

# Extracellular Vesicles from Carcinoma-Associated Fibroblasts Promote Epithelial-Mesenchymal Transition of Salivary Adenoid Cystic Carcinoma via Interleukin-6

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## Research Article

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## Abstract

Carcinoma-associated fibroblasts (CAF) play a pivotal role in cancer progression. Salivary adenoid cystic carcinoma (SACC) has a high tendency to invade and metastasize. Understanding how CAF interact with SACC cells is essential to develop new targeting therapies for SACC. Extracellular vesicles (EVs) play important roles in intercellular communication. However, the role of CAF-derived EVs in SACC invasion remains poorly understood. In this study, we show that CAF EVs promote the migration and invasion abilities of SACC cells. The expression of biomarkers of epithelial-mesenchymal transition (EMT) was higher in SACC cells treated with CAF EVs than in the negative controls, and high levels of IL-6 were detected in CAF and their EVs. Knockdown of IL-6 in CAF decreased invasive abilities and EMT biomarker expression in SACC cells induced by CAF EVs. CAF EV-associated IL6 promoted SACC EMT by activating JAK2/STAT3 signaling pathway. These findings suggest that targeting CAF-derived EVs may be an effective strategy for inhibiting SACC invasion.

## Background

Salivary adenoid cystic carcinoma (SACC) is a common malignancy of salivary glands [1, 2]. It is characterized by aggressive growth, nerve invasion, and high rate of metastasis to distant organs. Because of the highly invasive nature of SACC, the prognosis of patients is frequently poor. The five-year survival rate is approximately 35% and ten-year survival rate is approximately 15%[2, 3]. Therefore, understanding the mechanisms of SACC invasion and progression might provide new therapeutic targets.

The *in vivo* tumor microenvironment includes stromal components that play a critical role in cancer development and progression[4–6]. Stromal cells promote cancer invasion and metastasis by stimulating epithelial-mesenchymal transition (EMT)[7–9]. The tumor microenvironment is diverse and involves multiple cells, several cytokines, and the extracellular matrix (ECM). Carcinoma-associated fibroblasts (CAF) are the most important cells in tumor microenvironment. CAF play a significant role in cancer progression in various cancer types[10]. CAF are distinguished from other cells *in vitro* and *in vivo* by a group of biomarkers, such as  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), fibroblast-associated protein (FAP), and fibroblast-specific protein 1 (FSP1)[11]. CAF are considered activated fibroblasts resembling those involved in wound healing and inflammation. CAF can be originated from different cell types, including resident fibroblasts, epithelial cells through EMT, endothelial cells through endothelial-to-mesenchymal transition (EndMT), bone marrow-derived cells (BMDCs), adipocytes and stellate cells[12, 13]. During cancer growth and development, the cancer cells' "seed" with the encompassing microenvironment's "soil" including stromal cells, especially CAF[14, 15]. CAF can promote cancer progression by activating EMT through hedgehog (HH) pathway. CAF induce EMT via Hh pathway by expressing galectin-1, leading to enhanced invasion capability of gastric cancer cells[16]. In previous work from our group, we demonstrated that SACC-derived CAF induced cancer cell invasion by constructing an invasive track in ECM[17].

Extracellular vesicles (EVs) are nano-sized membranous vesicles. EVs carry biological cargo, such as proteins, microRNA, mRNA, and DNA[18]. EVs transfer their cargo to recipient cells, thereby altering the biological function of the recipient cells. CAF-derived EVs are involved in cancer progression and may serve as diagnostic and prognostic biomarker[19]. In prostate cancer, CAF EVs induced EMT of cancer cells by transferring miR-409 to cancer cells[20]. In colorectal cancer, CAF EVs contributed to the chemoresistance to 5-fluorouracil and oxaliplatin[21].

In this study, we demonstrated that CAF EVs are involved in the EMT of SACC and promote tumor invasion. CAF EVs promoted SACC cell invasion and EMT by delivering interleukin (IL)-6 and activating JAK2/STAT3 signaling pathway. The results suggest that CAF-derived EVs are potential targets for stroma-oriented therapy in SACC.

## Materials And Methods

### Cell culture

SACC-83 and SACC-LM were kind gifts from Peking University. Primary CAF cells, CAF-A1 and CAF-A2, were isolated from fresh tumor tissues of two SACC patients as described previously[17]. All of cells were cultured in Dulbecco modified Eagles medium (DMEM)/F12 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; ScienCell, USA), 100U/ml penicillin, and 100U/mL streptomycin (Gibco) with 5% CO<sub>2</sub> at 37 C°.

### EVs isolation

CAF cells were cultured in 75cm<sup>2</sup> flasks till cell confluence reached more than 80%. Then, they were washed with PBS and incubated with serum-free DMEM/F12 for 48 hours. The supernatant was collected as condition medium (CM) and stored at -80 C° until use. CM was sequentially centrifuged at 500 g, 2,500 g and 10,000 g and the supernatant was collected. Then EVs were isolated from the supernatant using Total Exosome Isolation Reagent (Invitrogen 4478359).

### Transmission electron microscopy (TEM)

EVs were evaluated for their morphology and size by TEM. Briefly, 40 µL of EV samples were placed on a film, then copper mesh is applied on it for 15 min, after that the copper was drained with filter paper and 3% of phosphotungstic acid was put on the mesh for 5 minutes. Finally filter paper was used to drain the negative dye solution and filter the mesh. Then the sample was observed under the electron microscope JEM-2000EX9 (JEOR, JAPAN).

### Wound healing assay

The migration abilities of both SACC-83 and SACC-LM cells were assessed by wound healing assay. Cells were seeded in 6-well plate till 100% confluence, then a straight scratch was made by using P1000 pipette tip. Then cells were washed with PBS and cultured with CAF EVs (10 µg/well) in serum free DMEM/F12 for another 48 hours. DMEM/F12 without EVs was used as a negative control. Images were recorded by

an inverted fluorescence microscope (Olympus IX71). The gap closure was evaluated as the cell migration rate. The relative closure rate of each sample was measured using Image-Pro Plus 6.0.

## Transwell invasion assay

We assessed the invasion ability of both SACC-83 and SACC-LM cells by transwell invasion assay. It was performed using the transwell chamber consisting of 6.5 mm diameter inserts (Corning Inc., USA). The membrane with 8.0  $\mu\text{m}$ -pore-size was coated with 1:10 diluted Matrigel (Corelle Life Science Co., Ltd). Briefly, SACC-83 and SACC-LM cells were seeded in the upper chamber and CAF EVs (10  $\mu\text{g}/\text{well}$ ) was added to the lower chamber for the induction. After 48 hours, the non-invading cells in the upper chamber were removed by cotton swaps and the cells that invaded to the bottom chamber were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet (coolaber science and technology), and photographed with an inverted microscope (Olympus IX 71). Finally, we took images from five random fields and counted the cells in each chamber to get the invaded cell number as the mean. The data represent at least three experiments (mean  $\pm$  standard error).

## Western blot

Protein was extracted using RIPA buffer (Anzure Sky Biological Technology Co., Ltd.) and protein concentration was detected by BCA assay kit (Beyotime Biotechnology). Subsequently, protein (20  $\mu\text{g}$ ) from each sample was separated by 12% SDS-polyacrylamide gel, transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (PIERCE), and probed with primary antibodies, including E-cadherin 1:4000 (20874-1-AP, Proteintech.), N-cadherin 1:800 (18203, Abcam), vimentin 1:1000 (10366-1-AP, Proteintech.), IL-6 1:2000 (9324, Abcam), CD81 1:200 (BM14579), CD63 1:200 (25682-1-AP, Proteintech.), HSP70 1:500 (32239, Santa Cruz), Calnexin 1:500 (10427-2-AP, Proteintech.), JAK2 1:800 (17670-1-AP, Proteintech.), STAT3 1:4000 (10253-2-AP, Proteintech.), p-JAK2 1:1000 ("phospho Y1007 + Y1008" 32101, Abcam), p-STAT3 1:2000 ("phospho S727" 32143, Abcam), and GAPDH 1:4000 (10494-1-1AP, Proteintech.). The membranes were blocked with skimmed milk (Becton, Dickinson, USA). Proteins were detected by horseradish peroxidase-conjugated antibody and goat anti-rabbit IgG (Boosten Company) at 1:2000 and 1:4000 dilution respectively with 1% BSA-PBS (including 0.05% TWEEN20) and revealed using the ECL system (Advantsa).

