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Cancer-associated fibroblast-secreted exosomes promote prostate cancer cell migration and invasion by the FGL1/SOX5 axis

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Summary. Exosomes secreted by cancer-associated fibroblasts (CAFs) play a critical role in cancer progression. This study aimed to explore the effects of CAF exosomes on prostate cancer (PC) cell metastasis. PC cells were treated with these exosomes, and their processes were evaluated using cell-counting kit-8 and Transwell assays. Exosome-regulated mRNAs were explored using quantitative real-time PCR. The relationship between FGL1 and SOX5 was analyzed using co-immunoprecipitation and fluorescence *in situ* hybridization (FISH) assays. The results of this study showed that exosomes derived from CAFs promoted PC cell viability, migration, and invasion. CAFs promoted PC cell viability and metastasis by releasing exosomes. Exosome treatment increased the levels of FGL1, which interacted with SOX5 and negatively regulated its expression. Rescue experiments demonstrated that CAF exosomes promoted the biological behaviors of PC cells by upregulating FGL1 and downregulating SOX5. Moreover, exosomes accelerated tumor growth by regulating the FGL1 level. In conclusion, CAF-derived exosomes promoted PC cell viability, migration, and invasion by elevating the FGL1/SOX5 axis, suggesting a novel strategy for the treatment of metastatic PC.

Key words: Prostate cancer, Exosome, Cancer-associated fibroblast, Migration, Invasion, FGL1

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Introduction

Prostate cancer (PC) is the third leading cause of cancer-related deaths, although it is the fifth most common cancer worldwide (Smyth et al., 2020). Despite a declining incidence, there are still approximately one million new cases diagnosed annually worldwide (Thrift and El-Serag, 2020). The five-year survival rate for early-stage PC exceeds 60% following surgical intervention; however, for advanced PC, the five-year survival rate ranges from 18% to 50%, depending on the dataset (Sexton et al., 2020). These disparities highlight the need for a deeper understanding of the pathogenesis of PC and the exploration of novel therapeutic strategies.

Cancer-associated fibroblasts (CAFs) reside within the tumor microenvironment (TME) and promote tumor cell proliferation, immune evasion, and drug resistance (Biffi and Tuveson, 2021). CAFs drive the progression of prostate cancer (PC) through paracrine signaling and modulation of the extracellular matrix (Ma et al., 2018). As such, CAFs represent attractive therapeutic targets for cancer. However, the efficacy of CAF-targeted approaches in clinical trials has been unsatisfactory, primarily due to the cellular heterogeneity of CAFs (Chen et al., 2021). Therefore, elucidating the complex mechanisms underlying CAF function is essential.

Exosomes are a subset of extracellular vesicles that function as mediators of intercellular communication (Kalluri and LeBleu, 2020). They carry a variety of components, including lipids, proteins, metabolites, and nucleic acids, which enable them to participate in cell-to-cell communication between primary tumor cells and the distant microenvironment, thereby promoting tumor metastasis (Wortzel et al., 2019; Zhang and Yu, 2019). Almost all cell types secrete exosomes. In the tumor microenvironment (TME), cancer-associated fibroblasts (CAFs) are key cells that secrete exosomes, which participate in the pathological processes of cancer (Yang et al., 2019a). CAF-secreted exosomes influence tumor cell behavior, promoting cell growth, invasion, and

migration, while inhibiting apoptosis (Li et al., 2021). However, the underlying mechanisms of CAF-derived exosomes have not been fully elucidated.

In this study, we clarified that CAF-derived exosomes influence PC cell migration and invasion by regulating the FGL1/SOX5 axis, therefore, providing a novel method to attenuate PC progression.

Materials and methods

Tissue samples

Prostate cancer (PC) tissues were obtained during surgery from patients at The First Affiliated Hospital of USTC. The study protocol was approved by the ethics committee of The First Affiliated Hospital of USTC, and written informed consent was obtained from all participants prior to surgery.

Animal model

All animal experiments were approved by the Animal Care and Use Committee of The First Affiliated Hospital of USTC and were conducted in accordance with the Guidelines for Experimental Animal Care and Use Organizations. Male BALB/c mice, aged 6-8 weeks, were purchased from Shanghai SLAC Laboratory (Zhejiang, China). The mice were housed in a room with controlled temperature and maintained on a 12-hour light/dark cycle with free access to water and standard rodent chow. In this study, mice were randomly divided into three groups (n=6 per group): control, exo + sh-NC, and exo + sh-FGL1. To establish a tumor-bearing mouse model, 22Rv1 cells (1×10^7 cells) transfected with sh-NC or sh-FGL1 were suspended in 100 µL of PBS and subcutaneously injected into the mice. One week later, the mice were intravenously injected with exosome solution (150 µg of exosomes in 100 µL of PBS) every other day for four weeks. Mice in the control group received subcutaneous injections of untransfected 22Rv1 cells and intravenous injections of 100 µL of PBS.Tumor volume was measured weekly using the formula: $volume=0.5\times length\times width^2$. At the end of the experiment, all mice were euthanized using 1.5% isoflurane in air, and the tumors were excised and weighed.

