Biogenic selenium nanoparticles alleviate intestinal epithelial barrier injury by regulating mitochondria-lysosome crosstalk

Xina Dou†, Lei Qiao†, Xiaofan Song, Jiajing Chang, Shanyao Pi, Xinyi Zhang, Xiaonan Zeng, Lixu Zhu, Chunlan Xu*

The Key Laboratory for Space Bioscience and Biotechnology, School of Life Sciences, Northwestern Polytechnical University, Xi'an, Shaanxi, 710072, China

1. Supplementary Methods

* Corresponding author：Chunlan Xu, Professor, School of Life Sciences, Northwestern Polytechnical University, 127 Youyixi Road Xi'an, Shaanxi, 710072, China. E-mail: clxu@nwpu.edu.cn. Telephone: +86-29-88460543, Fax: +86-29-88460332

†These authors contributed equally to this work.

1.1 Reagents

FITC-Dextran (4 kDa) and LPS were purchased from Sigma-Aldrich (St. Louis, MO). High glucose medium of Dulbecco's Modified Eagle Medium (DMEM), antibiotics (AB), 0.25% trypsin-EDTA, and fetal bovine serum (FBS) were purchased from Biological Industries (Shanghai, China). Annexin Vfluorescein isothiocyanate (FITC)/Propidium Iodide (PI) Cell Apoptosis Detection Kit, ROS Detection Kit, LysoSensor™ Green DND-189, LysoTracker Green DND-26, LysoTracker® Red CMXRos, Lyso-Tracker Red, Mito-Tracker Green and Fluo-4 AM were purchased from Yeasen Biotechnology Co., Ltd. (Shanghai, China). Hoechst 33342 staining kit, Cell Counting kit-8 (CCK-8), glutathione peroxidase (GPx) activity assay kit, total superoxide dismutase (T-SOD) assay kit, ATP Assay Kit and MMP Assay Kit were purchased from Beyotime Biotechnology (Shanghai, China). TRIzol reagent was purchased from Gibco-Invitrogen. ELISA kits for assaying interleukin-1β (IL-1β), interleukin-8 (IL-8), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) were purchased from Jianglai Biological Technology Co., Ltd. (Shanghai, China). Thioredoxin reductase (TrxR) activity assay kit, and malondialdehyde (MDA) assay kit were purchased from Solarbio Life Science (Beijing, China). RNAex pro reagent, evo M-MLV mix kit with gDNA clean and SYBR® green premix pro taq HS qPCR kit were purchased from TransGen Biotech (Beijing, China). Primary antibodies against occludin, claudin-1, β-actin, ZO-1, microtubuleassociated protein light chain 3 (LC3B), Rab7, Fis1, TBC1D15, Mucolipin-2 (MCOLN2), Cathepsin B, cysteine proteases 3 (caspase-3) and a horseradish peroxidase (HRP)-labelled secondary antibody were

procured from ABclonal Biotechnology (Wuhan, China). TBC1D15 siRNA and GP-transfect-Mate were procured from Shanghai GenePharma Co.,Ltd (Shanghai, China).

1.2 Apoptosis assays

Cell apoptosis and necrosis were detected by Annexin V-FITC/PI staining kit. IPEC-J2 cells were grouped and treated according to the method described in section 2.2. After the treatments, cells were washed twice with PBS (pH 7.4). Then 200 μL Binding Buffer, 10 μL Annexin V-FITC and 20 μL PI staining solution were added to each well. Cells were placed in darkness at room temperature for 10 min, and then immediately observed with a fluorescence microscope (Leica Microsystems, Germany).

Cell apoptosis was identified by Hoechst 33342 staining. First, IPEC-J2 cells were grouped and treated according to the method described in section 2.2. After the treatments, cells were washed twice with PBS (pH7.4) and then stained with Hoechst 33342 (100×) for 10 min at 37°C in darkness. Finally, cells were washed twice with PBS and then visualized with a fluorescence microscope (Leica Microsystems, Germany).

