

## Cancer-Associated Fibroblast Exosomal PWAR6 Promotes Colorectal Cancer Liver Metastasis through Metabolic Remodeling and Immune Suppression

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### Abstract

Hepatic metastasis secondary to colorectal cancer (CRC) remains a formidable clinical problem that dramatically shortens patient survival. Myofibroblastic cancer-associated fibroblasts (myCAFs) are among the most abundant stromal populations in the CRC tumor microenvironment and actively facilitate tumor growth and metastatic colonization by secreting exosomes. Single-cell profiling demonstrated a prominent accumulation of myCAFs within colorectal cancer liver metastases (CRLM). Sequencing of exosomal cargo revealed PWAR6 as the long non-coding RNA (lncRNA) showing the highest degree of upregulation in metastatic lesions. Complementary in vitro and in vivo experiments established that PWAR6 drives CRC cell stemness, motility, and glutamine acquisition. The underlying molecular pathway was dissected using RNA pulldown, RIP, and Co-IP assays focused on the PWAR6/NRF2/SLC38A2 axis in CRC progression, while flow cytometry quantified NK cell effector functions and cytotoxic potential. Clinically, elevated PWAR6 expression showed a robust association with higher 68Ga FAPI-PET/CT SUVmax values, particularly in patients with CRLM, where transcript levels were markedly higher than in non-metastatic cases and in normal colonic mucosa. Both regression modeling and survival analyses identified PWAR6 as an independent predictor of poor prognosis, with higher expression associated with inferior clinical outcomes. Mechanistically, PWAR6 enables immune escape by competitively binding Keap1, thereby stabilizing NRF2, which transcriptionally induces SLC38A2. The resulting increase in glutamine uptake by CRC cells simultaneously restricts glutamine availability to NK cells. Exosomal PWAR6 released by myCAFs acts as a central biomarker of CRC liver metastasis. Its specific inhibition by ASO-PWAR6, when combined with FAPI therapy, substantially attenuates metastatic progression in preclinical models and holds considerable promise for future clinical practice.

**Keywords:** Sexual satisfaction, Pregnant mothers, Healthy mothers, Candidate vaginitis

### Introduction

Colorectal cancer (CRC) is the third most frequently diagnosed cancer globally and the second leading contributor to cancer mortality [1]. Although advances in multimodal management have elevated the 5-year overall

survival (OS) rate for localized CRC above 90%, outcomes for patients with distant metastases remain poor, with 5-year OS rates below 15%. Notably, around 22% of individuals present with synchronous distant metastases upon initial CRC diagnosis [2, 3]. Therefore, identifying critical molecular mediators and pathways involved in metastatic dissemination is vital. Such insights could facilitate earlier detection, more effective therapies, and, ultimately, enhanced survival for CRC patients.

Cancer cell stemness and metabolic rewiring represent two fundamental drivers of malignancy. Stemness properties are central to CRC initiation and sustained growth, conferring self-renewal ability and tumor-

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forming capacity [4, 5]. Consistent with this, our earlier studies identified prominent stem-like characteristics in metastatic CRC organoids, where suppression of the key stemness regulator SOX2 substantially impaired organoid proliferation and metastatic behavior [6]. Given the established importance of stemness in CRC pathobiology, interventions that disrupt these features offer promising avenues to limit metastatic progression. Metabolic reprogramming likewise plays an essential role in fueling tumor expansion and spread. In CRC, altered utilization of glucose and glutamine stands out among the most common metabolic adaptations [7]. While glucose supports rapid energy production, glutamine functions as a critical anaplerotic fuel for the tricarboxylic acid (TCA) cycle and supplies precursors for lipid, nucleotide, and hexosamine biosynthesis [8]. Preliminary data from our group showed markedly elevated glutamate and related metabolites in CRC specimens, indicating hyperactive glutaminolysis in malignant cells [9]. Furthermore, glutamine restriction in the tumor milieu has been linked to enhanced stemness traits in diverse cancers, thereby accelerating disease advancement [10, 11]. These observations underscore how metabolic shifts within cancer cells influence not only local nutrient dynamics but also intrinsic stemness programs.

CRC cells also exhibit extensive crosstalk with the surrounding tumor microenvironment (TME), including cancer-associated fibroblasts (CAFs), various immune populations, and nutrient-rich components. CAFs, the dominant stromal cell type in the TME, exhibit heterogeneity and can be categorized into subtypes such as myofibroblastic CAFs (myCAFs), inflammatory CAFs (iCAFs), and antigen-presenting CAFs (apCAFs) based on marker expression and functional specialization. MyCAFs, marked by elevated  $\alpha$ -SMA, FAP, and Collagen I levels, drive extracellular matrix reorganization, tumor invasion, and desmoplastic reactions. These attributes position myCAFs as key contributors to malignant progression. Intercellular signaling between CAFs and CRC cells frequently occurs via exosomes [12]. Exosomes are small extracellular vesicles (sEVs), typically 30–200 nm in size, formed by the fusion of multivesicular endosomes with the plasma membrane. They shuttle proteins, lipids, and nucleic acids that profoundly alter the phenotypes of recipient cells [13]. For instance, studies by Hu *et al.* [14] showed that CAF-secreted exosomes containing miR-92a-3p are taken up by CRC cells, triggering epithelial-

mesenchymal transition (EMT) and enhancing metastatic potential. Deng *et al.* [15] similarly reported that exosomal lncRNA transfer from CAFs promotes chemoresistance in CRC. Our prior work further demonstrated that activated CAFs release higher levels of pro-inflammatory cytokines that promote CRC metastasis [16].

In addition, myCAFs actively reshape nutrient metabolism in both neoplastic cells and the wider TME. To meet the heightened glutamine requirements of tumor cells, myCAFs enhance their own anabolic programs and release glutamine into the extracellular space [17]. They can also suppress oxidative phosphorylation in cancer cells through exosome-dependent signals, thereby favoring glutamine-driven reductive carboxylation [18, 19]. Despite these findings, the detailed pathways by which myCAFs orchestrate glutamine homeostasis in the TME and modulate tumor stemness in CRC remain largely unclear, constituting a critical knowledge gap.

Immune cells within the TME, including macrophages, natural killer (NK) cells, and T lymphocytes, exert diverse effects on tumor development that depend on their activation state and functional polarization. NK cells, central to innate antitumor responses, recognize and destroy cells with reduced MHC-I expression by releasing granzyme and perforin. They form pores in target membranes via perforin, allowing Granzyme B to translocate and induce apoptosis [20]. Similar to tumor cells, NK cells require sufficient nutrients to maintain their effector functions and sustain antitumor activity [21]. However, aggressive nutrient scavenging by cancer cells can create a depleted microenvironment that impairs immune cell performance [22]. Glutamine shortage, for example, restricts proliferation of activated T cells and hinders M2 macrophage differentiation [23, 24]. The precise impact of glutamine limitation on NK cell activity in the TME, however, requires further clarification.

To explore these interactions, we isolated myCAFs from CRC liver metastases and paired normal fibroblasts (NFs) from adjacent non-malignant liver tissue. Comprehensive lncRNA profiling of exosomes from both cell types, combined with metabolomic analysis of the TME, identified PWAR6 as a myCAF-enriched lncRNA that modulates glutamine availability. We subsequently characterized the dynamics of competitive glutamine uptake between CRC cells and NK cells.