## RNA interference transfection

IL6-specific siRNA (si-IL6: TTCGGTCCAGTTGCCTTCT) was used to knockdown IL6 expression. A non-targeting siRNA (sINC: CGUACGCGGAAUACUUCGA) was used as a negative control. These products were purchased (RiboBio, China). These siRNA at the final concentration of 5 nmol/250  $\mu\text{L}$  were transfected into CAF cells with Lipofectamine 2000 reagent (Invitrogen, USA).

## Subcutaneous xenograft model

The use of BALB/c nude mice (3-4 weeks, female) were approved by the Institute Animal Care and Use Committee of Dalian Medical University. Animal experiments, transportation, and care were conducted in compliance with the relevant laws and the guidelines issued by the Ethical Committee of Dalian Medical

University. Mice were divided into three groups with five mice in each group: SACC-LM group was injected with  $2.25 \times 10^6$  cells/mouse; SACC-LM+CAF-A1 group was injected with  $2.0 \times 10^6$  SACC-LM and  $2.5 \times 10^5$  CAF-A1 per mouse; SACC-LM+CAF-A2 group was injected with  $2.0 \times 10^6$  SACC-LM and  $2.5 \times 10^5$  CAF-A2 per mouse. Cells were resuspended in 50 $\mu$ L PBS and 50 $\mu$ L Matrigel, then injected subcutaneously. The mice were raised for 5 weeks. Mice were anesthetized with 20% urethane when maximum tumor diameter reached to 15 mm. After euthanizing, the tumors were harvested and fixed with 4% paraformaldehyde and 30% sucrose solution overnight, then embedded into OCT and prepared into sections.

## Immunohistochemical staining

Immunohistochemical staining of xenografts was performed on 8- $\mu$ m-thickness sections using SPLink Detection Kits (SP-9000, ZSGB-BIO, China). The sections were rinsed with PBS and incubated with 3% hydrogen peroxide in methanol for blocking endogenous peroxidase activity. The nonspecific binding sites were blocked with 10% goat serum for 30 minutes. Sections were incubated with anti-human pan-CK (Merck Millipore), Vimentin (1:200, Proteintech), IL-6 (1:200, Proteintech), E-cadherin (1:200, Proteintech), N-cadherin (1:200, Abcam) overnight at 4°C. Immunoreactions were detected using 3, 3 $\bar{H}$ -diaminobenzidine. Nuclei were counterstained with hematoxylin. The integrated optical density and area of target distribution were measured with Image-Pro® Plus version 6.0.

## Statistical analyses

Statistical analysis was performed using SPSS 13.0 software. The one-way analysis of variance (ANOVA) is used to determine any statistically significant differences. Significance was defined at  $p < 0.05$ .

## Results

### CAF EVs promoted SACC migration and invasion

EVs were isolated from CAF conditioned medium (CM) (Fig. 1a). A cup-shaped morphology was observed by transmission electron microscopy (TEM) (Fig. 1b). Western blot confirmed the expression of CD63, CD81, and HSP70 in CAF-A1/A2 EVs (Fig. 1c). Calnexin, a calcium-binding protein expressed in endoplasmic reticulum membrane, showed negative expression in both EVs (Fig. 1c).

A wound healing assay was performed to investigate the effect of CAF EVs on the migration ability of SACC cells. CAF-A1/A2 EVs markedly promoted the migration of SACC-83 and SACC-LM cells compared with that in the negative control (Fig. 1d). A transwell invasion assay demonstrated that the invasion of both SACC-83 and SACC-LM cells increased significantly by CAF-A1/A2 EVs treatment significantly increased the invasion of both SACC-83 and SACC-LM cells compared with that in the control (Fig. 1e).

### CAF EVs induced EMT of SACC cells

Because EMT contributes to cancer invasion, we hypothesized that the increased migration and invasion abilities of SACC induced by CAF EVs were associated with the acquisition of an EMT state. The morphological changes and the intercellular junction between SACC cells were examined *in vitro*, and the

expression of EMT markers was assessed by western blotting. SACC-83 and SACC-LM cells were cultured in media supplemented with CAF-A1 or CAF-A2 EVs for three days, and serum-free medium was used as the control. The results showed that SACC-83 (Fig. 2a) and SACC-LM (Fig. 2b) cells treated with CAF-A1 and CAF-A2 EVs underwent morphological changes, including the development of elongated processes resembling the morphology of mesenchymal cells. Western blot analysis of the expression of EMT biomarkers showed that the epithelial biomarker E-cadherin was significantly downregulated, whereas the mesenchymal biomarkers vimentin and N-cadherin were upregulated in both SACC-83 and SACC-LM cells, compared with the control group (Fig. 2c-d). Overall, these data suggest that CAF EVs promote migration and invasion of SACC cells by modulating EMT.

## CAF EVs-associated IL-6 is critical in promoting SACC EMT

The findings showing that CAF EVs promoted the EMT of SACC cells prompted us to investigate the underlying mechanism. The results of cytoimmunofluorescence staining and western blotting showed high expression of IL-6 in CAF-A1 and CAF-A2 cells and their EVs (Fig. 3a). Because IL-6 plays an essential role in EMT of various cancers, we investigated whether CAF EVs-associated IL-6 promoted SACC EMT. First, CAF were transfected with a specific siRNA against IL-6 to knock down its expression, and EVs were isolated from the CM of transfected cells. SiIL-6 transfection significantly downregulated IL-6 in CAF-A1 and CAF-A2 cells as well as in their EVs compared with negative control (Fig. 3b). SACC cells were then treated with EVs from transfected CAF and migration and invasion were assessed. CAF siIL-6 EVs significantly inhibited the migration and invasion abilities of both SACC-83 and SACC-LM cells, as determined by the wound healing assay and Transwell invasion assay, respectively (Fig. 3c-d). Collectively, these results indicate that CAF EV-associated IL-6 plays an important role in the invasion and migration process in SACC.

Analysis of EMT biomarkers in SACC cells treated with CAF siIL-6 EVs showed that SACC-83 (Fig. 4a) and SACC-LM (Fig. 4b) cells maintained the morphological features of epithelial cells, characterized by tight intercellular junctions, compared with CAF EVs. In addition, CAF siIL-6 EVs induced an EMT phenotype characterized by increased E-cadherin expression and decreased N-cadherin and vimentin expression in SACC-83 (Fig. 4c) and SACC-LM (Fig. 4d) cells. These results suggest that IL-6 is critical for CAF EVs-induced SACC EMT.

## CAF EVs-associated IL-6 activates JAK2/STAT3 pathway in SACC cells

To investigate the potential mechanisms by which CAF EVs-associated IL-6 induced EMT of SACC-83 and SACC-LM cells, we measured the expression of total and phosphorylated JAK2 and STAT3 in SACC cells. Western blot analysis demonstrated that CAF-A1/A2 EVs upregulated p-STAT3 (p-STAT3 S727) and p-JAK2 (Y1007+Y1008) in SACC-83 cells significantly, whereas knockdown of IL6 in CAF-A1/A2 EVs decreased STAT3 and JAK2 phosphorylation (Fig. 5a). Similarly, CAF-A1/A2 EVs markedly upregulated

the expression of p-STAT3 and p-JAK2 in SACC-LM cells, whereas treatment of SACC-LM with CAF siLL-6 EVs downregulated the p-JAK2 and p-STAT3 (Fig. 5b).