CAF isolation

Cancer-associated fibroblasts (CAFs) were isolated from prostate cancer (PC) tissues. Briefly, PC tissues were washed with PBS and cut into small pieces. The tissues were then digested using type I collagenase (Gibco, Grand Island, NY) at 37°C for 1 hour. The cell suspension was filtered through a 70 µm filter, centrifuged at 200 g for 3 minutes, and the process was repeated twice. The supernatant was discarded, and the precipitated cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C in a humidified atmosphere with 5% CO_2. CAFs were allowed to adhere to the culture surface, and non-adherent cells were removed using sterile PBS. CAF cultures were maintained for 1-2 weeks.

Exosome isolation

Exosomes were isolated from the CAF culture medium using ultracentrifugation, as previously described (Li et al., 2018). Cell debris and impurities were removed from the culture medium by sequential centrifugation at 500g for 10 minutes, 2,000g for 20 minutes, and 10,000g for 30 minutes. Vesicles were then pelleted by ultracentrifugation at 100,000g for 90 minutes. The exosome pellet was washed with PBS and further purified by ultracentrifugation at 100,000 g for 90 minutes. Optionally, the exosomes were subjected to sucrose-gradient centrifugation for further purification. The isolated CAF exosomes were stored at -80°C until use. The exosomes were characterized using transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and Western blotting.

Transmission electron microscopy (TEM)

Exosome morphology was observed using transmission electron microscopy (TEM), as previously described (Zhang et al., 2020). The exosomes were fixed in 2.5% glutaraldehyde at 4°C overnight, followed by postfixation in 1% osmium tetroxide for 60 minutes. The samples were then dehydrated through a graded series of ethanol solutions and embedded in epoxy resin. Ultrathin sections were cut and mounted onto copper grids. After staining with 2% uranyl acetate for 10 minutes and Reynolds' lead citrate for 5 minutes, the exosomes were imaged using a TEM operated at 120 kV.

Nanoparticle tracking analysis (NTA)

Exosome size distribution was determined using nanoparticle tracking analysis (NTA) (Zhang et al., 2020). The exosomes were suspended and diluted in PBS, and samples were analyzed using the Nanosight NS300 system (Malvern Panalytical).

Western blot

The surface markers of exosomes, HSP70 and CD63, were detected via Western blot. Total protein was extracted from CAFs and exosomes using RIPA buffer and resolved using 10% SDS-PAGE, followed by transfer to PVDF membranes. The membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA) against HSP70 (#4872, 1:1000) and CD63 (#52090, 1:1000) at 4°C overnight. Subsequently, the membranes were incubated with a secondary antibody (#7074, 1:2000) at room temperature for 1 hour. The bands were visualized

using the SignalFire ECL reagent (Cell Signaling Technology).

Cell culture and treatment

Prostate cancer (PC) cell lines (22RV1 and VCaP; obtained from the Cell Bank of the Chinese Academy of Sciences) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO_2. CAF-secreted exosomes were used to treat 22RV1 and VCaP cells to explore the role of exosomes in PC. CAFs were co-cultured with 22RV1 or VCaP cells at a ratio of 3:1. Additionally, to inhibit exosome release, 10 μM of the exosome inhibitor GW4869 (Sigma-Aldrich, St. Louis, MO) was added to the culture medium.

Cell transfection

Short hairpin RNA (shRNA) targeting FGL1 (sh-FGL1), an SOX5 overexpression vector, and their respective negative controls (sh-NC and empty vector) were designed and synthesized by GenePharma (Shanghai). 22RV1 and VCaP cells were transfected with these vectors in six-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The transfection efficiency was assessed after 48 hours.

Determination of cell viability

Prostate cancer (PC) cells were seeded into 96-well plates and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24 hours. Then, 10 μL of the CCK-8 reagent from the CCK-8 kit (Dojindo, Tokyo) was added to each well and incubated with the cells for 2 hours. Absorbance was measured at 450 nm using a microplate reader.

Determination of cell migration and invasion

Cell migration was assessed using 24-well chambers with 8 μm pore inserts (Corning, Corning, NY), and cell invasion was assessed using chambers pre-coated with Matrigel (BD Biosciences, San Jose, CA). 22RV1 and VCaP cells were added to the upper chambers, and culture medium was added to the lower chambers. After incubating at 37°C for 24 hours, the migrated and invaded cells were fixed and stained with 0.1% crystal violet. The stained cells were photographed under a light microscope.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was extracted from prostate cancer (PC) cells using TRIzol reagent (Invitrogen). The RNA purity was assessed at a 260/280 nm absorbance ratio. Reverse transcription was performed using the Quant cDNA First-Strand Synthesis Kit (TIANGEN). Subsequently, the SuperReal Premix Plus (SYBR Green) (TIANGEN) was used for qPCR. mRNA expression was quantified using the $2^{-\Delta\Delta Ct}$ method, with GAPDH serving as the internal control.

