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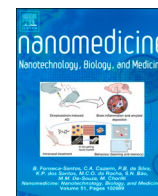
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Role of ESCCAL-1 in regulating exocytosis of AuNPs in human esophageal squamous carcinoma cells

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ABSTRACT

Exocytosis is a critical factor for designing efficient nanocarriers and determining cytotoxicity. However, the research on the exocytosis mechanism of nanoparticles, especially the role of long non-coding RNAs (lncRNAs), has not been reported. In this study, the exocytosis of AuNPs in the KYSE70 cells and the involved molecular pathways of exocytosis are analyzed. It demonstrates that nanoparticles underwent time-dependent release from the cells by exocytosis, and the release of β -hexosaminidase confirms that AuNPs are excreted through lysosomes. Mechanistic studies reveal that lncRNA ESCCAL-1 plays a vital role in controlling the exocytosis of AuNPs through activation of the MAPK pathway, including the phosphorylation of ERK and JNK. The study implies that the ESCCAL-1-mediated pathway plays an important role in the exocytosis of AuNPs in KYSE70 cells. This finding has implications for the role of ESCCAL-1 on the drug resistance of esophagus cancer by controlling lysosome-mediated exocytosis.

Introduction

In recent decades, with the rapid development of nanotechnology, nanomaterials have become increasingly widely used in various fields, especially in biomedicine.^{1,2} When nanoparticles are administered inside the body, nanoparticles will encounter the cells in tissues and organs, leading to cellular uptake. Intracellular nanoparticle content plays a decisive role in the therapeutic efficiency and biological toxicity of drug delivery and therapy; therefore, controlling nanoparticles' intracellular concentration and retention time of nanoparticles is an essential issue in nanomedicine applications.^{3,4} The concentration of intracellular nanoparticles is a highly dynamic process determined by cellular uptake and the cellular clearance mechanism, the process of exocytosis.^{5,6} Despite that nanoparticles' cellular retention and exocytosis and the underlying mechanisms have been studied in recent years, the role of exocytosis in tumor cells, especially drug resistance, is poorly understood.

Numerous studies have shown that nanoparticles enter the cell through endocytosis and eventually reach lysosomes through multiple

vesicular transportation and fusion mechanisms.⁷ For example, gold nanoparticles (AuNPs) uptake and transport mechanisms have been extensively studied.^{8–11} Qin et al. reported that AuNPs can be delivered to early endosomes, late endosomes, and lysosomes, with a large fraction trapped inside lysosomes.¹² The acidic environment of lysosomes plays a vital role in dissolving and degrading organic and highly soluble metal nanomaterials. However, recent studies have found that nanomaterials with low solubility are not degraded in lysosomes, and they can be transported to the plasma membrane and released from the cells by exocytosis.¹³ The exocytosis of gold nanoparticles from cells is a complex and dynamic process influenced by various factors such as size, shape, surface chemistry, cell type, and the experimental design. Chithrani et al. showed that removing protein-coated gold nanoparticles is size dependent; smaller ones (14 nm) are removed faster than bigger ones (50, 74 nm).¹⁴ Ho et al. found that the PEG shell prevents interparticle agglomeration or aggregation, which facilitates the exocytosis of gold nanoparticles.¹⁵ Interestingly, these NPs exit the cell predominantly via unconventional exocytosis, accompanied by enhanced secretion of sub-100 nm, CD81-enriched exosomes.¹⁶ Some studies

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showed that exocytosis is a highly dynamic process in nature, and the exocytosis rates are highest when cells are saturated with internalized nanoparticles.^{17,18} Other studies reported that the time of incubation with nanoparticles also influences exocytosis.^{19,20} Additionally, Ding et al. found that the removal mechanisms of gold nanoparticles with different sizes, shapes, and protein corona all involve lysosomes, and agglomeration inside cells impede exocytosis.²¹ Moreover, Zhu et al. found that 2D nanomaterials accumulate in lysosomes and are removed by exocytosis.²² Yanes et al. found that silica nanoparticles are localized in lysosomes that can be excreted through the Golgi complex and lysosomes²³; Ding et al. showed that AuNPs of different sizes and shapes are released through the lysosomal exocytosis pathway.²¹ Oh et al. showed surface chemistry played an important role in exocytosis; the cationic ones were retained in the cells for a relatively long time, likely due to their intracellular agglomeration.¹⁵ In contrast, the PEGylated GNPs migrated in the cytoplasm in the form of individual particles and exited the cells rapidly because the PEG coating mitigated interactions between GNPs and intracellular proteins. Additionally, their exocytosis pattern was not significantly governed by the size, particularly in the range from 10 to 40 nm.^{13,24} The study by Kim et al. demonstrated that a polyethylene glycol (PEG) coating facilitates the exocytosis of gold nanoparticles in macrophages, irrespective of size, emphasizing the role of surface functionality in exocytosis efficiency.²⁵ Furthermore, the involvement of lysosomal exocytosis in the excretion of mesoporous silica nanoparticles was presented by Yanes et al.²³ Understanding the mechanisms of exocytosis is crucial for optimizing the use of gold nanoparticles in biomedical applications. Regarding the signaling pathways, it has been documented that MAPK (mitogen-activated protein kinase) signaling may be involved in the lysosomal exocytosis of nanoparticles. Cui et al. reported that SWCNTs (single-walled carbon nanotubes) induce phosphorylation of PKC (protein kinase C) and MAPKs by activating P2X7R (purinergic ligand-gated ion channel 7 receptor), thereby triggering lysosomal exocytosis in macrophages.²⁶ Despite these progresses on nanomaterial exocytosis significantly improving our understanding of this issue, additional controlling mechanisms, especially the role of long noncoding RNAs (lncRNAs) on exocytosis, have not been elucidated.

lncRNAs have previously been shown to regulate the exocytosis of proteins. It has been reported that lncRNA regulates the exocytosis of insulin granules in β cells to regulate insulin secretion.²⁷ Among various lncRNAs, ESCCAL-1 is an oncogene discovered by our team,²⁸ which was highly overexpressed in esophagus squamous cancer cells (ESCC) and promotes cancer through various mechanisms, including drug resistance.^{29,30} However, it is presently unclear whether it plays a role in regulating the exocytosis of nanoparticles in esophageal cancer cells.