### CAF promote EMT by IL-6 in vivo

To determine whether CAF-derived IL-6 was involved with EMT of SACC *in vivo*, a subcutaneous xenograft model was established in BALB/c nude mice, which were divided into SACC-LM, SACC-LM+CAF-A1 and SACC-LM+CAF-A2 groups. By 12 weeks, the maximum tumor diameter reached to 15 mm. Survival analysis demonstrated that mice with SACC-LM+CAF-A1/A2 transplantation had lower survival times than those with SACC-LM transplantation solely[22]. Hematoxylin and eosin staining confirmed the cribriform growth pattern of SACC xenografts in the three groups (Fig. 6a). Immunohistochemical staining of pan-CK and vimentin identified tumor epithelial cells and mesenchymal cells (Fig. 6b-c). Both SACC-LM+CAF-A1 and SACC-LM+CAF-A2 groups contained more stromal components than SACC-LM group. IL-6 expression was higher in the SACC-LM+CAF-A1 and SACC-LM+CAF-A2 groups than in the SACC-LM group (Fig. 6d). IL-6 showed strong expression in tumor cells in SACC-LM+CAF-A1 and SACC-LM+CAF-A2 groups and weak expression in tumor cells in the SACC-LM group. We hypothesize that CAF-A1/A2 cells might deliver IL-6 to adjacent tumor cells via EVs.

Assessment of EMT markers showed that E-cadherin expression was lower and N-cadherin expression was higher in the SACC-LM+CAF-A1 and SACC-LM+CAF-A2 groups than in the SACC-LM group (Fig. 6e-f). These data suggest that CAF induce EMT of SACC *in vivo* by delivering IL-6 to tumor cells.

## Discussion

SACC is one of the most aggressive salivary gland cancers and it is characterized by an invasive growth pattern and distant metastasis[23, 24]. SACC invasion is related to the expression of several factors, such as EGFR, HIF-1 $\alpha$ , and survivin[25–27]. The tumor microenvironment is an essential mediator of the interaction between different cell types and it is involved in the regulation of tumor invasion. CAF are critical stromal components that promote cancer cell proliferation and invasion by expressing growth factors and chemokines and depositing tumorous ECM[28]. We recently demonstrated that CAF promote SACC lung metastasis by creating a pre-metastatic niche in the lung[22]. CAF promote cancer progression by transferring factors and genes to tumor cells via EVs, such as delivering CD9 in scirrhous-type gastric cancer[29] and delivering annexin A6 in pancreatic cancer[30]. In this study, we demonstrated that CAF EVs increase the migration and invasion abilities of SACC cells by transferring IL-6 to tumor cells.

Increasing evidence indicates that EMT is associated with cancer growth, invasion, angiogenesis, and metastasis[31]. In this study, SACC cells stimulated with CAF EVs acquired EMT-like characteristics with enhanced invasion ability. EVs can induce EMT by promoting the trans-differentiation of epithelial cancer cells into a mesenchymal phenotype mediated by multiple factors[32–34]. IL-6 is a multifunctional cytokine that has immunological and inflammatory functions, and high expression of IL-6 is detected in different epithelial tumors[35]. IL-6 is associated with EMT induction in different cancers[36]. CAF produced IL-6 *in vivo* in various cancers to induce growth and invasion through EMT[37, 38]. In the present

study, SACC-derived CAF secreted EVs expressing high levels of IL-6 were involved in the regulation of SACC EMT. A previous study demonstrated that IL-6 showed high expression in parotid gland SACCs, but not in submandibular gland SACCs[39]. Hoffmann et al. and his colleagues reported that IL-6 serum levels increased in some SACC patients, compared with healthy controls[40]. Besides IL-6, other cytokines may contribute to SACC invasion and metastasis, such as transforming growth factor- $\beta$ 1[41], macrophage migration inhibitory factor[42], and tumor necrosis factor receptor-associated factor 6[43], et al. Further studies are necessary to elucidate the exact roles of cytokines on SACC invasion.

IL-6 exerts its biological functions by binding to its receptor sIL-6R to activate JAK/STAT3 signaling pathway, which controls the occurrence and development of tumors[44–46]. In the present study, IL-6 expressed by CAF EVs activated the JAK2/STAT3 signaling pathway in SACC cells. Constitutive activation of JAK2/STAT3 is directly related to tumor progression via EMT induction. The present data indicated that the ability of IL-6 to stimulate SACC cell invasion is regulated, at least in part, by the JAK2/STAT3 pathway.

## Conclusions

The present study provides strong evidence that CAF EVs-associated IL-6 could induce SACC EMT via JAK2/STAT3 signaling pathway. Novel strategies targeting the tumor microenvironment may be essential approaches to the inhibition of SACC invasion. Moreover, CAF EVs carrying EMT-mediators might work as a molecular identifier and future drug targets for SACC.

## Declarations

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### Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

### Author contributions

Tingjiao Liu designed the study. Hyat Ahmed Ibrahim Al-raimi, Jing Kong, Yan Ran, Lei Zhu, Jiao Li, Xue Liu, Xuesong Yang, Yong Luo, Yao Lu, and Weidong Niu performed experiments. Hyat Ahmed Ibrahim Al-raimi, Jing Kong, and Yan Ran carried out data analyses and produced the initial draft of the manuscript. Bingcheng Lin reviewed the manuscript. Jing Kong and Tingjiao Liu contributed to drafting the manuscript. All authors have read and approved the final submitted manuscript.

## **Data availability**

All data generated or analyzed during this study are included in this article.

## **Ethics approval**

The use of human cells and samples was approved by the Ethics Committee of Dalian Medical University. Animal experiments, transportation, and care were conducted in compliance with the relevant laws and the guidelines issued by the Ethical Committee of Dalian Medical University.

## **Consent to participate**

Informed consent was obtained from all individual participants included in the study

## **Consent to publish**

There were no human research participants in this article."

