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Rab11 suppresses head and neck carcinoma by regulating EGFR and EpCAM exosome secretion

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ABSTRACT

Objectives: Rab11(Rab11a and Rab11b) localizes primarily along recycling endosomes in cells and is involved in various intracellular trafficking processes, including membrane receptor recycling and secretion of exosomes or small extracellular vesicles (EVs). Although Rab11 is closely associated with the progression and metastasis of various cancer types, little is known about Rab11' role in head and neck squamous cell carcinoma (HNSCC). In this study, we investigated the roles of Rab11a and Rab11b in HNSCC.

Methods: The clinical significance of Rab11 expression in HNSCC was investigated using a public database and tissue microarray analysis. Stable cell lines with loss and gain of Rab11a or Rab11b were originally established to investigate their roles in the proliferative, migratory, and invasive capabilities of HNSCC cells.

Results: Database analysis revealed a significant association between Rab11b mRNA expression and a favorable patient survival rate in HNSCC. Tissue microarray analysis revealed that Rab11b expression was the highest in normal tissues and gradually decreased across the stages of HNSCC progression. Overexpression of Rab11a or Rab11b resulted in a decrease in epidermal growth factor receptor (EGFR), Epithelial cell adhesion molecule (EpCAM) exosome secretion, and the migratory and invasive potential of HNSCC cells. The knockdown of Rab11a or Rab11b increased EpCAM/CD9 exosome secretion in addition to the migratory and invasive potential of HNSCC cells.

Conclusions: Rab11 suppresses HNSCC by regulating EGFR recycling and EpCAM exosome secretion in HNSCC cells. Our results indicate that Rab11b is a superior prognostic indicator of HNSCC and holds promise for developing novel therapeutic strategies.

1. Introduction

Most advanced cases of oral carcinoma are associated with jawbone invasion, which results in facial deformity, reduced quality of life (QOL), and poor prognoses. Hence, surgical treatment is mainly based on the extensive removal of the jawbone. The invasiveness of oral carcinoma cells into the jawbone is followed by their migration into the bone resorption area, where they are resorbed by osteoclast-secreted enzymes such as cathepsin K (CTSK), matrix metalloproteinase (MMP) [[1–3]]. Recently, in addition to CTSK and MMPs, the role of extracellular

Abbreviations: (CTSK), Cathepsin K; (EpCAM), Epithelial cell adhesion molecule; (EGFR), Epithelial growth factor receptor; (EV), Extracellular vesicle; (HNSCC), Head and neck squamous cell carcinoma; (MMP), Matrix metalloproteinase; (NVEP), Non-vesicular extracellular particles.

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vesicles (EVs) in cancer cell invasion and metastasis has been investigated. EVs can educate the tumor microenvironment and pre-/-pro-metastatic niche by transferring cargo molecules such as RNA, proteins, and lipids to recipient cells in local and distant tissues [4]. Many proteins are involved in the secretion/release of EVs, including Rab proteins — and Rab11 in particular. Epidermal growth factor receptor (EGFR) involves cell proliferation in cancer via EGFR signaling, with exosomal EGFR essential for the progression of HNSCC [[5,6]]. Epithelial cell adhesion molecule (EpCAM) is also a key factor in cancer progression, as exosomal and extracellular EpCAM promote cancer progression, including HNSCC [[7–10]]. Therefore, we hypothesized that Rab11 regulates cancer cells by regulating exosomal EpCAM and EGFR level.

The Rab family of proteins represents the largest branch of the Ras superfamily of small GTPases, and approximately 70 Rab GTPases have been identified in the human genome [11]. Rab GTPases control the vesicular transport system, which is composed of vesicle budding, uncoating, motility, and fusion within a target membrane site, and are important for ensuring that cargo is delivered to their correct destinations. They regulate the specificity and directionality of membrane trafficking signals by recruiting effectors, which come in many forms — such as sorting adaptors, cytoskeletal motor proteins, tethering factors, kinases, and phosphatases — to the vesicular membrane surface [[12–14]].

The abundant expression of Rab proteins is closely associated with cancer cell migration, invasion, metastasis, and even prognosis [15]. Indeed, Rab1A [[16,17]], Rab2A [18], Rab3D [19], Rab 4 [20], Rab5 [[20,21]], Rab8 [22], Rab11 [23], Rab17 [24], Rab 21 [25], Rab 23 [26], Rab25 [[27 [28–30]], Rab27B [31], Rab 31 [32], Rab35 [33], Rab37 [[34–36]], and Rab 38 [37] are involved in tumor migration, invasion, and metastasis.

It has been previously reported that Rab11a promotes breast cancer invasion by promoting EGFR recycling [38]. Structurally, Rab11 is classified into three isoforms, Rab11a, Rab11b, and Rab25. Of these, Rab11a is ubiquitously expressed, Rab11b is enriched only in the heart, brain, testes [39], polarized MDCK, and gastric parietal cells [40], while Rab25 is found only in epithelial cells [41]. Recently, it was reported that Rab11 expression influences cancer prognoses in various tissues [42–46]]. More recently, Rab11a expression was associated with the level of aggressiveness of a cancer via the regulation of EGFR signaling in lung squamous cell carcinoma [47]. On the other hand, the role of Rab proteins in the release of EVs from cells into the extracellular space has attracted attention — it has become clear that intracellular transport mechanisms play an important role in the secretion of these proteins and EVs [[48–51]]. Among these, Rab11 is an important factor in the control of EVs and cancer [48]. In the present study, we analyzed how Rab11 regulates intracellular trafficking and affects cancer cell growth and invasion.

2. Materials and methods

2.1. Antibodies and reagents

Dalbecco's modified Eagle's medium (DMEM) was purchased from WAKO (Osaka, Japan). Antibodies (Abs) of EpCAM (VU1D9), Rab11a and Rab11b were obtained from Cell Signaling Tech. (Massachusetts, USA). HRP-conjugated GAPDH monoclonal antibody was from Proteintech (IL 60018, USA). *Anti*-EGFR (ab32562) Abs and HRP-conjugated *anti*-β-actin antibody (ab49900) were purchased from Abcam (Cambridge, MA). Abs of CD9 (D252-3) were purchased from MBL (Tokyo, Japan).

2.2. Cell culture

Human HNSCC cell lines SAS, Ca9-22, OSC-19, HSC-2, HSC-3, and HSC-4 were obtained from JCRB Cell Bank (Osaka, Japan). Cells were cultured in DMEM supplemented with 10 % fetal bovine serum (FBS) (Gibco-BRL), 100 U/ml penicillin, and 100 μg/mL streptomycin in 5 % CO_2 and 95 % air humidified incubator at 37 °C.

2.3. Western blot (WB) analysis

WB was performed as described previously [52]. Briefly, whole cell lysates (WCL) were prepared using RIPA buffer (50 mM Tris-HCl [pH 8.0], 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 150 mM NaCl) supplemented with proteinase inhibitor cocktail (Sigma-Aldrich Tokyo, Japan). WCL (15–50 μg) were run on 10 % SDS-PAGE and then transferred to polyvinylidene fluoride membranes. The blots were blocked in Tris-buffered saline containing 0.05 % Tween-20 and 5 % skim milk for 1 h at room temperature (RT), and subsequently probed with various antibodies (Rab11a, Rab11b, EGFR, EpCAM; 1:1,000, CD9; 1:3,000, GAPDH & β-actin; 1:2000) at 4 °C overnight. After washed, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. WB visualization was achieved using a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA), according to the ECL substrate (Millipore, USA).