Co-Immunoprecipitation (Co-IP)

Prostate cancer (PC) cells were lysed using the lysis reagent. The lysate was incubated with anti-FGL1 or IgG at 4°C overnight. Protein A/G agarose magnetic beads were then added, and the mixture was incubated for an additional 3 hours. After elution of the magnetic beads, FGL1 and SOX5 were detected using Western blot with anti-FGL1 (ab37158, 1:200; Abcam, Cambridge, MA) and anti-SOX5 (ab94396, 1:1000; Abcam).

Immunofluorescence staining

To investigate the co-expression of SIRT5 and FGL1 in prostate cancer (PC) cells, immunofluorescence assays were conducted as follows. Cells grown on coverslips were fixed with 4% paraformaldehyde, permeabilized using 0.5% Triton X-100, and blocked with 5% bovine serum albumin. Subsequently, the cells were incubated overnight at 4°C with primary antibodies against SIRT5 and FGL1, followed by incubation with species-specific fluorophore-conjugated secondary antibodies (Alexa Fluor 488 for SIRT5 and Alexa Fluor 594 for FGL1). Nuclei were stained with DAPI. Fluorescent images were captured using a confocal laser scanning microscope, and image analysis was performed to quantify the expression levels and colocalization of SIRT5 and FGL1 using dedicated software.

Statistical analysis

All experiments were performed in triplicate. The results were analyzed using GraphPad Prism 8.0 software and are shown as mean ± SD. Comparisons were analyzed using the unpaired Student's t-test (two groups) or one-way ANOVA (multiple groups). $P<0.05$ represents a statistically significant difference.

Results

CAF-derived exosomes facilitate prostate cancer cell viability, migration, and invasion

To explore the role of CAF exosomes, we first isolated and identified exosomes from CAFs. As illustrated in Figure 1A, exosomes are characterized by bilayer membrane cup-shaped vesicles. Most exosomes were concentrated in the 100-200 nm diameter range (Fig. 1B). The levels of TSG101 and CD63 were higher in exosomes than in CAFs (Fig. 1C). These results

demonstrated that we had successfully isolated exosomes from CAFs.

We then investigated the effects of exosomes on prostate cancer (PC) cell phenotype. CAFs were co-cultured with PC cells and treated with the exosome inhibitor GW4869. Cellular processes were assessed. CAFs promoted the viability of 22RV1 and VCaP cells, and GW4869 abrogated this promotion. Additionally, GW4869 treatment suppressed the migration and invasion of 22RV1 and VCaP cells when co-cultured with CAFs (Fig. 2A-E). In conclusion, the results demonstrated that CAFs promoted PC cell progression by secreting exosomes.

Exosomes elevate the expression of FGL1

To explore the underlying mechanism, we selected five genes known to play important roles in prostate cancer (PC) development and examined their expression in PC cells. The results showed that CAF exosomes significantly upregulated FGL1 levels in both 22RV1 and VCaP cells (Fig. 3A,B). Therefore, we established shRNAs targeting FGL1 to investigate its function in PC. The results indicated that sh-FGL1 notably reduced

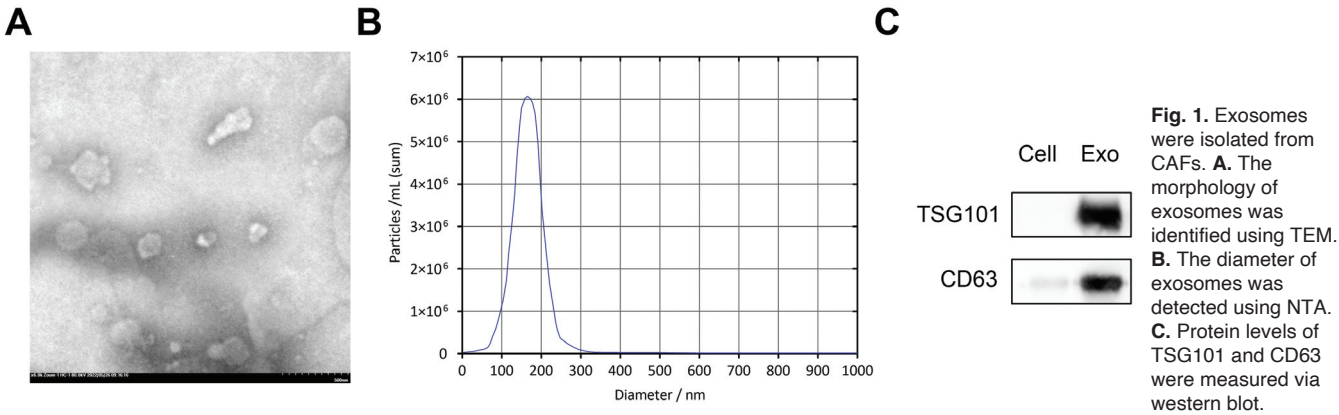

Fig. 1. Exosomes were isolated from CAFs. **A.** The morphology of exosomes was identified using TEM. **B.** The diameter of exosomes was detected using NTA. **C.** Protein levels of TSG101 and CD63 were measured via western blot.

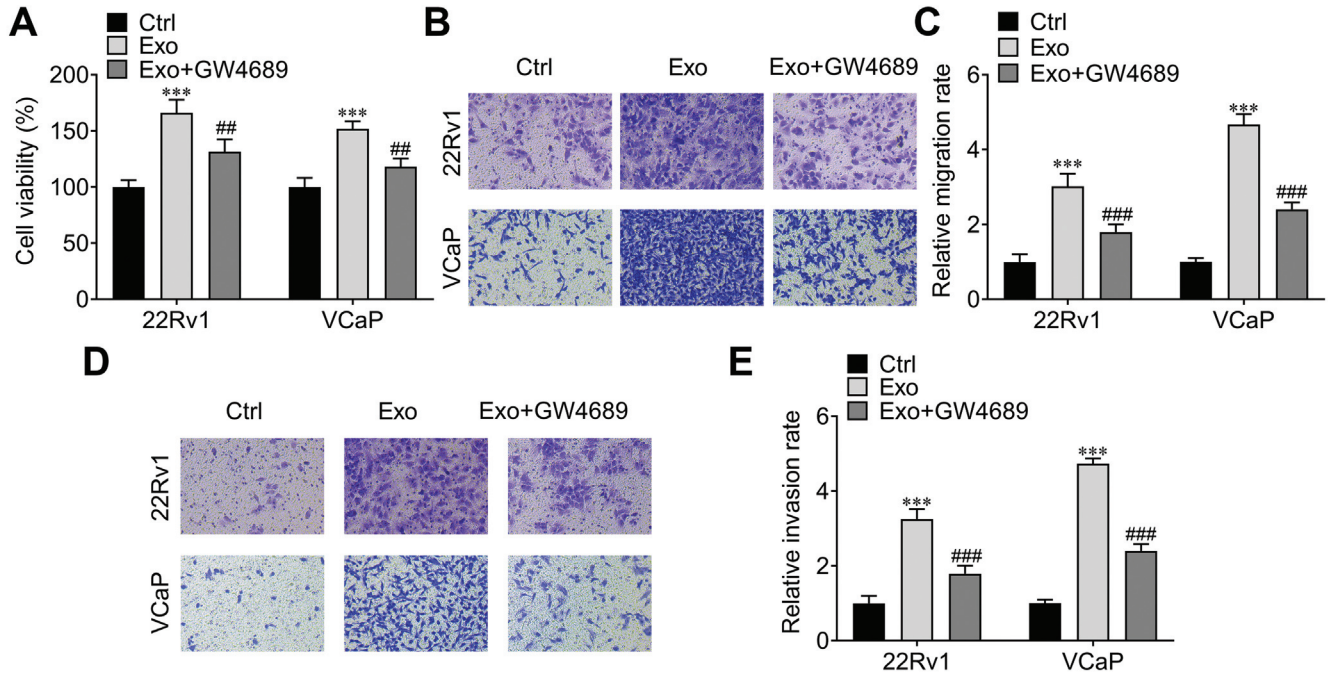

Fig. 2. CAF-derived exosomes facilitate PC cell viability, migration, and invasion. PC cells were treated with exosomes and GW4689, cell viability was assessed by the CCK-8 assay **(A)**; cell migration **(B, C)**, and invasion were analyzed by Transwell assays **(D, E)**. $^{***}P<0.001$. $^{###}P<0.001$. $^{##}P<0.01$.

the level of FGL1 (Fig. 3C). We then extracted exosomes from CAFs in which FGL1 had been silenced and added these exosomes to the culture of PC cells. Functional studies indicated that FGL1-depleted exosomes reversed the effects of normal exosomes (Fig. 3D-H). In summary, FGL1 was involved in the regulation of CAF exosomes in the biological behaviors of PC cells.

FGL1 interacts with SOX5 to regulate SOX5 expression

Furthermore, we identified the downstream factor of FGL1. We evaluated the expression of several genes that play critical roles in prostate cancer (PC) progression after FGL1 knockdown. It was found that FGL1 knockdown in PC cells notably inhibited the level of SOX5 in both 22RV1 and VCaP cells (Fig. 4A,B). In addition, FGL1 could interact with SOX5, as analyzed by co-immunoprecipitation (Co-IP) (Fig. 4C). The immunofluorescence assay showed that FGL1 was located in both the cell nucleus and cytoplasm, while SOX5 was localized in the cell nucleus, indicating that FGL1 and SOX5 interact in the nucleus. In summary, FGL1 acts as an upstream regulator of SOX5 in PC cells.