AuNPs were often used as a surrogate for nanoparticles, which have excellent physical and chemical properties, such as easy synthesis control, stability in solution, high biosafety, unique optoelectronic properties, and easy modification and detection³¹; it has been an attractive material used in drug delivery,^{32,33} photothermal therapy,³⁴ diagnosis,³⁵ and biosensing.^{36,37} In this study, we synthesized sodium citrate-modified gold nanoparticles (AuNPs) with an average diameter of 15 nm. This size was selected due to the finding that smaller AuNPs are rapidly exocytosed, so it is easier to study the effects of lncRNAs. We investigated the cellular uptake, transport, exocytosis, and molecular pathways that control the exocytosis of AuNPs in esophageal cancer cells. We found that after dose- and time-dependent uptake of AuNPs into the lysosomes, nanoparticles underwent time-dependent release from the cells by exocytosis. We also found that ESCCAL-1 promotes the exocytosis of AuNPs; further mechanistic studies revealed that ESCCAL-1 promotes the exocytosis of AuNPs by activating the MAPK pathway. This study will help to understand the exocytosis process of AuNPs and shed light on the role of ESCCAL-1 on the drug resistance of esophageal cancer cells by controlling lysosome-mediated exocytosis.

Materials and methods

Synthesis of AuNPs

We used the established method³⁸ to synthesize the citrate-coated AuNPs with average sizes of 15 nm. A 50 mL flask contained a 0.25 mM HAuCl₄ solution, while a separate 34.0 mM (1.0 wt %) Na₃Ct solution was prepared. The HAuCl₄ solution in the flask was heated on a hotplate with vigorous stirring and covered by a disposable Petri dish to prevent contamination and solvent evaporation. After reaching the boiling point, a specific volume of Na₃Ct solution was swiftly injected to achieve the desired particle size, controlled by the Na₃Ct to HAuCl₄ molar ratio. The synthesis concluded when the suspension's color stabilized, typically taking 2–5 min, and the sample was naturally cooled to room temperature.³⁹

Characterization of AuNPs

The shape and size of AuNPs were investigated using TEM (JEOL 2100, Japan). A Nanosizer (Zetasizer 3000 HS, UK) was used to measure the hydrodynamic size and the zeta potential.

Cell culture

The KYSE70 cells (human esophageal squamous cell carcinoma cells) were cultured at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂ with RPMI-1640 medium supplemented with 10 % FBS.

Cellular viability assay

The KYSE70 cells were seeded at a density of 5×10^4 cells per well in 96-well plates and incubated overnight. Then, cells were replaced with various concentrations of AuNPs (0, 5, 10, 20, 50 $\mu\text{g mL}^{-1}$) for 24 h and 48 h. After incubation, MTT solutions (0.5 mg mL⁻¹) were added to the cells and incubated for 4 h; then, the supernatant was removed, and 150 μL dimethylsulfoxide (DMSO) was added to each well, and a microplate reader measured the absorbance at a wavelength of 570 nm.

Uptake study and subcellular localization

KYSE70 cells were seeded in a 24-well tissue culture plate at a density of 2×10^5 cells per well and incubated overnight; for time-dependent cellular uptake, the cells were treated with AuNPs (500 μL , 20 $\mu\text{g mL}^{-1}$) for 1, 6, 12, and 24 h. For dose-dependent uptake, the cells were treated with various concentrations of AuNPs (5, 10, 20, 50 $\mu\text{g mL}^{-1}$) for 24 h; after incubation, cells were washed 3 times with D-Hanks to remove AuNPs weakly attached to the plasma membrane. Cells were harvested and incubated overnight in aqua regia to dissolve AuNPs; the amount of intracellular Au was detected by ICP-MS. KYSE70 was grown overnight on clean coverslips on a 24-well cell culture plate for dark-field imaging. The cells were incubated to allow uptake of AuNPs, then washed three times and fixed with 4 % paraformaldehyde for 30 min sealed. Dark-field images were captured at $\times 40$ magnification using the microscope Olympus BX43 equipped with a dark-field condenser ($0.8 < \text{NA} < 0.95$) and a $40\times$ objective lens ($\text{NA} = 0.65$). KYSE70 was treated with or without AuNP (20 $\mu\text{g mL}^{-1}$) for TEM imaging for 24 h. Subsequently, cells were collected and fixed; the samples were retained for TEM imaging.

Exocytosis study

KYSE70 were seeded in a 24-well culture plate with no or clean coverslips. After attachment, cells were treated with AuNPs (20 $\mu\text{g mL}^{-1}$) for 6 h. Then, the cells were washed three times, and the fresh medium without AuNPs was changed. For ICP-MS analysis (the amount of Au in the supernatants) and ELISA, the supernatant was collected at 6,

12, 24, and 48 h, and ELISA was used to detect β -hexosaminidase released into the supernatant following the instructions of the kit. For dark-field imaging, the cells were rinsed at 6, 12, 24, and 48 h, fixed with 4 % paraformaldehyde for 30 min, and then sealed.

Cell infection

ESCCAL-1 knockdown, ESCCAL-1-overexpressing plasmid lentivirus, and the control virus were purchased from GeneChem (Shanghai, China). Lentiviral infection was performed according to the manufacturer's protocol.

The knockdown efficiency and overexpression fold were analyzed by quantitative real-time PCR (qRT-PCR). To assess the effect of ESCCAL-1 on uptake and exocytosis, we divided cells into shRNA-NC (Non-targeting control shRNAs), shRNA-ESCCAL-1 (shRNAs for knockdown of ESCCAL-1), OE-NC (negative control vector), and OE (ESCCAL-1-overexpression lentiviral vector) groups. For the impact of ESCCAL-1 on uptake, each group of cells was treated with $20 \mu\text{g mL}^{-1}$ AuNPs for 24 h. After incubation, the cells were washed three times with D-Hanks to remove AuNPs weakly attached to the plasma membrane, and then harvested; for the impact of ESCCAL-1 on exocytosis and Lysosomal exocytosis, each group of cells was treated with AuNPs ($20 \mu\text{g mL}^{-1}$) for 6 h. Subsequently, the cells were washed three times, and fresh medium without AuNPs was added, and then the uptake and exocytosis were measured by ICP-MS, ELISA and dark field microscopy (DFM) as described previously.

qRT-PCR analysis

Total RNA was extracted from cells with TRIzol reagent (Thermo-Scientific, USA), reverse-transcribed into cDNA, and analyzed by real-time PCR on the ABI7500 system. Real-time PCR was performed at 95°C for 1 min, followed by 40 cycles of 95°C for 20 s, 60°C for 34 s, and 72°C for 30 s. All the experiments were repeated three times. The $2^{-\Delta\Delta\text{Ct}}$ method was used to measure the relative changes in gene expression using GAPDH as the endogenous control. Primers sequences used were as follows: ESCCAL-1: F-Primer: 5'-CCAGA-CAGCAGCAAAGCAAT-3', R-Primer: 5'-GGAAGCAGCAATGTGTCCAT-3'.

Western blot assay

Total proteins were extracted by RIPA lysis buffer (Beyotime, China) supplemented with protease inhibitor and phosphatase inhibitors; the lysate was subjected to SDS-PAGE electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5 % milk, followed by incubating with the primary antibody overnight and then incubating with the secondary antibody at room temperature for 2 h. The protein signal was detected using the enhanced chemiluminescence (ECL) kit (Beyotime Biotechnology, Shanghai, China). The primary antibodies used in this study were as follows: anti-GAPDH (Bioworld, 1:10000), anti-phospho-ERK, and anti-phospho-JNK (Cell Signaling Technology, 1:1000).