1.3 Antioxidant capacity evaluation

We use the reagent kit of Solarbo to detect the content of MDA (Cat# BC0025). Malondialdehyde (MDA) can be condensed with thiobarbituric acid (TBA) under acidic and high temperature conditions to form brownish red. Its maximum absorption wavelength is 532 nm. After colorimetry, the content of MDA in the sample can be estimated. The specific experimental methods as follows: add the MDA detection working solution, distilled water, sample and distilled water into a 1.5 mL centrifuge

tube according to the instructions. After the mixture is kept in a 100℃ water bath for 60 min (cover tightly to prevent water loss), put it in an ice bath for cooling, 10,000 g, normal temperature, centrifugation for 10 min. Suck 200 μL supernatant is put into a 96-well plate, and the absorbance of each sample at 532 nm and 600 nm is measured. Calculate the content of MDA according to the instructions.

We use the reagent kit of Solarbo to detect the activity of TrxR (Cat# BC1155). TrxR is a NADPH-dependent dimeric selenidase containing FAD domain, belonging to the pyridine nucleotide-disulfide oxidoreductase family, which together with thioredoxin and NADPH forms the thioredoxin system. TrxR is similar to GR in activity, catalyzing GSSG reduction to GSH, and is one of the key enzymes in glutathione oxidation-reduction cycle. TrxR catalyzes NADPH to reduce DTNB to produce TNB and $NADP^+$. TNB has a characteristic absorption peak at 412 nm, but reduced glutathione can also react with DTNB to produce TNB. Therefore, this kit uses 2-vinylpyridine to inhibit the original reduced glutathione in the sample. The TrxR activity can be calculated by measuring the increase rate of TNB at 412 nm wavelength. The specific test method is as follows: preheat the spectrophotometer or microplate reader for 30 min, adjust the wavelength to 412 nm, and use distilled water to zero. Reagent I is preheated at 37℃ for 30 min. Take 96-well plate, set blank well and experimental well, and add 20 μL Reagent II, 20 μL Reagent III, 160 μL Reagent 1. In addition, add to the test well respectively 20 μL Reagent II, 20 μL Reagent III, 140 μL Reagent I, 20 μL supernatant, quickly mix and measure the absorbance at 412 nm for 10 s, and take out the absorbance

at 412 nm quickly after a water bath at 37℃ for 5 min. Calculate the activity of TrxR according to the instructions.

We use the reagent kit of Beyotime to detect the activity of TrxR (Cat# S0109). This kit uses the classic nitroblue tetrazole (NBT) color method. Through the reaction system of xanthine and xanthine oxidase (XO), the superoxide anion (O^{2-}) is produced, and the nitroblue tetrazole is reduced to blue formazan, which has strong absorption at 560 nm. SOD can remove superoxide anion, thus inhibiting the formation of formazan. The darker the blue color of the reaction solution, the lower the activity of superoxide dismutase, and the higher the activity of enzyme. The activity level of superoxide dismutase can be calculated by colorimetric analysis. The specific test method is as follows: use 96-well plate to set sample well and blank control well. Add the sample to be tested and other reagents according to the amount shown in the instructions. Add reaction starting working fluid. Then fully mix. Incubate at 37ºC for 30 min. Determination of absorbance at 560 nm. Calculate the activity of SOD according to the instructions.

We use the reagent kit of Beyotime to detect the activity of Gpx (Cat# S0058). This kit uses an indirect determination method. Glutathione peroxidase (GPx) can catalyze GSH to produce GSSG, while glutathione reductase can use NADPH to catalyze GSSG to produce GSH. The activity level of glutathione peroxidase can be calculated by detecting the reduction of NADPH. The specific test method is as follows: use 96-well plate, add the test buffer, the sample to be tested and the GPx test working solution in turn, add 40 μL GPx test working solution, and incubate at room temperature for 15

min to consume the GSSG in the sample and eliminate the interference to the subsequent test. Add 10 μL of 30 mM peroxide reagent solution into each well and mix well. Immediately measure A_{340} with an appropriate enzyme marker or micro-ultraviolet spectrophotometer, and record it as 0 min reading value. Set the temperature at 25ºC and measure A_{340}. A_{340} shall be measured continuously for 5 min or automatically every 1 min. Record continuously for 5 min. Calculate the activity of Gpx according to the instructions.

1.4 Detection of ROS level

IPEC-J2 cells were grouped and treated according to the method described in section 2.2. After treatments, cells were cultured with 10 μM 2 ’-7 ’dichlorofluorescin diacetate (DCFH-DA) at 37°C for 20 min, and then washed twice with PBS (pH 7.4). Finally, fluorescence intensity was measured by an inverted fluorescence microscope.

1.5 MMP (Δψm) assay

MMP of experimental cells was detected by JC-1 staining. Cells were groups and treated according to the method described in section 2.2. After that, cells were washed twice with PBS (pH 7.4) and Then stained with JC-1 for 20 min. After discarding the JC-1 solution, cells were washed with JC-1 buffer solution. Finally, fluorescence images were captured by a laser scanning confocal microscope (Leicasp5, Leica Microsystems). Green monomeric JC-1 and red aggregated JC-1 were detected at 530 nm and 590 nm emission wavelength, respectively.

1.6 Lysosomal acidification evaluation

Cells were grouped and treated according to the method described in section 2.2.

Lysosomal acidification was measured in IPEC-J2 cells with 1 μM Lyso-Sensor Green DND-189 for 1 h at 37°C. Finally, images were taken with laser scanning confocal microscope. The fluorescence intensity of Lyso-Sensor Green DND-189 was calculated using the Image J 1.8.0 software.

1.7 Observation of SeNPs position in cell and mitochondrial and lysosomal ultrastructure

1×10^5 cells per well of IPEC-J2 were seeded into 6-well plates and cultured overnight. Then the cells were divided into LPS model group and SeNPs + LPS group. The treatment methods of SeNPs and LPS were the same as the method described in section 2.2. Subsequently, cells were washed twice with PBS and then collected into a 10 mL centrifuge tube. After centrifugation at 1,000 rpm for 10 min, cell pellets were resuspended in PBS and centrifuged at 4°C at 1,500 rpm for 10 min. Then, the cells were immobilized with 2.5% glutaraldehyde overnight at 4°C. After the samples were processed according to the standard procedures including staining, dehydration, embedding and sectioning, the position of SeNPs in cells, and mitochondrial and lysosomal ultrastructure were observed by a Hitachi HT7800 transmission electron microscope (Hitachi Ltd., Tokyo, Japan).

1.8 Colocalization of mitochondria and lysosomes

IPEC-J2 cells were grouped and treated according to the method described in section 2.2. Subsequently, the cells were incubated with Mito-Tracker Green fluorescent probe at 37℃ and 5% CO_2 for 45 min. After that, cells were washed twice with PBS (pH 7.4) and then incubated with Lyso-Tracker Red fluorescent probe at 37℃ and 5% CO_2 for

45 min and washed with PBS. Finally, mitochondria with green fluorescence and lysosomes with red fluorescence were observed under a confocal laser scanning microscope.

1.9 Detection of Ca^{2+} concentration in the cytoplasm

IPEC-J2 cells were grouped and treated according to the method described in section 2.2. Subsequently, discarded the medium. The cells were washed with HBSS and then cultured with Fluo-4AM for 30 min. Finally, the concentration of Ca^{2+} in the cytoplasm was detected by inverted fluorescence microscope.

1.10 Western blot analysis

The total protein was isolated using the RIPA buffer containing a mixture of protease and phosphatase inhibitors. And the protein concentration of samples was detected by the BCA assay kit according to the manufacturer's instrument. The protein samples were boiled in the loading buffer. and then the samples with equal volume and amount of protein were loaded onto the sodium dodecyl sulfate-polyacrylamide gel, and then transferred to the PVDF membrane. The membranes were incubated overnight with the primary antibodies for occludin, claudin-1, ZO-1, LC3B, Rab7, Fis1, TBC1D15, MCOLN2, Cathepsin B, caspase-3, and β-actin at 4℃ followed by incubation with the corresponding secondary antibody at room temperature for 1 h. Immunoreactive protein bands were visualized with the clarity Western ECL substrate kit using Tanon 5200 Multi (Shanghai, China) and quantified using the Image J 1.8.0 analyzer software.

1.11 Live cell time-lapse imaging

IPEC-J2 cells were grouped and treated according to the method described in section

2.2. Subsequently, the cells were incubated with 100 nM Mito-Tracker Red for 30 min, and washed twice with PBS (pH 7.4). Then, the cells were incubated with 50 nM Lyso-Tracker Green DND-26 for 2 h and washed twice with PBS. Finally, mitochondria with red fluorescence and lysosomes with green fluorescence were observed under confocal laser scanning microscope. The images of Mito-Tracker Red and Lyso-Tracker Green DND-26 were dynamically recorded at 561 nm for and 488 nm respectively. The images were analyzed using the Image J 1.8.0 software.

2. Supplementary Results

2.1 Figure legends

Figure S1 Establishment of LPS-induced intestinal epithelial cells injury model. (**A**) Different action time of LPS (n = 8). (**B**) Different concentrations of LPS (n = 8). (**C**) The morphology of IPEC-J2 cells (n = 6). (**D**-**F**) The IL-1β level in IPEC-J2 cells exposed to LPS for 24 h, 48 h and 72 h (n = 6). (**G**-**H**) The IL-1β level in IPEC-J2 cells exposed to LPS after administration of SeNPs for 12 h and 24 h (n = 6). All data are expressed as mean ± S.E.M., $^{*}P< 0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Figure S2 Effects of SeNPs on the cytotoxity in IPEC-J2 cells induced by LPS. (**A**) Cell apoptosis detected by Annexin V-FITC and PI staining. (**B**) Quantitative statistical analysis Results. All data are expressed as mean ± S.E.M., n=6, $^{*}P< 0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Figure S3 The expression level of TBC1D15 in IPEC-J2 cells transfected with TBC1D15 siRNA. (**A**-**B**) Screening of RNA sequences and quantitative analysis results (n = 3). (**C**-**D**) Screening of RNA concentration and quantitative analysis results (n = 3). (**E**-**F**) The expression levels of upstream and downstream genes in IPEC-J2 cells transfected with TBC1D15 siRNA and quantitative analysis results (n = 3). (**G**) The morphology of IPEC-J2 cells transfected with TBC1D15 siRNA (n = 6). (**H**) The cell viability of IPEC-J2 cells transfected with TBC1D15 siRNA (n = 8). All data are expressed as mean ± S.E.M., $^{*}P< 0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