Knockdown of FGL1 suppresses prostate cancer cell viability, migration, and invasion by regulating SOX5

A rescue experiment was performed to assess the role of FGL1 and SOX5 in prostate cancer (PC) cells. An SOX5 overexpression vector and an empty vector

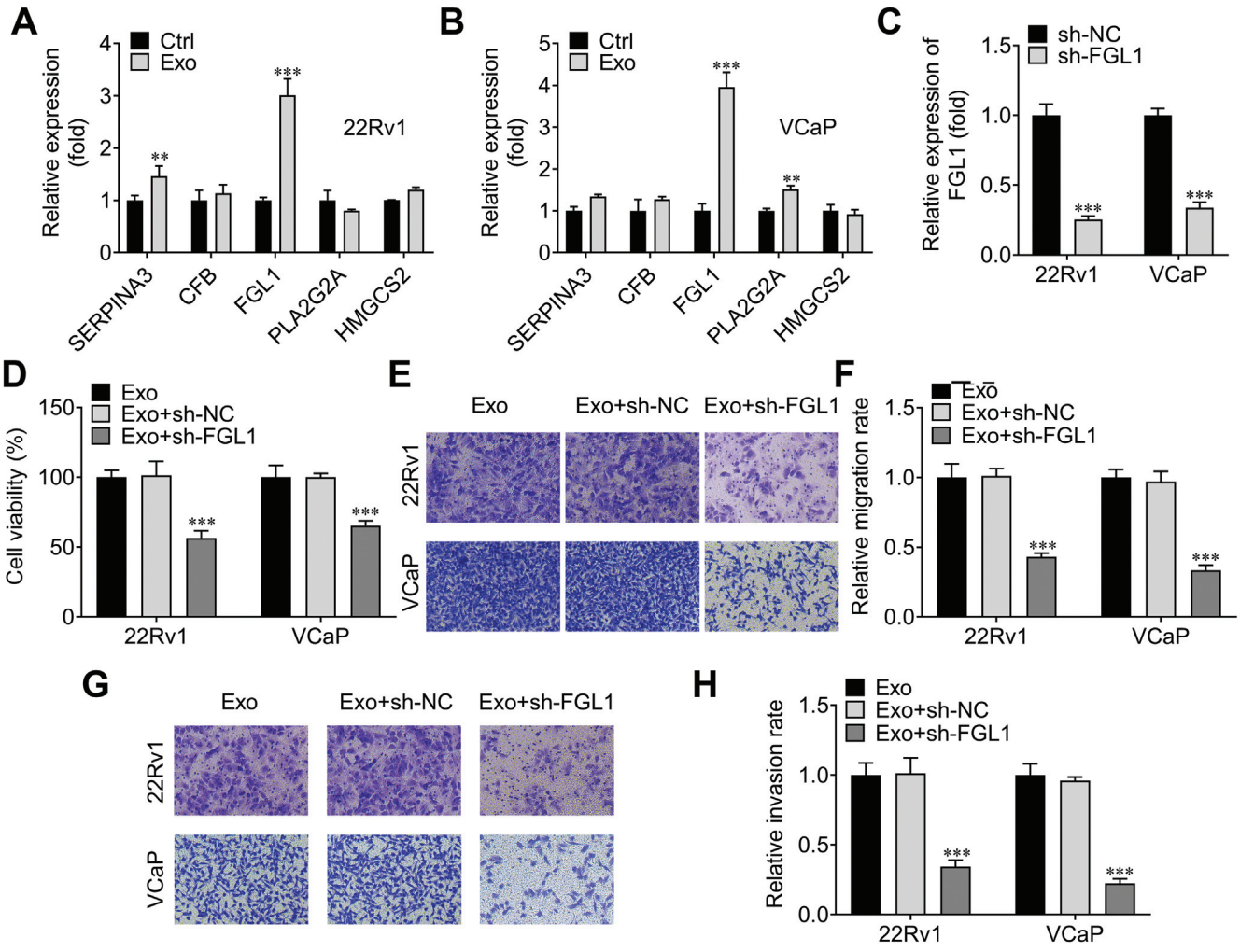

Fig. 3. Exosomes elevate the expression of FGL1. **A, B.** After exosome treatment, the expression of SERP/NA3, CFB, FGL1, PLA2G2A, and HMGCS2 was measured in PC cells using qPCR. **C.** The transfection efficiency was detected in PC cells after sh-NC and sh-FGL1 transfection. Transfected PC cells were treated with exosomes, cell viability was evaluated by CCK-8 **(D)**; cell migration **(E, F)** and invasion **(G, H)** were evaluated by Transwell assays. $^{**}P<0.01$. $^{***}P<0.001$.

were transfected into 22RV1 and VCaP cells, and SOX5 levels were upregulated following transfection with the SOX5 overexpression vector (Fig. 5A). Cell viability, migration, and invasion were all inhibited by FGL1 silencing, while overexpression of SOX5 reversed this inhibition (Fig. 5B-F). The data indicated that downregulation of the FGL1/SOX5 axis inhibited PC progression.