2.4. RNA interference

SAS cells were transfected with 5.0 μg control short hairpin (sh)RNA plasmid (sc-108060; Santa Cruz Biotechnology, Inc.) or human Rab11a and Rab11b shRNA plasmid (sc-44491-SH; Santa Cruz Biotechnology, Inc.) with the use of 4D-Nucleofector™ (Lonza Group, Ltd.). Two days later, cells were cultured in DMEM containing 10 % FBS for 5 days in the presence of 1.6 μg/mL puromycin dihydrochloride to select cells that stably expressed the shRNAs.

2.5. tissue microarray analysis

Expression of human Rab11b was analyzed in HNSCC tissue and a normal tissue microarray (#OR601c; US Biomax). For immunohistochemical (IHC) analysis, specimens were incubated with *anti*-Rab11b antibody (1:250) overnight at 4 °C. The slides were then treated with a streptavidin-biotin complex (EnVision System Labeled Polymer, HRP; Dako; Agilent Technologies, Inc.) for 60 min at a dilution of 1:100. The immunoreaction was visualized with the use of DAB substrate-chromogen solution (Dako Cytomation Liquid DAB Substrate Chromogen System; Dako; Agilent Technologies, Inc.). Cells were counted using a light microscope and evaluated.

2.6. xenograft of SAS cells to mice

SAS cells were transplanted subcutaneously to the backs and heads of 5 nude mice (BALB/c-nu/nu) at 1.0×10^6 cells per xenograft. As a control, 5 nude mice were xenografted by the above-mentioned method. Mice were fixed by perfusion with 10 % neutral buffered formalin. Tumors were excised, fixed by immersion in 10 % neutral buffered formalin, dehydrated, and embedded in paraffin. Paraffin sections were prepared for hematoxylin-eosin (HE) staining and IHC and observed with a light microscope. For the Negative Control, after blocking, the 2nd antibody of the mouse was applied and this was used as the Negative Control.

2.7. Immunohistochemistry (IHC)

Cells seeded and grown on glass coverslips were fixed with 4.0 % PFA in PBS for 1h at RT. After washing with PBS, fixed cells were permeabilized with 0.1 % Triton X-100 in PBS for 10 min. Cells were incubated sequentially with 10 % normal goat serum for 30 min and with primary antibodies (1:300) at 4 °C overnight. Cells were washed and stained with second antibodies, Alexa Fluor 594 goat anti-rat IgG or Alexa Fluor 594 goat anti-rabbit IgG (Cell Signaling Technology,

Danvers, MA, USA). Ultimately, nuclear staining with DAPI (Invitrogen Carlsbad, CA, USA) was carried out. The samples were visualized using a laser-scanning confocal imaging system (LSM 780 META; Carl Zeiss, AG, Jena, Germany) at Central Research Laboratory, Okayama University Medical School.

2.8. retrovirus construction and expression of human Rab11

Retrovirus construction and expression of human Rab11a and Rab11b were generated as the methods described previously [[52,53]]. Briefly, full-length cDNAs of human Rab11a and Rab11b were generated by PCR method employing cDNA originating from SAS cells. The primers were used for Rab11a (forward: 5′-GGACGAGCTGTACAAGGG CACCCGCGACGAGTAC-3′ and reverse: 5′- CTACCCGGTAGAATTCTTA GATGTTCTGACAGCACTGC-3′) and for Rab11b (forward: 5′- GGAC-GAGCTGTACAAGGGGACCCGGGACGACGAGTAC -3′ and reverse: 5′-CTACCCGGTAGAATTCTCACAGGTTCTGGCAGCAC -3′). Then, the cDNA(s) were amplified using Prime STAR GXL DNA polymerase (Takara, Tokyo). To generate GFP-Rab11a and Rab11b fusion protein, the amplified fragments were fused with linearized pMSCVpuro-GFP, gifted by Prof. Kosei Ito (Nagasaki University, Japan), using In-Fusion cloning kit (Clontech, Mountain View, CA, USA). pMSCVpuro-GFP was also used as a control vector. Vectors were transfected into HEK293T cells by using Lipofectamine 2000 (Life Technologies, Gaithersburg, MD, USA). After incubation at 37 °C in 5 % CO_2 for 48 h, the supernatants composed of viruses were collected and infected into SAS cells. The cells were cloned by puromycin (5 μg/mL) diluted in DMEM supplemented with 10 % FBS, and the medium was refreshed every 3 days. After 2 weeks of culture, puromycin-resistant cells were obtained.

2.9. cell viability assay

Cells seeded in 96-well cell culture plates were incubated with the Cell Counting Kit-8 (CCK-8: Dojindo, Kumamoto, Japan) for 1 h, and then the absorbance at 450 nm was measured with a microplate reader (HITACHI, Japan).

2.10. In vitro scratch assay

A total of 1.0×10^5 cells/well were seeded in 6-well plates. At 90 % confluence, an artificial wound was made using the head of a 200-μl pipette tip across the monolayer. The cells were then washed with PBS and cultured in a serum-free medium for 10 or 12 h. Wound width was measured microscopically at 0 and 10 or 12 h.

2.11. Cell invasion assay

The invasion assay was conducted with BioCoat Matrigel invasion chambers (Corning, NY, USA). Cells grown in a serum-free culture medium were applied to the upper chamber. In the lower chamber, a medium containing 10 % FBS as a chemoattractant was applied. After incubation for 48 h at 37 °C, the remaining cells were removed with cell scraper. The filters were then fixed with 10 % methanol for 10 min and stained with Diff Quik (Funakoshi, Japan). Cell counting was performed in four random microscope fields per well.

2.12. Preparation of EVs

EV fractions were prepared as described previously [[54–57]]. Briefly, the cell culture supernatant was centrifuged at 2000 g for 30 min and then at 10,000 g for 30 min at 4 °C. The supernatants were filtered with a 0.2-μm pore filter in a few experiments. The pass-through was concentrated using an ultrafiltration device for the molecular weight 100 K to separate an EV fraction and vesicle-free factors. The concentrate was applied to polymers of Total Exosome Isolation Reagent (Thermo Fisher Scientific, Carlsbad, CA). The EV fractions were suspended in PBS without calcium or magnesium (PBS (−)). For protein assay, 10 × RIPA buffer and 100 × a protease inhibitor cocktail (Sigma, St. Louis, MO) were added to the EV fraction. For WB, equal amounts of protein were applied to each lane.

2.13. Particle size distribution

A part of the EV fraction was diluted within PBS (−) to a volume up to 40 μL and then analyzed using a Zetasizer Nano ZSP (Malvern Panalytical, Malvern, UK) in a range of 0.3–10,000 nm-diameters, as described [[54,57]].

2.14. Transmission electron microscopy

Transmission electron microscopy (TEM) and particle diameter distribution analysis were carried out as described [[54,57]]. Briefly, a 400-mesh copper grid coated with formvar/carbon films was treated hydrophilically. EV suspension (5–10 μL) was placed on Parafilm®, and the grid was floated on the EV liquid and left for 15 min. The sample was negatively stained with 2 % uranyl acetate solution for 2 min. EVs and non-vesicular extracellular particles (NVEP) on the grid were visualized with 20,000 × magnification with an H-7650 transmission electron microscope (Hitachi).

2.15. Statistical analysis

Statistical significance was calculated using Microsoft Excel. Differences between two data sets were examined with a Welch's *t*-test, and more than three sets of data were examined with a Kruskal-Wallis test; values of $p < 0.05$ were considered to indicate statistical significance. Data were expressed as means ± S.D. unless otherwise specified.

3. Results

3.1. Rab11 expression in HNSCC

To determine whether HNSCC cells express the Rab11 protein, several HNSCC cell lines (SAS, Ca9-22, OSC-19, HSC2, HSC3 and HSC4) were examined at the protein level. Rab11a and Rab11b were expressed in all HNSCC cells examined (Fig. 1A). SAS cells that moderately expressed both Rab11a and Rab11b were selected for subsequent analysis. To investigate the tumor tissue localization of Rab11a and Rab11b in vivo, we next transplanted SAS cells into nude mice and examined their localization by IHC (Fig. 1B). Rab11 was expressed at the border between cancer and epithelial cells. However, no findings of cell morphology changes such as spindle shape, were observed upon knockdown or overexpression of Rab11 (Supplementary Fig. 1).

3.2. Rab11 affects cell migration and invasion in HNSCC

To determine whether Rab11 expression is associated with HNSCC invasion and migration, we first used Rab11 shRNA to generate Rab11a and Rab11b knockdown cell lines in SAS cells. Each shRNA efficiently suppressed the Rab11a and Rab11b knockdown in cell lines, but the generated cell lines had little effect on each other's Rab protein expression (Fig. 2A). These did not affect the proliferative potential of the suppressed sublines (Fig. 2B). Invasion assays were performed using Matrigel invasion chambers to examine the invasive abilities of Rab11a and Rab11b knockdown cells. The invasive capability of each Rab11-knockdown cell line was enhanced by the knockdown (Fig. 2C and D). To examine the effect of Rab11 suppression on the migratory ability of cancer cells, we performed a wound closure assay and found that Rab11 knockdown promoted cell migration and invasion (Fig. 2E and F).

To examine the migration and invasion abilities of SAS cells overexpressing Rab11a and Rab11b, the GFP-fused Rab11 cDNA was introduced into a retrovirus and expressed in SAS cells. The expression or

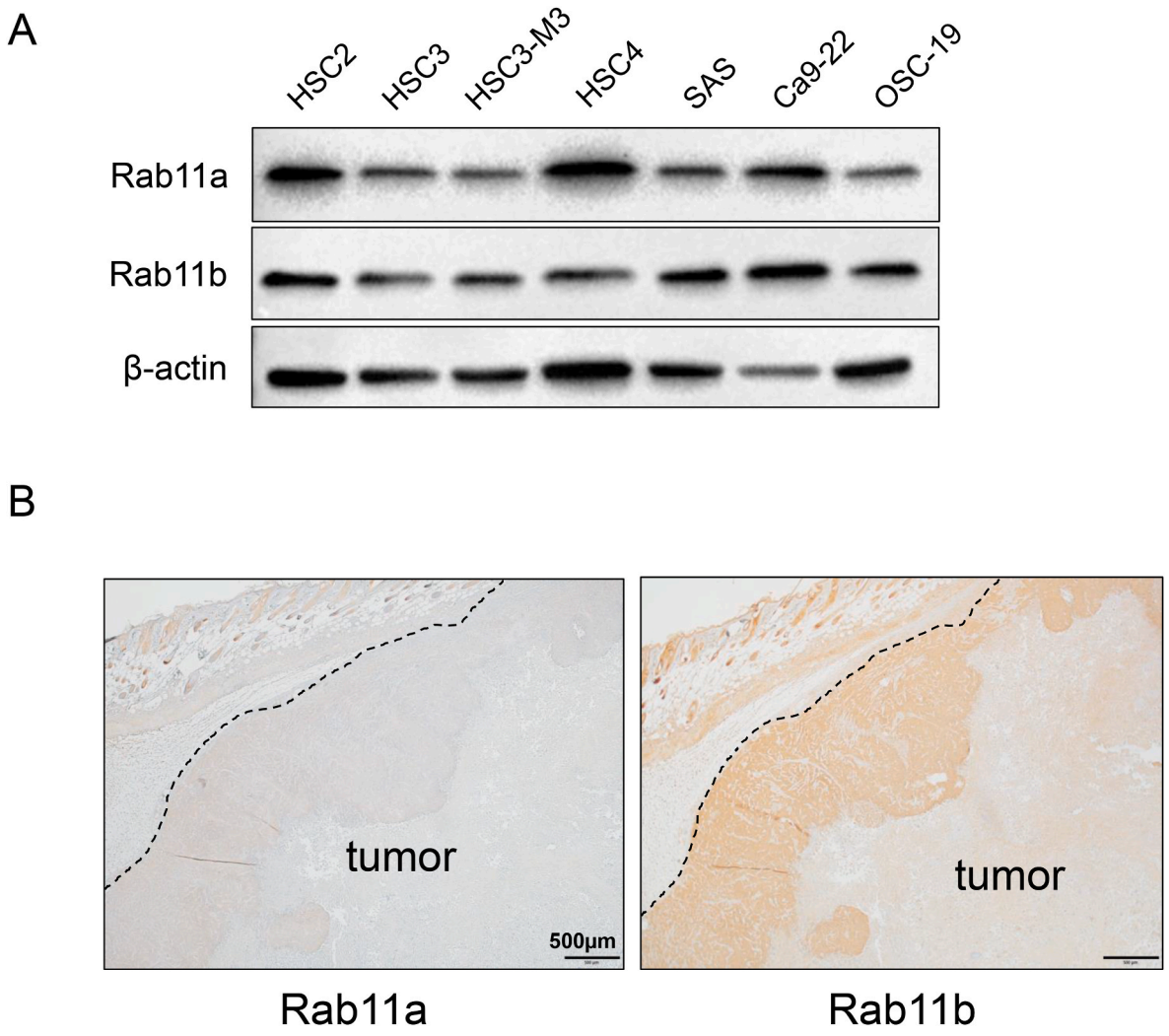

Fig. 1. The expression of Rab11 in HNSCC. (A) Detection of Rab11a/b in SAS, Ca9-22, OSC-19, HSC2, HSC3 and HSC4 cell lines. Cells were cultured in DMEM containing 10 % FBS. The whole cell lysates (WCL) were subjected to WB analysis with *anti*-Rab11a or Rab11b antibodies or β-actin-HRP as a loading control. (B) Rab11a and Rab11b IHC in mouse tumor xenograft model. SAS cells were transplanted into nude mice. Tumor tissues were stained using *anti*-Rab11a or Rab11b antibodies. Scale bar, 500 nm.

proliferation of these cell lines was not affected by each other (Fig. 3A and B). However, in contrast to the Rab11-repressed cell lines, both Rab11-overexpressing cell lines showed suppression of tumor invasiveness in the Matrigel invasion assay (Fig. 3C and D). Similarly, both Rab11b overexpressing cell lines showed a suppression of tumor invasiveness, although the Rab11a overexpressing cell lines showed no significant difference from the controls (Fig. 3E and F), indicating different roles for Rab11a and Rab11b.

