23242,P14094,A2ASQ1
GO:0035725	Sodium ion transmembrane transport	Biological process	P14094,Q8VDN2,A2ASQ1
GO:0086009	Membrane repolarization	Biological process	P23242,P14094,Q8VDN2
GO:0071804	Cellular potassium ion transport	Biological process	P14094,A2ASQ1,Q8VDN2
GO:0071260	Cellular response to mechanical stimulus	Biological process	P23242,Q02248,Q8VDN2

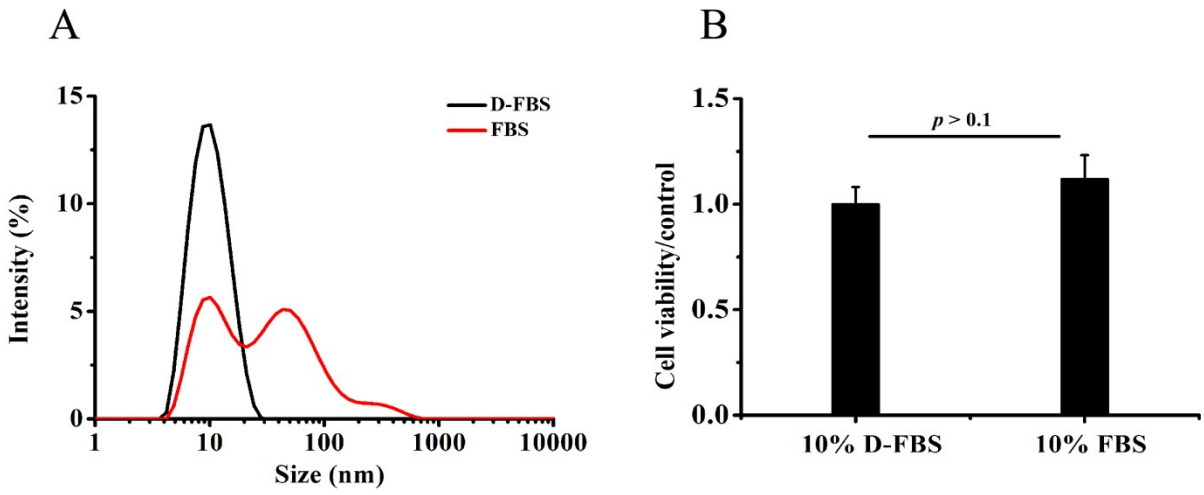
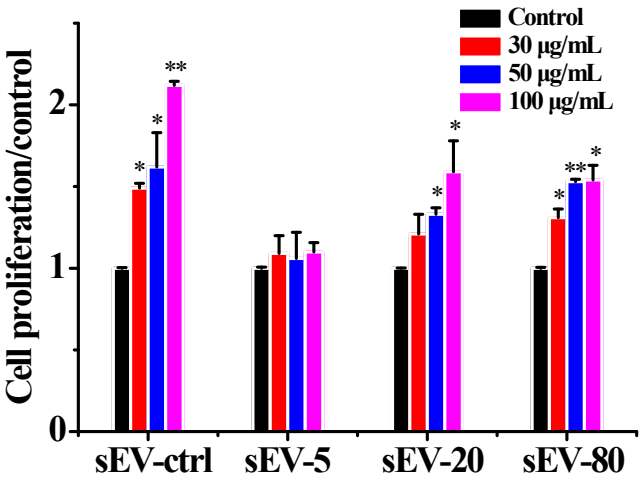


Fig. S11 Comparison of D-FBS with normal FBS. (A) Hydrodynamic sizes of particulate matters in FBS and D-FBS at 25 ℃. Several peaks appeared in the range of 1 nm - 400 nm in normal FBS sample, showing the existence of various EVs,[17] whereas, only a single peak with the intensity less than 15% was detected in D-FBS, indicating most of the EVs were removed. (B) Cell viabilities of 4T1 in DMEM medium supplemented with 10% FBS or 10% D-FBS (n = 3). No significant difference was observed between these two groups ($p > 0.1$).



419

420 **Fig. S12 The relative cell proliferation of 4T1 treated with sEVs** (n = 3). * p <

421 0.05, and ** p < 0.01 versus control.