## Materials and Methods

#### *Primary myCAF isolation from CRC tissues*

Colorectal cancer specimens were freshly procured post-resection and subjected to five washes in phosphate-buffered saline (PBS) supplemented with 20% antibiotic–antimycotic (Gibco, USA). Tissues were subsequently fragmented into small pieces with ophthalmic scissors and enzymatically digested with type IV collagenase (Yeast, China) for approximately 1 hour at 37 °C under agitation at 200 rpm. The resulting digest was passed through 70 µm cell strainers, after which cells were pelleted by centrifugation at 1500 rpm for 5 min and the supernatant discarded; this centrifugation and resuspension cycle was performed 2–3 times. Fluorescence-activated cell sorting (FACS) employed antibodies against CD45-APCCy7 (BD Biosciences, no. BD-557833), EpCAM-PerCP/Cy5.5 (BioLegend, catalog no. 324214), CD31-PECy7 (BioLegend, catalog no. 303118), FAP-APC (R&D Systems, no. MAB3715), and  $\alpha$ -SMA (BioLegend catalog no. 614851).

Cells lacking EpCAM, CD31, and CD45 expression, yet positive for FAP and  $\alpha$ -SMA, were classified as myCAFs. These purified myCAFs were resuspended in DMEM/F12 medium (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% antibiotics. Following plating, adherent fibroblasts were enriched after 24–48 h via differential adhesion as previously established. Established cultures were maintained with daily medium replacement using DMEM/F12 supplemented with FGF (PeproTech, USA). The isolated primary myCAFs exhibited characteristic expression of  $\alpha$ -SMA, FAP, and Collagen I. All procedures were performed with donor informed consent and received approval from the Ethics Committee of Fudan University Shanghai Cancer Center.

#### *Clinical tissue collection*

Tissue samples from the FUSCC cohorts 1, 2, and 3 were acquired at the Fudan University Shanghai Cancer Center and stored at –80 °C. Ethical approval for their utilization was granted by the Ethics Committee of Fudan University Shanghai Cancer Center (approval number: 050432-4-1911D).

#### *Exosome purification and validation*

Both CAFs and NFs were propagated in complete medium containing 10% exosome-depleted FBS (Gibco, USA). Culture supernatants were harvested after 72 h, once cells reached ~90% confluence. Sequential centrifugation steps included 3000 rpm for 10 min at 4

°C, followed by 10,000×g for 30 min at 4 °C to clear debris. The filtered supernatant (0.22 µm, Merck, USA) underwent ultracentrifugation at 120,000×g for 90 min (Thermo Fisher Scientific, USA). Isolated exosomes were validated by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA), and marker expression was confirmed for TSG101, HSP70, and Annexin. Exosomal RNA and proteins were isolated using the Total Exosome RNA and Protein Isolation Kit (Invitrogen, USA).

#### *Plasmid constructs, siRNA reagents, and stable line generation*

HA-ubiquitin, Myc-NRF2, and Flag-Keap1 expression plasmids were sourced from HANBIO (Wuhan, China). Lentiviral systems enabling PWAR6 overexpression (LvPWAR6) and silencing (shPWAR6) were obtained from Genechem (Shanghai, China). shRNA constructs targeting NRF2 and SLC38A2 were obtained from Ribobio (Guangzhou, China). Lentiviral vectors for Keap1 overexpression (LvKeap1) and knockdown (shKeap1) were likewise provided by Genechem (Shanghai, China). Transient transfections of plasmids and siRNAs into CRC cells were performed using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's guidelines.

#### *Multiplex immunofluorescence analysis*

Specimens were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 (Solar, Beijing) for 10 min, and blocked in 5% BSA (Sigma-Aldrich) in PBS for 1 h. Primary antibody staining was conducted overnight at 4 °C, initiated with anti-panCK (Proteintech). Subsequent steps included incubation with an HRP-linked secondary antibody (CST) for 1 h at room temperature, followed by Tyramide Signal Amplification (TSA) to generate green fluorescence. HRP activity was inactivated with 3% H<sub>2</sub>O<sub>2</sub>. The iterative process continued for anti-CD3 (Invitrogen, red), anti- $\alpha$ -SMA (CST, yellow), and anti-CD56 (Santa Cruz, pink), each with overnight incubation and TSA amplification. DAPI (blue) was applied for nuclear visualization. Slides were mounted with antifade reagent and examined by fluorescence microscopy.

#### *Transwell migration experiments*

Migration was evaluated in 24-well Transwell plates fitted with 8 µm pore inserts (Corning, USA). DLD1 and HCT116 cells were grown to 70–80% confluence,

detached with trypsin–EDTA (Gibco, USA), and resuspended at  $10^5$  cells/ml in serum-free medium (Gibco, USA). CAFs were prepared analogously and seeded at  $10^4$  cells/ml in complete serum-containing medium. Six hundred microliters of complete medium were placed in the lower chamber, followed by addition of CAF suspension and a 1 h attachment period. Inserts were positioned above the attached CAFs, and 100  $\mu$ L of tumor cell suspension was added to the upper chamber. Cultures were maintained at 37 °C with 5% CO<sub>2</sub> for 24–48 h. Non-migrated cells were wiped from the upper membrane surface with a cotton swab. Migrated cells on the underside were fixed in 4% paraformaldehyde for 15 min and stained with crystal violet for 10–15 min before microscopic quantification (Olympus).

#### *Organoid models and 3D invasion assay*

Fresh human colorectal carcinoma (CRC) specimens were harvested and prepared by carefully excising surplus adipose and non-viable tissue. The processed tissue was minced into small 1–2 mm<sup>3</sup> fragments and subjected to enzymatic dissociation using a solution containing Collagenase IV (1.5 mg/mL, Sigma-Aldrich, USA) and Dispase (0.1 mg/mL, Sigma-Aldrich, USA) at 37 °C for 30–60 minutes. Following digestion, the cell suspension was passed through a 70  $\mu$ m mesh filter, pelleted by centrifugation at 300 g for 5 minutes, and rinsed twice in Advanced DMEM/F-12 medium supplemented with 1% GlutaMAX (Gibco, USA), 10 mM HEPES (Gibco, USA), N2 supplement (Gibco, USA), B27 supplement (Gibco, USA), and 1 mM N-acetylcysteine (Gibco). The resulting cell pellet was resuspended in Matrigel (Corning, USA) to achieve a concentration of  $1 \times 10^4$  cells/ $\mu$ L. Aliquots of 50  $\mu$ L were then plated into each well of a 24-well plate and incubated at 37 °C for 30 minutes to allow gel polymerization. The organoids were maintained in Advanced DMEM/F-12 supplemented with 50 ng/mL EGF (PeproTech, USA), 100 ng/mL Noggin (PeproTech, USA), and 500 ng/mL R-spondin 1 (PeproTech, USA), with medium replacement performed every 2–3 days. Once established, the organoids were embedded within a neutralized 3D fibrillar rat tail collagen I matrix (3 mg/mL, Corning, USA) at a density of 1 organoid per  $\mu$ L and transferred to a 24-well glass-bottom plate. The collagen matrix was allowed to polymerize for 1 hour at 37 °C, after which complete organoid culture medium was added to the wells.

#### *3D co-culture of tumor organoids and CAFs*

Cancer-associated fibroblasts (CAFs) were initially plated at an optimal density onto the surface of a 24-well plate that had been pre-coated with a thin layer of Matrigel (Corning, USA). These cells were allowed to adhere and establish a confluent monolayer by overnight incubation in DMEM/F12 (Gibco, USA) containing 10% FBS (Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA).