## **References**

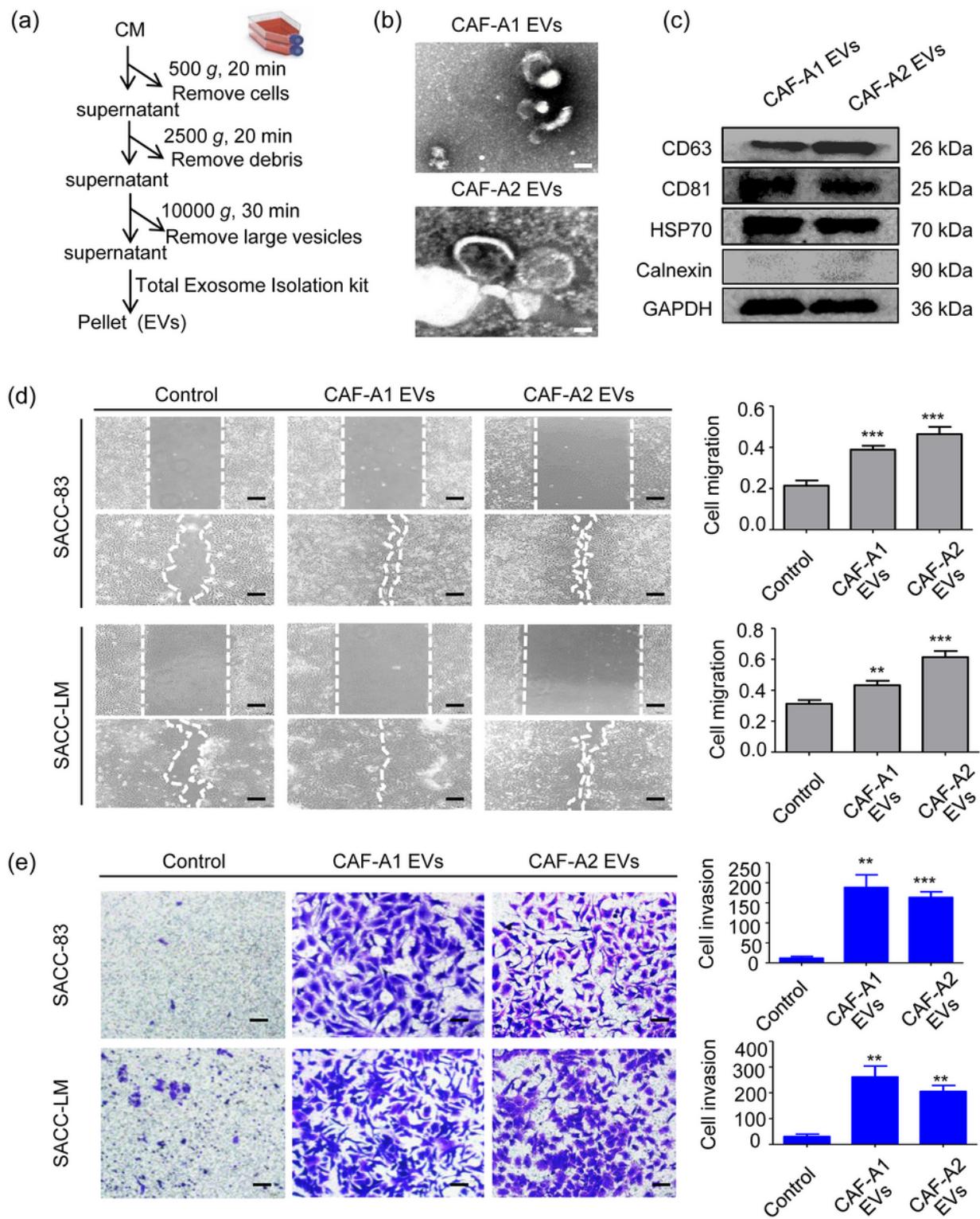
1. Hou J, Wang F, Liu X, Song M, Yin X. Tumor-derived exosomes enhance invasion and metastasis of salivary adenoid cystic carcinoma cells. *Journal of oral pathology & medicine: official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology*. 2018; 47(2):144–151. 10.1111/jop.12654.
2. Yang WW, Yang LQ, Zhao F, Chen CW, Xu LH, Fu J, Li SL, Ge XY. Epiregulin Promotes Lung Metastasis of Salivary Adenoid Cystic Carcinoma. *Theranostics*. 2017;7(15):3700–14. 10.7150/thno.19712.
3. Jia J, Zhang W, Liu JY, Chen G, Liu H, Zhong HY, Liu B, Cai Y, Zhang JL, Zhao YF. Epithelial mesenchymal transition is required for acquisition of anoikis resistance and metastatic potential in adenoid cystic carcinoma. *PloS one*. 2012;7(12):e51549. 10.1371/journal.pone.0051549.
4. Alkasalias T, Moyano-Galceran L, Arsenian-Henriksson M, Lehti K. Fibroblasts in the Tumor Microenvironment: Shield or Spear? *Int J Mol Sci*. 2018; 19(5). 10.3390/ijms19051532.
5. Dasari S, Fang Y, Mitra AK. Cancer Associated Fibroblasts: Naughty Neighbors That Drive Ovarian Cancer Progression. *Cancers*. 2018; 10(11). 10.3390/cancers10110406.
6. Sanford-Crane H, Abrego J, Sherman MH. Fibroblasts as Modulators of Local and Systemic Cancer Metabolism. *Cancers*. 2019; 11(5). 10.3390/cancers11050619.
7. Jia S, Wang W, Hu Z, Shan C, Wang L, Wu B, Yang Z, Yang X, Lei D. BDNF mediated TrkB activation contributes to the EMT progression and the poor prognosis in human salivary adenoid cystic carcinoma. *Oral Oncol*. 2015;51(1):64–70. 10.1016/j.oraloncology.2014.10.008.
8. Shan T, Chen S, Chen X, Lin WR, Li W, Ma J, Wu T, Ji H, Li Y, Cui X, et al. Prometastatic mechanisms of CAF-mediated EMT regulation in pancreatic cancer cells. *Int J Oncol*. 2017;50(1):121–8. 10.3892/ijo.2016.3779.

9. Izar B, Joyce CE, Goff S, Cho NL, Shah PM, Sharma G, Li J, Ibrahim N, Gold J, Hodi FS, et al. Bidirectional cross talk between patient-derived melanoma and cancer-associated fibroblasts promotes invasion and proliferation. *Pigment cell melanoma research*. 2016;29(6):656–68. 10.1111/pcmr.12513.
10. Sun Y, Fan X, Zhang Q, Shi X, Xu G, Zou C. Cancer-associated fibroblasts secrete FGF-1 to promote ovarian proliferation, migration, and invasion through the activation of FGF-1/FGFR4 signaling. *Tumour biology: the journal of the International Society for Oncodevelopmental Biology Medicine*. 2017;39(7):1010428317712592. 10.1177/1010428317712592.
11. Nurmik M, Ullmann P, Rodriguez F, Haan S, Letellier E. In search of definitions: Cancer-associated fibroblasts and their markers. *International journal of cancer*. 2020;146(4):895–905. 10.1002/ijc.32193.
12. Prakash J. Cancer-Associated Fibroblasts: Perspectives in Cancer Therapy. *Trends in cancer*. 2016;2(6):277–9. 10.1016/j.trecan.2016.04.005.
13. Bu L, Baba H, Yoshida N, Miyake K, Yasuda T, Uchihara T, Tan P, Ishimoto T. Biological heterogeneity and versatility of cancer-associated fibroblasts in the tumor microenvironment. *Oncogene*. 2019;38(25):4887–901. 10.1038/s41388-019-0765-y.
14. Bussard KM, Mutkus L, Stumpf K, Gomez-Manzano C, Marini FC. Tumor-associated stromal cells as key contributors to the tumor microenvironment. *Breast cancer research: BCR*. 2016;18(1):84. 10.1186/s13058-016-0740-2.
15. Kuzet SE, Gaggioli C. Fibroblast activation in cancer: when seed fertilizes soil. *Cell tissue research*. 2016;365(3):607–19. 10.1007/s00441-016-2467-x.
16. Chong Y, Tang D, Xiong Q, Jiang X, Xu C, Huang Y, Wang J, Zhou H, Shi Y, Wu X, et al. Galectin-1 from cancer-associated fibroblasts induces epithelial-mesenchymal transition through beta1 integrin-mediated upregulation of Gli1 in gastric cancer. *Journal of experimental clinical cancer research: CR*. 2016;35(1):175. 10.1186/s13046-016-0449-1.
17. Li J, Jia Z, Kong J, Zhang F, Fang S, Li X, Li W, Yang X, Luo Y, Lin B, et al. Carcinoma-Associated Fibroblasts Lead the Invasion of Salivary Gland Adenoid Cystic Carcinoma Cells by Creating an Invasive Track. *PloS one*. 2016;11(3):e0150247. 10.1371/journal.pone.0150247.
18. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol*. 2018;19(4):213–28. 10.1038/nrm.2017.125.
19. Shoucair I, Weber Mello F, Jabalee J, Maleki S, Garnis C. The Role of Cancer-Associated Fibroblasts and Extracellular Vesicles in Tumorigenesis. *Int J Mol Sci*. 2020; 21(18). 10.3390/ijms21186837.
20. Josson S, Gururajan M, Sung SY, Hu P, Shao C, Zhau HE, Liu C, Lichterman J, Duan P, Li Q, et al. Stromal fibroblast-derived miR-409 promotes epithelial-to-mesenchymal transition and prostate tumorigenesis. *Oncogene*. 2015;34(21):2690–9. 10.1038/onc.2014.212.
21. Deng X, Ruan H, Zhang X, Xu X, Zhu Y, Peng H, Zhang X, Kong F, Guan M. Long noncoding RNA CCAL transferred from fibroblasts by exosomes promotes chemoresistance of colorectal cancer cells. *International journal of cancer*. 2020;146(6):1700–16. 10.1002/ijc.32608.