3.3. Expression of EGFR was altered by knockdown and overexpression of Rab11

It is well known that EGFR, a receptor on the cell membrane, is involved in cancer growth, invasion, and migration. Therefore, to investigate whether Rab11 is involved in EGFR expression, we examined EGFR expression in Rab11 knockdown and overexpression systems. Rab11 knockdown showed a slight trend toward increased EGFR expression, but the difference was not significant (Fig. 5A). In contrast, Rab11 overexpression significantly decreased the expression of both Rab11a and Rab11b. In particular, the phenomenon of EGFR expression was more pronounced in Rab11a (Fig. 4B).

3.4. Rab11 inhibits EpCAM exosome secretion from HNSCC cells

To examine whether Rab11 is involved in exosome secretion or EV release, EVs were prepared from the culture supernatant of Rab11a and Rab11b knockdown and overexpression cell lines. EV morphology was observed using TEM, and particle size was measured using the Zetasizer Nano ZSP. The EV fractions contained a lipid bilayer (Fig. 5A, Supplementary Fig. 2A). The size of EVs from the Rab11a and Rab11b knockdown cell lines was 239.3 nm and 195.2 nm in diameter, respectively, which were larger than the control EVs (159.3 nm) (Fig. 5B). The EV marker proteins EpCAM and CD9 were also increased by Rab11a/b knockdown when compared to those in the control (Fig. 5 C, D).

Cup-shaped particles surrounded by lipid bilayers were observed in all the EV fractions, including the control (Fig. 6A). The size of EVs from Rab11a and Rab11b overexpressing cell lines was 154.8 nm and 143.7 nm, respectively, in diameter and nearly the same particle size of the control EVs (130.3 nm) (Fig. 6A and B, Supplementary Fig. 2B). The Rab11 overexpressing cell lines released almost the same amount of total EV protein from the same number of cells, although they released significantly reduced levels of the EV marker proteins EpCAM and CD9 compared to the controls (Fig. 6 C, D).

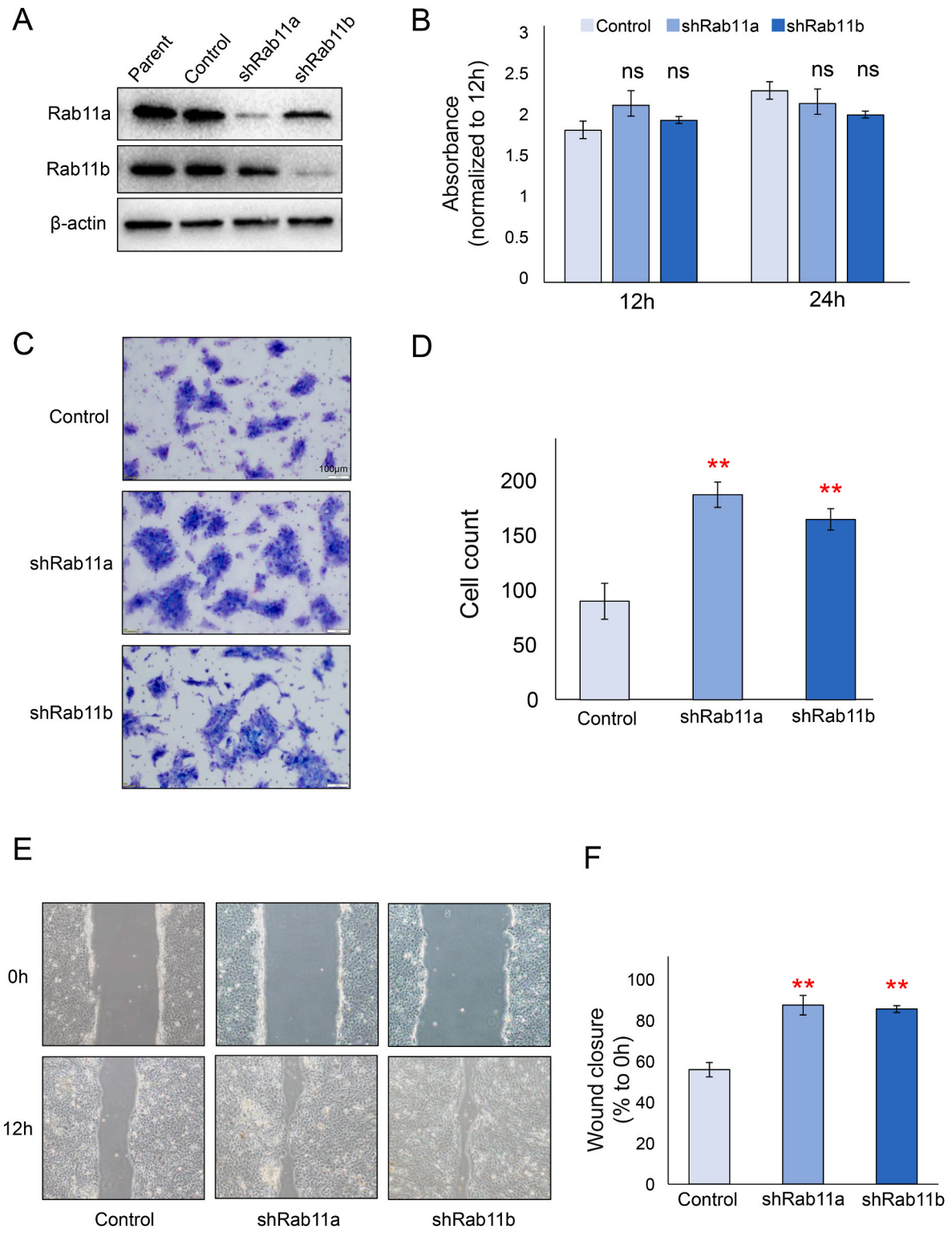

Fig. 2. The effects of Rab11a/b knockdown in SAS cells. (A) WB showing individual knockdown of Rab11a/b. SAS cells were transfected with a human Rab11a or Rab11b shRNA plasmid or a control vector. The whole cell lysates (WCL) were subjected to SDS-PAGE, followed by WB with *anti*-Rab11a or Rab11b antibodies or β-actin-HRP as a loading control. (B) Cell proliferation was detected by CCK-8 assay. (C, D) Invasion activities of knockdown cells. Cells were cultured in a 24-well Transwell chamber at 37 °C for 12 h, and cells migrated to the lower well were fixed, stained with Diff-Quik (C), and counted (D) under a light microscope. **p < 0.01, N = 3. (E, F) Wound healing assay. Wound width was taken photos (E) and measured (F) microscopically at 0 or 48 h **p < 0.01, N = 3.

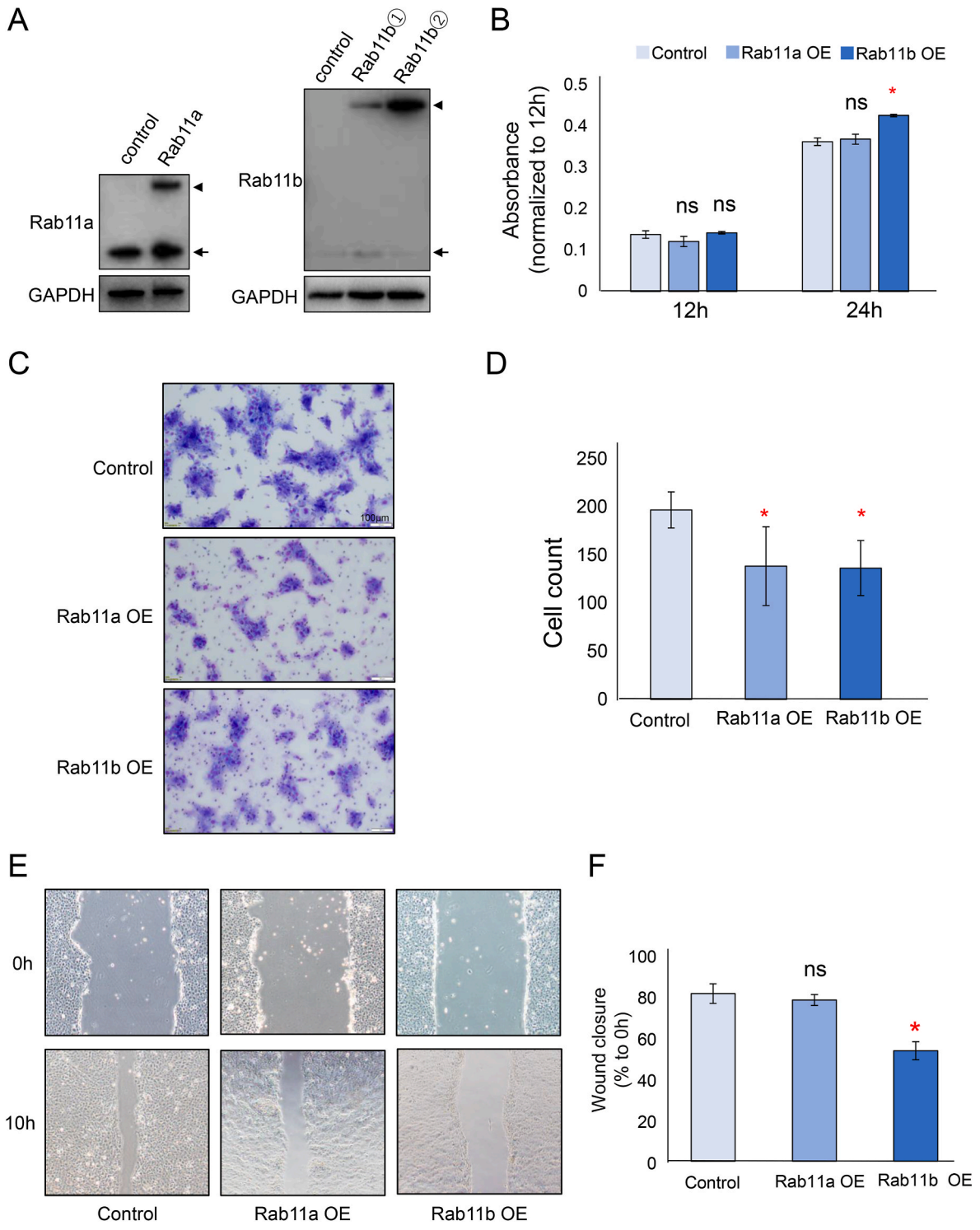

Fig. 3. The effects of Rab11 overexpression in SAS cells. (A) WB showing Rab11a/b overexpression. SAS cells were infected with a human Rab11a-GFP or Rab11b-GFP overexpression or a control virus. Arrow shows endogenous Rab11a and Rab11b, respectively and arrowhead shows fusion protein of GFP-Rab11a and GFP-Rab11b, respectively. GAPDH, loading control. (B) Cell proliferation detected by CCK-8 assay. *$p < 0.05$, $N = 3$. (C, D) Invasion activities of Rab11 overexpression cells. Cells in a 24-well Transwell chamber were incubated at 37 °C for 12 h, and cells that migrated to the lower well were fixed, stained with Diff-Quik (C), and counted (D) under a light microscope. *$p < 0.05$, $N = 3$. (E, F) Wound healing assay. Wound width was taken photos (E) and measured (F) microscopically at 0 and 48 h later. *$p < 0.05$, $N = 3$. ns: no significance.

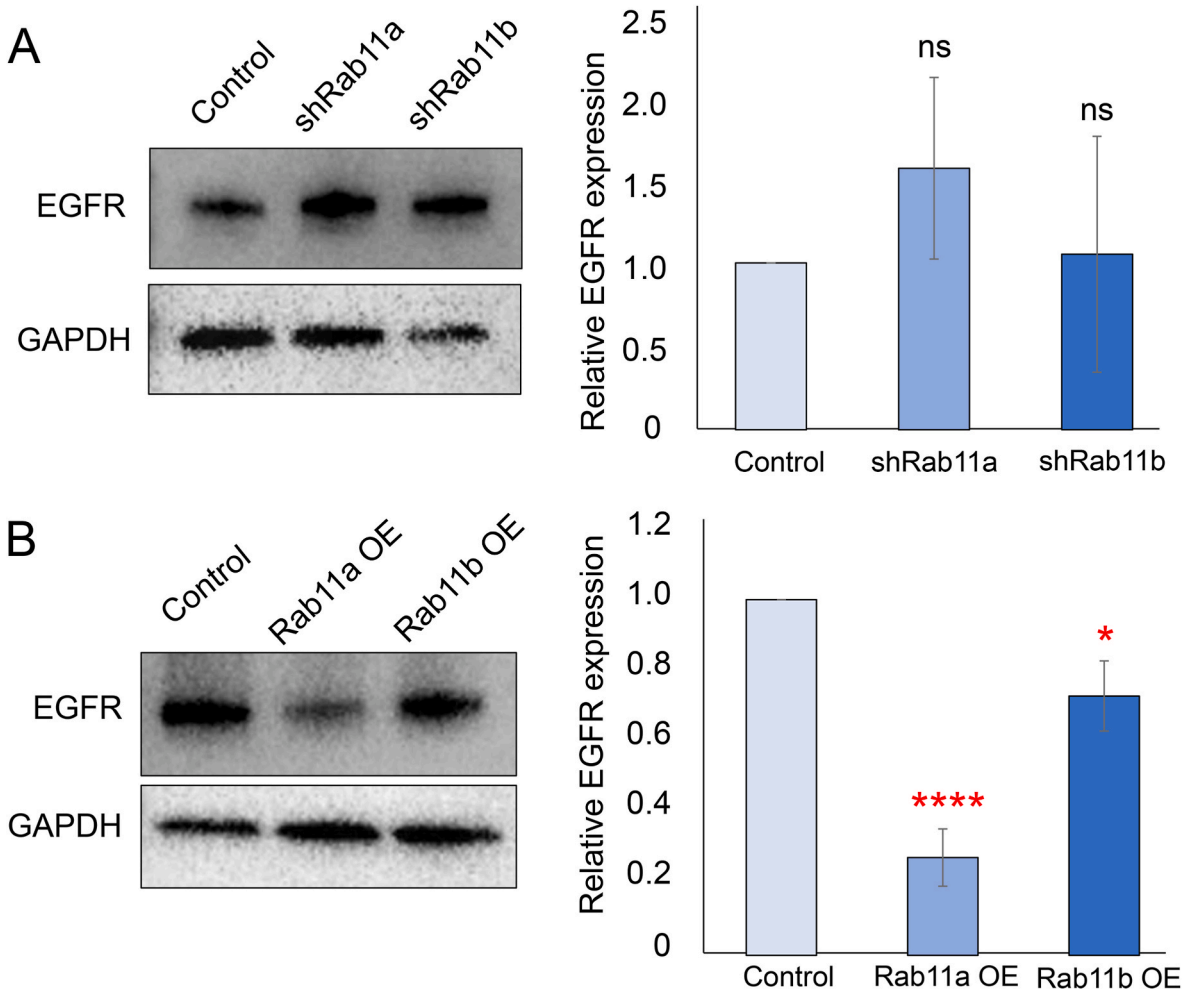

Fig. 4. The expression of EGFR in Rab11 knockdown and overexpression cells. (A) WB showing EGFR increased by Rab11a or Rab11b knockdown. Cells were incubated with DMEM containing 10 % FBS. Two days later, The WCL were subjected to SDS-PAGE, followed by WB with EGFR antibody. The experiments were three times and analyzed by using Image J software. Quantification is shown relative to the control. Three independent experiments were carried out. ns, no significance. (B) WB showing EGFR reduced by Rab11a or Rab11b overexpression. Cells were incubated with DMEM containing 10 % FBS. Two days later, The WCL were subjected to SDS-PAGE, followed by WB with EGFR antibody. The experiments were three times and analyzed by using Image J software. Quantification is shown relative to the control. Three independent experiments were carried out. *$p < 0.05$, ****$p < 0.0001$, $N = 3$.

3.5. Expression of Rab11 correlates with patients' survival periods in HNSCC

To investigate the involvement of Rab11a and Rab11b in HNSCC, we analyzed TCGA database. A Kaplan-Meier plot of the prognostic significance of Rab11 in HNSCC revealed that overall survival (OS) was longer in patients with lower Rab11b mRNA expression than in those with higher levels (Fig. 7A). This implies that low Rab11b expression is associated with a poor prognosis in HNSCC. In contrast, no significant differences were observed in Rab11a (Fig. 7A). To further investigate whether Rab11b influences HNSCC, we compared Rab11b expression between normal cells and HNSCC cells using tissue microarrays. Immunohistochemical analysis revealed lower levels of Rab11b in HNSCC tissues than in non-cancerous tissues (Fig. 7B). Furthermore, we quantified the tissue microarray results by scoring and found that Rab11b expression was lower in cancer cells than in normal cells (Fig. 7C).

4. Discussion

Rab proteins transport various intracellular proteins and lipids to the next organelle, extracellular secretions of EVs and NVEP, and free proteins. Of the Rab family, Rab11 is one of the oldest proteins and functions in various intracellular transport mechanisms via the recycling endosomal compartment. Notably, our study showed that Rab11 overexpression resulted in a reduction in EGFR expression in HNSCC cells (Fig. 4). EGFR mutations are one of the causes of poor patient prognosis in some cancer types, including lung and colorectal cancers, but not in HNSCC. EGFR genetic amplification, high expression, and mutations are involved in cancer growth, invasion, migration, and metastasis; however, the mechanisms of its transport to the cell membrane and recycling are still unknown. In general, Rab11 is associated with cancer growth and invasion by promoting high EGFR expression and enhancing the expression of various signaling factors. For example, in lung cancer [42, 43], hepatocellular carcinoma [44], colorectal carcinoma [45], and esophageal cancer [46], when Rab11 — and especially Rab11a — is overexpressed, the cancer becomes highly malignant and leads to a poor prognosis. Therefore, we hypothesized that high Rab11 expression in HNSCC, including oral cancer, would result in a poor prognosis. However, contrary to our expectations, knockdown of Rab11 in oral carcinoma cells promoted cancer cell growth and invasion, while overexpression of Rab11 suppressed them (Figs. 2 and 3). Consistently, the prognosis was also worse for those with lower Rab11 expression in terms of survival rates and tissue arrays from the database, and Rab11b expression was significantly lower in HNSCC, although Rab11a expression was not significant (Fig. 7). Other Rab proteins also differ in

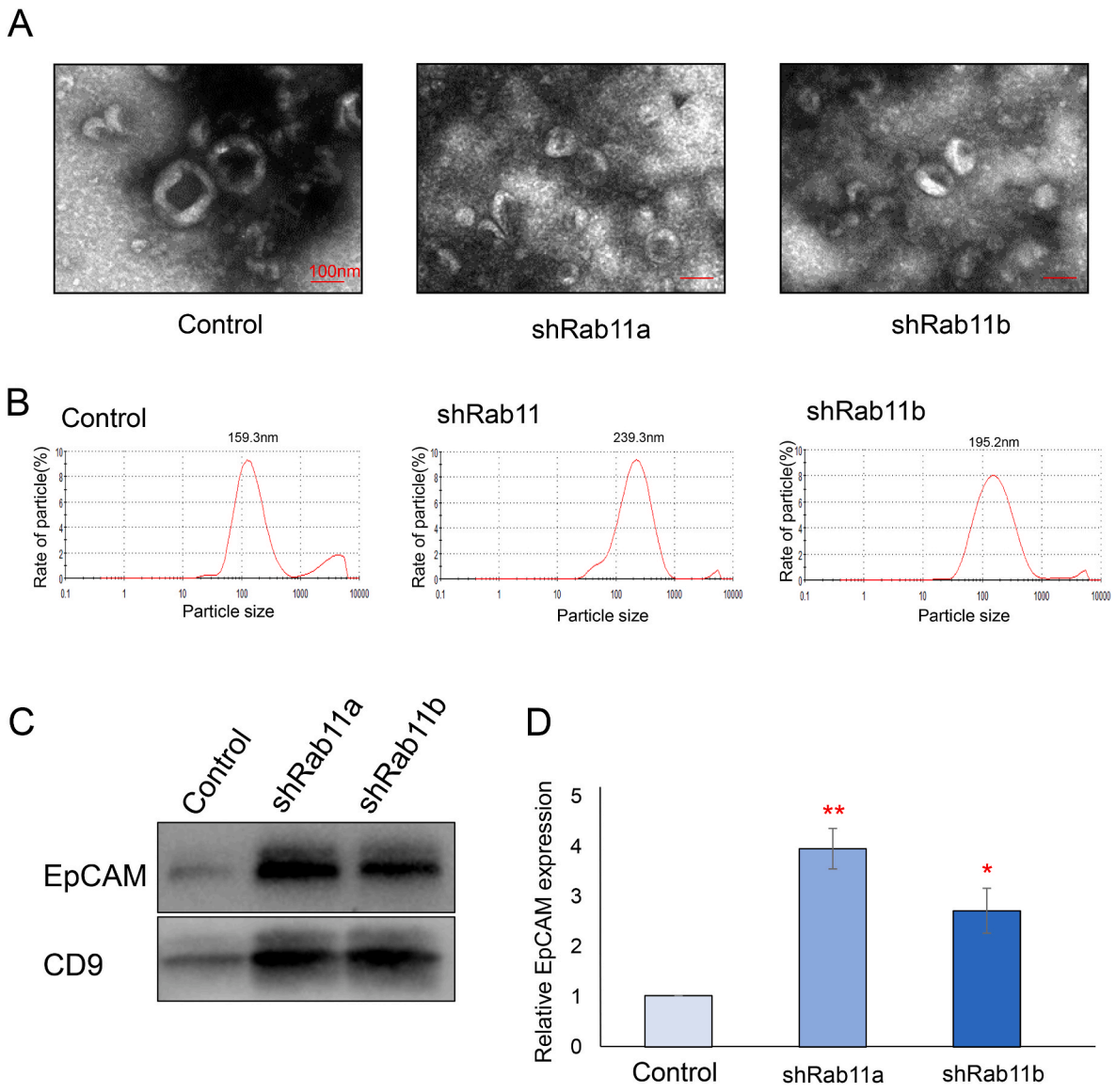

Fig. 5. The release of EpCAM + EVs from HNSCC cells was promoted by Rab11 knockdown. (A) Representative TEM images of EVs derived from SAS-based cell lines transfected with control shRNA, Rab11a or Rab11b shRNA. Scale bar, 100 nm. (B) Representative particle diameter distribution of EVs. Peak values were 150–250 nm. (C) WB showing EpCAM and CD9 in EVs. Equal amounts of protein were applied to each lane. (D) Relative levels of EpCAM expression in EVs. Image J was used to quantify WB bands in (C). Three independent experiments were carried out. *$p < 0.05$, $N = 3$.

cancer progression and prognosis depending on the tissue and organ. Rab25 acts as a promoter of cancer migration and invasion in ovarian and lung cancers [27] but as a suppressor in esophageal [28] and colon [29] cancers. Thus, the roles of Rab proteins in cancer invasion, migration, and prognosis vary among tissues and cancer types. Based on these reports and the present results, it is possible that Rab11 expression in the digestive system — particularly Rab11a, but not Rab11b — negatively regulates cancer progression.

In the present study, Rab11 overexpression decreased EGFR expression (Fig. 4). This is a similar manner to the lysosomal degradation mechanism of membrane receptors previously demonstrated in osteoclasts [[52,53]]. According to our current results, overexpression of Rab11 promoted the degradation pathway from endosomes to lysosomes rather than membrane receptor recycling to the membrane and inhibited osteoclast differentiation. However, unlike osteoclasts, suppression of Rab11 expression in cancer did not promote EGFR expression, suggesting that factors other than EGFR may be involved in cancer invasion and migration in the case of suppression of Rab11 expression.

Although MMPs and TIMP1 secreted by cancer cells are involved in cancer growth, invasion, and migration [3], their secretory mechanisms remain unknown. We recently showed that MMPs, including MMP3, are released with EVs and transferred into recipient cell nuclei [[58–60]]. In addition to MMPs and TIMP1, EVs have attracted attention for their role in vesicles secretion. Furthermore, intracellular vesicle transport mechanisms play an important role in the release of EVs and NVEP with these proteins. Indeed, Rab proteins such as Rab11, 27A, 31, and 35 play a central role in the intracellular vesicle and protein transport mechanisms [[48–51]]. In the current study, we found differences in the levels of key proteins (EpCAM and CD9) in small EVs inclusions upon knockdown or overexpression of Rab11 (Figs. 5 and 6). This may be due to changes in the proteins, nucleic acids, and lipids contained in or on EVs upon differential Rab11 expression. Differences in Rab11 expression may alter cancer cell migration and invasion. Further analyses of the number of EVs and their inclusion are necessary for future studies.

As for Rab effector proteins, the tissue-specific expression of each Rab protein varies. Future research should focus on Rab effector proteins for Rab in cancer cells. Currently, a precise explanation for the differences in the properties of Rab11a and Rab11b has not been obtained;

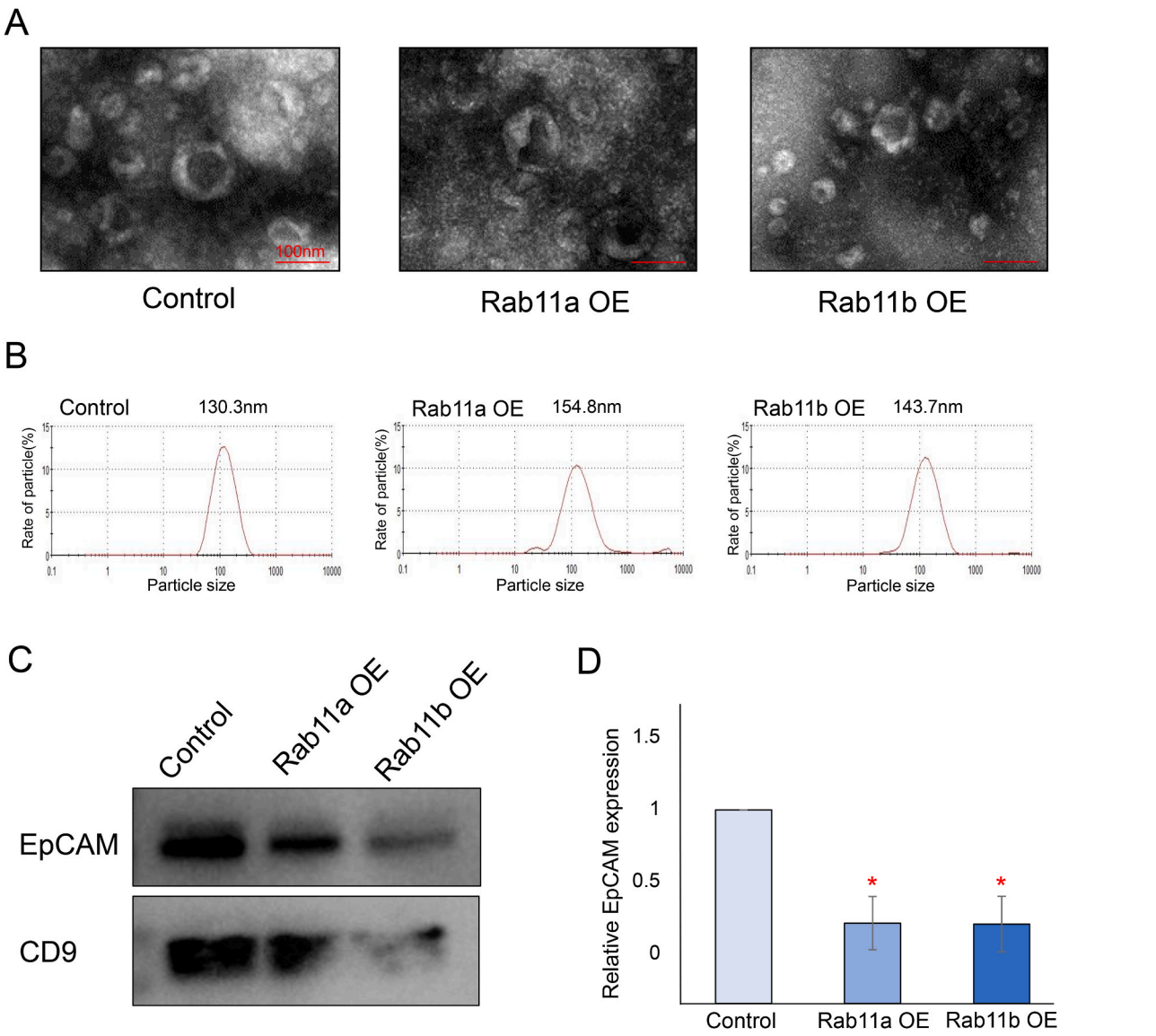

Fig. 6. The release of EpCAM + EVs from HNSCC cells was inhibited by Rab11 overexpression. (A) Representative TEM images of EVs derived from SAS-based cell lines overexpressed (OE) with control, Rab11a-GFP or Rab11b-GFP. Scale bar: 100 nm. (B) Representative particle diameter distribution of EVs. Peak values were 150–250 nm. (C) WB showing EpCAM and CD9 in EVs. Equal amounts of protein were applied to each lane. (D) Relative levels of EpCAM expression in EVs. Image-J was used to quantify WB bands in (C). Three independent experiments were carried out. *$p < 0.05$, $N = 3$.

however, it has been reported that a knockout of Rab11a in the brain has been reported to be compensated by Rab11b, but not in the intestine, suggesting that the specific expression of Rab11b may determine the difference in survival between Rab11a and Rab11b.

5. Conclusion

Rab11 acts as a tumor suppressor that controls tumor migration and invasion by regulating tumor receptor recycling on the plasma membrane. Furthermore, Rab11 inhibits exosome secretion in HNSCC cells. Rab11b expression levels correlated with better prognosis in HNSCC. Considering these things, Rab11a and Rab11b are potential prognostic indicators and promising targets for the development of novel therapeutic strategies for HNSCC.

Author contribution

Kunihiro Yoshida: data collection, data analysis, data interpretation, figures, writing—review and editing.
Kaung Htike: data analysis, figures, technical assistance.
Takanori Eguchi: funding acquisition, writing—review and editing.
Hotaka Kawai: data collection, data analysis.
Htoo Shwe Eain: data collection.
Manh Tien Tran: technical assistance.
Chiharu Sogawa: funding acquisition, technical assistance.
Koki Umemori: technical assistance, literature search.
Tatsuo Ogawa: technical assistance, literature search.
Hideka Kanemoto: technical assistance, literature search.
Kisho Ono: technical assistance, literature search.
Hitoshi Nagatsuka: data analysis, literature search.
Akira Sasaki: funding acquisition, literature search.
Soichiro Ibaragi: funding acquisition, literature search.
Kuniaki Okamoto: designed the experiments, data interpretation, funding acquisition, writing—review and editing.

Ethical statement

This article has not been published in whole or in part elsewhere. All

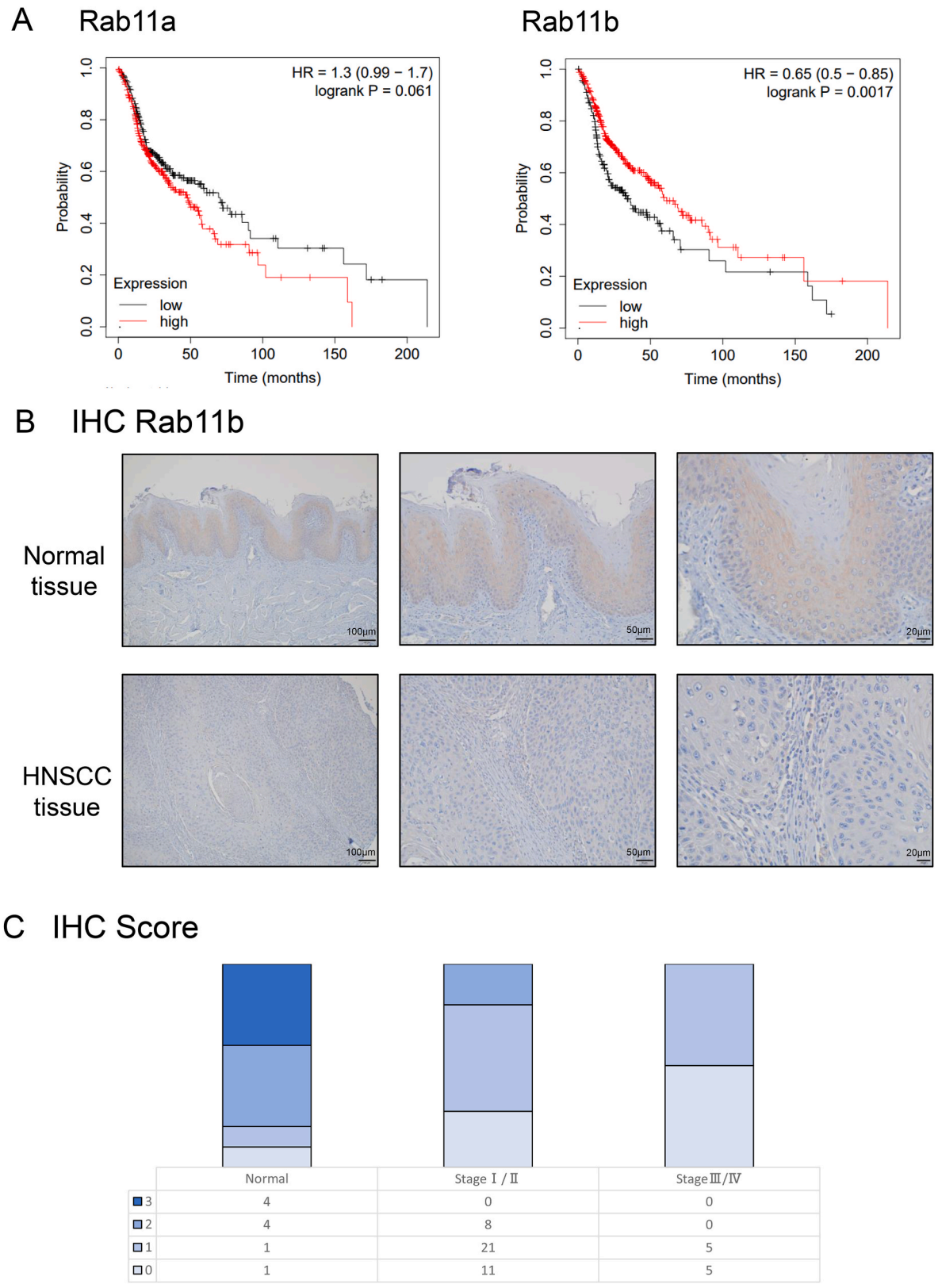

Fig. 7. Rab11 expression and prognostic values in patient-derived tumor specimens of HNSCC. (A) Kaplan-Meier survival analysis based on Rab11a or Rab11b expression in patients with HNSCC. Red lines, high expression group in Rab11a or Rab11b. Black lines, low expression group in Rab11a or Rab11b. (B) Immunohistochemistry (IHC) of human Rab11b in HNSCC vs. normal tissues using tissue microarray. (C) Rab11b IHC scores in normal vs. stage I/II and III/IV HNSCC specimens. The Rab11b -positive cells were counted using a light microscope and evaluated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

authors have agreed to have reviewed and approved the manuscript for submission.

All animal experiments were performed with the approval of the Animal Care and Use Committee of Okayama University (OKU-2023554).

Footnote

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.job.2023.11.007.

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