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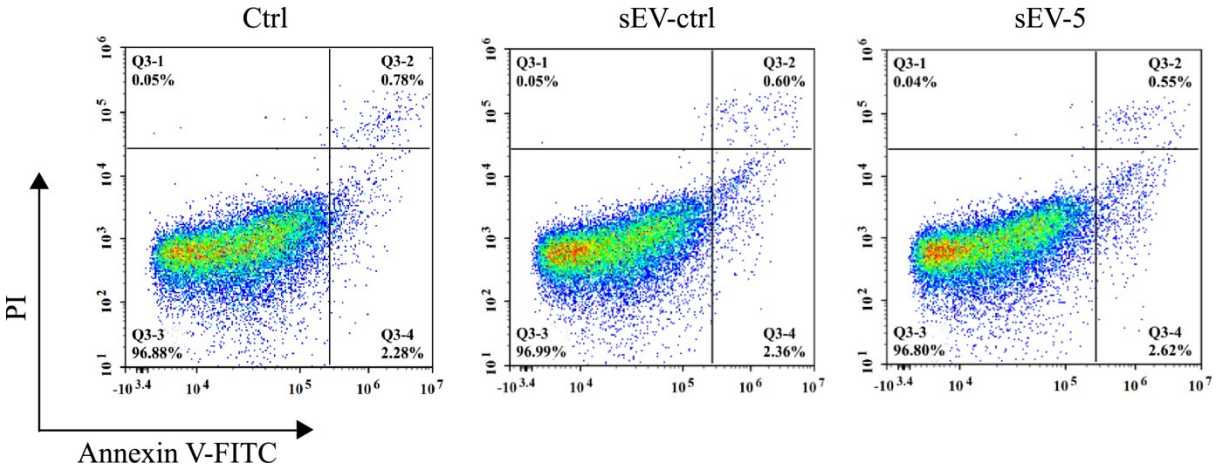

423

424 **Fig. S13 Cell apoptosis analysis of 4T1 cells in different groups.**

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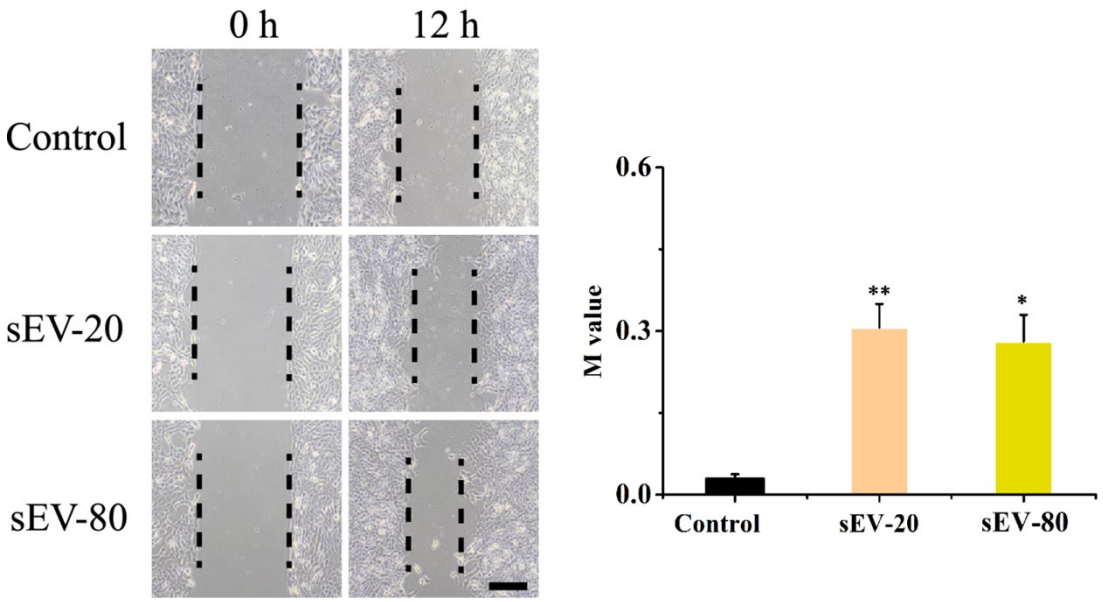

Fig. S14 Wound healing assays for 4T1 cells treated with sEV-20 and sEV-80 (n = 3). The images were obtained at 0 h and 12 h, and the scale bar represents 200 μm. *p < 0.05, **p < 0.01 versus control.

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