CAF-derived dxosomes promote tumor growth by mediating FGL1

22Rv1 cells were subcutaneously injected into nude mice to establish a xenograft model, which was then treated with exosomes. The results indicated that CAF-derived exosome treatment significantly promoted tumor growth *in vivo*. FGL1 silencing abrogated the tumor growth-promoting function induced by exosomes (Fig. 6A-C). These findings further confirmed the oncogenic role of FGL1 and demonstrated that CAF exosomes mediate tumor growth by regulating FGL1.

Discussion

The communication between cancer-associated fibroblasts (CAFs) and cancer cells can be significantly influenced by CAF-secreted exosomes. Activated CAFs within the tumor microenvironment (TME) promote tumor growth, metastasis, angiogenesis, and even drug resistance. Moreover, CAFs interact with tumor-related immune cells by secreting exosomes, facilitating immune escape (Seo et al., 2018; Mao et al., 2021). Numerous studies have focused on the impact of CAF exosomes on tumor cell phenotype and malignancy progression across various cancers, including pancreatic (Richards et al., 2017), colorectal (Ren et al., 2018), bladder (Goulet et al., 2019), and lung cancer (Liu et al., 2022). In prostate cancer (PC), CAF exosomes

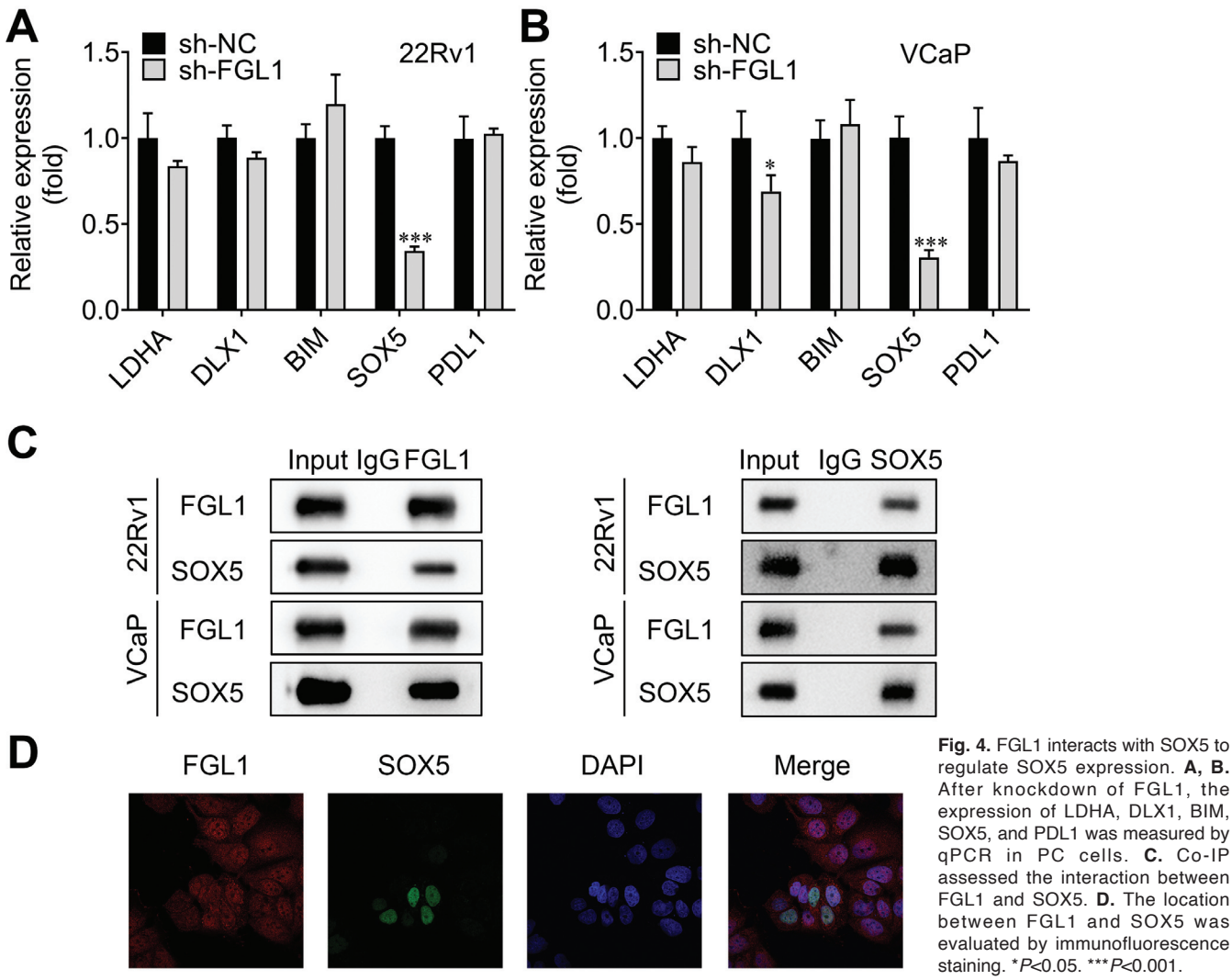

Fig. 4. FGL1 interacts with SOX5 to regulate SOX5 expression. **A, B.** After knockdown of FGL1, the expression of LDHA, DLX1, BIM, SOX5, and PDL1 was measured by qPCR in PC cells. **C.** Co-IP assessed the interaction between FGL1 and SOX5. **D.** The location between FGL1 and SOX5 was evaluated by immunofluorescence staining. **P*<0.05. ****P*<0.001.

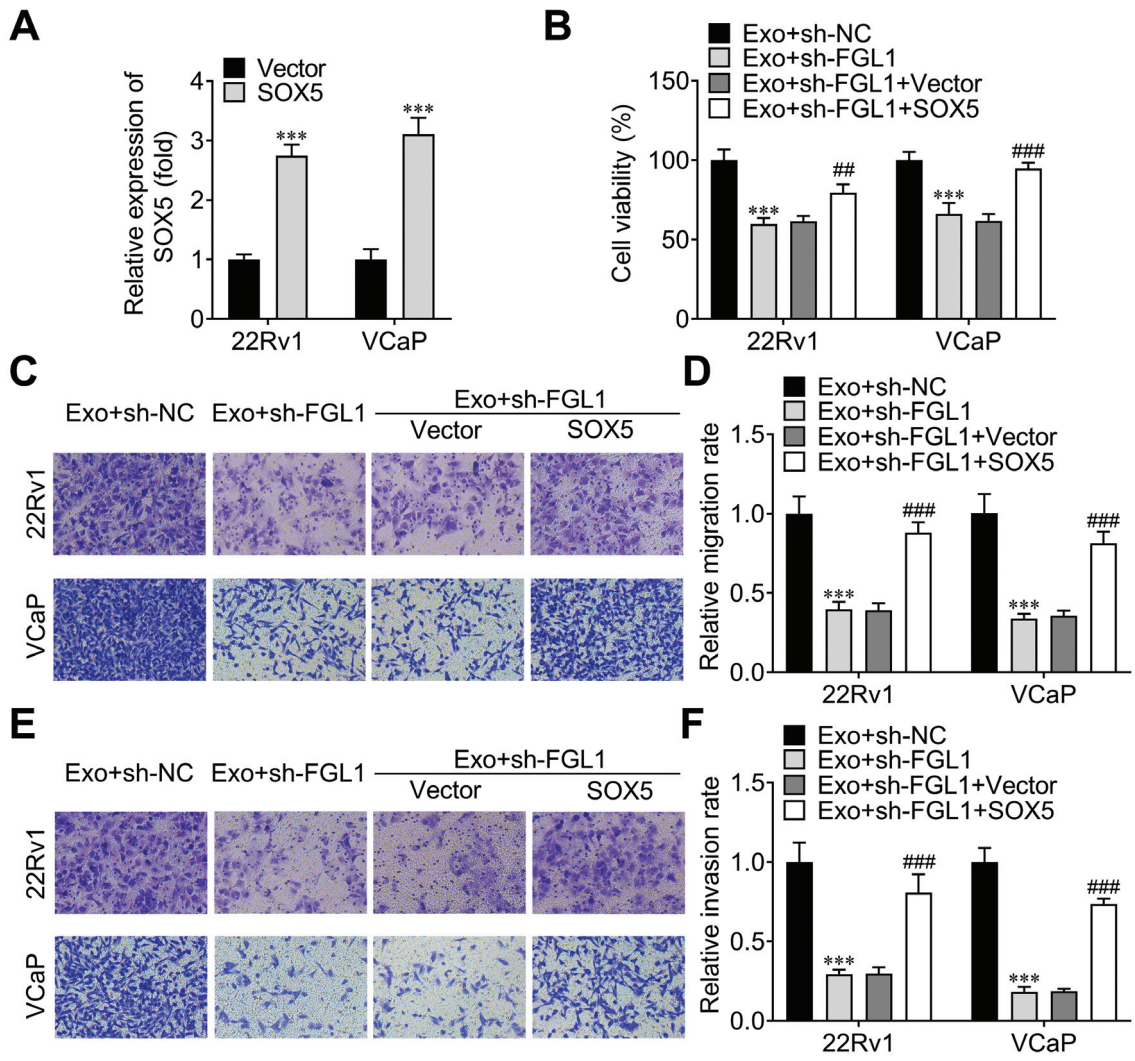

Fig. 5. Knockdown of FGL1 suppressed PC cell viability, migration, and invasion by regulating SOX5. **A.** Transfection efficiency was detected using qPCR after vector and SOX5 overexpressed vector transfection. FGL1 knockdown and SOX5 overexpressing PC cells were treated with exosomes, cell viability was measured by the CCK-8 assay **(B)**; A Transwell assay evaluated cell migration **(C, D)** and invasion **(E, F)**. $^{***}P<0.001$. $^{\#}P<0.05$. $^{\#\#}P<0.01$. $^{\#\#\#}P<0.001$.

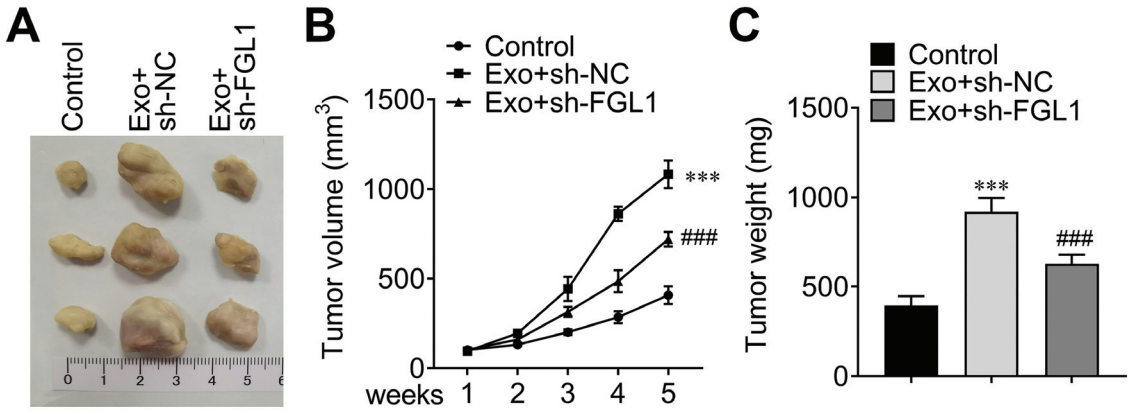

Fig. 6. CAF-derived exosomes promoted tumor growth by mediating FGL1. **A.** Tumors in mice were photographed. **B.** Tumor volume was detected weekly. **C.** Tumor weight. $^{***}P<0.001$. $^{\#\#\#}P<0.001$.

accelerate development and metastasis. For instance, CAF exosomes, whose secretion is promoted by cisplatin and paclitaxel, inhibit PC ferroptosis, thereby reducing chemosensitivity (Zhang et al., 2020). MiR-1290 is upregulated in PC cells and is delivered by CAF exosomes, promoting the malignant phenotype of PC cells (Wang et al., 2022). Additionally, CAF-secreted exosomal miR-423-5p promotes chemotherapy resistance in prostate cancer by targeting GREM2 through the TGF-β signaling pathway (Shan et al., 2022). As expected, in this study, we demonstrated that CAF-derived exosomes promoted cellular viability, migration, and invasion of PC cells, consistent with the findings from the aforementioned studies.

To explore the underlying mechanism of CAF-derived exosomes, we focused on multiple genes associated with prostate cancer (PC) progression. We identified that exosomes specifically elevated the levels of FGL1. Thus, we investigated the role of FGL1 in PC. FGL1 is expressed in almost all human tissues and organs, and it regulates cell growth, immune response, and metabolism (Hong et al., 2017). Because it can be induced by inflammation, researchers have also investigated the expression and role of FGL1 in inflammation-associated tumors (Sloot et al., 2018; Han and Yang, 2019). FGL1 has been shown to be upregulated in PC tissues and linked to tumor aggressiveness, particularly promoting migration and invasion (Tsai et al., 2014). In the present study, the results indicated that the knockdown of FGL1 suppressed the viability, migration, and invasion of PC cells and counteracted the effects of CAF exosomes. These findings suggest that CAF-derived exosomes facilitate PC progression by upregulating FGL1.

SRY-related high mobility group box 5 (SOX5) has been demonstrated to promote the progression of various cancer types by regulating cell growth, apoptosis, and differentiation. Eliminating SOX5 can inhibit the malignant behaviors of cancer cells (Chen et al., 2020; Zheng et al., 2020; Wang et al., 2023). In prostate cancer, Hu et al. (2018) demonstrated that SOX5 silencing inhibited the mesenchymal phenotype and cell migration ability. Yang et al. (2019b) confirmed that miR-139-5p inhibited PC by negatively regulating SOX5, which further alleviated the epithelial-mesenchymal transition development of PC cells. These results indicate that targeting the regulation of SOX5 expression may be an effective strategy to alleviate PC progression. In the present study, we found that FGL1 could interact with SOX5, which was negatively regulated by FGL1. Silencing of FGL1 inhibited the malignant phenotypes of PC cells by decreasing SOX5 expression. The data suggest that SOX5 acts as a tumor suppressor in PC.

In conclusion, our study first identified that CAF-derived exosomes promote prostate cancer (PC) cell growth and metastasis via the FGL1/SOX5 axis. The findings suggest that CAF exosomes are potential targets for the treatment of metastatic PC.

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

Author Contributions. YL conceived the study; LK conducted the experiments; XW analyzed the data; LK and YL were major contributors to writing the manuscript. All authors read and approved the final manuscript.

Ethics Approval. This study was approved by the First Affiliated Hospital of USTC. All experiments were performed in accordance with relevant guidelines and regulations.

Availability of data and materials. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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