The following day, the CAF culture medium was aspirated, and a co-culture matrix layer was overlaid onto the monolayer. This matrix was prepared as a 2:1 combination of Matrigel and Collagen I (Corning, USA), adjusted to a final concentration of 3 mg/mL. Patient-derived organoids (PDOs), dissociated into single cells or small cell clusters (corresponding to 2000–3000 organoid-forming units), were then carefully deposited as droplets onto this matrix layer.

Once the co-culture was assembled, the specialized co-culture medium—consisting of Advanced DMEM/F12 (Gibco, USA), 200 mM GlutaMAX (Gibco, USA), 1 M HEPES (Gibco, USA),  $1 \times$  B27 (Gibco, USA), 100 ng/mL FGF (PeproTech, USA), 50 ng/mL EGF (PeproTech, USA), and 5% RSPO1 (PeproTech, USA)—was gently introduced into each well without disturbing the positioning of the PDO layer above the CAFs.

The entire co-culture system was maintained at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Culture medium was renewed at regular intervals, and organoid development was closely observed. Upon completion of the co-culture period, organoid dimensions and morphological characteristics were quantified by microscopy or dedicated imaging software to assess CAF effects on PDO expansion.

#### *Sphere forming assay*

Cells were plated at a density of 1000 cells per well in 24-well ultra-low attachment plates (Corning, USA) using DMEM/F12 (Gibco, USA) medium supplemented with 4  $\mu$ g/mL heparin (Sigma-Aldrich, USA), 0.48  $\mu$ g/mL cortisol (Sigma-Aldrich, USA), 5 ng/mL EGF (Sigma-Aldrich, USA), 5 ng/mL FGF (Sigma-Aldrich, USA), and 10 ng/mL B12 (Sigma-Aldrich, USA). After 10 days in culture, the formed spheres were visualized, enumerated, and harvested under a microscope. In experiments involving CRC spheres, the spheres were enzymatically dissociated, replated, and then exposed to the designated experimental treatments.

*qRT-PCR assay*

Total RNA was isolated from cultured cells with TRIzol reagent (Invitrogen, USA), followed by DNase I treatment to eliminate residual genomic DNA. RNA quantity and quality were determined spectrophotometrically using a NanoDrop instrument. First-strand cDNA was generated from 1 µg of total RNA employing the HiScript II Q RT SuperMix for qPCR (Novozan, Nanjing). Quantitative real-time PCR reactions were carried out in a 20 µL volume containing 2× SYBR qPCR Master Mix (Novozan, Nanjing), 0.4 µM of each primer pair, and 100 ng of cDNA template. Gene-specific primers were used, with GAPDH serving as the housekeeping gene for normalization. Amplification was performed on an ABI 7500 Fast Real-Time PCR System under the following conditions: 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Product specificity was confirmed through melt curve analysis. Relative quantification of gene expression was calculated using the  $2^{-(\Delta\Delta Ct)}$  method. All data were processed with GraphPad Prism software, and differences were considered statistically significant at  $P < 0.05$ .

*Western blotting*

Proteins were isolated from cells, separated by SDS–polyacrylamide gel electrophoresis, and subsequently transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 2 h at room temperature using a blocking buffer. They were then probed overnight at 4 °C with specific primary antibodies, followed by incubation with secondary antibodies (1:10,000, Proteintech). Visualization of protein–antibody complexes was achieved with an ECL Kit (Yeasen, China).

*Pulldown assays*

Cells were harvested and disrupted in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, plus protease inhibitors) for 30 min on ice. After centrifugation, the supernatant was collected. Biotinylated PWAR6 probe (Ribobio, China) was coupled to streptavidin magnetic beads (CST, USA) by incubation at room temperature for 30 min, followed by washing to remove free probe. The prepared bead-probe complex was then mixed with the cell lysate and incubated overnight at 4 °C. The beads were washed thoroughly to remove nonspecifically

bound proteins. Bound proteins were released by heating the beads in elution buffer (100 mM Tris–HCl pH 8.0, 1% SDS) at 95 °C for 5 min. The eluted fractions were resolved by SDS-PAGE and analyzed via mass spectrometry.

*RNA immunoprecipitation*

For RNA immunoprecipitation (RIP), cells were lysed in RIP lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, supplemented with protease and RNase inhibitors). The lysates were incubated overnight at 4 °C with gentle rotation using magnetic beads pre-conjugated to NRF2 antibody (Proteintech, USA). After washing to remove unbound material, RNA was recovered from the immunoprecipitates with TRIzol reagent (Thermo Fisher Scientific, USA). cDNA was generated using the HiScript II Q RT SuperMix for qPCR (Novozan, Nanjing), and PWAR6 transcript levels were quantified by qPCR employing gene-specific primers and qPCR Master Mix (Novozan, Nanjing).

*CUT&Tag assay*

The CUT&Tag assay was conducted according to the manufacturer's instructions using the Hyperactive™ In-Situ ChIP Library Prep Kit for Illumina (TD901-TD902, Vazyme Biotech, China). Briefly, concanavalin A-coated magnetic beads (ConA beads) were mixed with resuspended cells and incubated at room temperature to capture the cells. Permeabilization of cell membranes was performed with Digitonin. Primary antibodies (NRF2 antibody [Proteintech, 16396-1-AP], Histone antibody [Proteintech, 17168-1-AP], and Normal IgG antibody [Proteintech, 30000-0-AP]), secondary antibody, and Hyperactive pA-Tn5 Transposase were added sequentially to the bead-bound cells. This enabled precise cleavage of DNA fragments associated with the target proteins by the transposase. The released DNA fragments were ligated with P5 and P7 adaptors, and the resulting libraries were amplified by PCR using P5 and P7 primers. Library quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed on the Illumina NovaSeq 6000 platform, yielding 150 bp paired-end reads for subsequent analysis.

*CFSE-based proliferation assay*

NK-92MI cells were prepared for the CFSE-based proliferation assay. Cells were suspended in pre-warmed

PBS at a density of  $1 \times 10^6$  cells/mL. CellTrace™ CFSE (Invitrogen, C34554) stock solution was diluted 1:2000 to reach a final concentration of 2.5  $\mu$ M. Labeling was performed by incubating the cells with CFSE for 20 min at 37 °C in a water bath, protected from light. Excess dye was removed by two washes with PBS, after which the labeled cells were transferred to complete culture medium. Following 2 days of incubation, proliferation was evaluated by measuring CFSE dilution using flow cytometry.

#### *Annexin V/7-AAD apoptosis assay*

Apoptosis in NK-92MI cells was determined with the PE Annexin V Apoptosis Detection Kit (Thermo Fisher Scientific, 88–8102-72) as per the manufacturer's protocol. In short, after 24 h of stimulation, cells were collected and resuspended in Binding Buffer at  $1 \times 10^6$  cells/mL. Staining was performed with PE-Annexin V and 7-AAD for 15 min at room temperature in the dark. Flow cytometric analysis was then conducted on the stained cells.

#### *Preparation of cell suspension from mouse samples*

Single-cell suspensions were generated from mouse tissues by first mincing the specimens into small pieces and then performing enzymatic digestion in HBSS containing collagenase IV (MCE, HY-E70005D), DNase I (MCE, HY-108882), and hyaluronidase (Sigma, H6254). Digestion was performed with gentle agitation at 37 °C for 1 h. Following enzymatic treatment, the suspension was passed through a 70  $\mu$ m cell strainer to remove undigested debris. The isolated cells were treated with RBC lysis buffer (Beyotime, C3702) to remove erythrocytes, then resuspended in PBS for downstream applications.