22. Kong J, Tian H, Zhang F, Zhang Z, Li J, Liu X, Li X, Liu J, Li X, Jin D, et al. Extracellular vesicles of carcinoma-associated fibroblasts creates a pre-metastatic niche in the lung through activating fibroblasts. *Mol Cancer*. 2019;18(1):175. 10.1186/s12943-019-1101-4.
23. Liu X, Yang X, Zhan C, Zhang Y, Hou J, Yin X. Perineural Invasion in Adenoid Cystic Carcinoma of the Salivary Glands: Where We Are and Where We Need to Go. *Front Oncol*. 2020;10:1493. 10.3389/fonc.2020.01493.
24. Singh FM, Mak SY, Bonington SC. Patterns of spread of head and neck adenoid cystic carcinoma. *Clin Radiol*. 2015;70(6):644–53. 10.1016/j.crad.2015.01.013.
25. Wang Y, Hu J, Wang Y, Ye W, Zhang X, Ju H, Xu D, Liu L, Ye D, Zhang L, et al. EGFR activation induced Snail-dependent EMT and myc-dependent PD-L1 in human salivary adenoid cystic carcinoma cells. *Cell cycle*. 2018;17(12):1457–70. 10.1080/15384101.2018.1489177.
26. Xiao C, Pan Y, Zeng X, Wang L, Li Z, Yan S, Wang H. Downregulation of hypoxia-inducible factor-1alpha inhibits growth, invasion, and angiogenesis of human salivary adenoid cystic carcinoma cells under hypoxia. *Oncol Rep*. 2018;40(3):1675–83. 10.3892/or.2018.6559.
27. Cai WY, Zhuang Y, Yan F, Li T, Song WT, Sun JH. Effect of survivin downregulation by simvastatin on the growth and invasion of salivary adenoid cystic carcinoma. *Mol Med Rep*. 2018;18(2):1939–46. 10.3892/mmr.2018.9204.
28. Sahai E, Astsaturov I, Cukierman E, DeNardo DG, Egeblad M, Evans RM, Fearon D, Greten FR, Hingorani SR, Hunter T, et al. A framework for advancing our understanding of cancer-associated fibroblasts. *Nat Rev Cancer*. 2020;20(3):174–86. 10.1038/s41568-019-0238-1.
29. Miki Y, Yashiro M, Okuno T, Kitayama K, Masuda G, Hirakawa K, Ohira M. CD9-positive exosomes from cancer-associated fibroblasts stimulate the migration ability of scirrhous-type gastric cancer cells. *British journal of cancer*. 2018;118(6):867–77. 10.1038/bjc.2017.487.
30. Leca J, Martinez S, Lac S, Nigri J, Secq V, Rubis M, Bressy C, Serge A, Lavaut MN, Dusetti N, et al. Cancer-associated fibroblast-derived annexin A6+ extracellular vesicles support pancreatic cancer aggressiveness. *The Journal of clinical investigation*. 2016;126(11):4140–56. 10.1172/JCI87734.
31. Pastushenko I, Blanpain C. EMT Transition States during Tumor Progression and Metastasis. *Trends Cell Biol*. 2019;29(3):212–26. 10.1016/j.tcb.2018.12.001.
32. Fujiwara T, Eguchi T, Sogawa C, Ono K, Murakami J, Ibaragi S, Asaumi JI, Calderwood SK, Okamoto K, Kozaki KI. Carcinogenic epithelial-mesenchymal transition initiated by oral cancer exosomes is inhibited by anti-EGFR antibody cetuximab. *Oral Oncol*. 2018;86:251–7. 10.1016/j.oraloncology.2018.09.030.
33. Chen Y, Zeng C, Zhan Y, Wang H, Jiang X, Li W. Aberrant low expression of p85alpha in stromal fibroblasts promotes breast cancer cell metastasis through exosome-mediated paracrine Wnt10b. *Oncogene*. 2017;36(33):4692–705. 10.1038/onc.2017.100.
34. D'Angelo E, Lindoso RS, Sensi F, Pucciarelli S, Bussolati B, Agostini M, Collino F. Intrinsic and Extrinsic Modulators of the Epithelial to Mesenchymal Transition: Driving the Fate of Tumor Microenvironment. *Front Oncol*. 2020;10:1122. 10.3389/fonc.2020.01122.

35. Schafer ZT, Brugge JS. IL-6 involvement in epithelial cancers. *J Clin Investig.* 2007;117(12):3660–3. 10.1172/JCI34237.
36. Abaurrea A, Araujo AM, Caffarel MM. The Role of the IL-6 Cytokine Family in Epithelial-Mesenchymal Plasticity in Cancer Progression. *Int J Mol Sci.* 2021; 22(15). 10.3390/ijms22158334.
37. Hugo HJ, Lebret S, Tomaskovic-Crook E, Ahmed N, Blick T, Newgreen DF, Thompson EW, Ackland ML. Contribution of Fibroblast and Mast Cell (Afferent) and Tumor (Efferent) IL-6 Effects within the Tumor Microenvironment. *Cancer microenvironment: official journal of the International Cancer Microenvironment Society.* 2012;5(1):83–93. 10.1007/s12307-012-0098-7.
38. Ebbing EA, van der Zalm AP, Steins A, Creemers A, Hermsen S, Rentenaar R, Klein M, Waasdorp C, Hooijer GKJ, Meijer SL, et al. Stromal-derived interleukin 6 drives epithelial-to-mesenchymal transition and therapy resistance in esophageal adenocarcinoma. *Proc Natl Acad Sci USA.* 2019;116(6):2237–42. 10.1073/pnas.1820459116.
39. Shin DY, Jang KS, Kim BY, Choi JE, Yoon H, Ko YH, Jeong HS. Comparison of adenoid cystic carcinomas arising from the parotid gland vs. the submandibular gland: focus on systemic metastasis and tumor-associated blood vessels. *Journal of oral pathology medicine: official publication of the International Association of Oral Pathologists the American Academy of Oral Pathology.* 2014;43(6):441–7. 10.1111/jop.12155.
40. Hoffmann TK, Sonkoly E, Homey B, Scheckenbach K, Gwosdz C, Bas M, Chaker A, Schirlau K, Whiteside TL. Aberrant cytokine expression in serum of patients with adenoid cystic carcinoma and squamous cell carcinoma of the head and neck. *Head Neck.* 2007;29(5):472–8. 10.1002/hed.20533.
41. Dong L, Wang YX, Li SL, Yu GY, Gan YH, Li D, Wang CY. TGF-beta1 promotes migration and invasion of salivary adenoid cystic carcinoma. *J Dent Res.* 2011;90(6):804–9. 10.1177/0022034511401407.
42. Zhang M, Li ZF, Wang HF, Wang SS, Yu XH, Wu JB, Pang X, Wu JS, Yang X, Tang YJ, et al. MIF promotes perineural invasion through EMT in salivary adenoid cystic carcinoma. *Mol Carcinog.* 2019;58(6):898–912. 10.1002/mc.22979.
43. Liang Y, Jiao J, Liang L, Zhang J, Lu Y, Xie H, Liang Q, Wan D, Duan L, Wu Y, et al: Tumor necrosis factor receptor-associated factor 6 mediated the promotion of salivary adenoid cystic carcinoma progression through Smad-p38-JNK signaling pathway induced by TGF-beta. *Journal of oral pathology & medicine: official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology.* 2018; 47(6):583–589. 10.1111/jop.12709.
44. Yan L, Xu F, Dai CL. Relationship between epithelial-to-mesenchymal transition and the inflammatory microenvironment of hepatocellular carcinoma. *Journal of experimental clinical cancer research: CR.* 2018;37(1):203. 10.1186/s13046-018-0887-z.
45. Johnson DE, O'Keefe RA, Grandis JR. Targeting the IL-6/JAK/STAT3 signalling axis in cancer. *Nature reviews. Clinical oncology.* 2018;15(4):234–48. 10.1038/nrclinonc.2018.8.
46. Wendt MK, Balanis N, Carlin CR, Schiemann WP. STAT3 and epithelial-mesenchymal transitions in carcinomas. *Jak-Stat.* 2014;3(1):e28975. 10.4161/jkst.28975.

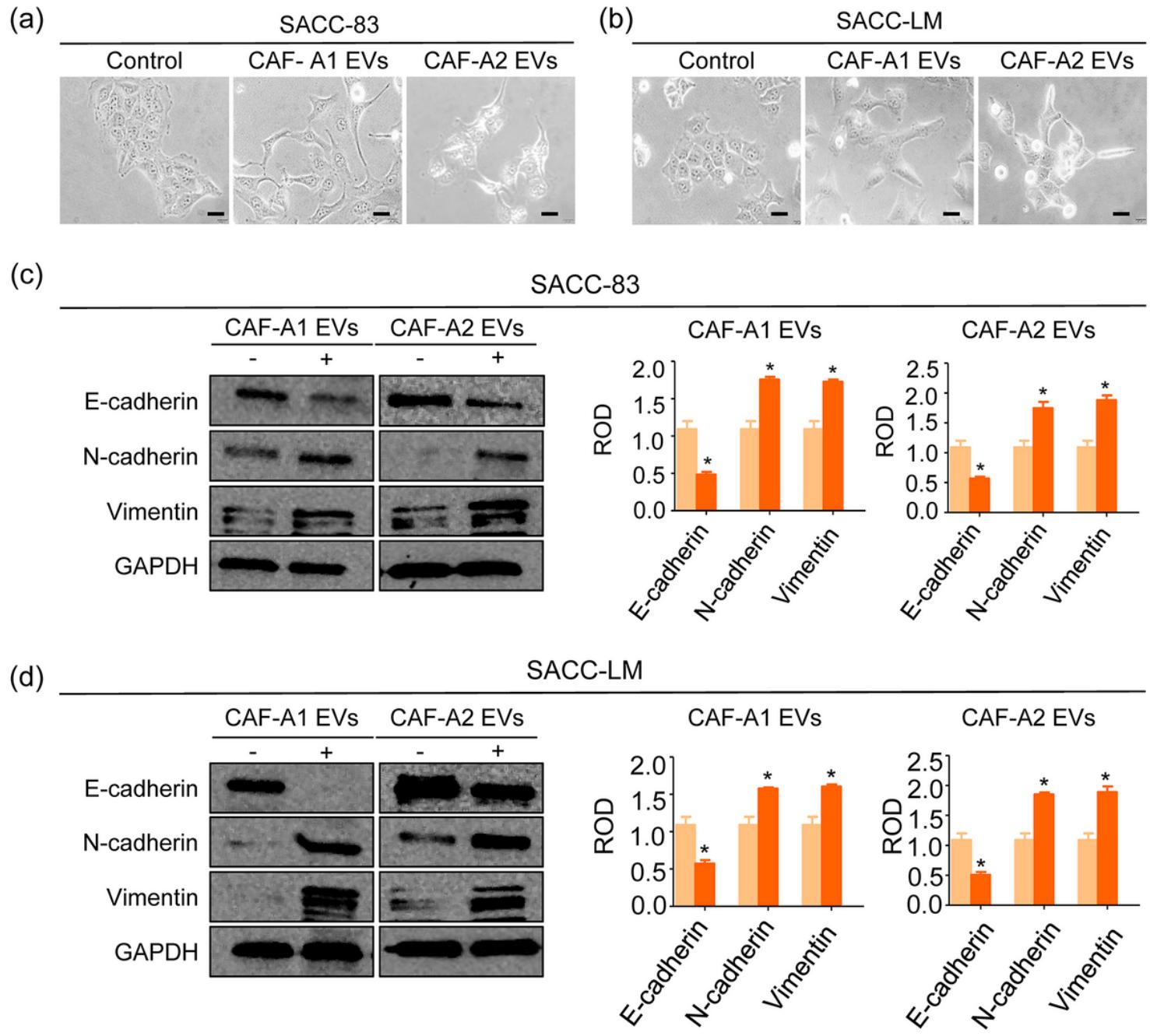
# Figures



**Figure 1**

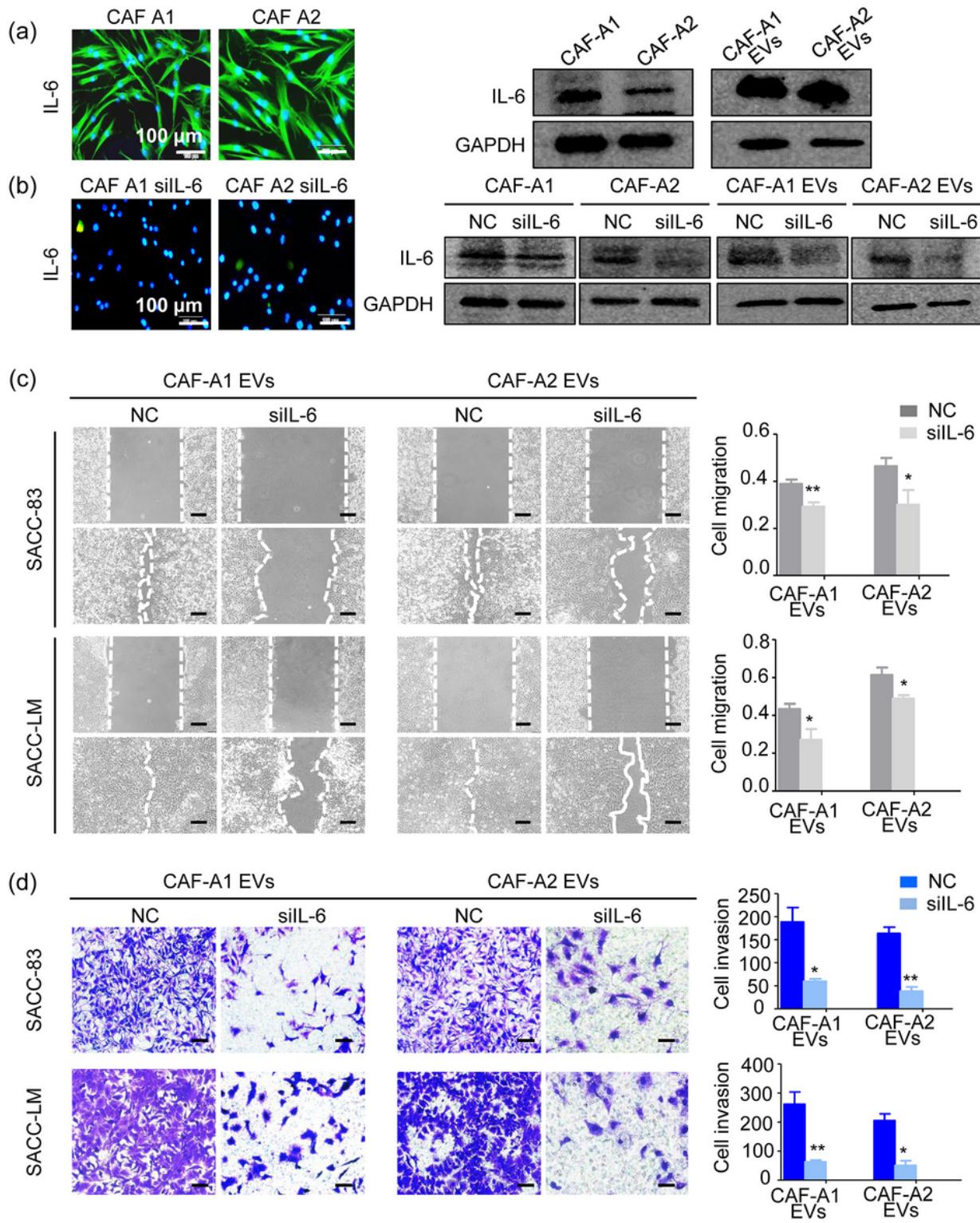
**CAF EVs promoted migration and invasion of SACC** (a) Steps of EVs isolation from conditioned media. (b) TEM images of CAF-A1 and CAF-A2 EVs. Scale bar = 100 nm. (c) Western blot examination of CD63, CD81, HSP70, and calnexin expression in CAF-A1 and CAF-A2 EVs. (d) Wound healing assay of SACC-83

and SACC-LM treated with or without CAF-A1 and CAF-A2 EVs. Left: representative images; Right: quantitative analysis. Scale bar = 100  $\mu$ m. \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . (e) Transwell invasion assay of SACC-83 and SACC-LM treated with or without CAF-A1 and CAF-A2 EVs. Left: representative images; Right: quantitative analysis. Scale bar = 50  $\mu$ m. \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .



**Figure 2**

**CAF EVs induced EMT of SACC** (a-b) Morphological changes of SACC-83 and SACC-LM cells induced by CAF-A1 and CAF-A2 EVs. (c-d) Western blot examination of the expression of E-cadherin, vimentin, and N-cadherin in SACC-83 and SACC-LM cells treated with or without CAF-A1 and CAF-A2 EVs. Left: representative images; Right: quantitative analysis. \*  $p < 0.05$ .



**Figure 3**

**CAF EVs-associated IL-6 regulate SACC invasion and migration** (a) Immunofluorescent staining and western blot analysis of the expression IL-6 in CAF-A1 and CAF-A2 cells and their EVs. (b) Knockdown of IL-6 in CAF-A1 and CAF-A2 cells and their EVs using siIL-6. (c) Wound healing assay of SACC-83 and SACC-LM cells treated with EVs isolated from CAF cells transfected with NC or siIL-6. Left: representative images; Right: quantitative analysis. Scale bar = 100  $\mu$ m. \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . (d) Transwell invasion

assay of SACC-83 and SACC-LM treated with EVs isolated from CAF transfected with NC or siIL-6. Left: representative images; Right: quantitative analysis. Scale bar = 50  $\mu$ m. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

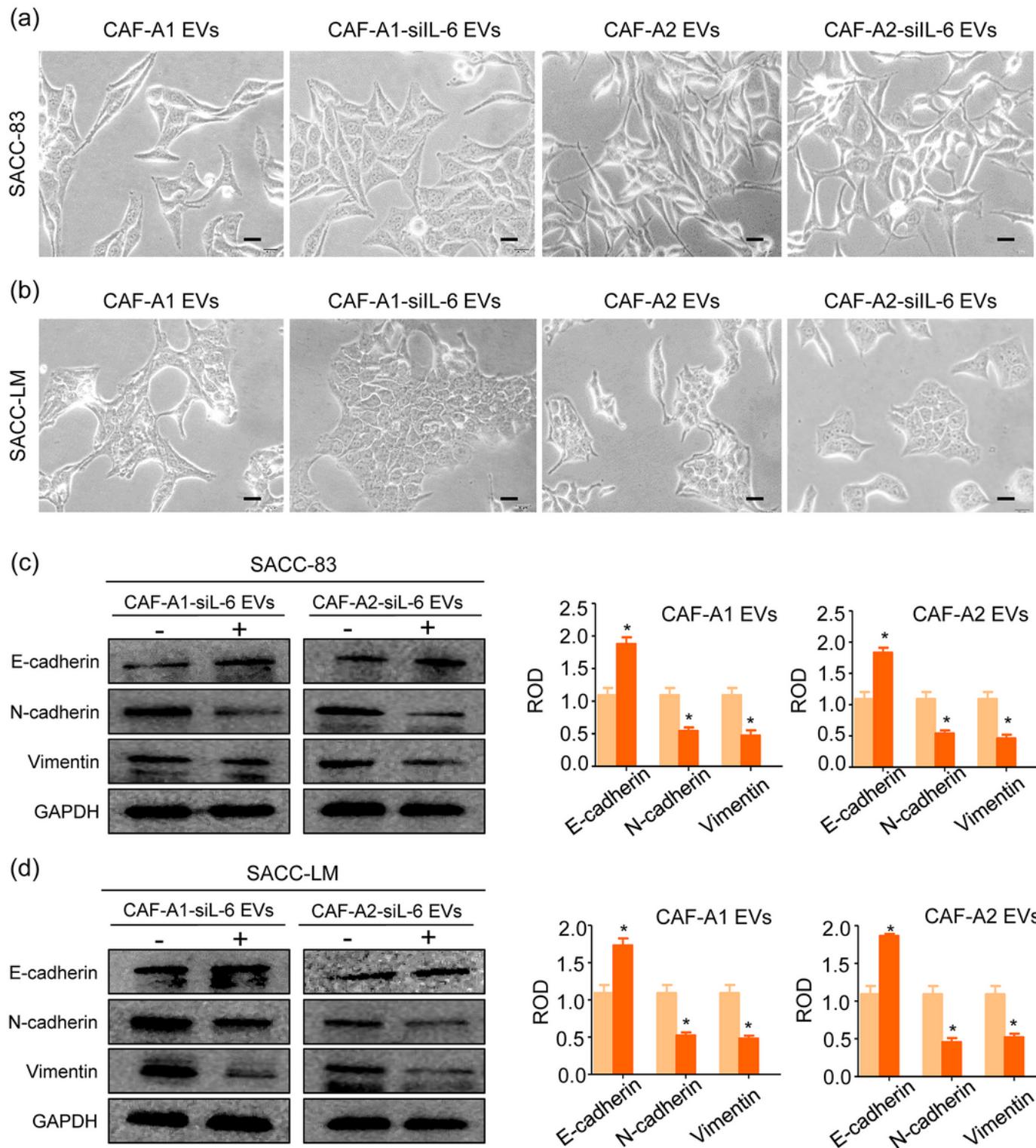
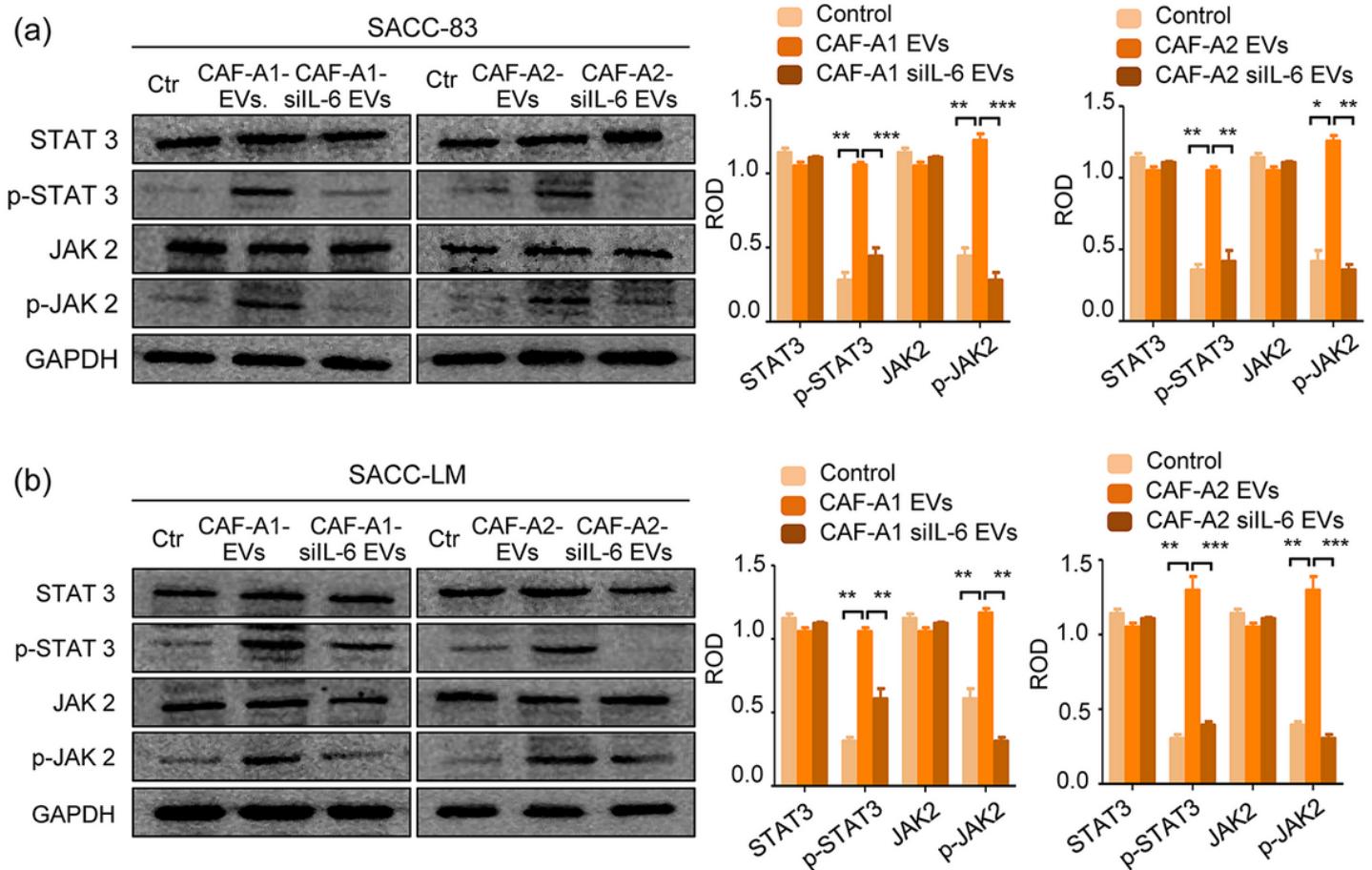


Figure 4

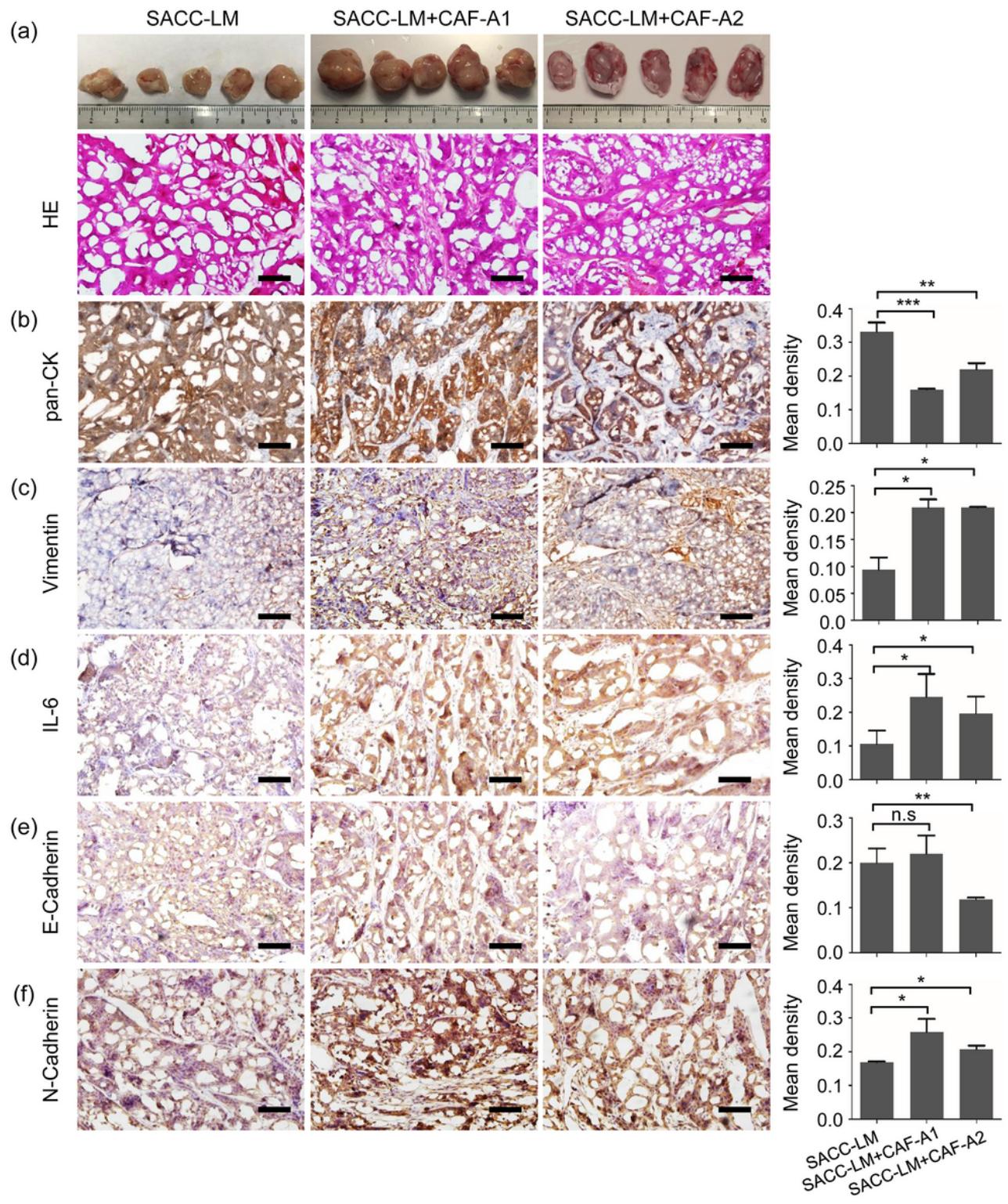
**Downregulation of IL-6 in CAF EVs inhibits EMT of SACC (A-B)** Morphological changes of SACC-83 (a) and SACC-LM (b) cells induced by EVs isolated from CAF transfected with NC or siIL-6. (c-d) Western blot

examination of the expression of E-cadherin, vimentin, and N-cadherin in SACC-83 (c) and SACC-LM (d) cells treated with EVs isolated from CAF transfected with NC or siIL-6. Left: representative images; Right: quantitative analysis. \*  $p < 0.05$ .



**Figure 5**

**CAF EVs induce EMT in SACC through the JAK2/STAT3 signaling pathway** (a-b) Western blot examination of the expression of total STAT3, p-STAT3, total JAK2, p-JAK2, or GAPDH in SACC-83 (a) or SACC-LM (b) cells with different treatments. Left: representative images; Right: quantitative analysis. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



**Figure 6**

**CAF promote EMT by IL-6 *in vivo*** (a) H&E staining of subcutaneous xenografts. (b-d) Immunohistochemical staining and quantification of pan-CK, vimentin and IL-6 in xenografts. (e-f) Immunohistochemical staining and quantification of E-cadherin and N-cadherin for ECM detection. Scale bar = 100  $\mu$ m. \* $p < 0.05$  \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .