

Oral cancer-derived extracellular vesicles biodistribute to lymph nodes, educating the metastatic niche.

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Abstract

Cancer extracellular vesicles (EVs) are crucial in establishing the tumor microenvironment (TME) and metastatic niches at distant organs. EVs of oral cancer, a major type of head and neck cancer, contribute to establishing the immunosuppressive TME and metastasis to lymph nodes (LNs). However, there have been limitations in methodology (cell culture media, in vivo tracking) and heterogeneity (cargo molecules, size, capacity) in EV research. Here, we generated a novel reporter system in which membrane-bound *Gaussia* luciferase (mbGLuc) is bound to EVs, thereby making EV tracking possible in vitro and in vivo. Screening of serum-free, xenofree media revealed that the Essential 8 medium most efficiently induces EV secretion among 5 medium types tested. LN-metastatic oral cancer cells and their EVs indeed biodistributed LNs in the xenograft mouse model. Proteomics of oral cancer EVs secreted by oral cancer cells (SAS, HSC-2, HSC-3, and M3) identified EpCAM signaling proteins (ADAM10/17, PSEN1, EGFR, ERK1/2, PD-L1/2, FAK1/2, AKT1/2/3, CTNNB1, and FHL2). Size-exclusion chromatography and immuno-electron microscopy revealed that LN-metastatic EVs are larger than low-metastatic EVs and contain EpCAM-rich large EVs (80–300 nm) and CD63-rich small EVs (30–100 nm). High EPCAM and CD63 expression were associated with poor prognosis in HNSC patients at early stages, whereas high ADAM10 and ADAM17 expression were associated with poor prognosis at stage III. Taken together, oral cancer EVs selectively biodistribute to LNs, where they educate metastatic niches, with EV-carried EpCAM signaling proteins contributing.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSC) ranks as the seventh most common malignancy worldwide, with approximately 890,000 new cases and 450,000 deaths annually, accounting for 4.5% of global cancer diagnoses and fatalities [1]. Oral squamous cell carcinoma (OSCC) is the most prevalent form of head and neck cancer and represents approximately 90% of all cases [2]. OSCC alone contributes to 3% of global malignancies and is the 17th leading cause of cancer-related mortality [3, 4]. OSCC cancerous lesions are usually found in the tongue, alveolar ridge, gingiva, floor of the mouth, cheeks, and palate. Despite recent advancements in many therapeutic strategies, HNSC remains associated with recurrence and progression. More than 50% of HNSC patients exhibit lymph node (LN) metastasis, one of the most common adverse prognostic factors in HNSC patients [4, 5]. The 5-year survival rate of primary HNSC patients is greater than 80%, but it falls to 40% with cervical LN metastasis and to 20% with distant metastases [6]. Cancer progression is often associated with extracellular molecules released by cancer cells into the milieu [7]. Such secreted molecules can educate cells in autocrine, paracrine, and/or endocrine manners, thereby inducing changes in tumor, stromal, endothelial, immune/inflammatory, and distant cells [8].

Extracellular vesicles (EVs) are small extracellular particles surrounded by lipid bilayers, ranging in diameter from 30 nm to 20 μ m, secreted by cells [9, 10]. EVs carry cell-derived cargo molecules, such as proteins, nucleic acids, lipids, and metabolites, either inside or on their surface. EVs carrying cargo molecules are found in body fluids, such as blood, saliva and urine, as well as in cell/tissue culture

supernatants. EVs are often surrounded by protein corona, including apolipoproteins and immunoglobulins, which are initially derived from body fluids such as blood plasma or serum [11, 12]. EV cargo molecules have been developed as prognostic biomarkers of diseases, including cancers, and as indicators of pathophysiological body status. Epithelial cell adhesion molecule (EpCAM), also known as CD326, and heat shock protein 90 (HSP90) are enriched in LN-metastatic oral cancer EVs and are associated with poor prognosis in HNSCs [13, 14]. Even CD9-EVs and CD63-EVs, general EV markers, were secreted in greater abundance by metastatic oral cancer cells than by lower-metastatic cells [13, 15]. Notably, EV-mediated intercellular molecular transfer is essential for intercellular communication and the fate of recipient cells [16, 17]. Through molecular transfer, cancer cell-derived EVs largely organize tumor-associated cells in the tumor microenvironment (TME) and distant cells to form a metastatic niche [18–20]. Our previous study showed that LN-metastatic oral cancer-EVs initiate epithelial-to-mesenchymal transition (EMT), promote cancer cell migration, invasion, and stem-like properties, and polarize tumor-associated macrophages (TAMs) toward tumor-supporting M2-type macrophages [15, 21].

EpCAM is a marker of EVs secreted by epithelial tumor cells, including squamous cell carcinoma and adenocarcinoma [13, 22, 23]. EpCAM is also known as a marker of cancer stem cells (CSCs) [15, 20, 23]. Recent studies revealed that EpCAM is cleaved by sheddases (such as ADAM10 or ADAM17), thereby generating extracellular EpCAM (EpEx), a ligand of epithelial growth factor receptor (EGFR), and EpCAM intracellular domain (EpICD), functioning as a transcription factor for stemness and EMT-related genes [24–27]. The EpEx activates EGFR-ERK signaling, stabilizing PD-L1 (programmed cell death ligand 1) by inhibiting its ubiquitination [24], thereby establishing an immune checkpoint with T cells. Here, we hypothesized that EpCAM signaling proteins can be carried by metastatic EVs, thereby educating the TME and metastatic niches. Indeed, we found that LN-metastatic oral cancer EVs are enriched with EpCAM and related proteins (EGFR, ADAMs, PSEN1, ERK, CTNNB1, FAK, AKT, PD-L1/2).

Methodology is very important in EV research. Fetal bovine serum (FBS) is often used in cell culture, although FBS-derived factors are often contaminated in the purified EV fraction [9]. We demonstrate that luciferase-based reporter systems address the contamination problem in EV research. The membrane-bound *Gaussia* luciferase (mbGLuc) is a reporter in which a PDGF transmembrane (TM) domain was fused with GLuc, so that mbGLuc binds to the EV membrane [28]. The mbGLuc reporter system has enabled in vivo imaging of EVs [29] and in vitro screening using cell culture supernatant without removing non-EV contents or serum [30]. In the present study, we developed a novel reporter cell system of human metastatic oral cancer. Using this, we screened five serum-free, xenofree media and found that the Essential 8 (E8) medium induces EV release from cells to a great extent. We also demonstrate that oral cancer EVs biodistribute to LNs to educate the metastatic niche, using the same mbGLuc reporter system.

Several major methods for EV separation have been established [9]. In the present study, we use phosphatidylserine affinity (PSA) purification [31], polymer-based precipitation (PBP) [13, 23], and size-exclusion chromatography (SEC) [32] methods, combined with the filter concentration (FC) method. The

PSA method involves binding phosphatidylserine (PS) on the surface of EVs to the Tim4 protein in a Ca^{2+} -dependent manner, followed by mild elution using a chelating agent. The PBP method precipitates EVs with EV-binding polymers. The SEC method separates EVs of various sizes, from large to small, using gel filtration. A combination of E8 medium, the FC-PSA or FC-PBP methods and proteomics identified over 5000 protein types, including EpCAM signaling proteins, across four oral cancer cell lines. The FC-SEC method separated EpCAM-positive large EVs (LEVs) and CD63-positive small EVs (SEVs), thereby more rigorously defining EV heterogeneity.

MATERIALS AND METHODS

Cell culture

Human oral squamous cell carcinoma cell lines (SAS, HSC-2, HSC-3) and a rapidly LN-metastatic subline HSC3-M3 (M3) were obtained from JCRB Cell Bank. These and M3/mbGLuc cells were maintained in DMEM (Wako, Osaka, Japan) supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA) and a 1% antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured in a humidified incubator with 5% CO_2 at 37°C. For EV analysis using ImmunoEM and proteomics, cells were cultured in Essential 8 (E8) xenofree, serum-free defined medium (Thermo Fisher Scientific, Gibco) containing DMEM/F12, bFGF, TGF β , insulin, transferrin, selenium, and ascorbic acid. For screening of xenofree, serum-free medium useful for EV study, DMEM/F-12, Essential 8, mTeSR1 (Stemcell Technologies, Vancouver, Canada), OncoPro Tumoroid Culture Medium (Thermo Fisher Scientific), and B-27-supplemented DMEM (Thermo Fisher Scientific, Gibco) were used.

Cloning of M3/mbGLuc reporter cells

For luminescent labeling of EVs, M3 cells were stably transduced with membrane-bound *Gaussia* luciferase (mbGLuc) reporter. A lentiviral reporter construct, CSCW-*Gaussia* luciferase-biotin acceptor-PDGFRB TM domain-IRES-GFP, was kindly gifted from Dr. Charles P. Lai [28, 33]. M3 cells were infected by spinfection with the viral solution. Infected/transduced stable cells were selected using puromycin. Single clones termed M3/mbGLuc cells were isolated by limiting dilution.

mbGLuc reporter assay

Gaussia luciferase activities were quantified using a *Gaussia* luciferase glow kit (Thermo Fisher Scientific). For cell culture, M3/mbGLuc cells were seeded at 2,500 cells/well in a 96-well plate. Cell culture supernatants or lysates (20 μL) were collected at 72 hours, and mixed with 50 μL of coelenterazine (CTZ) working solution in a white 96-well plate. Alternatively, 20 μL of an EV fraction was mixed with 50 μL of CTZ working solution. Luminescence was measured in a plate reader Gemini XP SOK (Molecular Devices, San Jose, CA, USA).

Separation of EVs

For EV separation, the modified polymer-based precipitation (PBP) method, the phosphatidyl serine affinity (PSA) purification method, or the size-exclusion chromatography (SEC) method [32] was combined with the filter concentration (FC) method [9, 13, 34] (Fig. 1). In common, cells were cultured in a 100-mm or 150-mm dish. The old medium was replaced with serum-free fresh medium (4 or 15 ml), such as Essential 8 or serum-free DMEM. The cell culture supernatant at 48 hours was collected and centrifuged at $2000 \times g$ for 30 min at 4°C to remove cells and blebbisomes. The supernatant was taken and centrifuged at $10,000 \times g$ for 30 min at 4°C to remove far larger EVs. For the FC method, the supernatant was concentrated by ultrafiltration using Amicon® Ultra-15 centrifugal devices (15 mL, 100 kDa; Merck, Burlington, MA) or Vivaspin 20 (100,000 MWCO PES; Sartorius, Gottingen, Germany) by centrifugation at $6000 \times g$ and 4°C for 15–60 min, several times in a fixed-angle rotor. For example, 40 ml was concentrated into 1 ml.

For the PBP method, the concentrate was incubated with an equal volume of Total Exosome Isolation Reagent (Thermo Fisher Scientific) at 4°C overnight, then centrifuged at $10,000 \times g$ for 60 min to precipitate EVs. For the PSA method, the concentrate was applied to MagCapture EV Isolation Kit PS for HTS (Fujifilm/Wako Pure Chemicals, Osaka, Japan) using a KingFisher Flex System (Thermo Fisher Scientific) or Auto-Pure Mini (Bio Medical Science, Tokyo, Japan). For immunoEM and proteomics of EVs, cells were cultured in fresh Essential 8 medium (15 mL per 150-mm dish) for 2 days during the proliferative phase. Culture supernatants were also collected at subconfluent and overconfluent stages. EVs were separated using the above-mentioned FC-PBP or FC-PSA method.

For the SEC method, the concentrate was applied to the qEV original/70 nm SEC column (Christchurch, New Zealand) after column equilibration. Fractions of pass-through were collected in order and concentrated into several groups (e.g., Fr. 1–3, 4–6...20–26) using Amicon Ultra centrifugal devices. The EV fraction was eluted or diluted in 100 μL phosphate-buffered saline (PBS) containing EV-Save (Fujifilm/Wako Pure Chemicals). Protein concentration was quantified using microBCA protein assay kit (Thermo Fisher Scientific).

Particle size distribution and counting

The EV fraction was diluted in PBS to a final volume of up to 1 mL. Nano tracking analysis (NTA) was performed using Nanosight NS300 (Malvern Panalytical, Malvern, UK) in a range of 0–1,000 nm. Alternatively, dynamic light scattering (DLS) was used as described previously [13, 15]. Briefly, we applied 40 μL of each concentrated fraction to a disposable solvent-resistant microcuvette (ZEN0040, Malvern Panalytical). Particle diameters ranging from 0 to 10,000 nm were analyzed using a Zetasizer nano ZSP (Malvern Panalytical) with Zetasizer Software version 7.03.

Immunolectron microscopy (immunoEM)

Transmission electron microscopy (TEM) grids, i.e., formvar/carbon-supported copper grids with a 400-mesh grid size, were hydrophilically treated. The EV fractions (10 μ L each) were placed onto Parafilm. The grids were floated on the EV fractions for 15 min. The samples were blocked in 1% bovine serum albumin (BSA) and 10% goat serum in PBS for 15 min and then treated with primary antibodies to EpCAM (Cell Signaling Technology, Danvers, MA, USA; CST2929s, VU1D9, mouse mAb, 1:800), CD9 (MBL, Tokyo, Japan, D252-3, mouse mAb, 1:50), and CD63 (Santa Cruz, Santa Cruz, CA, USA; sc-5275, mouse mAb, 1:25) in the blocking buffer overnight at 4°C. No primary antibody was used for the negative control. Samples were washed 5 times in 0.1% BSA in PBS and then treated with 10-nm colloidal gold particle-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (BBI solutions, Crumlin, Wales, United Kingdom; 1:50) secondary antibodies for 2 hours. Samples were washed 3 times with 0.1% BSA in PBS, fixed with 2% glutaraldehyde for 3 min, and washed once with distilled water. Samples were treated with 0.3% uranyl acetate and visualized at $\times 30,000$ or $\times 50,000$ magnifications with an H-7650 TEM (Hitachi, Tokyo, Japan) at the Central Research Laboratory, Okayama University Medical School. The number of molecules (antibody binding) per particle was counted and compared among the top 10 EVs.

Proteomics

EV proteins were analyzed with Liquid Chromatograph–tandem Mass Spectrometry (LC-MS/MS). Cells were cultured in Essential 8 medium, EVs were separated using the FC-PBP methods and analyzed using data-independent acquisition (DIA)-based proteomics. After reduction with 10 mM TCEP at 37°C for 30 min in PBS with 2% SDS and alkylation with 50 mM iodoacetamide at ambient temperature for 45 min, protein samples were digested with Trypsin/Lys-C Mix (Promega) at 47°C for 2 hours on S-Trap columns (ProtiFi, NY, USA). The resulting peptides were analyzed using Orbitrap Exploris 240 mass spectrometer (Thermo Scientific) combined with UltiMate 3000 RSLC nano-flow HPLC (Thermo Scientific). Peptides were enriched with μ -Precolumn (0.3 mm i.d. $\times$ 5 mm, 5 μ m, Thermo Scientific) and separated on AURORA column (0.075 mm i.d. $\times$ 250 mm, 1.6 μ m, Ion Opticks Pty Ltd, Australia) using the two-step gradient; 5–40% acetonitrile for 52 min, followed by 40–95% acetonitrile for 2 min in the presence of 0.1% formic acid. The analytical parameters for Full MS scan were set as follows: Orbitrap Resolution = 11,250, Scan range (m/z) = 495–745, Normalized AGC Target = 300%, Maximum injection time = 23 msec. The analytical parameters for DIA were set as follows; Precursor Mass Range (m/z) = 500–740, DIA Window Type = Auto, Isolation Window (m/z) = 4, Window Overlap (m/z) = 0, Number of Scan Events = 60, HCD Collision Energy = 30%, Orbitrap Resolution = 45,000, MS2 Scan Range (m/z) = 200–1800, Normalized AGC Target = 300%, Maximum Injection Time = 77 msec.

The mass spectrometric raw data files were converted to the mzML format using the msconvert software (Proteowizard). The resulting mzML files were analyzed using DIA-NN 1.9.2 software. Protein identification and quantification by DIA-NN were performed with mainly default search settings, except the precursor m/z range = 500–740 and fragment ion m/z range = 200–1800. The *Homo sapiens* protein sequence database in UniProt was used for the “FASTA digest for library-free search” function. The peptide identification filter was set at Precursor FDR (false discovery rate) < 1%. Log2 values of MS

scores were calculated. EV protein expression levels were normalized by dividing by the CD9 expression level.

Western Blotting

Western blotting was performed as described previously [32, 35]. Briefly, EV and non-EV fractions were prepared as described above. The FC-SEC-FC-based gathered fraction (Fr. 4–6, Fr. 7–9...Fr. 20–26), PBP-EVs (20 µg/20 µL), a non-EV fraction (20 µg/20 µL) or whole cell lysate (WCL) were mixed with 4 µL of a 6× SDS sample buffer with 9% β-mercaptoethanol and boiled at 95°C for 5 min. Briefly, we applied 20 µL of each mixture to 8%, 10%, or 12% polyacrylamide gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were transferred to PVDF membranes by wet transfer. Membranes were blocked in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T) and 5% Skim Milk (Fujifilm, Tokyo, Japan) for 1 hour at RT with shaking. Each membrane was incubated with primary antibodies to CD9 (Abcam, Cambridge, UK; ab92726, rabbit; 1:2000), CD63 (System Biosciences, Palo Alto, CA, USA; EXOAB-CD63A-1; rabbit, 1:1000), HSP90α (GeneTex, Irvine, CA; GTX109753, rabbit polyclonal, 1:5000), HSP90β (GeneTex; GTX101448, rabbit polyclonal, 1:1000), EpCAM (Abcam; E6V8Y, #93790, rabbit monoclonal, 1:2000), and β-actin (Sigma-Aldrich; rabbit, 1:200) overnight with shaking at 4°C. Afterward, secondary antibodies conjugated to horseradish peroxidase (HRP) (1:2500, Cell Signaling Technologies) were incubated with the membrane for 1 hour on a shaker at RT. Membranes were washed three times in TBS-T for 10 min at RT on a shaker, before and after the antibody reactions. Blots were visualized using an ECL Plus Western blotting substrate (Pierce, Rockford, IL, USA).

In vivo imaging

Xenograft of cells and EVs was performed as described previously [36]. Briefly, M3/mbGLuc cells (1×10^7 cells each) were subcutaneously injected into the backs of three 5-week-old female C.B-17 scid mice. A control mouse was not injected. Invasive tumors were formed in 8 weeks. CTZ-sol (NanoLight Technology, Norman, OK, USA) at a volume of 25 µg/100 µL ddH₂O was intraperitoneally injected into each mouse. Alternatively, 50 µg (/100 µl PBS) of EVs collected using the CF-PBP method from M3/mbGLuc cells were intraperitoneally injected into eight-week-old female BALB/c-nu/nu mice. CTZ-sol (25 µg/100 µl PBS) was injected intraperitoneally 40 min after EV injection. Luminescent signal distribution of mbGLuc in various organs was monitored using the in vivo imaging system (IVIS) at 5 min after CTZ injection. All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals approved by Okayama University and the Japanese Pharmacological Society. All experimental protocols were approved by the Okayama University Animal Experiment Licensing Committee (approval number: OKU-2023432).

Orthotopic lymph node metastasis model

Six- to eight-week-old severe combined immunodeficient (SCID) mice were used for orthotopic xenograft experiments. HSC-3-M3 cells (5×10^5 cells/50 µL) were inoculated into the lateral tongue under

anesthesia induced by intraperitoneal injection of pentobarbital (6 mg/mL). Four weeks after inoculation, mice were euthanized, and the tongues and cervical lymph nodes were harvested. Tumor-bearing tongue and draining cervical lymph nodes were harvested and fixed in 4% paraformaldehyde for 36–48 hours. Primary tumors were sectioned at the largest cross-sectional area. Tissues were dehydrated through a graded ethanol series (70–100%), cleared in xylene, and embedded in paraffin. Paraffin-embedded tissues were sectioned at a thickness of 3 μ m and subjected to hematoxylin and eosin (H&E) staining for histopathological evaluation. Cervical lymph node metastasis was assessed based on histological findings. All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals approved by Okayama University and the Japanese Pharmacological Society. All experimental protocols were approved by the Okayama University Animal Experiment Licensing Committee (approval number: OKU-2023432).

Immunohistochemistry (IHC)

IHC was performed as described previously [37]. Paraffin blocks of specimens were cut at 3- μ m thickness. The tissue slides were deparaffinized in xylene and gradually rehydrated through a series of ethanol solutions from 100% to 70%. Antigen retrieval was performed by microwave heating in 0.01 M citrate buffer (pH 6.0, sodium citrate dihydrate and citric acid; Sigma-Aldrich, USA) at 100°C for 1 min. Tissue slides were washed with distilled water, then blocked with Block Ace (DS Pharma Biomedical, Osaka, Japan) for 20 min at RT. The slides were incubated overnight at 4°C with the primary EpCAM antibody (rabbit, 1:300, Cell Signaling Technology, 93790). Slides were then washed with 0.05 M TBS solution, followed by incubation for 60 min at RT with secondary antibody, anti-rabbit IgG (donkey)-Alexa Fluor 647 (A31573, Thermo Fisher Scientific). Sections were stained with 4',6-diamidino-2-phenylindole (DAPI) and covered with cover glasses. Stained sections were observed with a BZ-X700 all-in-one fluorescence microscope (Keyence, Osaka, Japan) with a $\times$ 40 lens. Pseudo-colors, brightness, contrast, and image merging were adjusted using the Keyence BZ-X800 Analyzer. OSCC tissues resected at Okayama University Hospital Oral Surgery were examined. The study was approved by the Okayama University Human Ethics Committee (Approval number: 1608–018), and informed consent was obtained from all patients.

Gene expression in patient-derived samples

mRNA expression in HNSC patient-derived tumors and normal tissues was analyzed using GEPIA (<http://gepia.cancer-pku.cn>) based on TCGA and GTEx datasets. Protein and mRNA expression in HNSC tumors, cell lines, and normal tissues was analyzed using the Human Protein Atlas database (<https://www.proteinatlas.org>). Kaplan-Meier analyses were performed using Kaplan-Meier (KM) Plotter (<https://kmplot.com>) [38].

Statistics

Statistical significance calculations and graph generation were performed using Prism 8 (GraphPad Software) and Excel (Microsoft). The difference between the two data sets was examined using an unpaired *t*-test. $p < 0.05$ was considered statistically significant. For KM analysis, the log-rank test was

used; log-rank p-values and hazard ratios (HRs) were calculated using the KM plotter. $p < 0.05$ was considered statistically significant.

RESULTS

Extracellular vesicle membrane-bound Gaussia luciferase reporter system of oral cancer cells

To efficiently monitor and track EVs in screening systems or in vivo imaging, we created a novel reporter system in which mbGLuc was overexpressed in HSC3-M3 cells and then secreted with EVs (Fig. 2A). This mbGLuc system labels all membrane-surrounded EVs, including exosomes, microvesicles, apoptotic bodies, large oncosomes, and matrix-bound EVs. This system is advantageous for quantifying EVs in cell culture supernatants, including those containing serum, without EV separation/purification steps. M3/mbGLuc cells were simultaneously labeled with enhanced green fluorescent protein (GFP) and continuously expressed EpCAM (Fig. 2B, Figure S1), a biomarker of cancer stem cells (CSC) and cancer EVs [13, 15, 23]. EpCAM was well-stained on/around the cell surface, suggesting that EpCAM + EVs could be trapped in the cell surface ECM. The overexpressed mbGLuc was well quantified in cell lysates and culture supernatants without separating EVs from other factors (Fig. 2C). The mbGLuc activity levels in the separated EVs using the FC-PBP method were proportional to the EV protein concentrations (Fig. 2D).

A limitation of EV research is the cell culture medium containing FBS. In this case, bovine-derived EVs contaminate the medium, so EV-free serum or serum-free medium has been used. However, it is time-consuming, labor-intensive or expensive. Moreover, the composition of serum is unknown, and serum-free culture is stressful for cells. To overcome these limitations, we screened serum-free, xenofree media that efficiently induce EV secretion, using the mbGLuc system. Essential 8 (E8) medium significantly increased EV-mbGLuc activities, indicating that E8 efficiently induced EV secretion compared with other serum-free media (DMEM/F12, mTeSR1, OncoPro, and B-27) (Fig. 2E).

These data indicate that the mbGLuc reporter system and E8 medium are markedly useful for EV research.

Highly metastatic oral cancer cells secrete EpCAM-rich and CD63-rich EVs.

It has been shown that M3 cells migrated and metastasized more rapidly to LNs than the parental HSC-3 cells [15]. Interestingly, the size of small EVs from M3 cells is larger than that of those from HSC-3 cells [13, 15]. Moreover, expression levels of EpCAM, CD63, CD9, EGFR and HSP90 in M3-SEVs were higher than those in HSC3-SEVs [13, 15]. Therefore, we hypothesized that M3-SEVs contain higher levels of EpCAM or CD63 than HSC3-SEVs. To test this hypothesis, we performed immunoEM on EVs secreted by HSC-3 and M3 cells.

M3-SEVs tended to be larger than HSC3-SEVs (Fig. 3A–C, Figure S2). One to nine CD9 molecules were bound on the single EVs of HSC-3 (average 4.4, max 7 molecules) and M3 cells (average 5.4, max 9

molecules) (Fig. 3A, B). The number of CD63 molecules on the surface of single EVs was significantly higher in M3-EVs (average 8.2, max 14 molecules) than in HSC3-EVs (average 3.8, max 5 molecules) (Fig. 3C, D). The number of EpCAM molecules on the surface of single EVs was significantly higher in M3-EVs (average 6.0, max 16 molecules) than in HSC3-EVs (average 2.2, max 4 molecules) (Fig. 3E, F). These data indicate that rapidly metastatic oral cancer SEVs are larger and contain more EpCAM and CD63 molecules.

To investigate the correlation between HNSC patients' survival time and the gene expression of CD9, CD63, and EpCAM, we next performed a Kaplan-Meier analysis. High *CD9* expression was associated with a poor prognosis in HNSC, especially at stage II (HR = 2.61, $p = 0.032$) and stage I + II+III (HR = 1.89, $p = 0.021$) (Fig. 3G, H). High *CD63* expression was associated with a poor prognosis in HNSC, especially at stage I (HR = 8.59, $p = 0.026$) and stage I + II (HR = 3.75, $p = 0.00039$) (Fig. 3I, J). High *EPCAM* expression was associated with poor prognosis in HNSC, especially at stage II (HR = 2.19, $p = 0.066$) and stage I + II+III (HR = 2.01, $p = 0.016$) (Fig. 3K, L).

These data indicate that rapidly metastatic oral cancer SEVs are larger and contain more EpCAM and CD63 molecules. Also, higher levels of EV-EpCAM and EV-CD63 can predict rapid LN metastasis in HNSCs.

EpCAM signaling proteins are contained in oral cancer EVs.

EVs can transfer proteins to recipient cells. EpCAM is a single-pass TM protein, but its cleavage by ADAM17/TACE and presenilin (PSEN) generates EpEx and EplCD, which involve signaling and transcription of pluripotent stem cell genes [25, 39, 40]. EpEx functions as an EGFR ligand that counteracts EGF-mediated EMT by modulating phospho-ERK1/2 in head and neck cancers [26]. Therefore, we hypothesized that EpCAM-EGFR signaling proteins were contained in oral cancer EVs and transferred to form a metastatic niche. Proteomics revealed that oral cancer SEVs contained TM proteins, including EpCAM, EGFR, ADAM10, ADAM17, PSEN1, PD-L1, and PD-L2, and cytoplasmic proteins, including ERK1 (MAPK1), ERK2 (MAPK3), β -catenin (CTNNB1), focal adhesion kinases (FAK1/2), AKT1/2/3, and FHL2 (Fig. 4A). On the other hand, oral cancer EVs did not contain nuclear transcription factors (FOXO3, HIF1A, and TCF/LEFs), PSEN2, and PI3K/PIK3CA.

We next examined whether EPCAM gene expression was increased in HNSC tumors compared with normal tissues, and its stage dependency. Indeed, EPCAM gene expression levels increased with HNSC stage progression (Fig. 4B). EPCAM gene expression levels in HNSC tumor tissues were significantly higher than in normal tissues (Fig. 4C, Figure S3A). ADAM10 and ADAM17 gene expression levels in HNSC tumor tissues tended to be higher than in normal tissues (Fig. 4D, E; Figures S4, S5). High expression of ADAM10 (HR = 3.26, $p = 0.0019$) and ADAM17 (HR = 2.93, $p = 0.0032$) mRNA was associated with shorter survival time in HNSC patients, indicating that ADAM10 and ADAM17 are prognostic biomarkers in HNSC. The expression of EpCAM was found in both the primary lesions of oral cancer and metastatic LNs, in both lung metastasis-positive and negative cases (Fig. 4H).

These data indicate that HNSC tumors express high levels of EpCAM with stage progression and release EVs containing EpCAM signaling proteins, which can be transferred to educate the TME and metastatic niches.

Metastatic oral cancer cells release EpCAM-positive large EVs and CD63-positive small EVs

Oral cancer EV markers (or exosome markers) have been established, including CD9, CD63, EpCAM, EGFR, and HSP90 [15, 17, 23, 51]. Recent studies have addressed heterogeneity in EVs, but the extent of this heterogeneity remains unclear. The FC-SEC method is useful for efficiently separating and characterizing EV populations of different sizes. A previous study identified CD9 + large EVs (100–200 nm) and CD63 + HSP90+ small EVs (30–50 nm) secreted by macrophages using the FC-SEC method [32]. In the present study, we used the FC-SEC to characterize EVs released by metastatic oral cancer cells. To simplify the measurement process, we combined and concentrated the EV fractions (Fr.) into Fr. 1–3 (not containing EVs), Fr. 4–6, 7–9, 10–12, and so on, using the same concentration factor (15:1) for all fractions (Fig. 5A). These combined fractions were then used to analyze the EV particle-size distribution and protein expression. The fraction 4–6 contained large EVs (150–300 nm), although no EV marker was detected in this fraction. Another large EV (120–200 nm, Fr. 7–9) fraction contained the cancer EV marker EpCAM (Fig. 5B, C; Figures S6, S7). Consistently, EpCAM was detected in large EVs in immunoEM. A class I EV marker CD9 was found in large and small EVs (50–200 nm, Fr. 7–12) from M3 cells, whereas this appeared to shift into smaller EVs (30–200 nm, Fr. 7–26) of M3/mbGLuc cells. Consistently, CD9 was detected in small and large EVs in immunoEM. HSP90 α and HSP90 β were contained in large and small EVs (30–200 nm, Fr. 7–26). CD63 was detected only in small EVs (30–100 nm, Fr. 13–24) but not in large EVs (Fig. 5E). Fraction 25–30 can contain HSP90-positive exomeres and CD63-negative.

These data indicate that EpCAM identifies large EVs (80–300 nm), whereas CD63 marks small EVs (30–100 nm) of metastatic oral cancer cells, while CD9, HSP90, and β -actin broadly marked large and small EVs (Fig. 5F).

Biodistribution of oral cancer-derived EVs to lymph nodes

It has been shown that tumor EVs educate distant organs (bone marrow, lungs, or liver) to form pro- and pre-metastatic niches (PMN) [41, 42]. Oral cancers (or HNSC) tend to metastasize to LNs, including cervical and axillary LNs [43–47]. Therefore, we next investigated whether metastatic oral cancer cells (M3/mbGLuc) and their EVs biodistribute to distant organs, such as LNs, using a xenograft mouse model. To detect metastasis of oral cancer cells labeled with mbGLuc, we subcutaneously xenografted M3/mbGLuc cells into the back of mice (Fig. 4, top). Invasive carcinomas formed in the primary dorsal, secondary cervical, and splenic regions by the M3/mbGLuc cells (Fig. 4, left). The mbGLuc signal was also detected in the bladder, indicating that EVs were excreted during urination. To detect the biodistribution of mbGLuc-labeled metastatic EVs, we transplanted metastatic EVs purified from

M3/mbGLuc cells into the abdominal cavities of mice. The mbGLuc-labeled EVs were biodistributed to axillary LNs and liver, modeling metastatic niche formation by HNSC-derived EVs (Fig. 4, right).

These data indicate that metastatic oral cancer cell-derived EVs biodistribute to the LNs, forming a PMN, and that the mbGLuc reporter system enables tracking of EVs that educate distant organs in vivo.

Tongue cancer metastasize to lymph node in orthotopic xenograft.

The origin of HSC-3 and M3 cells is tongue cancer, which often metastasize to LNs. It has been shown that the orthotopic xenograft better reflects the anatomical and biological context of tumor growth and lymphatic dissemination, compared with conventional subcutaneous models [48, 49]. To investigate cervical LN metastasis in OSCC, we established an orthotopic xenograft model by inoculating HSC-3-M3 cells into the lateral tongue of SCID mice (Fig. 7A). Tumor formation was consistently observed at the primary injection site. Four weeks after inoculation, macroscopic examination revealed primary tongue tumors and enlargement of cervical LNs, suggestive of metastatic dissemination (Fig. 7B).

Histopathological analysis confirmed the presence of tumor cells in both the primary tongue lesions and cervical LNs (Fig. 7C). The metastatic lymph nodes exhibited poorly differentiated tumor morphology, comparable to the primary tumors (Fig. 7C). These findings indicate that this orthotopic model reliably recapitulates cervical LN metastasis of oral cancer.

DISCUSSION

In this study, we newly established a useful reporter system (M3/mbGLuc) to study metastatic oral cancer EVs. Using this system, we found that E8 medium induced EV secretion and that metastatic oral cancer EVs biodistributed to LNs, thereby educating the PMN (Fig. 6). EpCAM was expressed in primary oral cancer, secreted with EVs that also contained related proteins (ADAM10/17, PSEN1, EGFR...), and detected in LNs (Fig. 4). The capacity (largeness) of metastatic EVs to carry large numbers of proteins (such as EpCAM and CD63) can contribute to metastatic niche formation (Figs. 3 and 5). We also demonstrated EV heterogeneity, including EpCAM-LEVs and CD63-SEVs, which should play different roles in cancer progression.

The EV field has been significantly advanced by the development of sensitive reporter systems that enable the tracking of these nanoscale carriers in living organisms. Our study used an mbGLuc reporter, a methodology that builds on the pioneering work of Lai et al. [28, 29], to study the behavior of oral cancer EVs. The strength of our mbGLuc system lies in its practical utility in addressing two common experimental hurdles. First, it enabled direct quantification of EV secretion into complex cell culture media without prior EV isolation. Second, and more critically, the mbGLuc reporter provides a powerful method to monitor EV fate in vivo. Our application of this system directly visualized the organ-specific trafficking of oral cancer EVs. The non-invasive bioluminescence imaging clearly demonstrated the accumulation of EVs derived from metastatic cells in LNs, which was a key finding in head and neck cancer metastasis. This evidence supported the concept that tumor-derived EVs can induce specific metastatic niches. By using mbGLuc activity to guide EV production in vitro and reveal their targeted

biodistribution in vivo, we could subsequently analyze the protein cargo of these LN-tropic vesicles. Thus, the mbGLuc system bridges the gap between observing where EVs go and what they might do.

Our experiments demonstrated that several EV separation methods were useful for specific purposes. We compared the FC-PBP and FC-PSA methods using proteomics and obtained similar results, but PBP tended to collect higher protein levels than PSA. However, PD-L1 in EVs was detected only in PSA-EVs, not in PBP-EVs, indicating that PD-L1 is present in PS-positive EVs but not in PS-negative EVs. The PBP-EVs were useful for in vivo biodistribution analysis. Notably, the FC-SEC method enabled the separation of EpCAM-LEVs (80–300 nm) and CD63-SEVs (30–100 nm), whose functional differences are of interest. The choice of method for isolating EVs directly affects the purity, yield, and function of the vesicles obtained. For this study, investigating how oral cancer EVs educate LN metastatic niches, we required a method that provided sufficient purity while preserving natural EV function for our in vivo tracking and proteomic profiling. Among prevalent techniques, each presents distinct advantages and disadvantages [9]. PBP offers high yield but co-isolates abundant contaminants such as lipoproteins and protein aggregates, which could confound proteomic data and functional assays [50]. However, our CF-PBP method resulted in similar proteomics data with CF-PSA method, indicating that PBP is useful when combined with other methods. Affinity purification provides exquisite subtype specificity but yields limited material and introduces selection bias, making it less suitable for global profiling or studies of heterogeneous EV populations [51]. Indeed, our study captured PS-positive EVs, not PS-negative EVs [31]. Tangential flow filtration (TFF) excels at processing large volumes but offers a size-based resolution similar to other methods [52]. In this context, SEC is often favored for functional studies due to its gentle, buffer-based operation, which effectively removes soluble protein contaminants while maintaining EV biological activity [53]. Combination of the FC method and E8 medium with these methods largely increase the yield and purity of EVs. Our methodological approach followed a strategy validated in the relevant study, which successfully employed SEC followed by FC to isolate exosomes from macrophage-like cell conditioned medium [32]. We demonstrated that these FC-SEC-purified exosomes retained potent biological activity, significantly affecting the viability of recipient oral cancer cells in co-culture experiments. This study confirmed that the FC-SEC can prepare functionally competent EV preparations suitable for studying EV-mediated interaction in the oral cancer TME. Guided by this study, our present work relied on obtaining functionally intact vesicles. Our subsequent finding that metastatic cells secrete larger EpCAM-rich EVs (80–300 nm) alongside smaller CD63-rich EVs (30–100 nm) highlighted the importance of using a method such as FC-SEC that preserves these native sizes and surface protein heterogeneity. This key heterogeneity might be affected by methods with lower purity or those that selectively capture only one subpopulation. In summary, for studies focused on EV function and native heterogeneity in complex settings like metastatic niche formation, the SEC-based method offers a validated compromise. It provided the necessary balance of purity and preserved bioactivity, as confirmed by the functional and molecular data in our works.

Our study investigated canonical EV markers and novel EV proteins in oral cancer. Our proteomics revealed > 5000 protein types, but here we focused on several, including EpCAM signaling proteins. These include category 1a (multi-pass TM proteins: CD9, CD63, PSEN1), 1b (single-pass TM proteins:

EpCAM, EGFR, E-cadherin, ADAM10, ADAM17, PD-L1, PD-L2), and 2b (promiscuous incorporation into EVs: HSPs, beta-actin, vimentin, ERK1/2, CTNNB1, FAK1/2, AKT1/2/3, FHL2) proteins, according to the Minimal Information for Studies of Extracellular Vesicles 2023 (MISEV2023) [9]. Accurate characterization of EVs and their subtypes is fundamental to elucidating their specific roles in tumor progression. This relies on identifying EV-associated markers. The updated MISEV2023 guideline provides a crucial framework, advocating a shift from oversimplified classification based on a single marker to a comprehensive, multi-parametric characterization involving transmembrane, cytosolic, and luminal proteins [54]. Traditionally, EV subtypes have been distinguished by markers linked to their biogenesis pathways. Tetraspanins (CD9, CD63, CD81...) and ESCRT components (TSG101, ALIX: category 2a) are frequently used as canonical exosome markers, reflecting an endosomal origin. It is critical to recognize that these proteins are not merely passive identifiers but have active functional roles. For instance, the tetraspanin CD63 was deeply involved in EV cargo sorting and EV-mediated processes that drive tumor progression, angiogenesis, and metastasis [41]. Similarly, surface exposure of PS and specific integrins is often associated with microvesicles shed from the plasma membrane [55, 56]. However, MISEV2023 recommended against definitively classifying EVs into specific biogenesis pathways based solely on protein markers, due to their significant overlap and context-dependent expression. Instead, it proposed operational definitions based on physical characteristics (e.g., small, medium, and large EVs) and detailed compositional analysis. Our observation of EpCAM enrichment on larger EVs from metastatic oral cancer cells exemplifies this concept. The heterogeneity of EVs, reflected in their size and molecular composition, is directly linked to their functional capacity in PMN formation. Our study also touches on the mechanism by which EV heterogeneity contributes to cancer progression. Our SEC and immunoEM revealed that oral cancer cells secrete EpCAM-LEVs (80–300 nm) and CD63-SEVs (30–100 nm). The CD63-SEVs were also secreted by macrophages [32], indicating that this is generalizable and universal. In contrast, CD9 and HSP90 are found in both LEVs and SEVs. HSPs are classified into category 2b, cytosolic proteins found in all EVs, promiscuous incorporation into EV, and possibly non-vesicular extracellular particles (NVEPs) [9]. HSP90 is also found in exomeres (non-EVs, ~ 35–50 nm) [9, 57]. Nevertheless, our data show relatively novel EV components, including category 1a (PSEN1), 1b (ADAM10, ADAM17, PD-L1, PD-L2), and 2b (ERK1/2, CTNNB1, FAK1/2, AKT1/2/3, FHL2) proteins.

Our EV proteomics data suggest that oral cancer EVs contain EpCAM signaling proteins (EGFR, ADAM10, ADAM17, CTNNB1, PSEN1, AKT1/2/3, ERK1/2, PD-L1/2 and FHL2) and can transfer them to educate the TME and metastatic niches. Oral cancer EVs alter recipient cells, including cancer cells themselves, epithelial cells, cancer-associated fibroblasts (CAFs), TAMs, tumor vascular endothelial cells (TECs), and LECs [8, 20]. It has been shown that cleavage of EpCAM by ADAM10/17 (aka sheddases, α -secretases or TACE (TNF-alpha-converting enzyme)) that function with β -secretase (BACE, beta-site amyloid precursor protein cleaving enzyme) and γ -secretase (a complex of presenilin 1 or 2 (PSEN1/2), nicastrin, presenilin enhancer 2 (PEN-2), and APH-1) generates EpEx, a ligand of EGFR and EpICD that forms a transcription factor complex [25, 39, 58]. EpEx binding to EGFR triggers ERK activation (phosphorylation), which inhibits PD-L1 degradation, establishing the immune checkpoint with T cells and activating sheddases

(ADAM17 and γ -secretase) as a positive feedback loop [24]. EpEx-EGFR binding also activates the PI3K-AKT-FOXO3a signaling pathway, thereby inhibiting pro-apoptotic genes [24]. Simultaneously, EpICD forms a transcription factor complex with β -catenin and FHL2, which activates stemness genes (OCT4, SOX2...) and EMT-related genes (Vimentin, Snail, Slug) [24]. Indeed, the EpCAM neutralizing antibody blocked EGFR-ERK signaling, thereby degrading PD-L1 and inhibiting sheddase activation; blocked EGFR-PI3K-AKT signaling, thereby activating FOXO3a to induce pro-apoptotic gene expression; and blocked EpICD activity, thereby inhibiting stemness and EMT-related genes [24]. Therefore, EV-contained EpCAM, sheddases, and EGFR, found in our study, can generate an EpEx-EGFR complex that activates EGFR-ERK signaling, thereby establishing the immune checkpoint via PD-L1/2, and activates EGFR-PI3K-AKT signaling, thereby inhibiting pro-apoptotic genes in cancer cells and epithelial cells. Simultaneously, EV-bound EpCAM and sheddases can generate EpICD that activates stemness and EMT-related genes in cancer cells, CAFs, and epithelial cells. Consistently, it has been shown that tumor-derived exosomal ADAM17 promotes PMN formation by enhancing vascular permeability in colorectal cancer [59]. Thus, oral cancer EVs contain EpCAM signaling proteins that educate TME and metastatic niches.

Our data also indicate that LN-metastatic EVs are larger than low-metastatic EVs, thereby carrying more molecules, such as EpCAM and CD63, and so efficiently educate the TME and metastatic niches. The association of EpCAM and CD63 with poor prognosis in early-stage HNSC patients suggests that these EV subpopulations are clinically relevant biomarkers, a notion supported by another article that emphasizes the diagnostic potential of EVs in oral cancer [54]. Our data also suggest that high levels of ADAM10 and ADAM17 in EVs are prognostic biomarkers for oral cancers. In cancer, the most clinically significant EV markers are frequently not constitutive proteins but rather cancer-specific functional molecules that govern organotropic tropism and niche remodeling. Seminal work by Hoshino et al. established the foundational principle that integrin profiles on tumor-derived exosomes dictate organ-specific metastasis [41]. The translational potential of analyzing these EV-associated signatures is considerable. Detecting EV-borne proteins and non-coding RNAs (e.g., miR-24-3p) in liquid biopsies from patients with oral cancer offers a promising avenue for early detection, risk stratification, and therapy monitoring [60, 61]. In summary, moving forward in the study of EV markers for oral cancer requires a shift from simply listing common markers to understanding how specific molecular complexes control EV homing and niche remodeling. Targeting these key surface markers or their effects offers a promising therapeutic strategy to block PMN formation and stop metastasis in oral cancer.

The formation of PMNs in cervical LNs is a critical early event in oral cancer metastasis. EVs have emerged as pivotal mediators in this process, serving as messengers that prime distant sites for tumor cell colonization [62]. Our findings demonstrated the selective biodistribution of oral cancer-derived EVs to LNs in vivo, consistent with this concept. The cargo of oral cancer EVs can modulate PMN formation. Our proteomic analyses, which identified EpCAM signaling network proteins within EVs from metastatic cells, suggested a mechanism beyond cell adhesion. These EV-bound signaling packages can be delivered to lymphatic endothelial cells (LECs) and LN stromal cells, including fibroblastic reticular cells (FRCs), thereby driving phenotypic changes. Indeed, EV-associated laminin gamma2 (LAMG2) has been shown to enhance lymphangiogenic tube formation via integrin alpha3 (ITGA3)-dependent uptake by

LECs [46]. Furthermore, the Shh/RhoA signaling pathway mediated by oral cancer EVs can promote angiogenesis, thereby supporting a vascular network conducive to metastasis [63]. Furthermore, EV-mediated regulation of key factors such as VEGF-C, a master regulator of lymphangiogenesis, is a conserved mechanism across cancers, as observed in pancreatic models [64]. This expanded and remodeled lymphatic network is a hallmark of the PMN. A study revealed that head and neck cancer EVs can induce PD-L1 expression on FRCs through the IFNGR1/JAK1/STAT1 axis, effectively crippling anti-tumor T-cell responses within the LN [65]. This immunosuppressive role is a critical feature of tumor-derived EVs, as highlighted in recent classifications that emphasize their contribution to immune evasion [8]. In summary, EV-based PMN formation in cervical LNs is a coordinated, multi-step process: 1) targeted EV homing, 2) induction of local immunosuppression via stromal cell modulation, and 3) stimulation of lymphangiogenesis to enhance vascular access [66]. These EV-driven processes collectively and systemically alter the LN microenvironment to be more permissive for the survival and outgrowth of disseminated tumor cells, thereby facilitating metastatic colonization.

CONCLUSIONS

Firstly, we developed a novel reporter cell system of human metastatic oral cancer. Using this, we discovered that the Essential 8 medium induces extensive EV release from cells and that oral cancer cells and their EVs biodistribute to lymph nodes, thereby educating the PMN. Secondly, using the FC-SEC method, we successfully separated EpCAM-LEVs and CD63-SEVs, which are prognostic biomarkers in HNSC. EpCAM signaling proteins (ADAM10/17, PSEN1...) were present in oral cancer EVs, which can also be transferred to educate the PMN.

Abbreviations

ADAM a disintegrin and metalloproteinase

CSC cancer stem cell

CTNNB1 catenin beta 1 (β -catenin)

E8 Essential 8

EGFR epidermal growth factor receptor

EM electron microscopy

EpCAM epithelial cell adhesion molecule

EpEx EpCAM extracellular domain

EpICD EpCAM intracellular domain

ERK extracellular signal-regulated kinase

EV extracellular vesicle

FC filter concentration

HNSC head and neck squamous cell carcinoma

HR hazard ratio

HSC human oral squamous cell carcinoma cell line

HSP heat shock protein

LEC lymphatic endothelial cell

LN lymph node

M3 HSC-3 lymph-node-metastatic number 3 (HSC-3-M3)

MAPK mitogen-activated protein kinase

mbGLuc membrane-bound *Gaussia* luciferase

OSCC oral squamous cell carcinoma

PBP polymer-based precipitation

PD-L programmed cell death-ligand

PMN pre- / pro-metastatic niche

PS phosphatidylserine

PSA phosphatidylserine affinity

PSEN presenilin

SEC size exclusion chromatography

TACE TNF-alpha-converting enzyme

TM transmembrane

TME tumor microenvironment

Declarations

Ethics approval and consent to participate

All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals approved by Okayama University and the Japanese Pharmacological Society. All experimental protocols were approved by the Okayama University Animal Experiment Licensing Committee (approval number: OKU-2023432). All methods are reported following ARRIVE guidelines (<https://arriveguidelines.org>) for the reporting of animal experiments.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available in supplemental data or from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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AUTHORS' CONTRIBUTIONS

Conceptualization, T.E.; methodology, Y.L., K.Y., K.On., H.K., K.U. and T.E.; investigation, Y.L. (mbGLuc reporter assay, EV separation and analyses, WB), K.Y. (EV separation, in vivo imaging), K.On. (orthotopic model), L.X. (reporter assay for medium screening), H.S. (proteomics), H.S.E. (IHC), T.E. (immunoEM, dry analyses); validation, M.W.O. (animal experiment), L.X. (dry analyses, animal experiment); data curation, T.E., K.On., and K.U.; formal analysis, Y.L. and T.E.; funding acquisition, T.E., K.U. and Y.L.; resources, T.E. (reporter cells), K.U. (EV separation system, proteomics system), H.K. (patient-derived samples) and K.Ok.; writing—original draft preparation, T.E., Y.L., and K.On.; writing—editing, T.E.; visualization, T.E. (graphics); supervision, T.E., H.K. and K.U.; project administration, T.E.; All authors have read and agreed to the manuscript.

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Figures

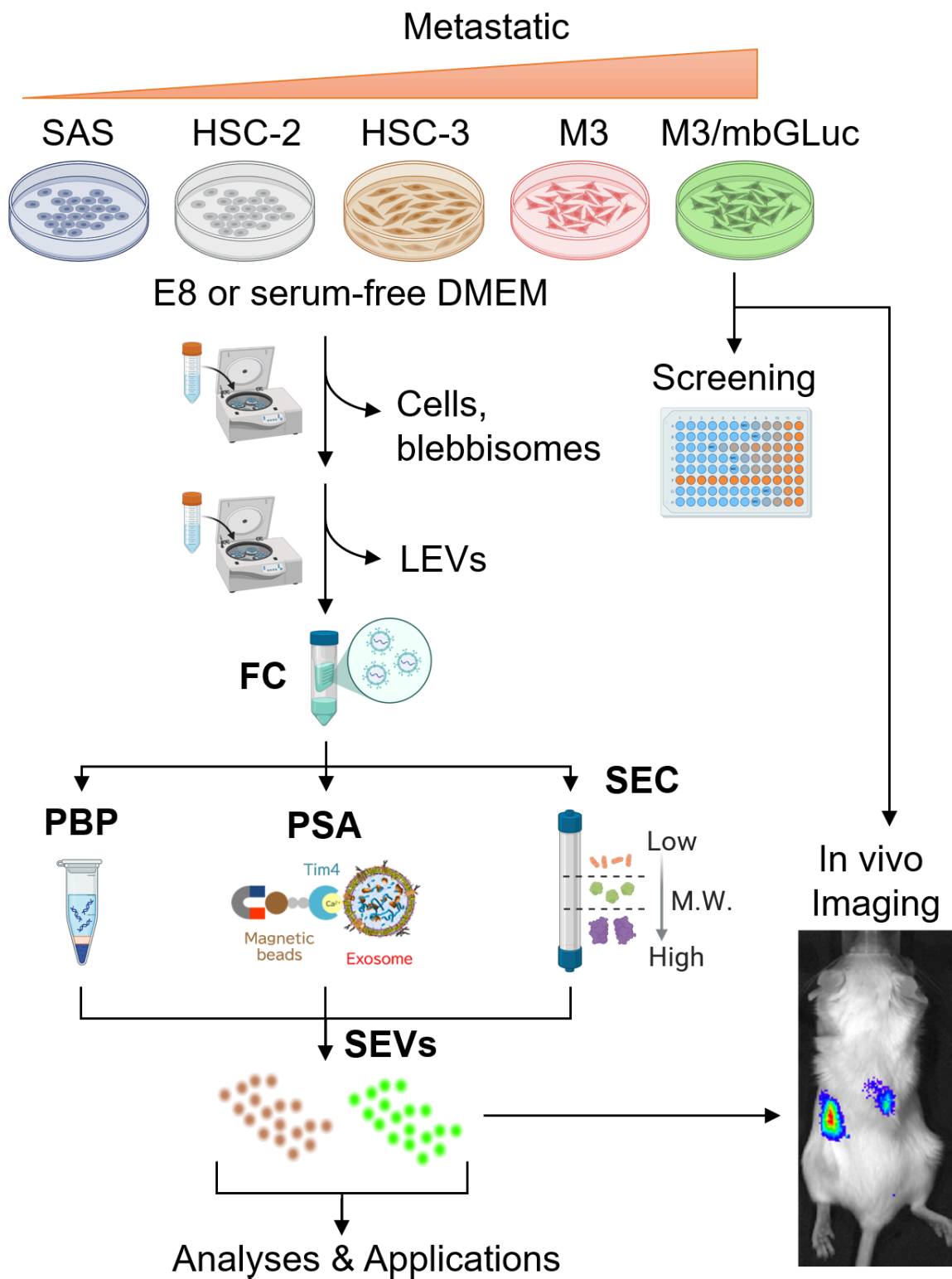


Figure 1

EV separation methods. Cells were cultured in xenofree conditions in Essential 8 medium or serum-free DMEM. EVs were separated using the FC-PBP, FC-PSA and FC-SEC methods. M3/mbGLuc cells were used for screening and in vivo imaging.

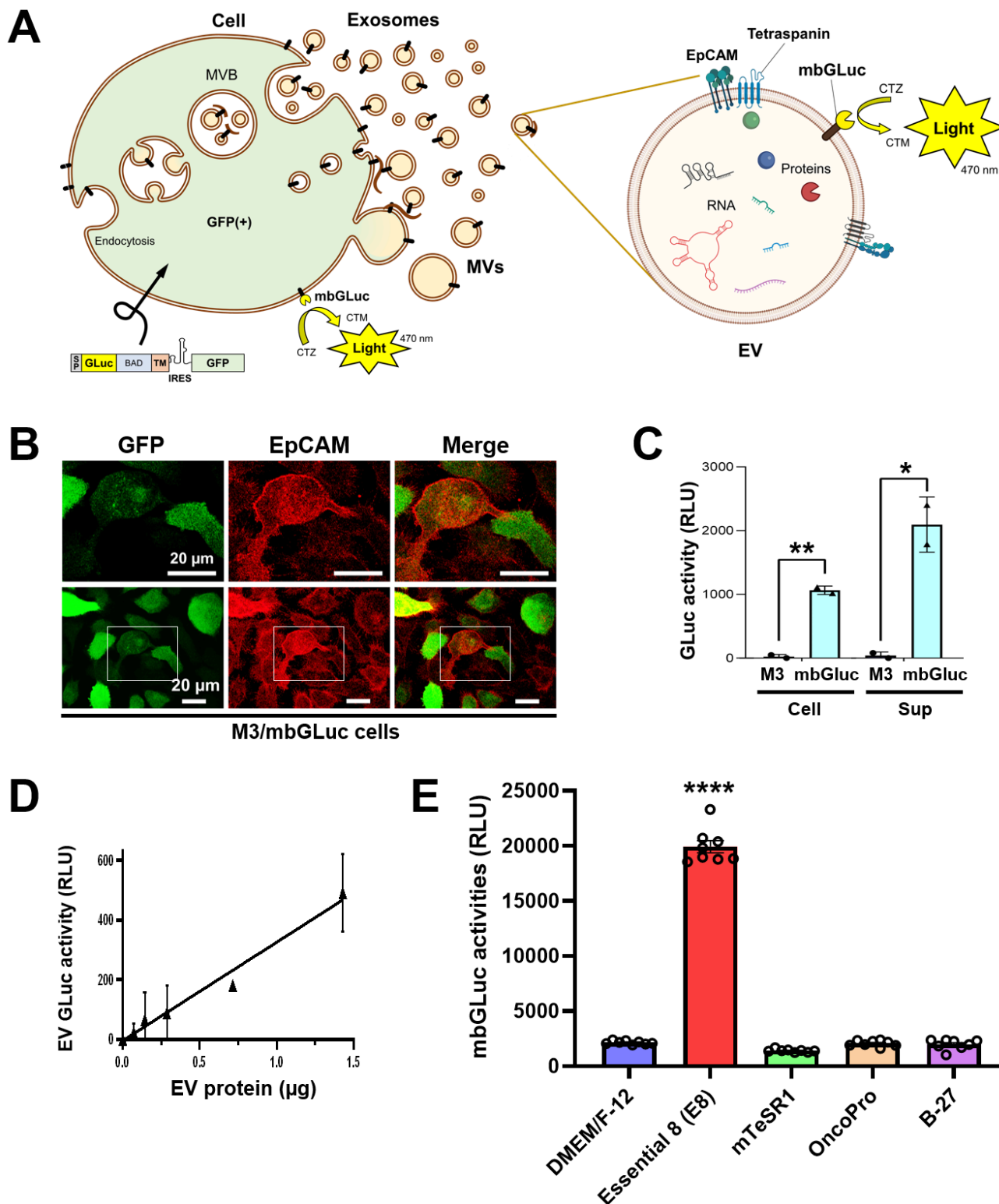


Figure 2

Extracellular vesicle membrane-bound *Gaussia* luciferase (mbGLuc) reporter system of oral cancer cells. (A) The cells (left) expressed mbGLuc and released mbGLuc-EVs (right). ECM proteins can bind with both exosomes and microvesicles to generate secretory matrix-bound EVs. (B) GFP and EpCAM expression in M3/mbGLuc cells. (C) GLuc activity in the cell-culture supernatant (Sup) and cell lysate of M3/mbGLuc cells and HSC3 (ctrl) cells. Cells were cultured in DMEM containing 10% FBS. Data are

mean $\pm$ S.D. $n = 2$, * $p < 0.05$, ** $p < 0.01$, unpaired t-tests. (D) Positive correlation between EV GLuc activity and protein concentration. Cells were cultured in serum-free DMEM. EVs were separated using the CF-PBP method. Data are mean $\pm$ S.D., $n = 2$. (E) Screening of xenofree, serum-free media that efficiently induce EV secretion. Data are mean $\pm$ S.E.M., **** $p < 0.0001$ (vs. all other media), $n = 8$, unpaired t-tests.

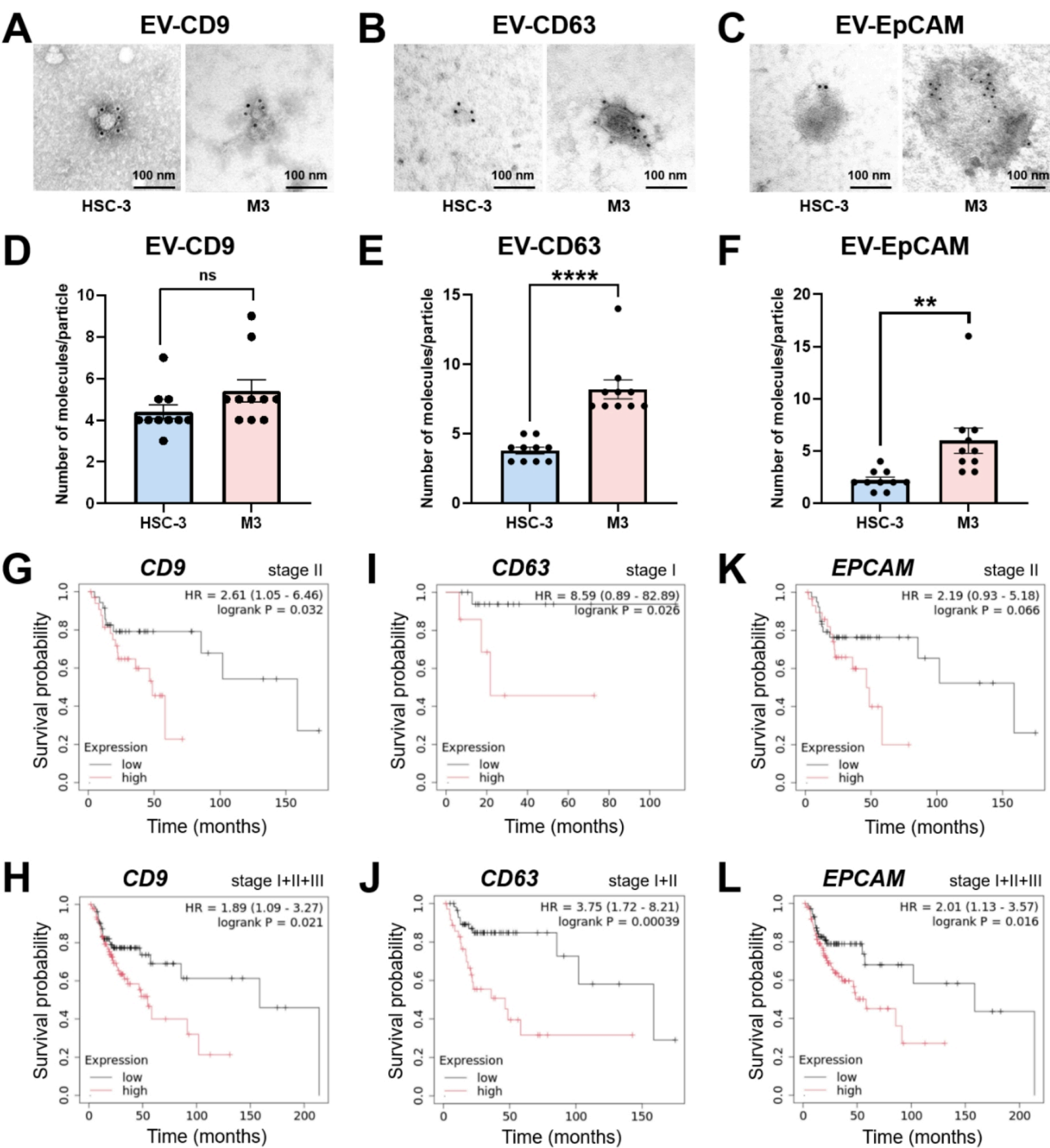


Figure 3

Rapidly metastatic oral cancer EVs contain higher levels of EpCAM and CD63. (A–F) Immunoelectron microscopy (immunoEM) of CD9, CD63 and EpCAM on EV surfaces of HSC-3 and M3 cells. Cells were cultured in E8 medium. EVs were separated using the FC-PBP method. Representative immunoEM images (A–C) and the number of molecules per EVs (D–F) are shown. Scale bars, 100 nm. The top 10 EVs were analyzed for counting (n = 10). Data were expressed as means $\pm$ S.E.M. ns, not significant. ****p < 0.0001, **p = 0.0016, unpaired t-test. (G–L) Kaplan-Meier survival analysis of the high or low gene expression of CD9 (G, H), CD63 (I, J) and EpCAM (K, L) in HNSC. (G) CD9 at stage II (hazard ratio (HR) = 2.61; p = 0.032), n = 35 (low), n = 34 (high). (H) CD9 at stage I+II+III (HR = 1.89; p = 0.021), n = 80 (low), n = 92 (high). (I) CD63 at stage I (HR = 8.59; p = 0.026), n = 18 (low), n = 7 (high). (J) CD63 at stage I+II (HR = 3.75; p = 0.00039), n = 58 (low), n = 36 (high). (K) EPCAM at stage II (HR = 2.19; p = 0.066), n = 39 (low), n = 30 (high). (L) EPCAM at stage I+II+III (HR = 2.01; p = 0.016), n = 72 (low), n = 100 (high).

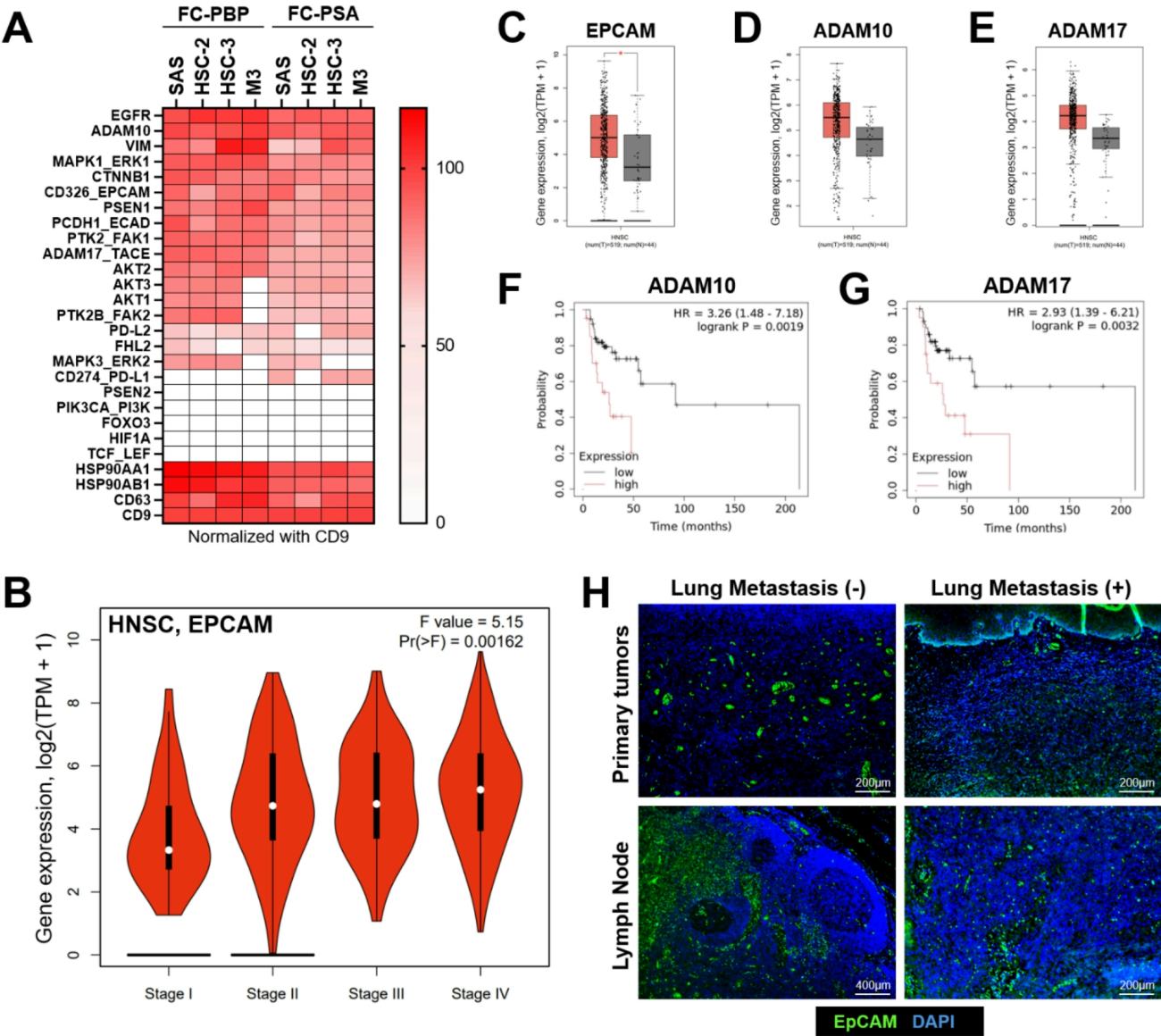


Figure 4

EpCAM signaling proteins are contained in oral cancer EVs. (A) A heat map showing that oral cancer EVs harbor EpCAM and its related proteins (EGFR, ADAM10/17, ERK1/2, CTNNB1, PSEN1, FAK1/2, AKT1/2/3, PD-L1/2, etc.). Cells were cultured in Essential 8 medium. Small EVs were separated using the FC-PBP and FC-PSA methods. (B) Violin plots showing HNSC stage-dependent increase of *EPCAM* mRNA expression. (C–E) Box-and-whisker plot showing *EPCAM* (C), *ADAM10* (D), and *ADAM17* (E) mRNA expression in HNSC (red, n = 519 cases) and normal tissues (gray, n = 44 cases). *p < 0.01. (F, G) Kaplan-Meier plotting of stage III HNSC patients' survival with high (red) vs. low (black) *ADAM10* (F) or *ADAM17* (G) expression. (F) HR = 3.26, p = 0.0019, n = 57 (low) and 21 (high). (G) HR = 2.93, p = 0.0032, n = 58 (low) and 20 (high). (E) Representative IHC images showing EpCAM expression in primary oral tumors and LNs in the cases with or without lung metastasis.

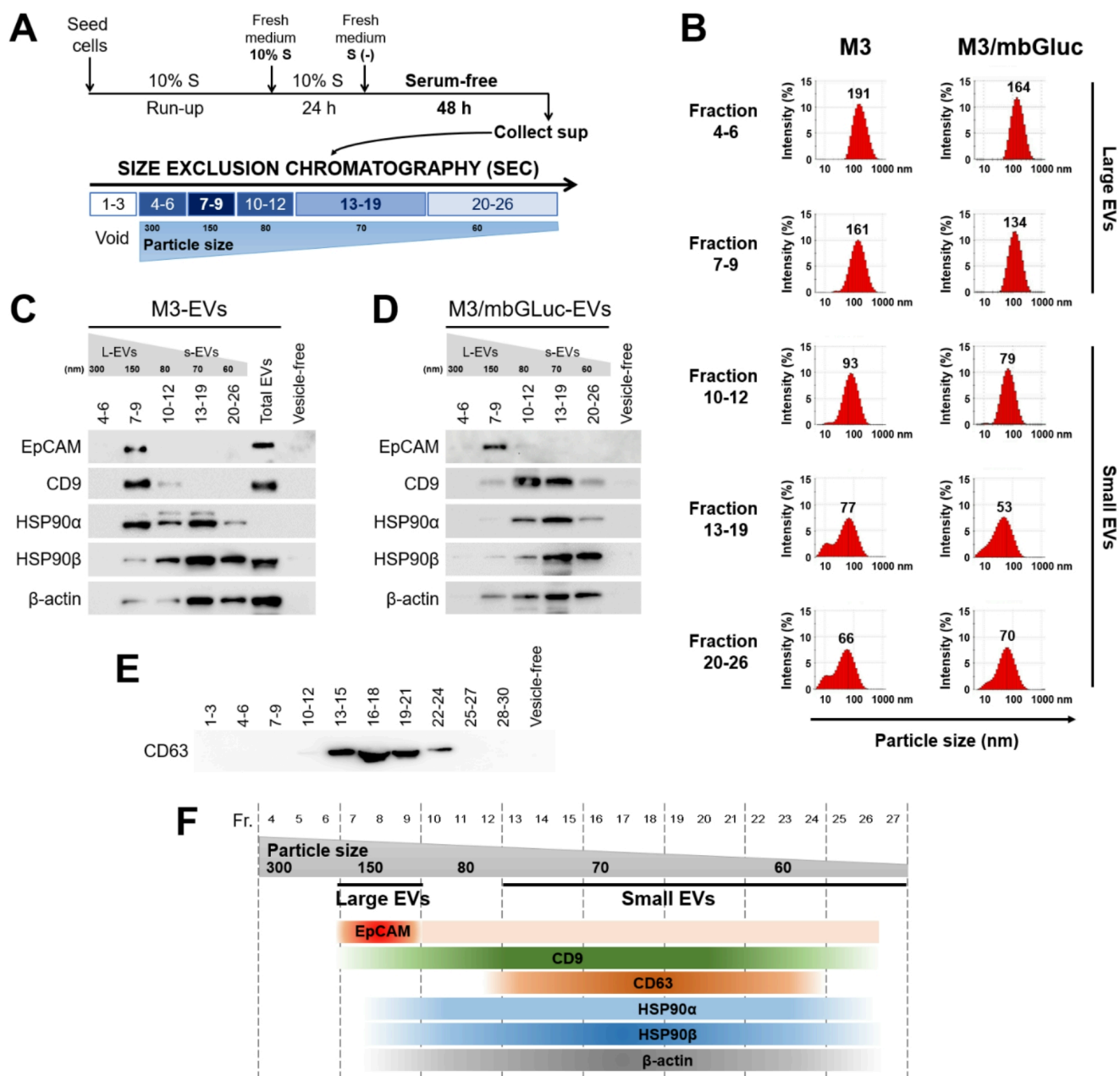


Figure 5

Metastatic oral cancer cells release EpCAM-positive large EVs and CD63-positive small EVs. (A) A workflow of the SEC. (B) Particle size distributions of the SEC fractions. (C, D) Western blotting of CD9, EpCAM, HSP90α, HSP90β, and β-actin in EVs released by M3 (C) and M3/mbGLuc cells (D). (E) Western blotting of CD63 found in small EVs (Fr. 13–24) secreted by M3/mbGLuc cells. (F) A schematic showing the presence of EV markers in large and small EVs.

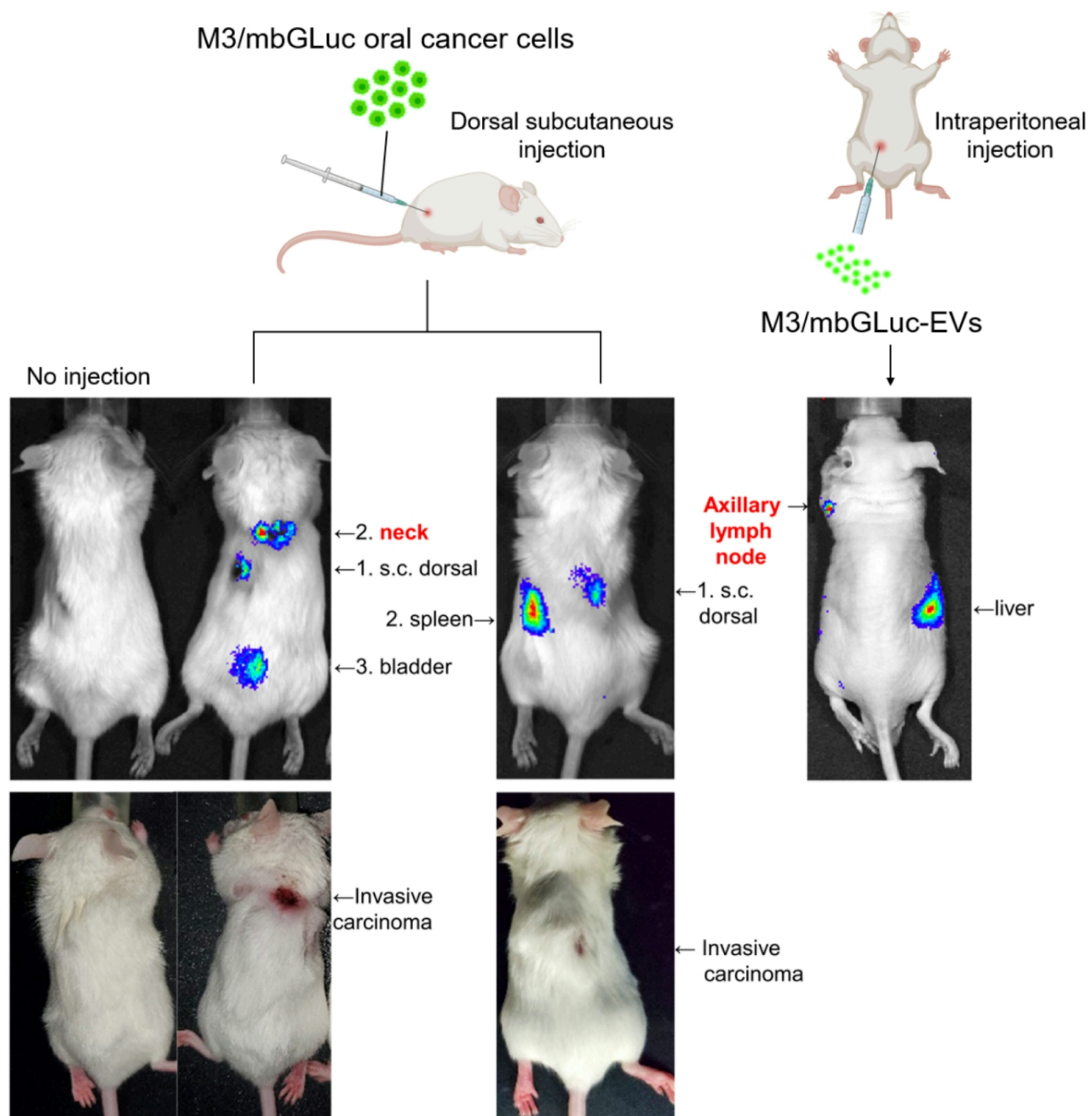


Figure 6

Biodistribution of oral cancer-derived EVs to lymph nodes. Cells were cultured in serum-free DMEM. EVs were separated using the FC-PBP method. Top, schemes of injection methods of M3/mbGLuc oral cancer cells and their EVs. Bottom, in vivo imaging showing the biodistribution of oral cancer cells and their EVs. M3/mbGLuc cells were subcutaneously xenografted to the dorsal wall of the mice (left). EVs were collected from M3/mbGLuc cells and injected into the abdominal cavity of the mice (right).

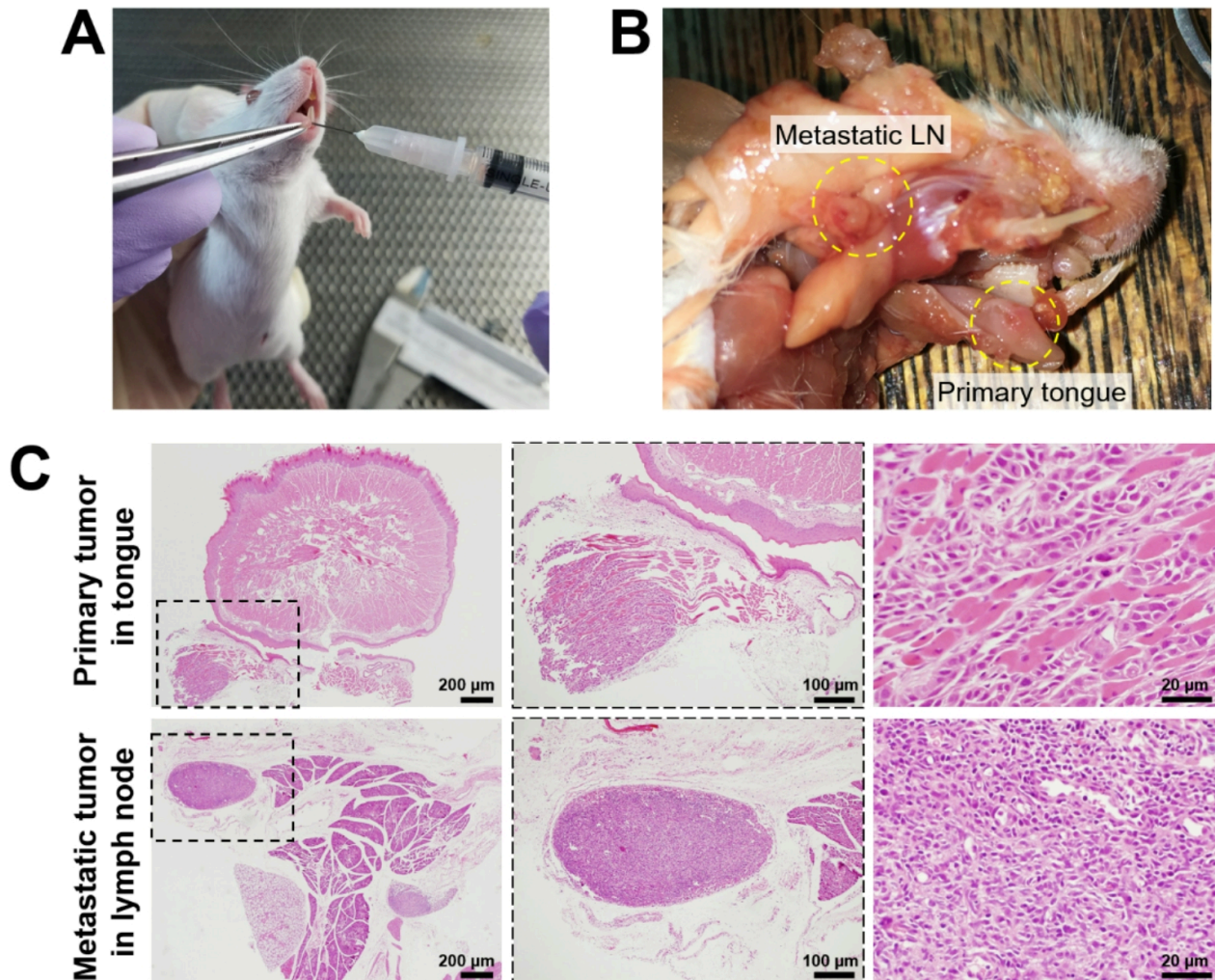


Figure 7

Tongue cancer metastasize to cervical lymph node in orthotopic xenograft. (A) Representative image of orthotopic inoculation of M3 cells into the lateral tongue of a SCID mouse. (B) Macroscopic view at necropsy showing primary tongue tumor and enlarged cervical lymph nodes (dashed circles). (C) H&E staining of the primary tongue tumor (top panels) and metastatic cervical lymph node (bottom panels). Left panels show low-magnification images of the entire tissue sections, middle panels show magnified views of the boxed regions, and right panels show high-magnification images. Both primary and metastatic lesions exhibit poorly differentiated carcinoma morphology. Scale bars, 200 μm (left), 100 μm (middle), and 20 μm (right).

Supplementary Files

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