#### *Flow cytometry and functional analysis of immune cells*

Immune cell populations isolated from mouse tissues were analyzed by flow cytometry. Cells were initially incubated with blocking buffer (Biolegend, 101320) for 15 min and then subjected to viability staining (Biolegend, 423105). Surface marker staining was performed to characterize specific immune subsets using the following gating strategy: NK cells (CD45+, NK1.1+, CD3-), CD8+ T cells (CD45+, CD8+, CD3+), CD4+ T cells (CD45+, CD4+, CD3+), macrophages (CD45+, CD11b+, Ly6g-, F4/80hi), and B cells (CD45+, CD19+).

Functional evaluation of immune cells involved stimulation with Cell Activation Cocktail (BioLegend, 423303) for 4 h. After stimulation, nonspecific binding was minimized by treatment with blocking solution (BioLegend, 101320). Cells were stained for viability using the Zombie NIR™ Fixable Viability Kit (BioLegend, 423105), followed by surface marker staining with fluorochrome-conjugated antibodies. The cells were then fixed with Fixation Buffer (BioLegend, 420801) and stained for intracellular cytokines with appropriate antibodies. Finally, flow cytometry was performed to assess the activation status and functional capacity of immune populations.

#### *LDH cytotoxicity assay*

Cytotoxicity was measured using an LDH cytotoxicity kit (C0016, Beyotime). Target cells (HCT116 and DLD1) were seeded at  $5 \times 10^4$  cells/mL and co-cultured for 2 h with NK-92MI effector cells at effector-to-target (E: T) ratios of 1:1, 2:1, 5:1, and 10:1 in 96-well plates. Culture supernatants were harvested after the incubation period and processed according to the manufacturer's instructions to quantify LDH release as an indicator of cytotoxicity.

#### *Animal models*

Female C57BL/6 mice (5 weeks old) were acquired from the Shanghai Experimental Animal Center and maintained in standard housing conditions in compliance with protocols approved by the Institutional Animal Care and Use Committee of Fudan University Shanghai Cancer Center (approval number: FUSCC-IACUC-S20210022).

Subcutaneous xenograft and splenic injection models were established to investigate tumor growth and metastatic potential in vivo, respectively. In the subcutaneous model, a suspension containing  $1 \times 10^6$  CAFs combined with MC38 cells in 100  $\mu$ L PBS was injected into the right flank of each mouse. Tumor volumes were monitored and recorded at regular intervals. For the liver metastasis model,  $1 \times 10^6$  CAFs and MC38 cells suspended in 100  $\mu$ L PBS were injected into the spleen. The spleen was retained in place for 5 min to facilitate cell dissemination toward the liver before surgical removal to prevent primary tumor development at the injection site.

Beginning 7 days after cell inoculation, tumor-bearing mice were treated with shRNA delivered intraperitoneally at 1.5 mg/kg/week using the

Invivofectamine 3.0 reagent (Thermo Fisher Scientific, IVF3001). The shRNA was properly complexed with the delivery vehicle before injection. Additionally, scrambled ASO or ASO-PWAR6 inhibitor (10 nmol per injection, RiboBio) was administered via tail vein every 3 days. FAPI (20 µg per injection, MCE) was given orally twice daily. All animals were euthanized at 6 weeks post-injection; tumors were excised and weighed, and collected tissues underwent histopathological evaluation.

#### Development of myCAF score

The myCAF score was constructed using 596 genes previously reported by Herrera *et al.* [25] as strongly associated with the myCAF phenotype in colorectal cancer. The Lasso Cox regression method was applied to select 40 genes for the final score. Patients were classified into high- and low-myCAF groups based on the median score. The prognostic value and biological relevance of this score were validated by integrating clinicopathological features, gene ontology (GO) analysis, and Kaplan–Meier (K-M) survival analysis across both the TCGA and meta-GEO cohorts. Statistical comparisons were performed using the Log-Rank test for survival data, the Wilcoxon test for differences in gene expression between groups, and the chi-squared test for categorical variables.

#### Non-coding RNA analysis in GSE39582

Data on non-coding RNA sequencing, particularly regarding PWAR6, are publicly accessible only in the GSE39582 dataset and are not available in the TCGA database. Accordingly, evaluation of the association between PWAR6 expression levels and the myCAF score was restricted to the GSE39582 dataset. Group differences were examined for statistical significance employing Student's t-test or one-way analysis of variance (ANOVA).

#### Statistical analysis

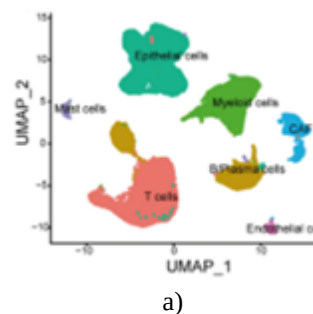
The findings reported herein derive from at least three independent experiments. Results are expressed as mean values ± standard error of the mean (s.e.m.). All statistical computations were executed using either SPSS 22.0 (SPSS) or GraphPad Prism 9.0 (GraphPad) software. Intergroup comparisons were conducted via Student's t-test or one-way analysis of variance (ANOVA). The chi-squared test served to examine relationships between PWAR6 expression and various clinicopathological parameters. Univariate and multivariate Cox

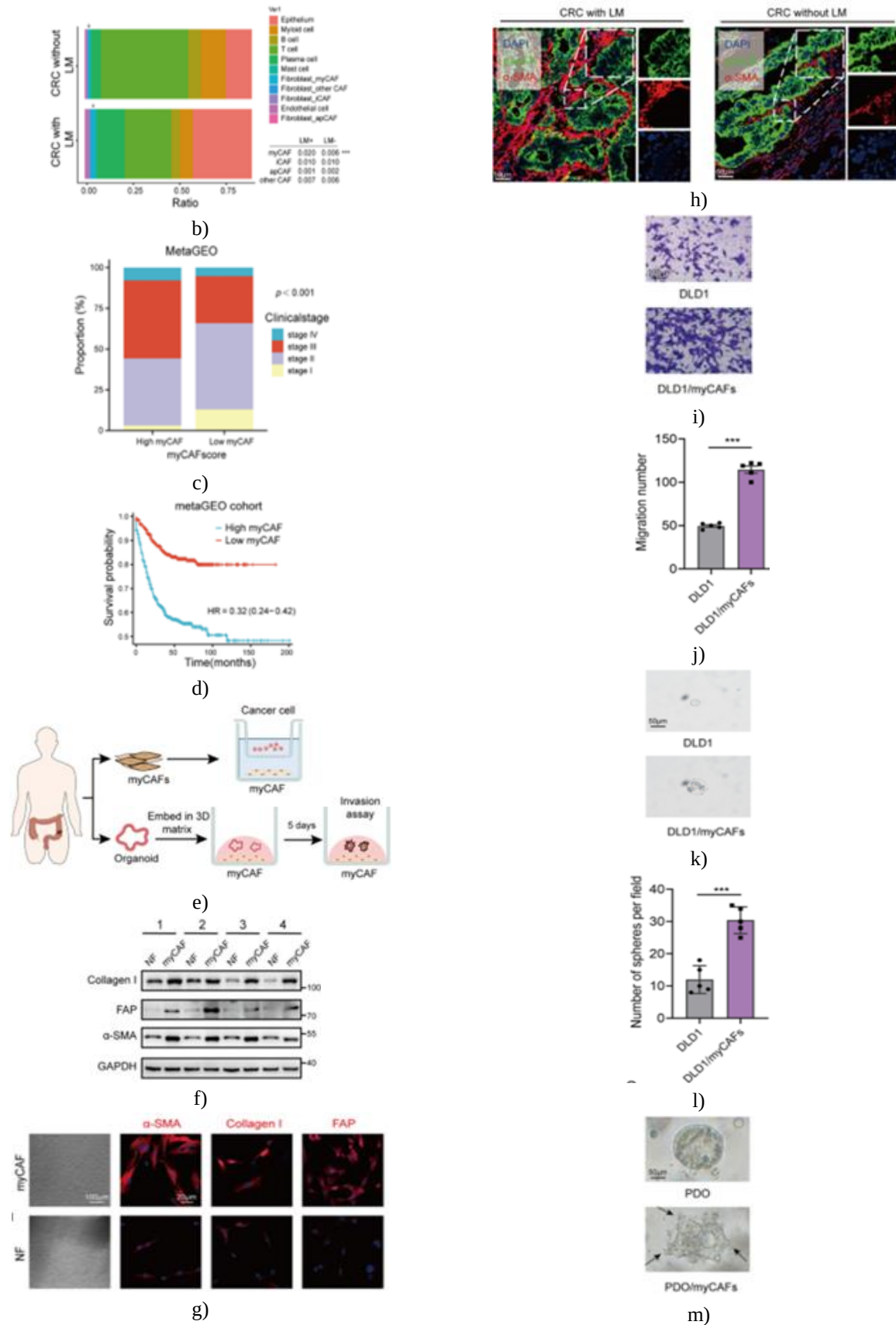
proportional hazards regression analyses were used to assess the influence of PWAR6 expression, along with additional clinical variables, on patient survival, and hazard ratios (HRs) were calculated. Spearman's correlation coefficient was utilized to determine variable associations. Overall survival (OS) probabilities were estimated using the Kaplan–Meier method and compared using the log-rank test. Significance thresholds were set at \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001.

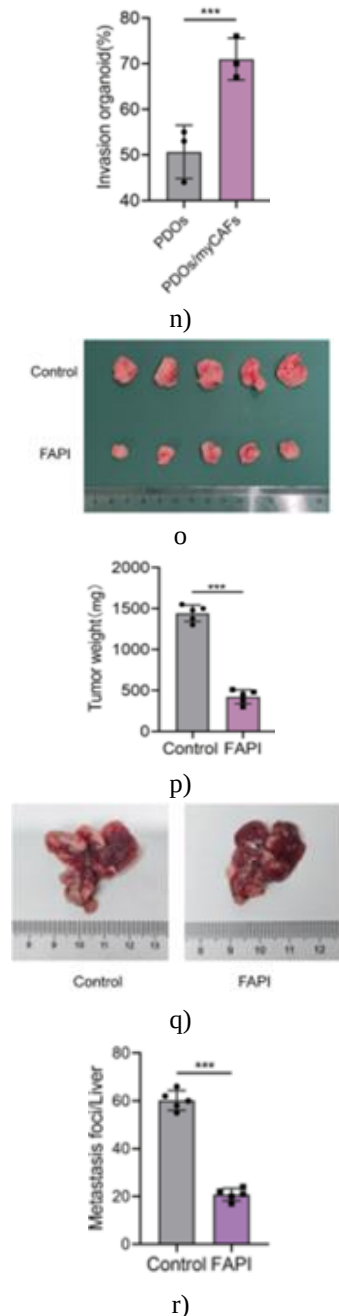
## Results and Discussion

#### *myCAFs enhanced CRC cell stemness and promoted migration*

To elucidate the involvement of cancer-associated fibroblasts (CAFs) in colorectal cancer (CRC) pathobiology, single-cell RNA sequencing datasets GSE166555 (tumor and paired normal tissues from 12 non-metastatic CRC patients) and GSE178318 (primary tumors, liver metastases, and blood samples from 6 patients with liver metastases) were examined. Clustering of single-cell profiles indicated increased representation of CAFs among individuals with liver metastasis (CRLM) compared with those without metastatic disease. In particular, the myCAF subset displayed substantial enrichment in CRLM specimens, while iCAFs, apCAFs, and remaining CAF populations showed comparatively modest variations (**Figures 1a and 1b**). A myCAF score was derived from bulk RNA-seq data in both meta-GEO and TCGA cohorts to quantify myCAF abundance. Patients exhibiting high myCAF scores were more commonly diagnosed with advanced-stage disease (stages III and IV), in contrast to those with low scores who presented predominantly with early-stage tumors (stages I and II). Kaplan–Meier survival analysis further revealed that elevated myCAF scores correlated with markedly inferior clinical outcomes in CRC (**Figures 1c and 1d**).







**Figure 1.** myCAFs enhanced CRC cell stemness and promoted migration

(a) Uniform manifold approximation and projection (UMAP) plot illustrating the eight primary cell populations. Each dot corresponds to an individual cell, with distinct colors denoting different cell types ( $n = 18$  patients), (b) Clustering results from single-cell transcriptomic data sourced from GSE166555 and GSE178318 ( $n = 18$  patients), (c) Distribution of tumor stages among patients stratified by high versus low

myCAF scores, derived from the meta-GEO database ( $n = 913$  patients), (d) Kaplan–Meier survival curves for patients in the high-myCAF and low-myCAF score groups, based on meta-GEO data ( $n = 913$  patients), (e) Schematic diagram outlining the isolation of myCAFs and patient-derived organoids (PDOs) from CRC tissues and the subsequent evaluation of invasive properties following co-culture, (f) Western blot analysis depicting expression of  $\alpha$ -SMA, FAP, and Collagen I proteins in normal fibroblasts (NFs) and myCAFs obtained from CRC patients (isolated from four patients), (g) Immunofluorescence staining for  $\alpha$ -SMA, FAP, and Collagen I in NFs and myCAFs from CRC patients. Scale bars = 100  $\mu$ m (left) and 20  $\mu$ m (right) ( $n = 3$ , representative of three biological replicates), (h) Representative  $\alpha$ -SMA immunohistochemical staining in CRC tissue sections from the FUSCC cohort 2. Scale bar = 50  $\mu$ m ( $n = 3$ , representative of three biological replicates), (i, j) Assessment of DLD1 cell invasion by Transwell assay following co-culture with myCAFs. Scale bar = 100  $\mu$ m (representative images; data from five biological replicates), (k, l) Sphere formation assay measuring stemness of DLD1 cells after co-culture with myCAFs. Scale bar = 100  $\mu$ m (representative images; data from five biological replicates), (m, n) Three-dimensional invasion assay of PDOs co-cultured with myCAFs. Scale bar = 50  $\mu$ m (representative images; data from three replicates), (o, p) Representative photographs of subcutaneous tumors in mice receiving FAPI treatment by oral gavage ( $n = 5$  mice per group), (q, r) In vivo evaluation of liver metastasis following splenic injection to determine the influence of FAPI on metastatic spread ( $n = 5$  mice per group). Data represent mean  $\pm$  s.e.m. Statistical tests: chi-squared test for panels (b, c); log-rank test for panel (d); two-tailed Student's  $t$ -test for panels (I–R). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

To further elucidate the impact of myCAFs on colorectal cancer (CRC) advancement, myCAFs and normal fibroblasts (NFs) were successfully isolated from fresh CRC specimens and matched non-tumoral tissues. Patient-derived organoids were also generated from the same CRC tissues according to standardized protocols (**Figure 1e**). Validation of fibroblast identity was achieved through western blot analysis of canonical myCAF markers such as  $\alpha$ -SMA, FAP, and Collagen I [26] (**Figure 1f**). The isolated myCAFs displayed markedly elevated expression of these proteins relative to NFs. Morphological characterization of both

fibroblast populations was performed via light microscopy and immunofluorescence staining. Both cell types exhibited comparable spindle-like appearances under phase-contrast microscopy (**Figure 1g**), (left). Nevertheless, immunofluorescence imaging demonstrated stronger signal intensity for the marker proteins in myCAFs (**Figure 1g**), (right). In addition, multiplex immunofluorescence staining was conducted on clinical CRC tissue sections from patients with and without liver metastasis to quantify myCAF abundance within the tumor microenvironment. This analysis corroborated the preferential accumulation of myCAFs in CRLM cases, as evidenced by intense  $\alpha$ -SMA positivity (red fluorescence) (**Figure 1h**).