Statistical analysis

Data in this article are presented as means $\pm$ SD (standard deviation). The statistical significance of the two groups was compared using Student's t-test. Statistical analyses and data plotting were performed using SPSS 20.0 and GraphPad 8.0 software. A P -value < 0.05 was considered to indicate statistical significance.

Results

Characterization of AuNPs

We prepared the citrate-coated AuNPs using previously established methods. Transmission electron microscopy (TEM) images show uniform sizes and shapes for all AuNPs (Fig. 1a). Fig. 1b shows a detailed distribution of sizes. Hydrodynamic size and zeta potential measurements were conducted to confirm the formation of a uniform and stable dispersion of AuNPs in ultrapure water and culture medium. As presented in Table 1, the average hydrated particle diameter of AuNPs was determined to be 21.1 nm, while the ζ potential was measured as -38.7 mV. In the culture medium, the average hydrated diameter of AuNPs increased to 46.4 nm, and the ζ potential decreased to approximately -8.4 mV. The above results show that the AuNPs were stable and well-dispersed in aqueous solution and culture medium.

Cell viability and endocytosis after AuNPs treatment

MTT was used to evaluate the cytotoxicity of AuNPs on KYSE70 cells and to ensure that the cells were healthy after incubation with AuNPs. Cell viability was maintained above 90 % after 24 h or 48 h incubation with AuNPs concentrations ranging from $0 \mu\text{g mL}^{-1}$ to $50 \mu\text{g mL}^{-1}$ (Fig. 2a), suggesting that there was no significant toxicity associated with AuNPs in the concentration range of 0 – $50 \mu\text{g mL}^{-1}$. In addition, TEM images revealed that many granular AuNPs were present in lysosomes, indicating that AuNPs were internalized by KYSE70 cells and localized to lysosomes (Fig. 2b and c).

Dose and time-dependence uptake and intracellular transport

Fig. 3a illustrates a dose-dependence of cellular uptake of AuNPs. According to the results, increasing AuNP concentrations increased intracellular gold content within the concentration range of 5 – $50 \mu\text{g mL}^{-1}$. For the subsequent tests, a concentration of $20 \mu\text{g mL}^{-1}$ was selected. In Fig. 3b, the endocytosis curves of AuNPs in KYSE70 cells show increased uptake with time. We visually confirmed AuNPs uptake with cells by dark-field microscopy imaging. As seen in Fig. 3c–f, the levels of AuNPs in cells gradually rise with incubation time or concentration, which agrees with the quantitative results of ICP-MS. From the TEM images in Fig. 3g, we observed the AuNPs distributed in early/late endosomes, as well as lysosomes which revealed that AuNPs might be transported through vesicles to early/late endosomes and lysosomes after endocytosis. We also found that AuNPs were present on both lysosomes and cell membranes, suggesting that AuNPs might be excreted through lysosomal exocytosis.

Time-dependence exocytosis of AuNPs and release of lysosomal enzymes

To quantitatively investigate the exocytosis of AuNPs, we cultured

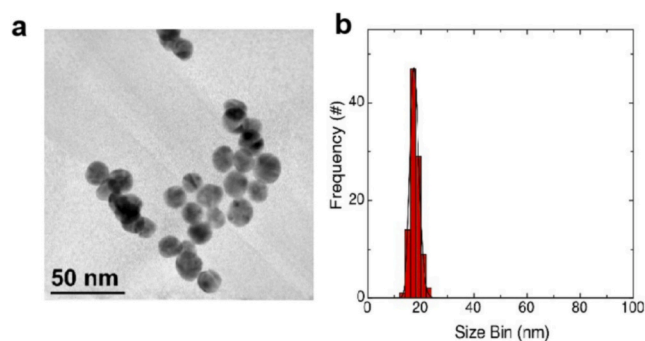


Fig. 1. Characterization of AuNPs. (a) AuNPs image captured by TEM. (b) Particle size distribution.

Table 1
Hydrodynamic sizes and zeta potentials of AuNPs in Milli-Q ultra-pure water and culture medium.

	Size (nm)	Zeta potential (mV)
Water	21.1 ± 0.3	−38.7 ± 2.9
RPMI	46.4 ± 0.6	−8.4 ± 2.2

KYSE70 in the presence of AuNPs ($20\text{ }\mu\text{g mL}^{-1}$) for 6 h. The trend of exocytosis of nanoparticles with time in Fig. 4a showed that within 0–48 h, the concentration of extracellular AuNPs increased, and the increasing rate gradually slowed, indicating that the exocytosis efficiency decreased with time. DFM confirmed the exocytosis of AuNPs in KYSE70 cells (Fig. 4b–c), which showed that the content of intracellular AuNPs decreased with time. However, the exocytosis reached equilibrium after 24 h, and the content of intracellular AuNPs changed slowly, in agreement with the results of ICP-MS. The exocytosis of AuNPs appears to correlate with lysosomal exocytosis (Fig. 4d). β -hexosaminidase is an enzyme in the lysosome; we can detect lysosomal exocytosis by measuring the amount of this enzyme released into the medium. The results showed that the exocytosis patterns of the AuNPs were similar to that of β -hexosaminidase, suggesting that the AuNPs mainly exit KYSE70

cells via lysosomal exocytosis.

LncRNA ESCCAL-1 promotes exocytosis of AuNPs in KYSE70 cells

Previously, we have reported many functions of ESCCAL-1 in esophageal cancer, including controlling cell proliferation, migration, inflammation, and cell death. Here, to determine whether ESCCAL-1 plays a role in exocytosis, we performed knockdown or overexpression of ESCCAL-1 in KYSE70 cells; the result is shown in Fig. 5a and d, the knockdown decreased the level of ESCCAL-1 mRNA by 54.0 %, and overexpression resulted in a 30.39-fold increase in ESCCAL-1 mRNA levels intracellularly. Then, we evaluate the effect of ESCCAL-1 on the uptake and exocytosis of AuNPs; the results indicate that knockdown and overexpression had no noticeable effect on the cellular uptake of AuNPs. However, the exocytosis of AuNPs has significantly decreased after knockdown and increased after overexpression of lncRNA ESCCAL-1 (Fig. 5c and f), and at the same time, the amount of β -hexosaminidase in the supernatant also decreased after knockdown (Fig. 5k). The results of DFM further verified the above finding (Fig. 5g–j). Based on these results, we can see that there was no significant difference in the uptake of AuNPs between groups (Fig. 5b and e). However, compared with the control group, the exocytosis of the knockdown group was significantly

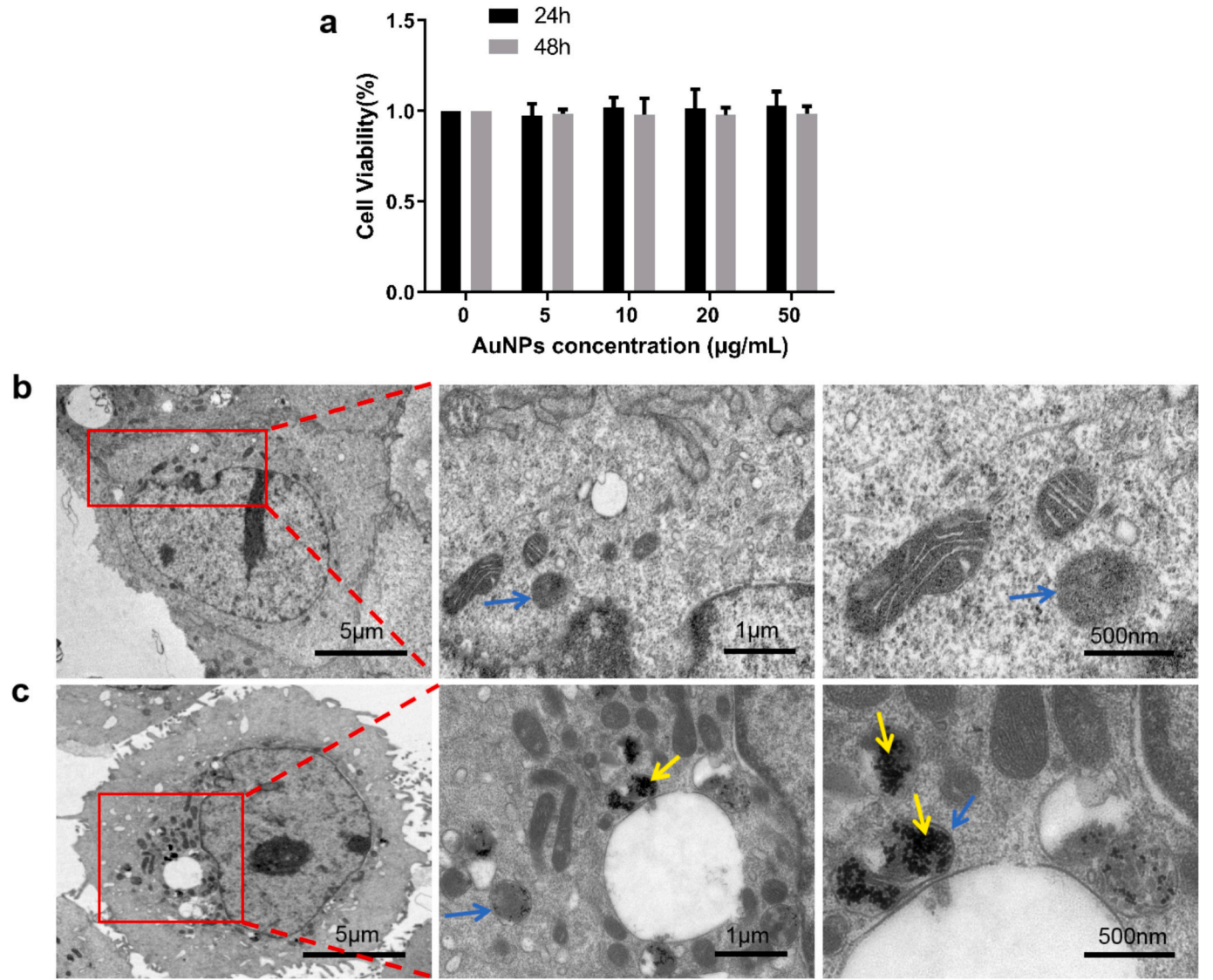


Fig. 2. Cell viability and endocytosis after AuNPs treatment. (a) Effect of AuNPs on the cell viability of KYSE70 cells. (b) TEM imaging of KYSE70 after incubation with no AuNPs for 24 h. (c) TEM imaging of KYSE70 after incubation with $20\text{ }\mu\text{g mL}^{-1}$ AuNPs for 24 h. Blue arrows indicate lysosomes, and yellow arrows for AuNPs.

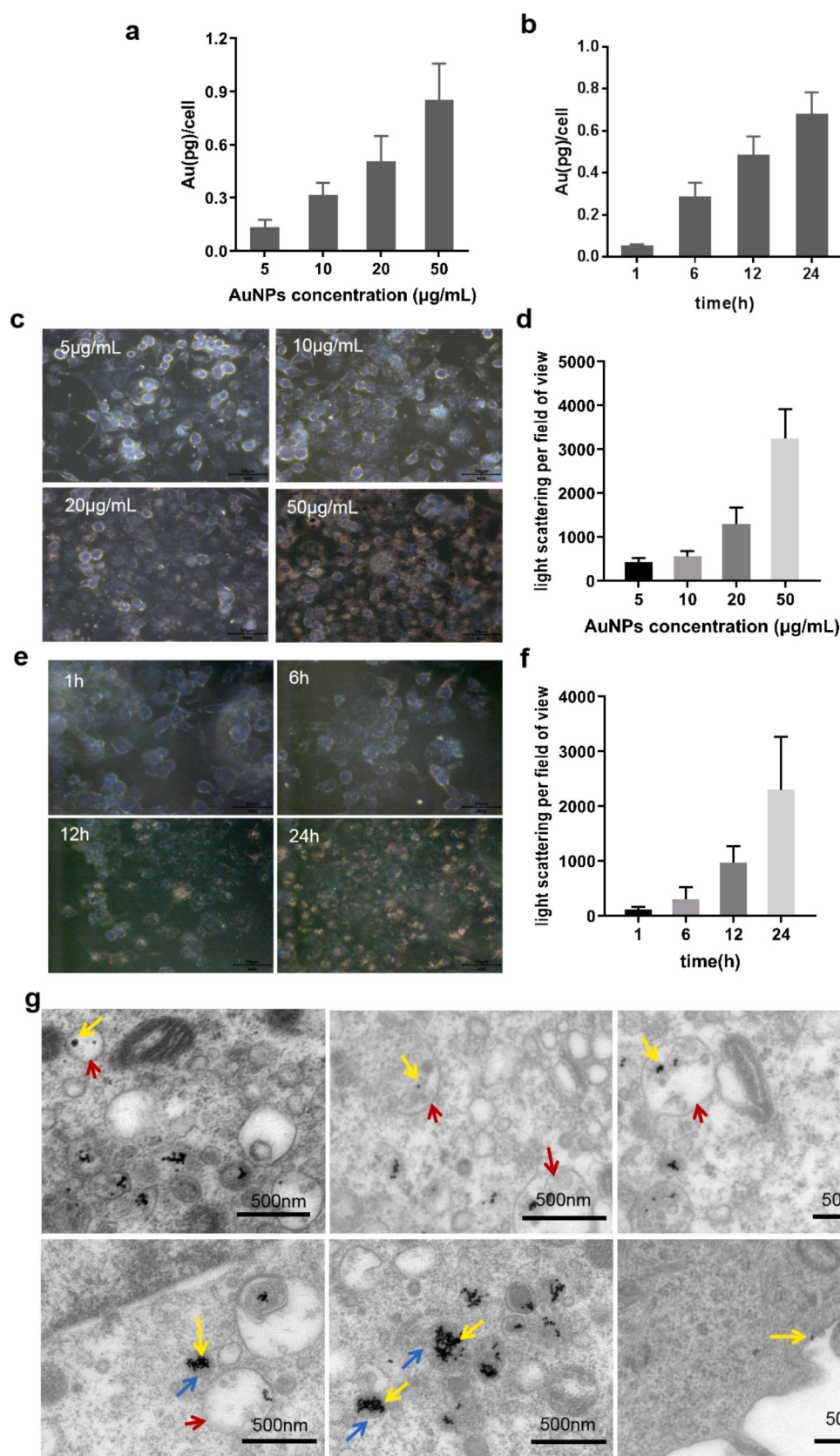


Fig. 3. Uptake of AuNPs and subcellular localization. (a) Dose-dependent uptake of AuNPs determined by ICP-MS. (b) Time-dependent uptake of AuNPs determined by ICP-MS. (c and d) Darkfield microscopic images of AuNPs dose-dependent uptake. (e and f) Darkfield microscopic images of AuNPs time-dependent uptake. (g) Electron microscope of intracellular transport of AuNPs. Red arrows indicate early or late endosomes, blue arrows indicate lysosomes and yellow arrows indicate AuNPs.

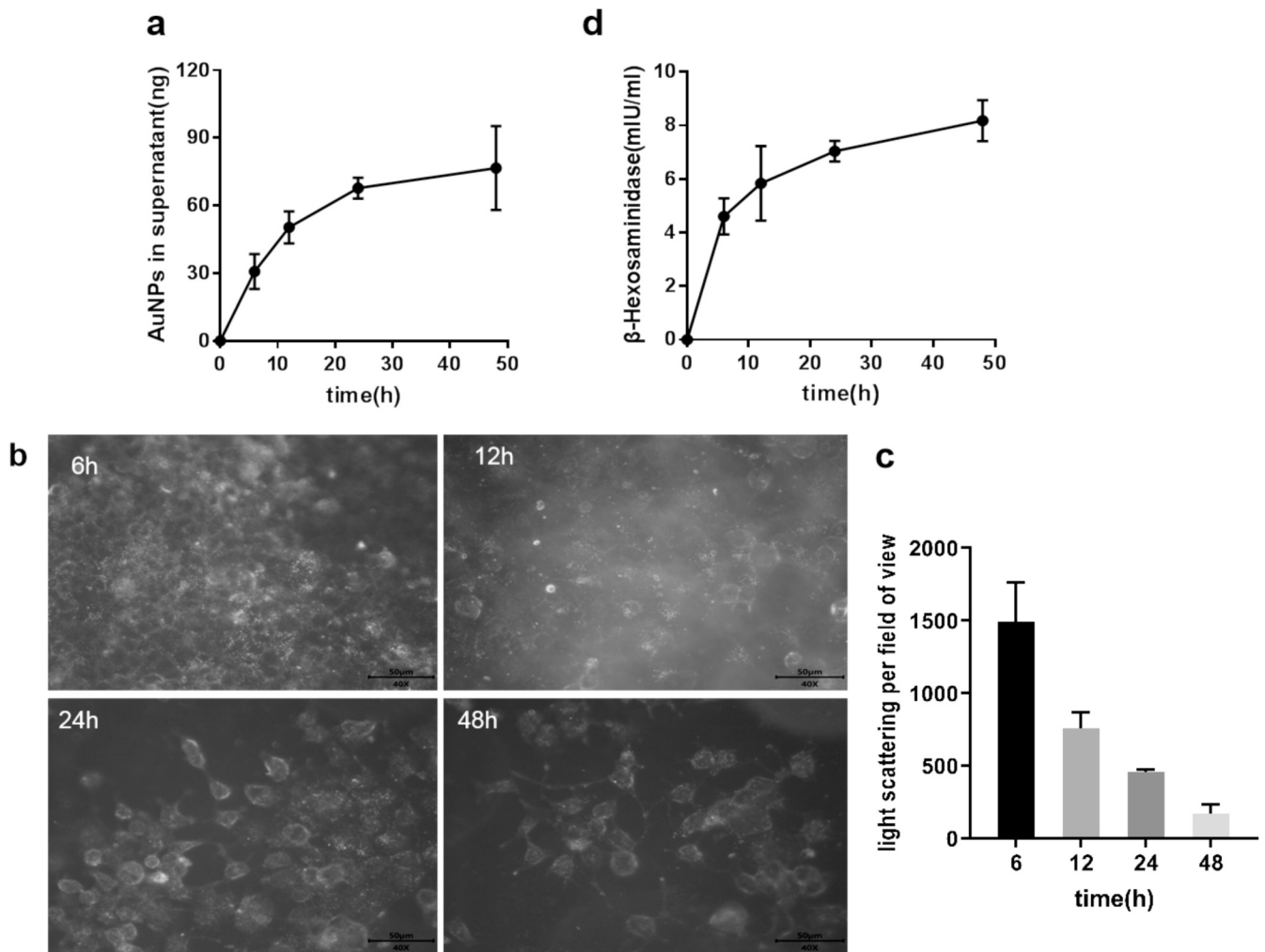


Fig. 4. Exocytosis of AuNPs. (a) Changes of AuNP content in the supernatant with culture time. (b and c) Darkfield microscopic image of cells clearing AuNPs. (d) The release of β -Hexosamine enzyme in the culture supernatant with time.

reduced, and that of the overexpression group was increased. These results indicate that lncRNA ESCCAL-1 can promote the exocytosis of AuNPs in KYSE70 cells.

AuNPs induced activation of mitogen-activated protein kinases (MAPK) pathways in KYSE70

Studies have shown that the MAPK signaling pathway plays a role in lysosomal exocytosis. Since our previous experimental results show that AuNPs can trigger lysosomal exocytosis, we verified whether AuNPs activate the MAPK pathway by western blot analysis. After exposing cells to AuNPs, ERK and JNK phosphorylation levels increased compared to the control (Fig. 6).

LncRNA ESCCAL-1 activates MAPK signaling pathway

To study the role of ESCCAL-1 in activating the MAPK pathway, we knocked down and overexpressed the gene of lncRNA ESCCAL-1 in KYSE70 cells. The western blotting results (Fig. 7) show that ERK and JNK phosphorylation levels were decreased after lncRNA ESCCAL-1 knockdown and increased after overexpression relative to the control.

LncRNA ESCCAL-1 promotes the exocytosis of AuNPs in KYSE70 cells by activating MAPK signaling pathway

Recent research shows that activating the MAPK pathway has been involved in lysosomal exocytosis. The above results suggest that the lncRNA ESCCAL-1 regulates the exocytosis of AuNPs. Thus, we speculate that lncRNA affects the exocytosis of AuNPs in KYSE70 cells by activating the MAPK pathway. To verify this point, we performed a western blot analysis. When we exposed the ESCCAL-1 knocked-down cells to AuNPs, ERK and JNK phosphorylation levels almost returned to that of the control group. In contrast, when we exposed the ESCCAL-1 overexpression cells to AuNPs, the ERK and JNK phosphorylation levels were higher than those in the other groups (Fig. 8a–b). ERK inhibitor (U0126) and JNK inhibitor (SP600125) were used to inhibit the activities of ERK and JNK, respectively. As shown in Fig. 8c, the results indicate that overexpression of ESCCAL-1 can enhance the activation of ERK and JNK, as well as the exocytosis of AuNPs. U0126 and SP600125 both suppressed the exocytosis induced by the upregulation of lncRNA ESCCAL-1. These findings suggest that lncRNA ESCCAL-1 facilitated the exocytosis of AuNPs in KYSE70 cells through the activating of the ERK and JNK signaling pathways.

Discussions

Exocytosis is important because the uptake and exocytosis of

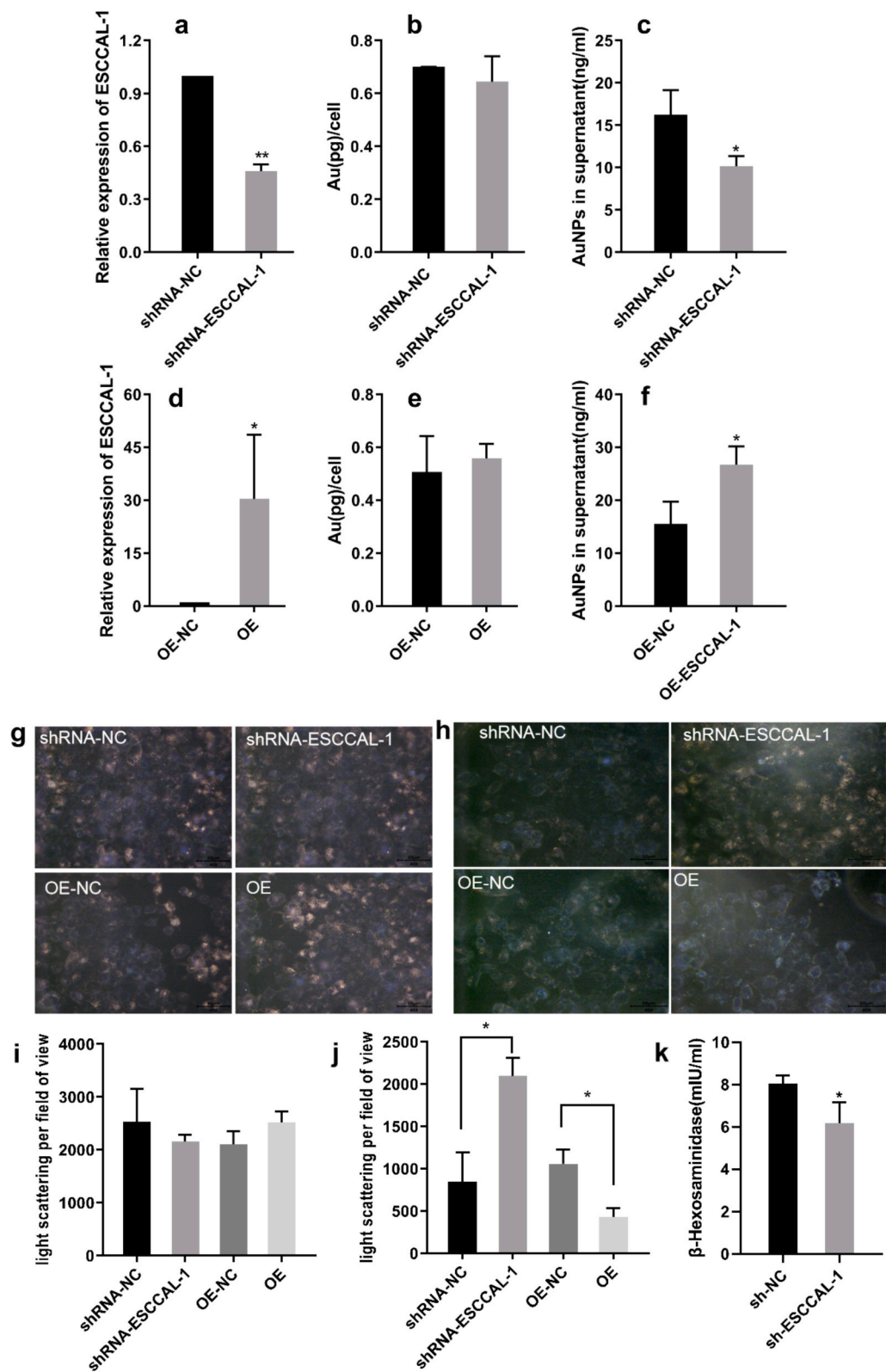


Fig. 5. Effect of LncRNA ESCCAL-1 on uptake and exocytosis of AuNPs in KYSE70 cells. (a) Expression level of LncRNA ESCCAL-1 in KYSE70 cells after knockdown of LncRNA ESCCAL-1. (b) The uptake of AuNPs by KYSE70 cells after knockdown of LncRNA ESCCAL-1. (c) The exocytosis of AuNPs by KYSE70 cells after knockdown of LncRNA ESCCAL-1. (d) Expression level of LncRNA ESCCAL-1 in KYSE70 cells after overexpression of LncRNA ESCCAL-1. (e) The uptake of AuNPs by KYSE70 cells after overexpression of LncRNA ESCCAL-1. (f) The exocytosis of AuNPs by KYSE70 cells after overexpression of LncRNA ESCCAL-1. (g and i) Darkfield microscopic images of AuNPs uptake by KYSE70 cells with LncRNA ESCCAL-1 knockdown or overexpression. (h and j) Darkfield microscopic images of AuNPs exocytosis by KYSE70 cells with LncRNA ESCCAL-1 knockdown or overexpression. (k) The release of β -Hexosamine enzyme in the culture supernatant by KYSE70 cells after knockdown of LncRNA ESCCAL-1.

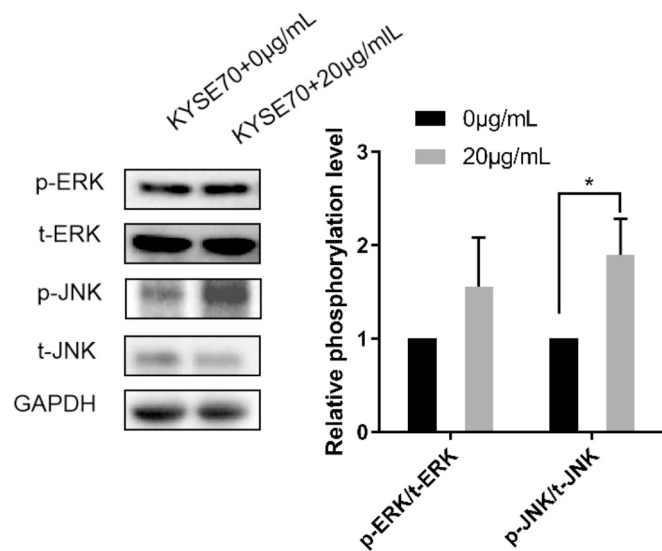


Fig. 6. AuNPs-Induced activation of the MAPK pathway. KYSE70 cells were treated with 20 $\mu\text{g mL}^{-1}$ AuNPs, and western blotting was performed to detect the phosphorylation of ERK and JNK. GAPDH was used as a control. Protein levels were quantified by density measurement of blots.

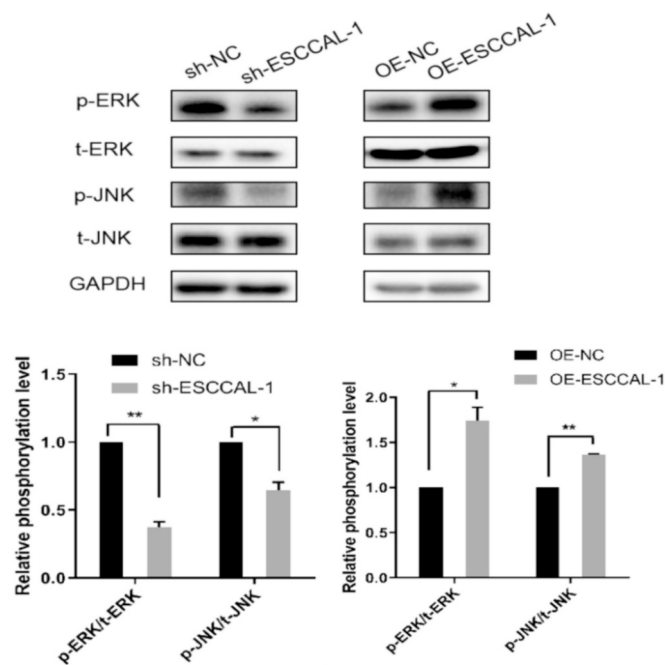


Fig. 7. ESCCAL-1 activation of the MAPK pathway. Knockdown or overexpression of lncRNA ESCCAL-1 was conducted in KYSE70 cells. Western blotting was performed to detect the effects of the lncRNA on the phosphorylation of ERK and JNK. GAPDH was used as a control. Protein levels were quantified by density measurement of blots.

nanoparticles are directly related to the intracellular residence time, detection accuracy, and therapeutic efficiency of nanomedicine.⁴⁰ Although many studies have focused on nanoparticles' cellular uptake and exocytosis behavior, detailed mechanisms, especially the role of lncRNAs, have not been reported before.^{15,41–43} Here, we prepared the citrate-coated AuNPs with an average particle size of 15 nm; for subsequent studies, we determined the cytotoxicity of AuNPs in the KYSE70 cells to establish a safe concentration of 20 $\mu\text{g mL}^{-1}$ for the subsequent in vitro optimization studies. We then investigated the uptake and

exocytosis of AuNPs. We have demonstrated that AuNPs uptake occurs in a concentration and time-dependent manner. These results are consistent with previously reported results on the uptake kinetics of AuNPs.²⁴ Exocytosis increases with time, reaching a plateau after 24 h, similar to the secretion of β -hexosaminidase, a lysosomal enzyme, indicating that these nanoparticles are mainly excluded from the KYSE70 cell by lysosomal exocytosis. Other studies have drawn similar conclusions.¹⁴ Therefore, we confirmed that KYSE70 cells ingested AuNPs and excreted them mainly through lysosomal exocytosis.

lncRNA ESCCAL-1 is the oncogene identified in our laboratory. Our previous works have revealed that ESCCAL-1 promotes esophageal squamous cell carcinoma (ESCC) through multiple mechanisms. Cui et al. reported that ESCCAL-1 promotes ESCC cell cycle progression by interacting with galectin-1.⁴⁴ Liu et al. found that ESCCAL-1 promotes ESCC by downregulating the APOBEC3G negative regulator.²⁹ However, it has yet to be reported whether lncRNA ESCCAL-1 plays a role in regulating exocytosis. In this study, we explored the effect of ESCCAL-1 on uptake and exocytosis; the results have indicated that knockdown of ESCCAL-1 inhibits the exocytosis process but not the uptake process in the KYSE70 cells, whereas overexpression of ESCCAL-1 resulted in the consistent results. These results show that ESCCAL-1 can regulate the exocytosis of AuNPs in KYSE70 cells for the first time. Here, we studied the mechanism underlying its role in controlling exocytosis.

MAPKs are serine/threonine-specific protein kinases, including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK), and p38 kinases.^{45–47} Studies have shown that activation of MAPK is involved in lysosomal exocytosis.^{48–50} However, it is unclear whether ESCCAL-1 promotes the exocytosis of AuNPs in KYSE70 cells by activating MAPK. This study found that AuNPs could activate JNK signaling while ESCCAL-1 could activate both the ERK and JNK signaling. When exposed, the lncRNA ESCCAL-1 knocked down cells to AuNPs, and the levels of ERK and JNK phosphorylation largely returned to that of the control group. The above results strongly suggest that AuNPs induce phosphorylation of ERK/MAPK (increased activation by not statistically significant) and JNK/MAPK via ESCCAL-1. Thus, AuNPs can induce lysosomal exocytosis, and ESCCAL-1 promotes exocytosis of AuNPs in KYSE70 cells by activating ERK and JNK signaling pathways. This finding contradicts previous studies that AuNPs inhibited p42/44 MAPK (also called ERK1 and ERK2) in the tumor⁵¹ and inhibited p38 MAPK in the fibrotic kidney.⁵² The differences may be due to the physicochemical properties of AuNP, cell types, and experimental conditions. Additional experiments are warranted to resolve the discrepancy. The detailed underlying mechanisms of how ESCCAL-1 induced inflammatory pathways still need further investigation.

Clinical chemotherapy resistance is the main reason for tumor treatment failure. Studies have shown that when drug resistance occurs, the intracellular drug concentration decreases (mainly due to increased pumping), and increasing the intracellular drug concentration can achieve better treatment results. Recent studies have shown that lysosomal accumulation of chemotherapeutics leads these anticancer drugs away from their cellular target sites and mediates drug resistance.^{53–56} The mechanism of lysosomal accumulation involves the hydrophobic weak-base anticancer drugs being extensively sequestered in acidic lysosomes. This current study found that overexpression of lncRNA ESCCAL-1 increases the exocytosis of AuNPs. Although we do not have direct evidence on the role of exocytosis in the drug sensitivity of ESCC cells, we know elevated ESCCAL-1 expression is linked to poor prognosis (development of drug resistance). In our preliminary data, we found that ESCCAL-1 knockdown (Fig. S1) induced enhanced sensitivity to different chemotherapeutic drugs, including 5-FU, Diamminedichloroplatinum (DDP), and TAXOL (Fig. S2). This led to the increased activation of Caspase 3 (Fig. S3) and decreased activation of BCL-2, suggesting ESCCAL-1 plays an important role in apoptosis induced by the drugs. This current study also found that ESCCAL-1 promotes the exocytosis of AuNPs by activating ERK/MAPK and JNK/MAPK, and we are curious about whether it can achieve better therapeutic effects by

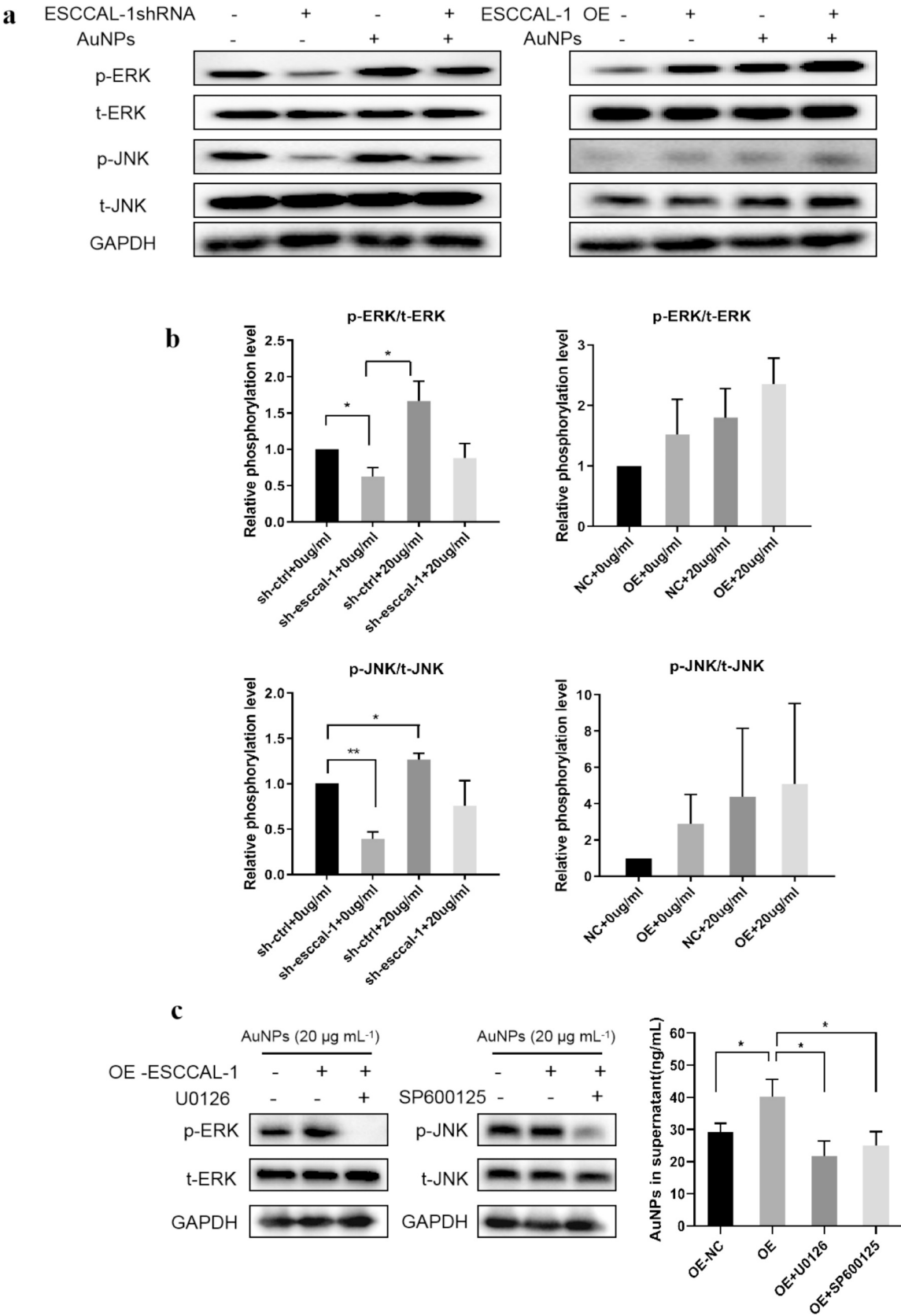


Fig. 8. lncRNA affects the exocytosis of AuNPs through activation of the MAPK pathway. Knockdown or overexpression of lncRNA ESCCAL-1 was conducted in KYSE70 cells. (a–b) Western blotting was performed to detect the effects of the lncRNA on the phosphorylation of ERK and JNK in the presence or absence of AuNPs, GAPDH was used as a control. Protein levels were quantified by density measurement of blots. (c) Cells were treated with U0126 (an MEK1/2 inhibitor; 20 μ M) and SP600125 (a JNK inhibitor; 20 μ M) to inhibit the activities of ERK and JNK. ICP-MS was employed to measure the exocytosis of AuNPs.

inhibiting exocytosis by blocking the ERK/MAPK and JNK/MAPK pathways or inhibit the lysosomal functions. Future studies are needed to clarify these points.

Conclusion

We studied the cellular uptake, localization, and exocytosis of AuNPs and the involved mechanisms. We demonstrate that AuNPs localize to lysosomes after internalization and are excreted through lysosome exocytosis in KYSE70 cells. We also found that overexpression of lncRNA ESCCAL-1 promoted exocytosis of AuNPs in KYSE70 cells and revealed that ESCCAL-1 regulates the exocytosis of AuNPs by activating ERK/MAPK and JNK/MAPK. This study provides insight into the control of intracellular retention and exocytosis of AuNPs. This finding sheds light on the potential role of ESCCAL-1 on the drug resistance of esophagus cancer by controlling lysosome-mediated exocytosis.

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CRediT authorship contribution statement

Fenfen Gong: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Yuanbo Cui:** Writing – review & editing, Investigation. **Pengju Lv:** Visualization, Investigation. **Jia Liu:** Visualization. **Xiaoyan Sun:** Investigation. **Pengli Han:** Resources. **Lijuan Zhou:** Methodology. **Tian Xia:** Writing – review & editing, Funding acquisition, Conceptualization. **Wei Cao:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

No potential conflict of interest was reported by the author(s).

Data availability

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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