2.2 Results

Figure S1

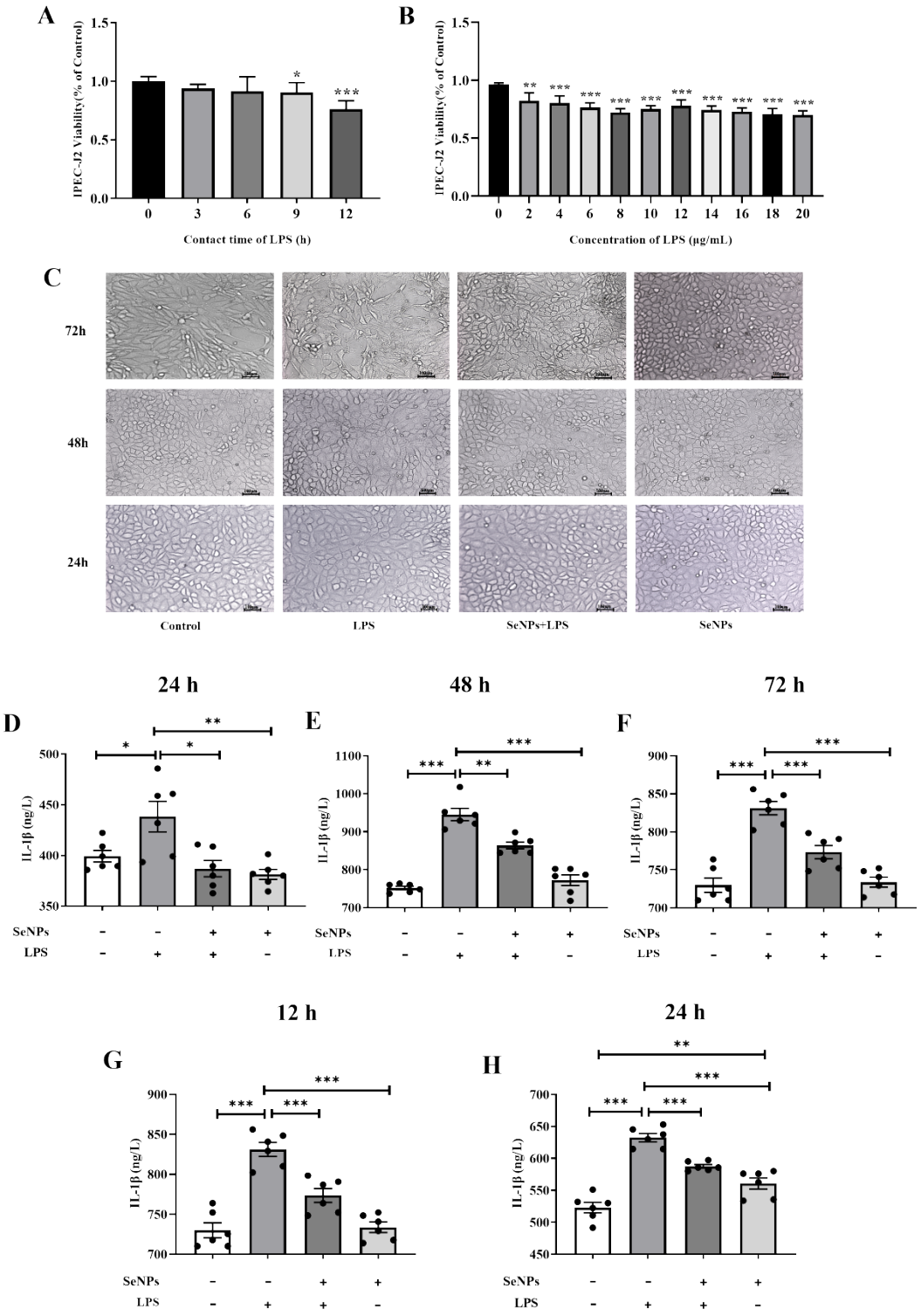


Figure S2

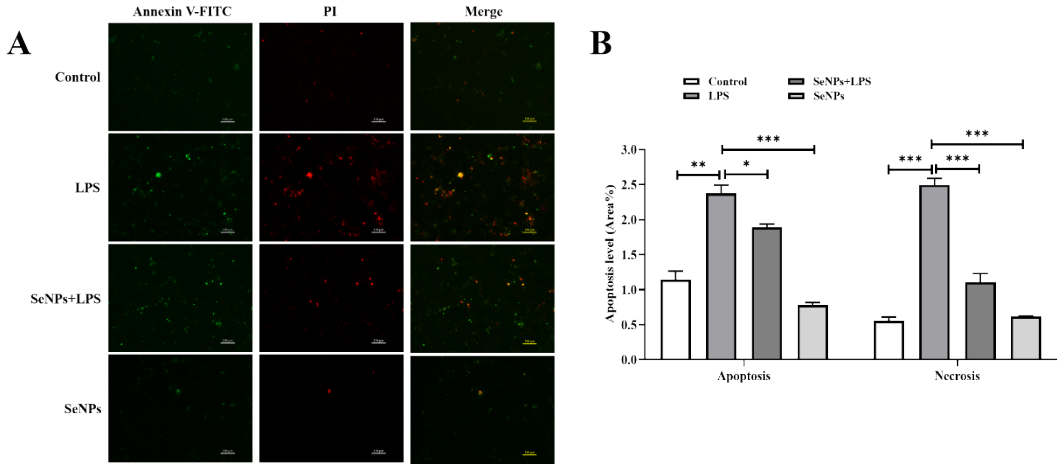
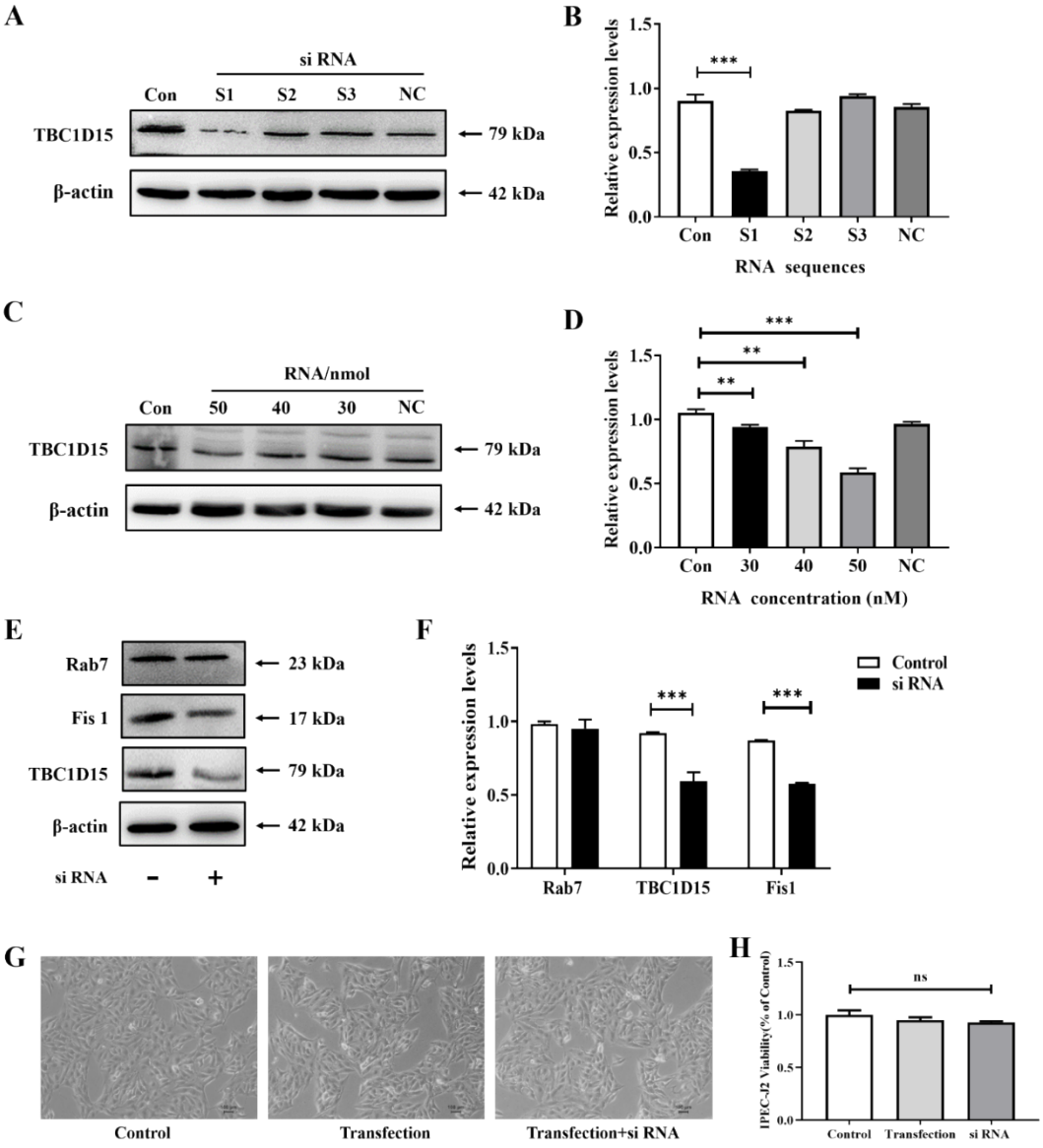

A
Annexin V-FITC
PI
Merge
Control
LPS
SeNPs+LPS
SeNPs
B
Control
LPS
SeNPs+LPS
SeNPs
Apoptosis level (Area%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
***
***
***
***
Apoptosis
Necrosis

Figure S3


A
si RNA
Con
S1
S2
S3
NC
TBC1D15
β-actin
79 kDa
42 kDa
B
Relative expression levels
1.5
1.0
0.5
0.0
***
Con
S1
S2
S3
NC
RNA sequences
C
RNA/nmol
Con
50
40
30
NC
TBC1D15
β-actin
79 kDa
42 kDa
D
***
**
**
Relative expression levels
Con
30
40
50
NC
RNA concentration (nM)
E
Rab7
Fis 1
TBC1D15
β-actin
23 kDa
17 kDa
79 kDa
42 kDa
si RNA
-
+
F
Control
si RNA
Relative expression levels
***
***
Rab7
TBC1D15
Fis1
G
Control
Transfection
Transfection+si RNA
H
IPEC-J2 Viability(% of Control)
ns
Control
Transfection
si RNA