Next, the functional effects of myCAFs on CRC cells were examined using both established cell lines and organoid systems to mimic interactions within the tumor microenvironment. For cell line studies, a transwell-based non-contact co-culture model was implemented with two CRC lines and primary myCAFs, following the optimized procedures described by Liu *et al.* [27] (**Figure 1e**). Co-culture with myCAFs resulted in substantially increased cell migration, enhanced sphere-forming ability, and upregulated stemness-related gene expression in CRC cells. These findings indicate that myCAFs promote stem-like properties and metastatic behavior of CRC cells in vitro (**Figures 1i-1l**). Furthermore, myCAFs were integrated into the three-dimensional collagen matrix surrounding patient-derived organoids (PDOs) using established techniques. PDOs grown in the presence of myCAFs displayed significantly greater invasive outgrowth into the surrounding matrix compared with control PDOs (**Figures 1m and 1n**). Collectively, these in vitro experiments provide robust support for myCAFs' capacity to potentiate the invasive and metastatic traits of CRC cells.

To confirm these effects in vivo, subcutaneous xenograft and liver metastasis models were established in immunodeficient mice. CRC cells were co-injected with myCAFs, after which animals were randomized into either a Fibroblast Activation Protein Inhibitor (FAP) treatment group or a vehicle (saline) control group. FAP is abundantly expressed in the tumor microenvironment stromal compartment, particularly within myCAFs. FAP has shown promise in suppressing fibroblast activation and in imaging metastatic lesions across multiple malignancies [28]. Mice in the treatment arm received oral FAPI, whereas control animals were given

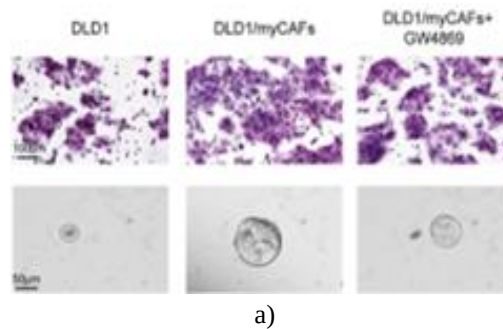
saline. Treatment with FAPI led to a notable reduction in both subcutaneous tumor volume and the extent of liver metastatic burden compared with the saline-treated cohort (**Figures 1o and 1r**).

Taken together, these results underscore the pivotal role of myCAFs in CRC metastasis and highlight promising strategies to target myCAF–CRC cell interactions and impede disease progression.

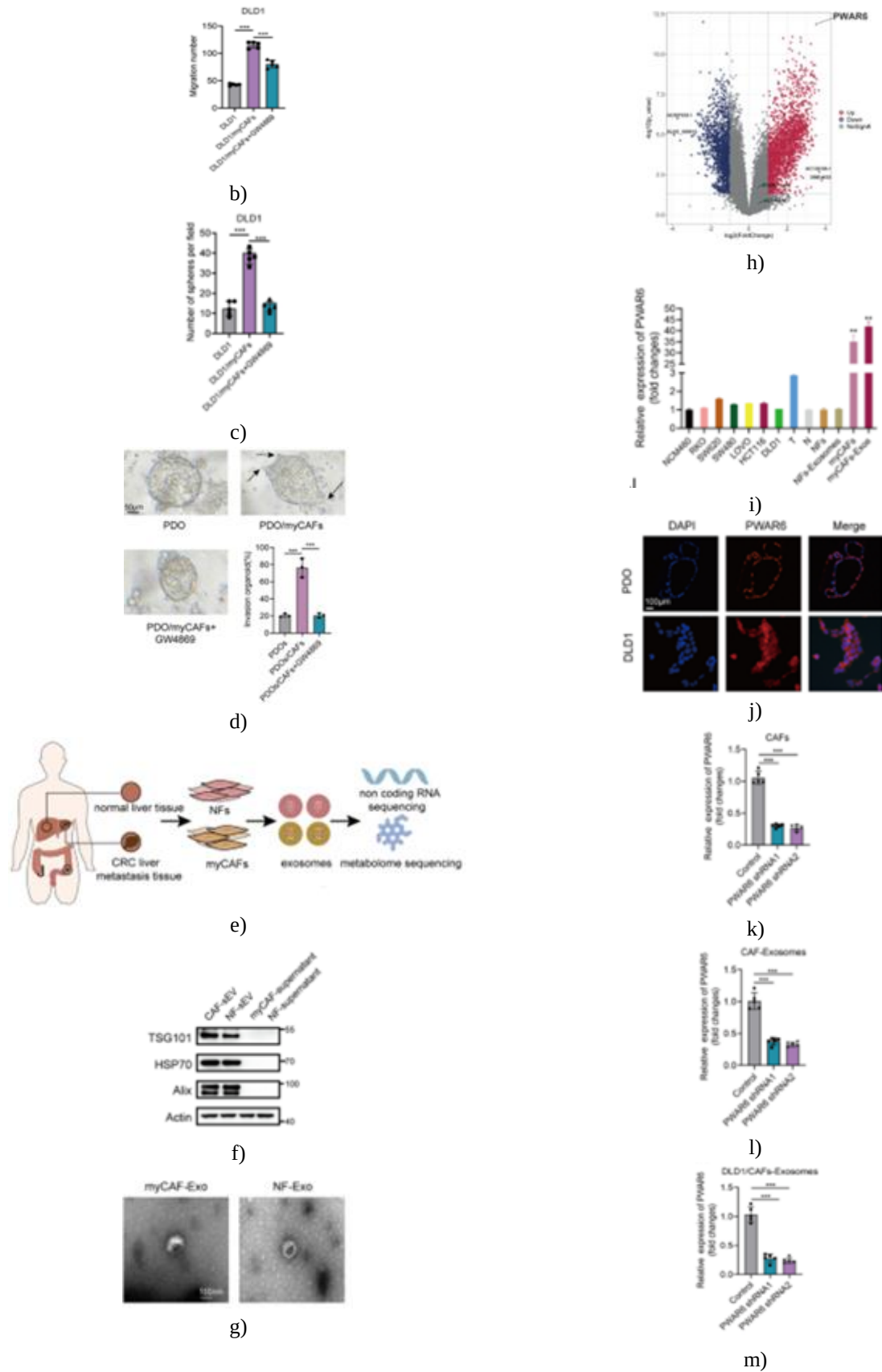
#### *myCAF-derived exosomal PWAR6 enhanced cell stemness and promoted CRC cell migration*

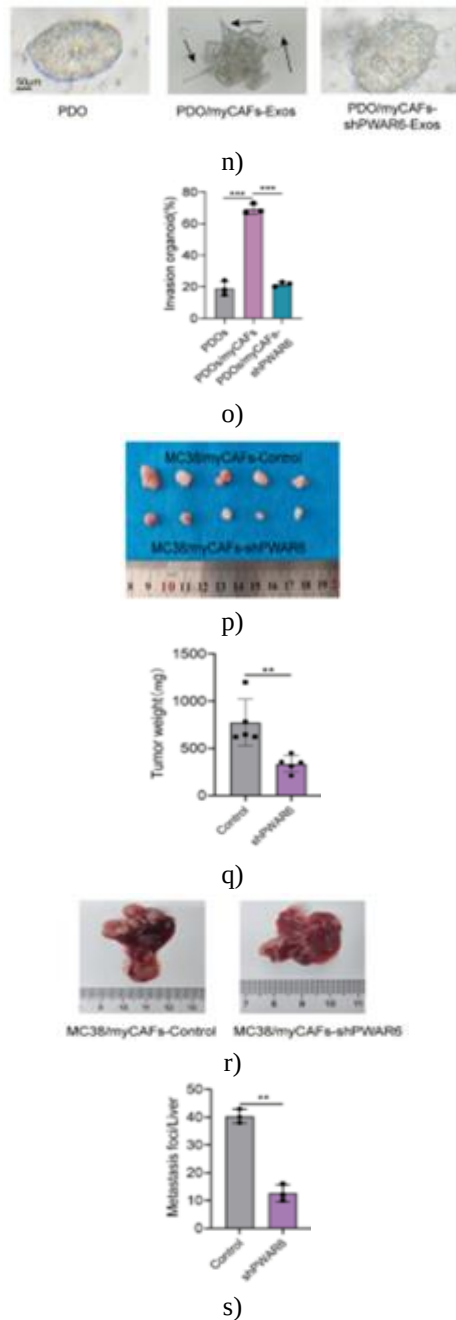
Consistent with prior observations, exosomes represent a major mechanism through which myCAFs interact with other constituents of the tumor microenvironment. Applying the established myCAF score, we conducted differential expression analysis and functional pathway enrichment on meta-GEO cohort samples divided into high- and low-myCAF score groups. Gene sets associated with exosome production and release were prominently enriched in samples exhibiting high myCAF scores.

To examine the potential involvement of exosomes in the previously observed phenotypes, myCAFs and CRC cells were co-cultured in the presence of the exosome biogenesis inhibitor GW4869. Notably, pharmacological blockade with GW4869 effectively abrogated the myCAF-induced increases in CRC cell migration and stemness, as evidenced by Transwell migration and sphere-formation assays (**Figures 2a–2c**). In complementary experiments, exosomes purified from myCAFs were incubated with DLD1 and HCT116 cells, resulting in enhanced migratory ability and stem-like characteristics. Similarly, in 3D PDO invasion models, addition of GW4869 markedly suppressed the invasive behavior promoted by myCAFs (**Figure 2d**). These findings indicate that myCAFs drive CRC cell metastasis and stemness largely through exosomal transfer.



a)





**Figure 2.** myCAF-derived exosome PWAR6 enhanced glutamine uptake. It promoted CRC cell migration

(a–c) Analysis of DLD1 cell invasion and stemness following treatment with GW4869 in co-culture, evaluated by Transwell and sphere formation assays. Scale bars = 100  $\mu$ m (upper panels) and 50  $\mu$ m (lower panels) (representative images; data from five biological replicates), (d) Three-dimensional organoid invasion assay assessing the effect of GW4869 treatment on PDO

invasiveness. Scale bar = 50  $\mu$ m (representative images; data from three biological replicates), (e) Overview of the experimental pipeline involving isolation of myCAFs from liver metastatic lesions and NFs from adjacent normal tissue in CRLM patients, followed by exosome extraction for lncRNA sequencing and metabolomics profiling, (f) Confirmation of exosomal markers TSG101, HSP70, and Alix by western blotting ( $n = 3$ , representative of three biological replicates), (g) Representative transmission electron micrographs of exosomes released by myCAFs and NFs. Scale bar = 100 nm ( $n = 3$ , representative of three biological replicates), (h) Identification of the top 100 most differentially expressed lncRNAs (up- and downregulated) in exosomes from myCAFs versus paired NFs isolated from CRLM tissues, (i) PWAR6 expression levels measured across the normal colonic epithelial cell line NCM460, multiple CRC cell lines, tumor tissues (T), matched normal intestinal tissues (N), NFs, NF-derived exosomes, myCAFs, and myCAF-derived exosomes ( $n = 3$  technical replicates, representative of three biological replicates), (j) In situ hybridization demonstrating PWAR6 localization in both nuclear and cytoplasmic compartments of CRC cells and PDOs. Scale bar = 100  $\mu$ m ( $n = 3$ , representative of three biological replicates), (k) Validation of PWAR6 knockdown efficiency in myCAFs ( $n = 5$ , representative of three biological replicates), (l) Reduced PWAR6 content in exosomes following knockdown in source myCAFs ( $n = 5$ , representative of three biological replicates), (m) Decreased PWAR6 levels in DLD1 cells after exposure to exosomes from PWAR6-depleted myCAFs ( $n = 5$ , representative of three biological replicates), (n, o) PDO 3D invasion assay following PWAR6 knockdown in myCAFs. Scale bar = 50  $\mu$ m (representative images; data from three biological replicates), (p, q) In vivo subcutaneous tumor growth was significantly attenuated by PWAR6 knockdown ( $n = 5$  mice per group), and (r, s) Liver metastatic burden was markedly diminished following PWAR6 knockdown ( $n = 3$  mice per group). Data are expressed as mean  $\pm$  s.e.m. Statistical tests: one-way ANOVA for panels (a–d), (i), and (k–o); two-tailed Student's t-test for panels (p–s). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

To clarify the molecular mechanisms by which myCAF-derived exosomes (CAF-Exo) affect CRC cells, five matched pairs of colorectal liver metastasis (CRLM) tissues and neighboring normal liver parenchyma were procured. Normal fibroblasts (NFs) and myCAFs were

isolated from these tissues. Exosomes were subsequently purified from the conditioned culture media of myCAFs and NFs via ultracentrifugation [29] (**Figure 2e**). Confirmation of exosome isolation was achieved through western blotting for established exosomal proteins TSG101, HSP70, and Annexin [30] (**Figure 2f**). Transmission electron microscopy (TEM) further visualized the exosomes as typical closed, round vesicles with characteristic membrane structures secreted by both fibroblast populations (**Figure 2g**).

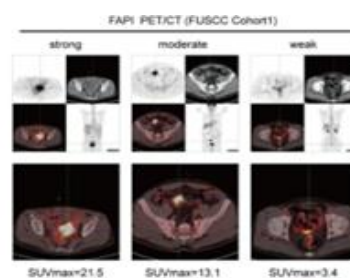
Non-coding RNA sequencing of exosomes from myCAFs (myCAF-Exo) and normal fibroblasts (NF-Exo) was carried out. PWAR6 emerged as the lncRNA with the highest degree of upregulation in myCAF-Exo compared with NF-Exo (**Figure 2h**). Quantitative validation across multiple sample types showed consistently elevated PWAR6 levels in myCAFs, myCAF-derived exosomes, and CRC tumor tissues. In contrast, expression remained low and comparable in NFs, NF-derived exosomes, CRC cell lines, and the normal intestinal epithelial cell line (**Figure 2i**). Supporting data from the GSE41568 dataset indicated significantly increased PWAR6 expression in metastatic CRC lesions relative to primary tumors. Collectively, these observations demonstrate prominent upregulation of PWAR6 in myCAFs and their exosomes, suggesting its role in driving CRC progression. FISH analysis in patient-derived organoids and CRC cell lines further localized PWAR6 predominantly to the nucleus (**Figure 2j**).

To define the functional contribution of exosomal PWAR6, PWAR6 expression was silenced in myCAFs using short hairpin RNA (shRNA) (**Figure 2k**). This resulted in a parallel reduction in PWAR6 abundance in secreted exosomes (**Figure 2l**). Co-culture studies were then conducted by incubating CRC cell lines and PDOs with either PWAR6-knockdown myCAFs or control myCAFs (**Figure 2m**). PDOs exposed to PWAR6-deficient myCAFs displayed substantially impaired invasive outgrowth within the 3D matrix (**Figures 2n and 2o**). CRC cells similarly showed attenuated migration and sphere-forming ability when co-cultured with PWAR6-knockdown myCAFs, along with lowered expression of stemness-associated and epithelial-mesenchymal transition genes. These in vitro observations establish that PWAR6 packaged in myCAF-derived exosomes augments stemness and metastatic traits in colorectal cancer cells.

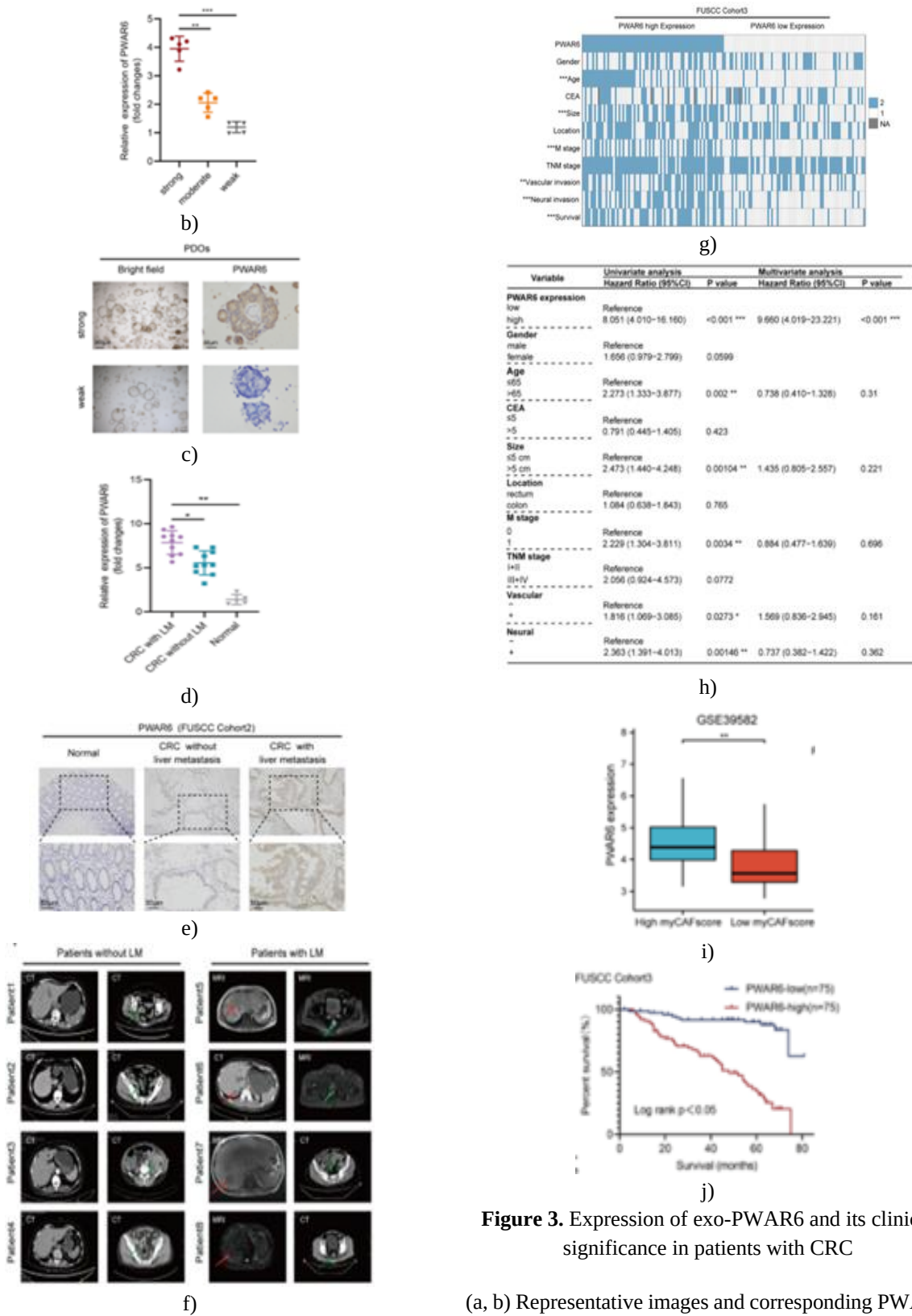
In vivo experiments involved co-culturing MC38 cells with control or PWAR6-knockdown myCAFs, followed by subcutaneous or intrasplenic injection into C57BL/6 mice. Mice in the PWAR6-knockdown group exhibited markedly smaller subcutaneous tumors and significantly reduced liver metastatic burden and weight compared with controls (**Figures 2p–2s**). Immunohistochemical examination of metastatic liver tissues demonstrated reduced levels of mesenchymal markers Vimentin and N-cadherin, coupled with elevated expression of the epithelial marker E-cadherin in the shPWAR6 group. In conclusion, the present study demonstrates that PWAR6 conveyed by myCAF-derived exosomes promotes stemness and metastatic dissemination of CRC cells in both in vitro and in vivo settings.

#### *Expression of exo-PWAR6 and its clinical significance in CRC patients*

As previously outlined, FAPI is a promising radiotracer for detecting myCAFs and tumor sites [31]. In recent years, 68Ga-FAPI positron emission tomography (68Ga-FAPI-PET) has been introduced and clinically applied to identify metastases and facilitate tumor diagnosis [32]. In this investigation, PWAR6 expression was systematically examined in tissue specimens from 15 patients at the Fudan University Shanghai Cancer Center (FUSCC Cohort 1) with varying 68Ga-FAPI-PET SUVmax values. PWAR6 levels were substantially elevated in individuals with high SUVmax and notably reduced in those with low SUVmax (**Figures 3a and 3b**). Organoids derived from these patient samples were also evaluated. Tissues from patients with higher 68Ga-FAPI-PET SUVmax produced more abundant organoids (**Figure 3c**), (left) and displayed increased PWAR6 positivity by in situ hybridization (**Figure 3c**), (right). Consistent with these observations, analysis of the GSE39582 dataset revealed significantly higher PWAR6 expression in samples characterized by elevated myCAF scores (**Figure 3i**).



a)



**Figure 3.** Expression of exo-PWAR6 and its clinical significance in patients with CRC

(a, b) Representative images and corresponding PWAR6 expression levels in patients grouped according to 68Ga-FAPI-PET SUVmax values (n = 15 patients), (c)

Representative images of patient-derived organoids (PDOs) and PWAR6 in situ hybridization staining from tissues of patients with different 68Ga-FAPI-PET SUVmax values. Scale bars = 50  $\mu$ m (n = 15 patients), (d, e) Representative in situ hybridization images and PWAR6 expression quantification in samples from healthy donors (enteritis) and CRC patients with or without liver metastasis. Scale bars = 50  $\mu$ m (n = 25 patients), (f) Representative CT or MRI scans of CRC patients with and without liver metastasis (n = 25 patients). (g) