

ORIGINAL ARTICLE

Epidermal growth factor/epidermal growth factor receptor signaling blockage inhibits tumor cell-derived exosome uptake by oral squamous cell carcinoma through macropinocytosis

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Abstract

Various cell types secrete exosomes into their surrounding extracellular space, which consequently affect the function and activity of recipient cells. Numerous studies have showed that tumor cell-derived exosomes play important roles in tumor growth and progression. Although a variety of endocytic pathways are reportedly involved in the cellular uptake of exosomes, detailed mechanisms remain unknown. The present study demonstrated that treatment with recombinant epidermal growth factor (EGF) time- and dose-dependently promoted cellular uptake of oral squamous cell carcinoma (OSCC) cell-derived exosomes into OSCC cells themselves. Conversely, EGF receptor (EGFR) knockdown and treatment with EGFR inhibitors, including erlotinib and cetuximab, abrogated OSCC cell uptake of exosomes. The macropinocytosis inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA) blocked the effects of active EGF/EGFR signaling on uptake of OSCC cell-derived exosomes. These EGFR inhibitors also suppressed OSCC cell-derived exosome-induced proliferation, migration, invasion, stemness, and chemoresistance of OSCC cells. Taken together, the data presented herein suggest that EGFR inhibitors might inhibit the malignant potential of OSCC cells through direct inhibition of not only EGFR downstream signaling pathway but also cellular uptake of OSCC cell-derived exosomes through macropinocytosis.

KEYWORDS

EGF, EGFR, exosome, macropinocytosis, oral squamous cell carcinoma

Abbreviations: 5-FU, 5-fluorouracil; BCA, bicinchoninic acid; BSA, bovine serum albumin; CCK-8, Cell Counting Kit-8; CDDP, cis-diamminedichloroplatinum; CSF-1, colony-stimulating factor-1; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; ERK, extracellular signal regulated kinase; FBS, fetal bovine serum; JAK, Janus Kinase; mAbs, monoclonal antibodies; MFI, mean fluorescence intensity; miRNA, micro RNA; NAC, N-acetyl-L-cysteine; OSCC, oral squamous cell carcinoma; PBS, phosphate buffered saline; PI3K, phosphatidylinositol 3-kinase; rEGF, recombinant EGF; ROS, reactive oxygen species; siRNA, small interfering RNA; SOD, superoxide dismutase; STAT, Signal transducer and activator of transcription; TKIs, tyrosine kinase inhibitors; TRX, thioredoxin.

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1 | INTRODUCTION

Over 350 000 cases of oral cancer are reported annually worldwide. Almost half of these individuals subsequently die of their disease.¹ Among all defined histological types of oral cancer, more than 90% are oral squamous cell carcinomas (OSCC). Despite recent therapeutic advances, significant recurrence rates in OSCC are still observed, with approximately 40% of patients developing cervical lymph node metastases.² Therefore, OSCC is still characterized as having a poor prognosis and low survival rates, and improvements in the treatment for oral cancer are necessary.^{3,4}

Exosomes, which are small membrane vesicles released by various cells, including tumor cells, can be taken up by parent and recipient cells, consequently affecting their function and activity through included materials, such as lipids, proteins, and nucleic acids from their cell of origin.⁵ Studies have shown that tumor-derived exosomes affect tumorigenesis, tumor progression, and metastasis by exhibiting immunosuppressive properties, facilitating tumor invasion and metastasis, stimulating tumor cell proliferation, or inducing drug resistance.^{6,7} We also previously demonstrated that OSCC cell-derived exosomes taken up by OSCC cells themselves significantly promoted proliferation, migration, invasion, and growth of tumor xenografts implanted into nude mice through the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt, MAPK/extracellular signal regulated kinase (ERK), JNK-1/2 pathways.⁸ Therefore, the aforementioned findings suggest that tumor cell-secreted exosomes can be therapeutic targets in OSCC.

Concerning the therapeutic potential of targeting tumor cell-secreted exosomes, evidence has shown that blocking of exosome production, secretion, and uptake may be effective for cancer therapy. For instance, studies have revealed that blockade of Rab27a, a small GTPase, suppressed the secretion of exosomes and decreased primary tumor growth.^{9,10} Exosomes are taken up through a variety of endocytic pathways, including clathrin-dependent endocytosis, caveolin-mediated uptake, macropinocytosis, phagocytosis, lipid raft-mediated internalization, membrane fusion, and protein interactions.¹¹ Among these endocytic pathways, macropinocytosis allows for the uptake of large amounts of nutrients and other components, including exosomes, through actin cytoskeleton rearrangement followed by membrane ruffle formation via the activation of the Rho family of GTPases, such as Rac1, and phosphoinositide signaling.¹² Macropinocytosis can also occur in response to the stimulation of growth factors, such as epidermal growth factor (EGF), macrophage colony-stimulating factor-1 (CSF-1), and platelet-derived growth factor, as well as the activation of oncogenic Ras.¹³⁻¹⁵ Therefore, identifying and suppressing the exosome uptake pathway specific to tumor cells may contribute toward suppressing cancer progression.

The EGF receptor (EGFR) is overexpressed in OSCC, with the EGF-EGFR signaling pathway regulating the malignant potential of OSCC cells and influencing anticancer drug resistance, progression, and poor prognosis of patients with OSCC.¹⁶⁻¹⁸ Accordingly, anti-EGFR agents, including monoclonal antibodies (mAbs) and small-molecule tyrosine kinase inhibitors (TKI), have been developed, with cetuximab currently approved for the treatment of patients with

advanced OSCC in Japan.^{19,20} However, it remains unknown whether these anti-EGFR agents suppress the uptake of tumor cell-derived exosomes through EGF-EGFR signaling-regulated macropinocytosis and inhibit the progression of OSCC.

Therefore, the present study examined whether OSCC cells themselves take up OSCC cell-derived exosomes through macropinocytosis via EGF-EGFR signaling pathway activation. Furthermore, this study explored the inhibitory effects of anti-EGFR agents on exosome uptake through macropinocytosis and their role in tumor development and progression using in vitro assays.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

HSC-2, -3, -4, and SAS were obtained from the RIKEN BioResource Center and cultured in DMEM (Nissui Pharmaceutical) supplemented with 10% (v/v) FBS, 10 mmol/L of glutamine, 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Invitrogen) at 37°C in a humidified 5% CO₂/95% air atmosphere. EGFR-specific small interfering RNA (siRNA) was synthesized by Ambion. Transfection was performed using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). 5-Fluorouracil (FU) and cisplatin (CDDP) were obtained from Sigma-Aldrich (Merck KGaA); recombinant human EGF was obtained from PeproTech; and erlotinib was from Cayman chemical. Cetuximab (Erbix) was purchased from Merck Serono. 5-(N-ethyl-N-isopropyl) amiloride (EIPA) and N-acetyl-L-cysteine (NAC) were supplied by Sigma-Aldrich.

2.2 | Exosome isolation

Oral squamous cell carcinoma cells (2×10^6 cells/10 cm dish) were cultured in conventional culture medium for 24 hours. The medium was then replaced with DMEM supplemented with 5% (v/v) exosome-depleted FBS (System Biosciences, LLC) for 48 hours, after which the exosomes were isolated using the Total Exosome Isolation Kit (Invitrogen; Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, cell culture supernatants were harvested and centrifuged at 2000 g for 30 minutes to remove cells and cell debris. Next, the reagent was added to the supernatants, and the mixture was refrigerated overnight. The mixture was then centrifuged at 10 000 g for 60 minutes, and the supernatants were removed. The exosome pellet was resuspended in PBS, and the protein concentration was determined using a bicinchoninic acid (BCA) assay kit (Pierce Biotechnology).

2.3 | Exosome labeling and cellular uptake

The purified exosomes were labeled PKH26 or PKH67 (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol.

Briefly, 1 μ L of PKH26 or PKH67 was added to 100 μ g of exosome pellet in 200 μ L (total volume) of diluent C and incubated for 5 minutes at room temperature. The labeling reaction was stopped by adding an equal volume of FBS, after which the samples were ultracentrifuged at 10 000 g for 60 minutes at 4°C. After removing the supernatant, the pellets were resuspended in PBS.

To assess the cellular uptake of PKH26-labeled exosomes or FITC-dextran through confocal laser microscopy, a total of 1×10^4 cells/well HSC-4 cells were initially cultured in Nunc Lab-Tek 8-well chamber slides (Thermo Fisher Scientific) for 24 hours to achieve complete adhesion. Next, the cells were treated with PKH26-labeled exosomes or FITC-dextran (Sigma-Aldrich) with or without 10 μ mol/L of erlotinib, 100 μ g/mL of cetuximab, or 25 μ mol/L of EIPA for 24 hours. After incubation, cells were washed twice with PBS and mounted using SlowFade Diamond Antifade Mountant with DAPI (Invitrogen). Images were then captured using a Fluoview FV-1000D confocal laser scanning microscope (Olympus) at 400 \times magnification.

To assess cellular uptake of exosomes via flow cytometry, a total of 1×10^5 cells/well OSCC cells were cultured in 12-well microplates (Corning) for 24 hours to achieve complete adhesion. Next, the cells were treated with PKH67-labeled exosomes or FITC-dextran with or without 10 μ mol/L of erlotinib, 100 μ g/mL of cetuximab, or 25 μ mol/L of EIPA for 24 hours. After incubation, the cells were washed twice with PBS, and cellular uptake of OSCC-derived exosomes and FITC-dextran was analyzed on a FACScan cytometer using CELLQUEST (Becton Dickinson).

2.4 | Protein extraction and western blot analysis

Total cells from cell cultures and proteins from HSC-4-derived exosomes were lysed using radioimmunoprecipitation assay buffer (50 mmol/L; Tris pH 7.4, 150 mmol/L of NaCl, 1 mmol/L of EDTA, 0.25% sodium deoxycholate, 1.0% NP-40, and protease inhibitors). Protein concentrations were then determined using a BCA assay. The extracted proteins (50 μ g/lane) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. Thereafter, the membranes were blocked in Tris-buffered saline containing 5% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20 at 4°C overnight and then probed with primary antibodies against CD9 (1:1,000; cat. no. ab92726; Abcam), CD63 (1:1,000; cat. no. sc-5275; Santa Cruz Biotechnology), calnexin (1:200; cat. no. MAB3126; EMD Millipore), cytochrome C (1:1,000; cat. no. 556433; BD Biosciences), phosphorylated (Tyr1068) EGFR (p-EGFR; 1:1000; cat. no. cs3777; Cell Signaling Technology), EGFR (1:1000; cat. no. cs4267; Cell Signaling Technology), phosphorylated (Ser473) AKT (p-AKT; 1:2000; cat. no. cs4060; Cell Signaling Technology), AKT (1:2000; cat. no. cs4691; Cell Signaling Technology), phosphorylated (Thr202/Tyr204) ERK (p-ERK; 1:2000; cat. no. cs4370; Cell Signaling Technology), ERK (1:2000; cat. no. cs4695; Cell Signaling Technology, Inc), E-cadherin (1:1000; cat. no. cs3915; Cell Signaling Technology), N-cadherin

(1:1000; cat. no. BD610920; BD Transduction Laboratories.), vimentin (1:1000; cat. no. cs3932; Cell Signaling Technology), CD44 (1:1000; cat. no. 15675-1-AP; Proteintech Group), Oct4 (1:1000; cat. no. 11263-1-AP; Proteintech Group), SOD2 (1:1000; cat. no. BD611580; BD Transduction Laboratories.), SOD1, catalase, and thioredoxin (1:250; cat. no. ab179843; Abcam), and β -actin (1:500; cat. no. ab8226; Abcam). After incubating the signals with horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (cat. no. NA9310) or HRP-conjugated anti-rabbit IgG secondary antibody (cat. no. NA9340) (both 1:2000; both from GE Healthcare) for 1 hour at room temperature, signal detection was performed using an enhanced chemiluminescence system (GE Healthcare).

2.5 | Cell proliferation assay

Cell proliferation was analyzed using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies) assay. A total of 5×10^3 cells/well HSC-4 cells were seeded in 96-well culture plates and cultured for 24 hours followed by incubation with HSC-4-derived exosomes with or without 100 μ mol/L of 5-fluorouracil (5-FU), 25 μ mol/L of CDDP, 10 μ mol/L of erlotinib, 100 μ g/mL of cetuximab, or 5 mmol/L of NAC for 24 hours. Thereafter, 10 μ L of CCK-8 solution was added to each well followed by incubation for an additional 2 hours at 37°C. Absorbance was then measured at 450 nm using a microplate reader. Each experiment was performed in triplicate.

2.6 | Migration assay

The migratory potential of the cells was examined using the CytoSelect 24-well cell migration assay (Cell Biolabs). Briefly, a total of 2.5×10^5 cells/well HSC-4 cells were seeded into 24-well plates containing proprietary-treated plastic inserts and maintained in culture for 24 hours. After the inserts were removed, the cells were treated with HSC-4-derived exosomes with or without 10 μ mol/L of erlotinib or 100 μ g/mL of cetuximab for 10 hours at 37°C. After staining with 0.5% crystal violet in 10% ethanol for 10 minutes at room temperature, the percentage of closure in the wound field was determined using light microscopy at 40 \times magnification. Each experiment was performed in triplicate.

2.7 | Invasion assay

The invasive potential of the cells was evaluated using the BioCoat Matrigel Invasion Chamber kit (BD Biosciences). Briefly, HSC-4 cells were added to the Transwell insert chamber containing a filter coated with Matrigel at a density of 1.5×10^2 cells/ μ L. In the lower compartment, 750 μ L of DMEM containing 10% FBS was used as the chemoattractant. The HSC-4 cells were incubated with HSC-4-derived exosomes with or without 10 μ mol/L

of erlotinib or 100 $\mu\text{g}/\text{mL}$ of cetuximab for 24 hours at 37°C. After removing the inserts, non-invading cancer cells remaining on the upper side of the filter were scraped off. Cells that invaded the lower side of the filter were then stained with the Diff-Quick solution (Sysmex Corporation) at room temperature for 10 minutes, observed under a light microscope, and counted over five randomly selected fields at 200 \times magnification. Each experiment was performed in triplicate.

2.8 | Shere formation assay

To allow sphere formation, 1×10^3 cells were seeded into the 96-well U-bottom low adherence plates (Thermo Fisher Scientific Nunc, cat. no. 174925) and cultured with or without 100 $\mu\text{g}/\text{mL}$ of HSC-4-derived exosomes, 10 $\mu\text{mol}/\text{L}$ of erlotinib, or 100 $\mu\text{g}/\text{mL}$ of cetuximab for 7 days. The surface area was analyzed using a Keyence BZ-X800 fluorescence microscope and BZ-X800 Analyzer software (Keyence) following the manufacturer instructions. Each experiment was performed in triplicate.

2.9 | Colony formation assay

To allow colony formation, 1×10^3 cells were seeded in six-well culture plates and cultured with or without 100 $\mu\text{g}/\text{mL}$ of HSC-4-derived exosomes, 100 $\mu\text{mol}/\text{L}$ of 5-FU, 25 $\mu\text{mol}/\text{L}$ of CDDP, 10 $\mu\text{mol}/\text{L}$ of erlotinib, or 100 $\mu\text{g}/\text{mL}$ of cetuximab for 24 hours at 37°C. Then, the medium was replaced with DMEM supplemented with 10% (v/v) FBS and cultured for 7 days. The colony number in each well was counted under a light microscope. Each experiment was performed in triplicate.

2.10 | Flow cytometry

For the stemness assay, cells with an anti-CD44-PE/Cy7 antibody (BioLegend, cat. no. 372810) and submitted for analysis on a FACScan cytometer using FlowJo software (version 10; BD Biosciences). For the apoptosis assay, cells were stained with propidium iodide and FITC-conjugated annexin V and analyzed on a FACScan cytometer using FlowJo software (version 10; BD Biosciences). Each experiment was performed in triplicate.

2.11 | Measurement of intracellular reactive oxygen species levels

Cells were incubated with 5 $\mu\text{mol}/\text{L}$ DCFH-DA (dichlorodihydrofluorescein diacetate) (Molecular Probes) for 1 hour at 37°C. Then, the cells were treated with or without 100 $\mu\text{g}/\text{mL}$ of HSC-4-derived exosomes, 100 $\mu\text{mol}/\text{L}$ of 5-FU, or 25 $\mu\text{mol}/\text{L}$ of CDDP for 24 hours and analyzed using a ThermoLab System Fluoroskan Ascent FL (Thermo Electron).

2.12 | Statistical analysis

Data are presented as means \pm standard deviations. Statistical differences among experimental conditions were determined using one-way analysis of variance (ANOVA) or two-way ANOVA followed by Tukey's multiple comparisons test. All statistical analyses were performed using BellCurve for Excel (Social Survey Research Information), with $P < .05$ indicating statistical significance.

3 | RESULTS

3.1 | Time- and dose-dependent uptake of HSC-4-derived exosomes by HSC-4 cells themselves

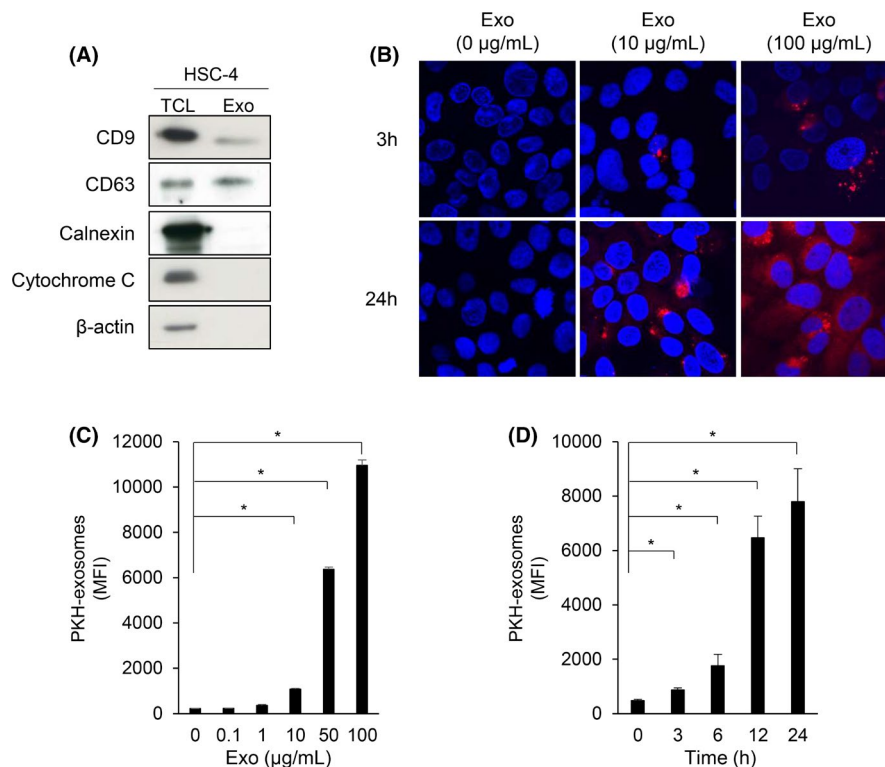
HSC-4-derived exosomes were initially characterized using western blot analysis. Accordingly, HSC-4-derived exosomes expressed CD9 and CD63, which are used as exosomal markers (Figure 1A). Conversely, calnexin and cytochrome C were not detectable in the exosomal lysates (Figure 1A). To study the uptake of isolated exosomes, HSC-4-derived exosomes were treated with PKH26 and PKH67, two fluorescent dyes with long aliphatic tails that are incorporated into the lipid membrane of exosomes. After incubating HSC-4 cells with PKH26-labeled HSC-4-derived exosomes, confocal laser microscopy revealed PKH26-positive granules in the cytoplasm of HSC-4 cells. Notably, HSC-4 cells treated with 100 $\mu\text{g}/\text{mL}$ of HSC-4-derived exosomes at 24 hours had more diffuse granules in their cytosol compared to those treated with 10 $\mu\text{g}/\text{mL}$ of exosomes at 3 hours (Figure 1B). Flow cytometric analysis also revealed a dose- and time-dependent increase in the uptake of both PKH67-labeled HSC-4-derived exosomes by HSC-4 cells (Figure 1C,D). The aforementioned data suggested that HSC-4 cells themselves dose- and time-dependently take up HSC-4-derived exosomes.

3.2 | HSC-4-derived exosomes promote the malignant potential of HSC-4 cells

To determine the autocrine or paracrine effects of HSC-4-derived exosomes, we determined the effects of HSC-4-derived exosomes on the malignant potential of HSC-4 cells themselves. Accordingly, HSC-4-derived exosomes dose-dependently facilitated proliferation (Figure 2A) and migration of HSC-4 cells (Figure 2B). The increase in the number of HSC-4 cells invading the Matrigel depended on the concentration of the HSC-4-derived exosomes (Figure 2C). Furthermore, treatment with 100 $\mu\text{g}/\text{mL}$ of exosomes increased the population of cancer stem-like cells expressing CD44 and the size of spheres (Figure 2D,E).

We previously reported that OSCC-derived exosomes promoted the malignant potential of OSCC cells through the activation of AKT and ERK signaling pathways, which are involved in the survival of various cell types. In this experiment, HSC-4-derived exosomes also

FIGURE 1 Characterization and cellular internalization of HSC-4-derived exosomes. A, TCL and Exo lysates of HSC-4 cells were analyzed by western blotting. B, HSC-4 cells were incubated with or without PKH26-labeled Exo (red) derived from HSC-4 cells for 3 and 24 h and analyzed using confocal microscopy. Nuclei were stained with DAPI (blue) (400 \times magnification). C and D, Uptake of PKH67-labeled HSC-4-derived exosomes was analyzed using flow cytometry at different Exo doses for 24 h (C) and at different time-points in the presence of 100 μ g/mL HSC-4-derived Exo (D). CD9, CD9 antigen; CD63, CD63 antigen; Exo, exosomes; MFI, mean fluorescence intensity; TCL, total cell lysate



promoted the phosphorylation of AKT and ERK but not EGFR. Apart from the promotion of migration, invasion, and stemness of HSC-4 cells, these exosomes increased the expression of mesenchymal markers, including N-cadherin and vimentin, and stemness markers, such as CD44 and Oct4 (Figure 2F). The aforementioned results suggested that OSCC cells may increase their malignant behavior through the secretion and uptake of OSCC cell-derived exosomes.

3.3 | HSC-4-derived exosomes attenuate the chemosensitivity of HSC-4 cells

HSC-4-derived exosomes also significantly attenuated the 5-FU- or CDDP-mediated inhibition of HSC-4 proliferation and colony formation ability (Figure 3A,B). Furthermore, the treatment of HSC-4 cells with 5-FU or CDDP increased the percentage of apoptotic cells, which was inhibited after adding HSC-4-derived exosomes to the cell culture (Figure 3C). To elucidate the mechanism of OSCC cell-derived exosome-induced chemoresistance, we analyzed the involvement of intracellular reactive oxygen species (ROS). Treatment with chemotherapeutic drugs induced the generation of ROS in HSC-4 cells, but the effects were inhibited by HSC-4-derived exosomes (Figure 3D). HSC-4-derived exosomes induced the expression of some antioxidative enzymes including catalase, SOD1, and thioredoxin regardless of the presence of chemotherapeutic drugs (Figure 3E). Next, we examined the effects of an antioxidant NAC to investigate whether the decrease in intracellular ROS levels induced by HSC-4-derived exosomes regulated the chemosensitivity of HSC-4 cells. NAC prohibited chemotherapeutic drug-induced antiproliferative effects and apoptosis (Figure 3F,G), and the effects were promoted by adding

HSC-4-derived exosomes. The aforementioned results suggested that OSCC cell-derived exosomes may decrease the chemosensitivity of OSCC cells through the attenuation of the effects of chemotherapeutic drugs on intracellular ROS regulation.

3.4 | Epidermal growth factor/epidermal growth factor receptor signaling regulates exosome uptake in HSC-4 cells

To verify whether OSCC cell-derived exosomes are taken by OSCC cells through EGF/EGFR signaling activation, both control and *EGFR*-knockdown HSC-4 cells were treated with recombinant human EGF. Accordingly, rhEGF treatment time- and dose-dependently increased the uptake of HSC-4-derived exosomes (Figure 4A,C). Conversely, *EGFR* knockdown suppressed the internalization of exosomes both with and without rhEGF (Figure 4A,C). Concerning the activation of EGFR signaling, rhEGF treatment activated the phosphorylation of EGFR and its downstream signaling pathways AKT and ERK at 1 hour, with the activation status being maintained until 6 hours in control cells and then gradually decreasing until 24 hours (Figure 4B). In *EGFR* knockdown cells, EGFR phosphorylation was induced only at 1 and 3 hours, with the level of AKT and ERK phosphorylation being lower compared to control cells (Figure 4B). rhEGF treatment dose-dependently induced EGFR, AKT, and ERK phosphorylation at 24 hours, although no dose-dependent activation of EGFR signaling was observed in *EGFR*-knockdown cells (Figure 4D). The aforementioned results suggested that HSC-4 cells uptake their own exosomes through the activation of the EGF/EGFR signal pathways.

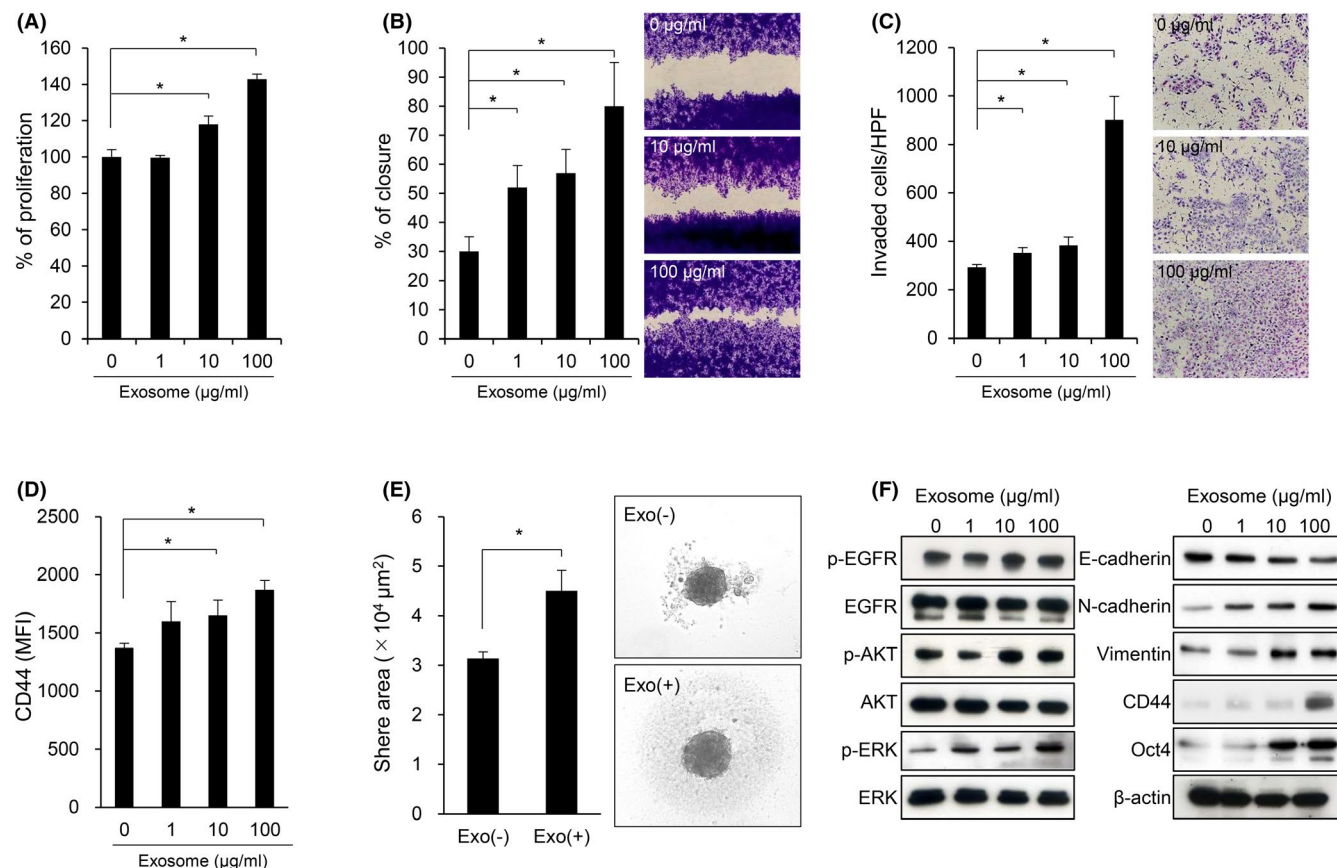


FIGURE 2 Effects of HSC-4-derived exosomes on the malignant potential of HSC-4 cells. HSC-4 cells were incubated with or without HSC-4-derived Exo (1, 10, or 100 µg/mL) for 24 h. A, The viability was assessed using Cell Counting Kit-8 assay. B, Wound healing assay to determine the migratory capacity of HSC-4 cells treated with HSC-4-derived Exo. Magnification, 40 \times . C, Invasion assay to determine the invasive capacity of HSC-4 cells treated with HSC-4-derived Exo. Magnification, 100 \times . D, Expression of CD44 of HSC-4 cells was analyzed using flow cytometry. E, Sphere area and phase-contrast photomicrographs of spheres formed from HSC-4 cells growing in low-adhesive 96-well culture plates. F, TCL from HSC-4 cells treated with HSC-4-derived Exo were analyzed using western blotting. * $P < .05$ vs cells in the absence of Exo. Exo, exosomes; MFI, mean fluorescence intensity

3.5 | Involvement of macropinocytosis with epidermal growth factor/epidermal growth factor receptor signaling-regulated uptake of exosomes in HSC-4 cells

To verify whether OSCC cell-derived exosomes were taken up by OSCC cells through the activation of the macropinocytosis pathway via EGFR stimulation, HSC-4 cells were treated with two types of EGFR inhibitors: erlotinib (an EGF receptor tyrosine kinase inhibitor) and cetuximab (a recombinant human/mouse chimeric EGFR monoclonal antibody) with or without rhEGF for 24 hours. PKH26-positive granules were observed in the cytoplasm of HSC-4 cells and appeared more diffuse during confocal laser microscopy in the presence of rhEGF (Figure 5A). Co-localization of PKH26-labeled HSC4-derived exosomes and FITC-dextran, a probe for the macropinocytosis pathway, was observed 24 hours after treatment. Conversely, treatment with the macropinocytosis inhibitor EIPA inhibited the uptake of both exosomes and dextran. In the presence of erlotinib and cetuximab, the internalization of PKH26-labeled exosomes was also suppressed.

To thoroughly determine the inhibitory effects of EGFR inhibitors on macropinocytosis, the time course of dextran uptake was verified using flow cytometry. rhEGF treatment time-dependently increased the uptake of dextran, with a 1.8-fold higher uptake of dextran having been observed at 24 hours compared to the control, suggesting that EGF/EGFR signaling activated macropinocytosis in HSC-4 cells (Figure 5B). Although no obvious suppressive effects of EIPA on dextran uptake were observed until 6 hours, treatment for 24 hours inhibited both EGF-induced and control dextran uptake. Erlotinib showed the strongest inhibitory effect on dextran uptake, completely abolishing rhEGF-induced uptake. Conversely, cetuximab time-dependently suppressed dextran uptake, albeit weakly, regardless of the presence of rhEGF.

Thereafter, the time course of HSC-4-derived exosome uptake was verified using flow cytometry. Accordingly, rhEGF increased exosome uptake by 1.8-fold at 24 hours (Figure 5C). Remarkable suppressive effects by EIPA on exosome uptake were observed at 24 hours. Erlotinib also showed strong inhibitory effects on exosome uptake at 24 hours, completely abolishing rhEGF-induced effects. In contrast, cetuximab time-dependently suppressed

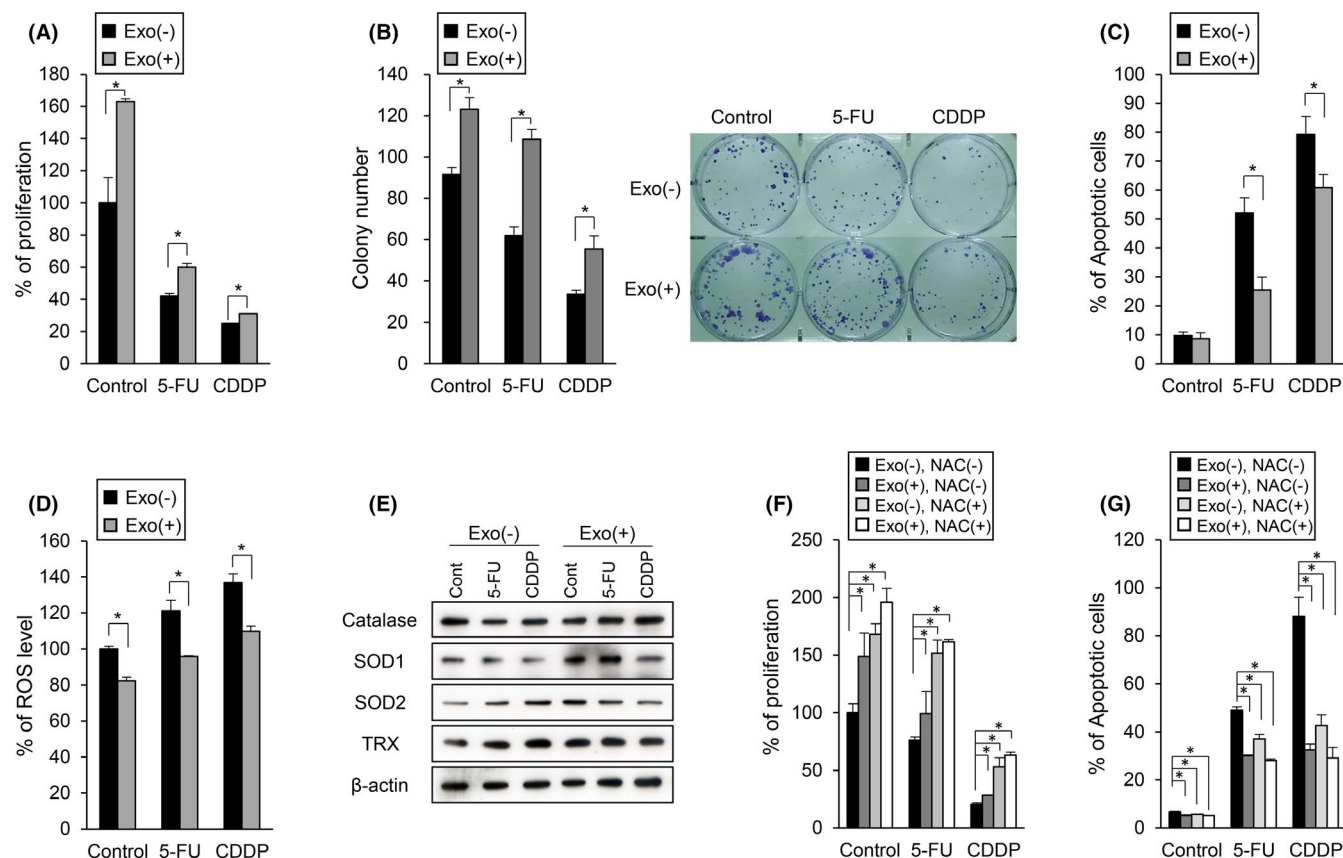


FIGURE 3 Effects of HSC-4-derived exosomes on the chemosensitivity of HSC-4 cells. A, HSC-4 cells were incubated with 100 μ g/mL HSC-4-derived Exo for 24 h with or without 100 μ mol/L of 5-FU or 25 μ mol/L of CDDP, after which cell proliferation was assessed using the Cell Counting Kit-8 assay. B, The effect of HSC-4-derived Exo on colony formation in HSC-4 cells. Colony numbers and representative images are shown. C, Apoptosis was evaluated using flow cytometry. D, The intracellular ROS levels were estimated by fluorometer after DCFH-DA staining. E, TCL from HSC-4 cells were analyzed using western blotting. F, HSC-4 cells were incubated with 100 μ g/mL HSC-4-derived Exo for 24 h with or without 100 μ mol/L of 5-FU, 25 μ mol/L of CDDP, or 5 mM of NAC, after which cell proliferation was assessed using the Cell Counting Kit-8 assay. G, Apoptosis was evaluated using from cytometry. * P < .05 vs cells in the absence of Exo. CDDP, cis-diamminedichloroplatinum; Exo, exosomes; 5-FU, 5-fluorouracil; MFI, mean fluorescence intensity; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species; SOD, superoxide dismutase; TRX, thioredoxin

exosome uptake at 24 hours, albeit weakly, regardless of the presence of rhEGF, suggesting that HSC-4 cells uptake exosomes through the activation of macropinocytosis via EGF/EGFR signaling. Moreover, we showed that erlotinib more effectively inhibited exosome uptake compared to cetuximab. In other OSCC cells, including SAS, HSC-2, and HSC-3 cells, rhEGF treatment also increased the uptake of dextran and OSCC-derived exosomes. In contrast, EIPA and EGFR inhibitors suppressed the uptake of dextran and OSCC-derived exosomes both with and without rhEGF (Figure S1).

Furthermore, we investigated whether EGFR inhibitors suppressed EGF-activated signal transduction. As shown in Figure 5D, an increase in EGFR, ERK, and AKT phosphorylation was observed in HSC-4 cells treated with rhEGF for 24 hours. Although both EGFR inhibitors suppressed EGFR phosphorylation, erlotinib alone completely abolished AKT and ERK phosphorylation. In the presence of rhEGF, no obvious suppressive effects on AKT and ERK phosphorylation were observed with cetuximab treatment. Although EIPA slightly suppressed EGFR phosphorylation both with and without rhEGF, EIPA did not influence

the phosphorylation status of AKT and ERK. The aforementioned results suggested that OSCC cells uptake their own exosomes through the macropinocytosis pathway via EGFR stimulation.

3.6 | Suppression of exosome-induced malignant potential of HSC-4 cells by epidermal growth factor receptor inhibitors

To examine whether the inhibition of OSCC cell-derived exosome uptake through EGFR inhibitors was involved with OSCC cell behavior, HSC-4 cells were treated with EGFR inhibitors in the presence or absence of OSCC cell-derived exosomes in vitro. Each inhibitor inhibited cell proliferation even in the absence of exosomes while also suppressing exosome treatment-induced proliferation (Figure 6A). Furthermore, treatment with each inhibitor suppressed HSC-4 cell migration, invasion, and stemness both with and without HSC-4-derived exosomes (Figure 6B-E).

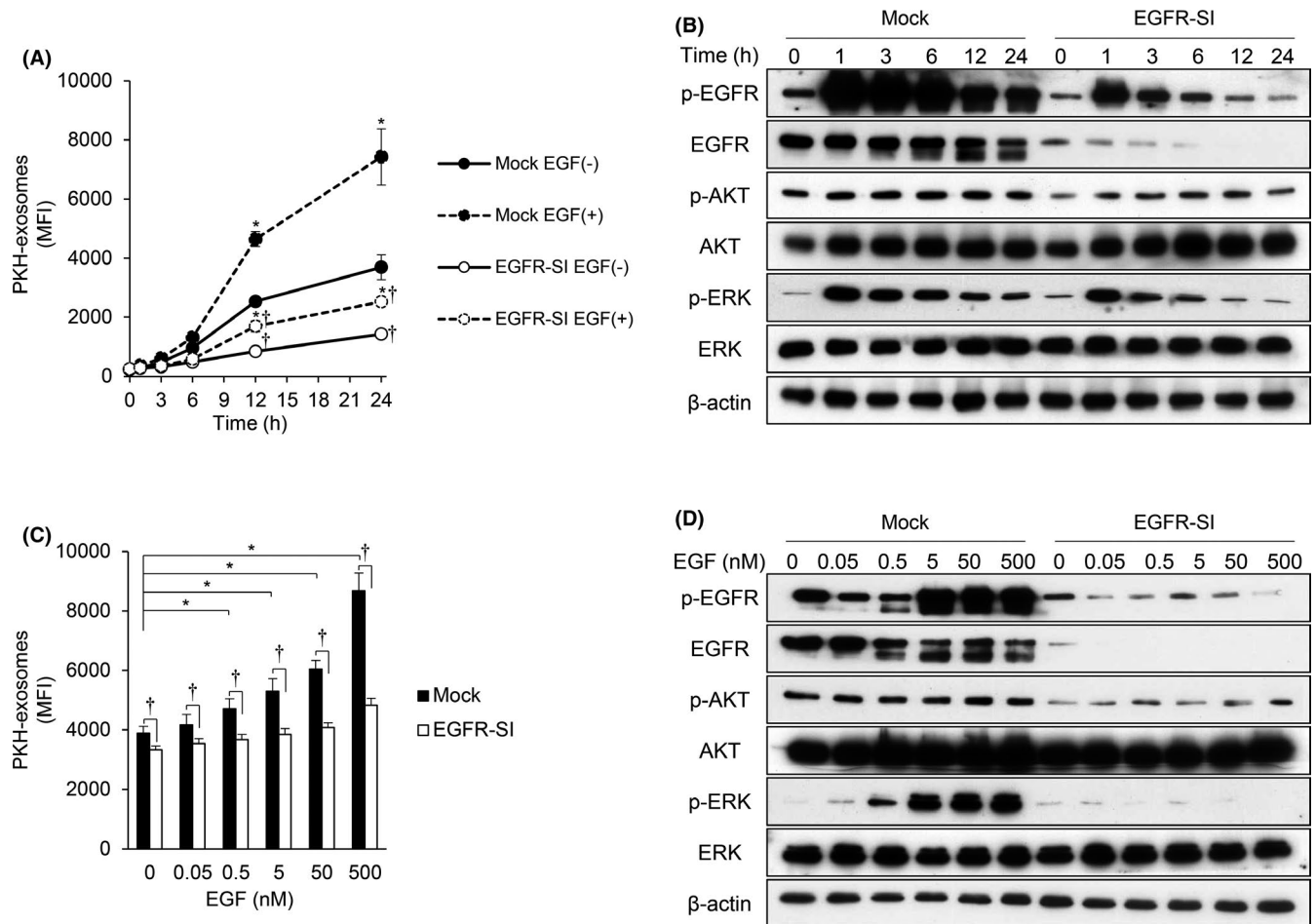


FIGURE 4 Effects of epidermal growth factor (EGF)/epidermal growth factor receptor (EGFR) signaling on the cellular internalization of HSC-4-derived exosomes. **A**, HSC-4 cells were transiently transfected with *EGFR* siRNA and cultured for 48 h. Next, cells were cultured with 25 μ M PKH26-labeled Exo derived from HSC-4 cells for the indicated times with or without 500 nmol/L of rhEGF and analyzed using flow cytometry. **B**, Total cell lysate (TCL) from control and *EGFR*-siRNA transfected cells treated with 500 nmol/L of rhEGF for the indicated times were analyzed using western blotting. **C**, HSC-4 cells were cultured with 25 μ M PKH67-labeled Exo derived from HSC-4 cells at different rhEGF concentrations for 24 h and analyzed using flow cytometry. **D**, TCL from control and *EGFR*-siRNA transfected cells treated with different concentrations of rhEGF for 24 h were analyzed using western blotting. * $P < .05$ vs cells in the absence of rhEGF and † $P < .05$ vs mock-transfected cells

3.7 | Suppression of exosome-induced chemoresistance of HSC-4 cells by epidermal growth factor receptor inhibitors

Treatment with erlotinib and cetuximab also had profound inhibitory effects on proliferation and colony formation ability in the presence or absence of HSC-4-derived exosomes (Figure 7A,B). Furthermore, treatment with each EGFR inhibitor increased the percentage of apoptotic cells and abolished the antiapoptotic effects induced by HSC-4-derived exosomes (Figure 7C).

4 | DISCUSSION

Cancer cells secrete aberrantly large amounts of exosomes that are taken up by various types of cells, including cancer cell themselves, stromal cells, and immune cells, which constitute the tumor

microenvironment. Evidence has shown that the molecular and genetic contents of cancer cell-secreted exosomes can influence the phenotype of the incorporated cells through the activation of intracellular signal pathways, as well as promote cancer progression and poor prognosis.²¹ Our group, along with other researchers, had previously reported that OSCC cell-secreted exosomes were taken up by OSCC cells themselves, subsequently promoting proliferation, migration, and invasion leading to cancer progression.^{8,22,23} In our experiments, other human oral squamous cell carcinoma cell lines, such as HSC-4, also secreted exosomes that promoted their malignant potential through the uptake of self-derived exosomes. The aforementioned findings suggested that OSCC cells can acquire more malignant biological behavior by taking up their own exosomes. Therefore, therapeutic approaches aimed at inhibiting the formation, secretion, and incorporation of tumor cell-secreted exosomes can prevent OSCC development.

The molecular mechanisms of the materials in OSCC cell-derived exosomes that regulate the malignant potential of OSCC cells remain

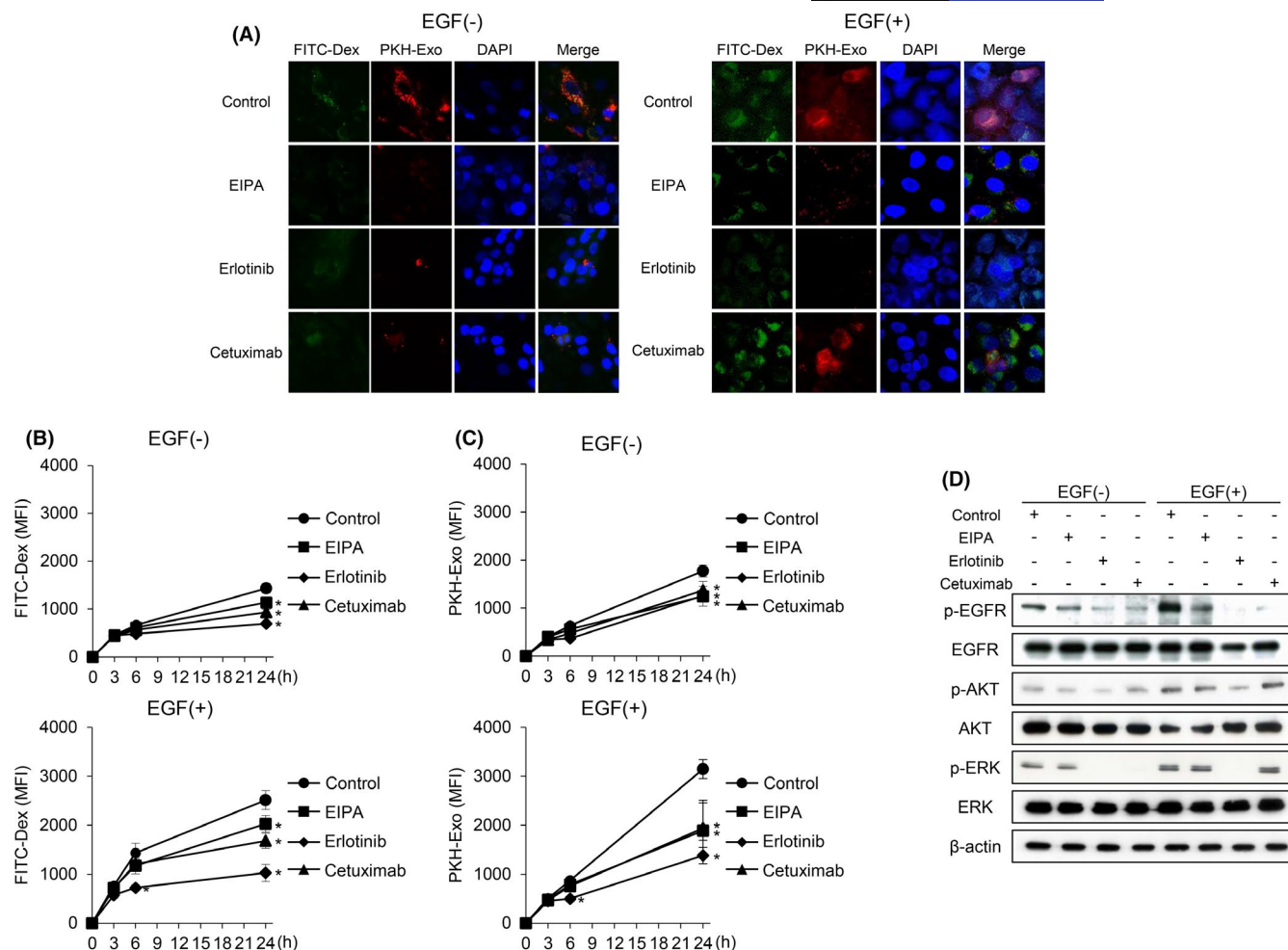


FIGURE 5 Effects of EIPA and EGFR inhibitors on the uptake of HSC-4-derived exosomes. A, HSC-4 cells were treated with 0.5 mg/mL FITC-dextran or 25 μ g/mL PKH26-labeled Exo derived from HSC-4 cells with or without 500 nmol/L of rhEGF, 25 μ mol/L of EIPA, 10 μ mol/L of erlotinib, or 100 μ g/mL of cetuximab for 24 h. After incubation, cellular uptake was analyzed using confocal microscopy. B and C, Internalization of FITC-dextran (B) or PKH67-labeled Exo (C) was analyzed using flow cytometry after treatment for 3, 6, and 24 h. $^*P < .05$ vs cells in the absence of EIPA or EGFR inhibitors. D, TCL from control and rhEGF-treated cells with or without EIPA or EGFR inhibitors for 24 h were analyzed using western blotting. Dex, dextran; EGFR, epidermal growth factor receptor; Exo, exosomes; MFI, mean fluorescence intensity

to be fully elucidated. Among the bioactive molecules of exosomes, transferred regulatory miRNAs can epigenetically alter gene function in the recipient cell. Because it has been reported that some miRNA contained in tumor cell-derived exosomes, such as miR-21, miR-342-3p, and miR-1246, promote cancer progression through activation of AKT and ERK signaling pathways, these exosome-containing miRNAs may also be involved in the promotion of malignant potential in OSCC cells.^{22,24} Furthermore, various mechanisms of chemoresistance induced by OSCC cell-derived exosomes were reported, such as shuttling of miRNA content, drug efflux, alteration of vesicular pH, anti-apoptotic signaling, modulation of DNA damage repair, immunomodulation, epithelial-to-mesenchymal transition, and maintenance of tumor by cancer stem cells.²⁵ Chemotherapeutic drug-induced cytotoxicity and apoptosis are also regulated by ROS generation. It has also been shown that exosomes conferred chemoresistance to pancreatic cancer cells by promoting ROS detoxification, through lateral transfer of SOD2 and catalase transcripts.²⁶ We

showed that HSC-4-derived exosomes induced the expression of some antioxidant enzymes and decreased the intracellular ROS level in HSC-4 cells. These results suggested that OSCC cells may acquire malignant potential and chemoresistance through transfer of OSCC cell-derived exosomal miRNAs or transcripts.

The EGFR is often overexpressed in OSCC cells, while the activation of EGF/EGFR signaling can promote malignant potential, such as proliferation, migration, invasion, epithelial-mesenchymal transition, and cancer stem-like cell properties through the activation of downstream signal cascades, such as the RAS/ERK, PI3K/AKT, and the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway.²⁷⁻²⁹ EGF can also stimulate the induction of macropinocytosis, an actin-driven endocytic process whereby membrane ruffles fold back onto the plasma membrane to form large (>0.2 μ m in diameter) endocytic organelles called macropinosomes. Cellular uptake of exosomes through macropinocytosis which is induced by the activation of EGF-EGFR signaling or

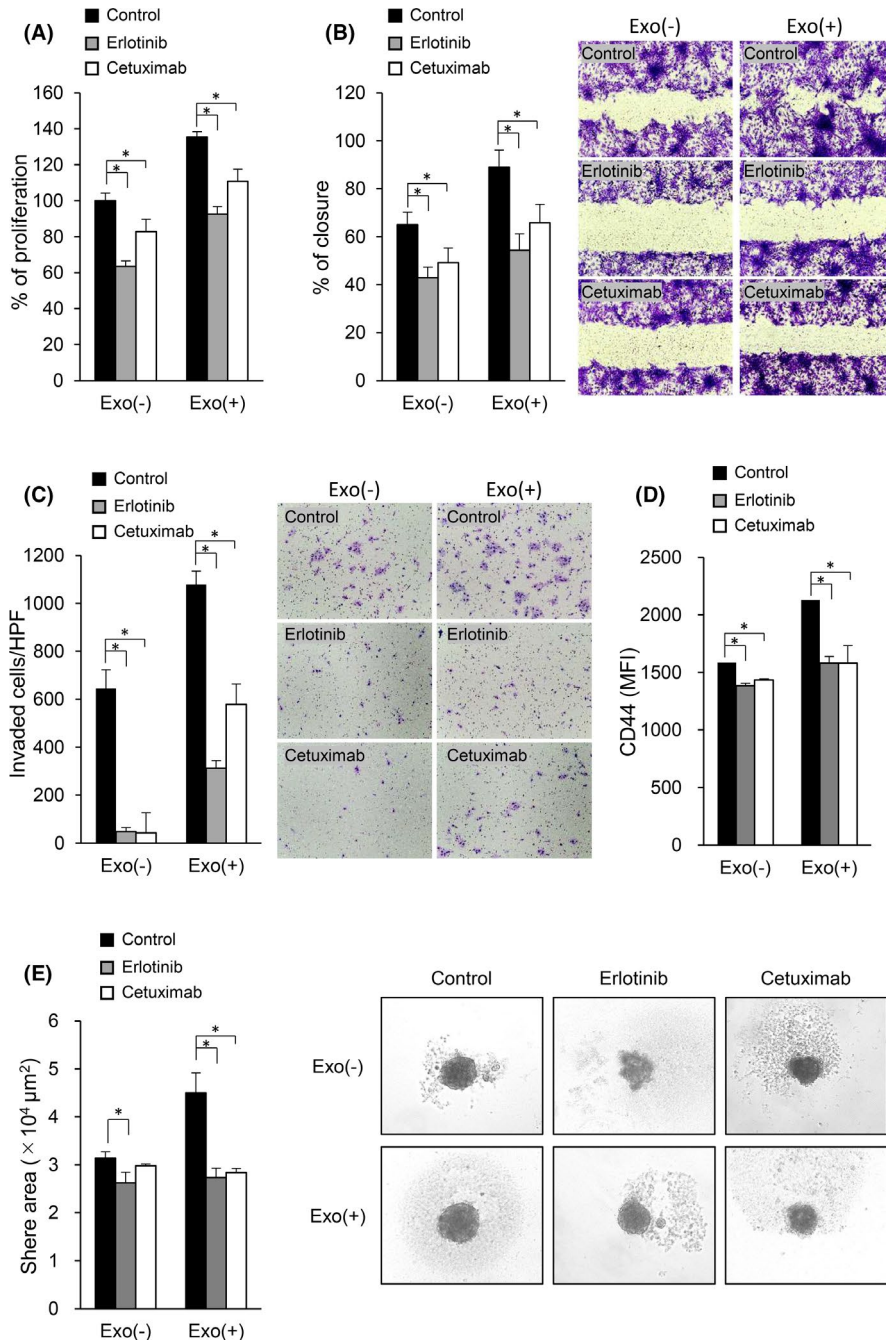
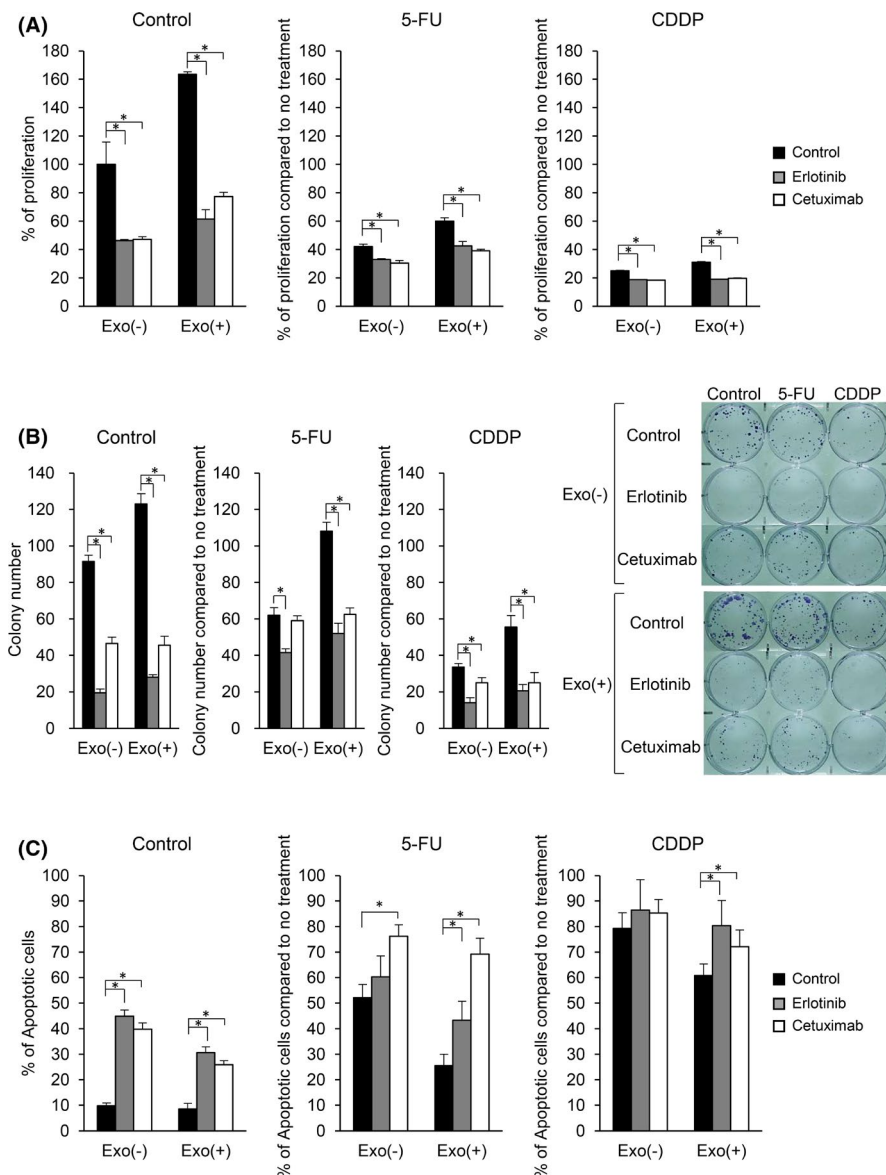


FIGURE 6 Effects of epidermal growth factor receptor (EGFR) inhibitors on HSC-4-derived exosome-induced malignant potential of HSC-4 cells. Control and 100 $\mu\text{g}/\text{mL}$ of HSC-4-derived Exo-treated HSC-4 cells were incubated with or without 10 $\mu\text{mol}/\text{L}$ of erlotinib or 100 $\mu\text{g}/\text{mL}$ of cetuximab for 24 h. A, Viability was assessed using the Cell Counting Kit-8 assay. B, A wound healing assay was performed to determine the migratory capacity of HSC-4 cells. C, An invasion assay was performed to determine the invasive capacity of HSC-4 cells. D, Expression of CD44 of HSC-4 cells was analyzed using flow cytometry. E, Sphere area and phase-contrast photomicrographs of spheres formed from HSC-4 cells growing in low-adhesive 96-well culture plates. * $P < .05$ vs cells in the absence of EGFR inhibitors. Exo, exosomes

oncogenic Ras was reported in A431 human epidermoid carcinoma cells and non-small cell lung cancer cells.^{13,30} We showed that EGF/EGFR signaling stimulation in HSC-4 cells following rhEGF treatment time- and dose-dependently increased the uptake of HSC-4-derived exosomes. Conversely, EGFR knockdown of HSC-4 cells through of EGFR-siRNA transfection remarkably suppressed the uptake of HSC-4-derived exosomes. Furthermore, FITC-dextran and PKH-26-labeled HSC-4-derived exosomes had been found to be co-localized in the cytosol of HSC-4 cells, with two types of EGFR inhibitors, erlotinib and cetuximab, as well as EIPA (a macropinocytosis inhibitor) suppressing exosome uptake with or without rhEGF. The aforementioned findings suggested that EGF/EGFR signaling-induced macropinocytosis might be involved in the uptake of OSCC cell-derived

exosomes by OSCC cells themselves. Anti-EGFR therapeutic agents (mAb and TKI) are currently being developed for the treatment of patients with advanced OSCC,^{19,20} while cetuximab has become a standard therapeutic regimen in the treatment of oral cancer.³¹⁻³³ In our experiments, both types of anti-EGFR agents inhibited the proliferation, migration, invasion, stemness, and chemoresistance of OSCC cells that had taken up OSCC cell-derived exosomes, suggesting their potential efficacy for not only direct blockage of EGF/EGFR downstream signal cascades, such as ERK and AKT-induced malignant transformation of OSCC cells, but also inhibition of exosome uptake via macropinocytosis. Multiple studies have shown that blocking exosomal uptake can be a potentially effective method for cancer therapy.³⁴ Various endocytosis inhibitors, including heparin,

FIGURE 7 Effects of epidermal growth factor receptor (EGFR) inhibitors on HSC-4-derived exosome-induced chemoresistance of HSC-4 cells. **A**, Control and 100 $\mu\text{g}/\text{mL}$ of HSC-4-derived Exo-treated cells were incubated with 100 $\mu\text{mol}/\text{L}$ of 5-FU, 25 $\mu\text{mol}/\text{L}$ of CDDP, 10 $\mu\text{mol}/\text{L}$ of erlotinib, or 100 $\mu\text{g}/\text{mL}$ of cetuximab for 24 h, after which cell proliferation was assessed using the Cell Counting Kit-8 assay. **B**, The effect of EGFR inhibitors on colony formation in HSC-4 cells. Colony numbers and representative images are shown. **C**, Apoptosis was evaluated using flow cytometry. $P < .05$ vs cells in the absence of EGFR inhibitors. CDDP, cis-diamminedichloroplatinum; Exo, exosomes; 5-FU, 5-fluorouracil



methyl- β cyclodextrin, and genistein, have been reported to abrogate exosome endocytosis in cancer cells and inhibit phosphorylation of intracellular signaling pathways, such as ERK.^{8,35-37} Although cytochalasin D, dimethyl amiloride, and EIPA have been reported as macropinocytosis inhibitors, clinical trials must verify their efficacy and complications before these inhibitors can be clinically applied, which requires a considerable amount of time.³⁷⁻³⁹ As such, anti-EGFR agents may be an effective therapeutic strategy given that they have already been clinically applied in the treatment of patients with OSCC and their side effects and complications have been clearly determined.

In our experiments, erlotinib had stronger suppressive effects on OSCC-derived exosome-induced malignant potential regardless of the presence of rhEGF. Conversely, cetuximab had weak effects in the presence of large amounts of rhEGF. When a large amount of EGF is present in the tumor environment, administering massive amounts of anti-EGFR agents may be necessary to block EGF/EGFR signaling. It has also been shown that EGFR was abundantly

expressed in cancer cell-derived exosomes, which could activate survival-stimulating pathways, such as the MAPK and AKT pathway, by transporting functional EGFR.⁴⁰ Therefore, the activation of the AKT and ERK pathways induced by HSC-4-derived exosomes may be related to the transport of EGFR expressed in OSCC cell-derived exosomes into OSCC cells, with TKI inhibiting the activation of internalized EGFR. These results suggested that TKI may be more effective than monoclonal antibodies for the treatment of patients with OSCC. Furthermore, some studies have shown that tumor cells secrete exosomes containing EGFR that regulate the signaling pathway of endothelial cells, monocytes, and T cells in the tumor microenvironment.⁴¹⁻⁴³ Zhang H et al reported that EGFR contained in gastric cancer cells exosomes promoted liver metastasis by inducing hepatocyte growth factor via the suppression of miR-26a/b in liver stromal cells.⁴⁴ Moreover, it was shown that analysis of the content of exosomal EGFR expression or EGFR mutation may allow prediction of tumorigenesis, clinical stage, therapeutic effects of EGFR inhibitors, or prognosis using clinical specimens such as liquid biopsy

samples, including plasma, serum, saliva, or urine.⁴⁴⁻⁴⁸ Because it remains unknown whether cancer development and progression depend on the promotion of tumor cell-derived EGFR-overexpressing or EGFR-mutated exosomes uptake by tumor cells themselves clinically, further detailed analysis is required.

To conclude, our findings demonstrated that EGF/EGFR signaling pathway facilitated the uptake of tumor cell-derived exosomes via macropinocytosis and enhanced the malignant potential of OSCC cells. Anti-EGFR agents may be effective for the treatment of patients with OSCC by blocking not only the direct EGF/EGFR signaling pathway but also uptake of tumor cell-derived exosomes through macropinocytosis. Further investigations are needed to determine whether antagonizing EGF/EGFR signaling might be an effective therapeutic strategy.

DISCLOSURE

The authors have no conflicts of interest to declare.

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REFERENCES

- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394-424.
- Okura M, Aikawa T, Sawai NY, Iida S, Kogo M. Decision analysis and treatment threshold in a management for the N0 neck of the oral cavity carcinoma. *Oral Oncol*. 2009;45:908-911.
- Mydlarz WK, Hennessey PT, Califano JA. Advances and perspectives in the molecular diagnosis of head and neck cancer. *Expert Opin Med Diagn*. 2010;4:53-65.
- da Silva SD, Ferlito A, Takes RP, et al. Advances and applications of oral cancer basic research. *Oral Oncol*. 2011;47:783-791.
- Mathivanan S, Ji H, Simpson RJ. Exosomes: extra-cellular organelles important in intercellular communication. *J Proteomics*. 2010;73:1907-1920.
- Lu Y, Zheng Z, Yuan Y, et al. The emerging role of exosomes in oral squamous cell carcinoma. *Front Cell Dev Biol*. 2021;9:628103.
- Cao J, Zhang M, Xie F, et al. Exosomes in head and neck cancer: roles, mechanisms and applications. *Cancer Lett*. 2020;494:7-16.
- Sento S, Sasabe E, Yamamoto T. Application of a persistent heparin treatment inhibits the malignant potential of oral squamous carcinoma cells induced by tumor cell-derived exosomes. *PLoS One*. 2016;11:e0148454.
- Bobrie A, Krumeich S, Reyat F, et al. Rab27a supports exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can promote tumor progression. *Cancer Res*. 2012;72:4920-4930.
- Li J, Chen J, Wang S, et al. Blockage of transferred exosome-shuttled miR-494 inhibits melanoma growth and metastasis. *J Cell Physiol*. 2019;234:15763-15774.
- Mulcahy LA, Pink RC, Carter DR. Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles*. 2014;3:24641.
- Lim JP, Gleeson PA. Macropinocytosis: an endocytic pathway for internalizing large gulps. *Immunol Cell Biol*. 2011;89:836-843.
- Nakase I, Kobayashi NB, Takatani-Nakase T, Yoshida T. Active macropinocytosis induction by stimulation of epidermal growth factor receptor and oncogenic Ras expression potentiates cellular uptake efficacy of exosomes. *Sci Rep*. 2015;5:10300.
- Yoshida S, Gaeta I, Pacitto R, et al. Differential signaling during macropinocytosis in response to M-CSF and PMA in macrophages. *Front Physiol*. 2015;6:8.
- Dharmawardhane S, Schürmann A, Sells MA, et al. Regulation of macropinocytosis by p21-activated Kinase-1. *Mol Biol Cell*. 2000;11:3341-3352.
- Ang KK, Berkey BA, Tu X, et al. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer Res*. 2002;62:7350-7356.
- Chung CH, Ely K, McGavran L, et al. Increased epidermal growth factor receptor gene copy number is associated with poor prognosis in head and neck squamous cell carcinomas. *J Clin Oncol*. 2006;24:4170-4176.
- Temam S, Kawaguchi H, El-Naggar AK, et al. Epidermal growth factor receptor copy number alterations correlate with poor clinical outcome in patients with head and neck squamous cancer. *J Clin Oncol*. 2007;25:2164-2170.
- Mahipal A, Kothari N, Gupta S. Epidermal growth factor receptor inhibitors: coming of age. *Cancer Control*. 2014;21:74-79.
- Loeffler-Ragg J, Schwentner I, Sprinzl GM, Zwierzina H. EGFR inhibition as a therapy for head and neck squamous cell carcinoma. *Expert Opin Investig Drugs*. 2008;17:1517-1531.
- Whiteside TL. Tumor-derived exosomes and their role in cancer progression. *Adv Clin Chem*. 2016;74:103-141.
- Sakha S, Muramatsu T, Ueda K, Inazawa J. Exosomal microRNA miR-1246 induces cell motility and invasion through the regulation of DENND2D in oral squamous cell carcinoma. *Sci Rep*. 2016;6:38750.
- Fujiwara T, Eguchi T, Sogawa C, et al. Carcinogenic epithelial-mesenchymal transition initiated by oral cancer exosomes is inhibited by anti-EGFR antibody cetuximab. *Oral Oncol*. 2018;86:251-257.
- Ma Q, Wu H, Xiao Y, et al. Upregulation of exosomal microRNA-21 in pancreatic stellate cells promotes pancreatic cancer cell migration and enhances Ras/ERK pathway activity. *Int J Oncol*. 2020;56:1025-1033.
- Law ZJ, Khoo XH, Lim PT, et al. Extracellular vesicle-mediated chemoresistance in oral squamous cell carcinoma. *Front Mol Biosci*. 2021;8:629888.
- Patel GK, Khan MA, Bhardwaj A, et al. Exosomes confer chemoresistance to pancreatic cancer cells by promoting ROS detoxification and miR-155-mediated suppression of key gemcitabine-metabolising enzyme, DCK. *Br J Cancer*. 2017;116:609-619.
- Ohnishi Y, Yasui H, Kakudo K, Nozaki M. Regulation of cell migration via the EGFR signaling pathway in oral squamous cell carcinoma cells. *Oncol Lett*. 2017;13:930-936.
- Xu Q, Zhang Q, Ishida Y, et al. EGF induces epithelial-mesenchymal transition and cancer stem-like cell properties in human oral cancer cells via promoting Warburg effect. *Oncotarget*. 2017;8:9557-9571.
- Lv XX, Zheng XY, Yu JJ, et al. EGFR enhances the stemness and progression of oral cancer through inhibiting autophagic degradation of SOX2. *Cancer Med*. 2020;9:1131-1140.
- Takenaka T, Nakai S, Katayama M, et al. Effects of gefitinib treatment on cellular uptake of extracellular vesicles in EGFR-mutant non-small cell lung cancer cells. *Int J Pharm*. 2019;572:118762.
- Mehra R, Serebriiskii IG, Dunbrack RL, et al. Protein-intrinsic and signaling network-based sources of resistance to EGFR and ErbB family-targeted therapies in head and neck cancer. *Drug Resist Updat*. 2011;14:260-279.
- Pickhard AC, Margraf J, Knopf A, et al. Inhibition of radiation induced migration of human head and neck squamous cell carcinoma cells by blocking of EGF receptor pathways. *BMC Cancer*. 2011;11:388.
- Psyrrri A, Seiwert TY, Jimeno A. Molecular pathways in head and neck cancer: EGFR, PI3K, and more. *Am Soc Clin Oncol Educ Book*. 2013;33:246-255.

34. Hayatudin R, Fong Z, Ming LC, et al. Overcoming chemoresistance via extracellular vesicle inhibition. *Front Mol Biosci.* 2021;8:629874.
35. Christianson HC, Svensson KJ, van Kuppevelt TH, Li JP, Belting M. Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc Natl Acad Sci USA.* 2013;110:17380-17385.
36. Kosgodage US, Trindade RP, Thompson PR, Inal J, Lange S. Chloramidine/Bisindolylmaleimide-I-mediated inhibition of exosome and microvesicle release and enhanced efficacy of cancer chemotherapy. *Int J Mol Sci.* 2017;18:1007.
37. Costa Verdera H, Gitz-Francois JJ, Schiffelers RM, Vader P. Cellular uptake of extracellular vesicles is mediated by clathrin-independent endocytosis and macropinocytosis. *J Control Release.* 2017;266:100-108.
38. Chalmin F, Ladoire S, Mignot G, et al. Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. *J Clin Invest.* 2010;120:457-471.
39. Lin HP, Singla B, Ghoshal P, et al. Identification of novel macropinocytosis inhibitors using a rational screen of Food and Drug Administration-approved drugs. *Br J Pharmacol.* 2018;175:3640-3655.
40. Al-Nedawi K, Meehan B, Kerbel RS, et al. Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. *Proc Natl Acad Sci USA.* 2009;106:3794-3799.
41. Huang SH, Li Y, Zhang J, et al. Epidermal growth factor receptor-containing exosomes induce tumor-specific regulatory T cells. *Cancer Investig.* 2013;31:330-335.
42. Song X, Ding Y, Liu G, et al. Cancer cell-derived exosomes induce mitogen-activated protein kinase-dependent monocyte survival by transport of functional receptor tyrosine kinases. *J Biol Chem.* 2016;291:8453-8464.
43. Sanderson MP, Keller S, Alonso A, et al. Generation of novel, secreted epidermal growth factor receptor (EGFR/ErbB1) isoforms via metal loprotease-dependent ectodomain shedding and exosome secretion. *J Cell Biochem.* 2008;103:1783-1797.
44. Zhang H, Deng T, Liu R, et al. Exosome-delivered EGFR regulates liver microenvironment to promote gastric cancer liver metastasis. *Nat Commun.* 2017;8:15016.
45. Castellanos-Rizaldos E, Grimm DG, Tadigotla V, et al. Exosome-based detection of EGFR T790M in plasma from non-small cell lung cancer patients. *Clin Cancer Res.* 2018;24:2944-2950.
46. Okuma Y, Morikawa K, Tanaka H, et al. Prospective exosome-focused translational research for afatinib study of non-small cell lung cancer patients expressing EGFR (EXTRA study). *Thorac Cancer.* 2019;10:395-400.
47. Castellanos-Rizaldos E, Zhang X, Tadigotla VR, et al. Exosome-based detection of activating and resistance EGFR mutations from plasma of non-small cell lung cancer patients. *Oncotarget.* 2019;10:2911-2920.
48. Kim Y, Shin S, Lee KA. Exosome-based detection of EGFR T790M in plasma and pleural fluid of prospectively enrolled non-small cell lung cancer patients after first-line tyrosine kinase inhibitor therapy. *Cancer Cell Int.* 2021;21:50.

SUPPORTING INFORMATION

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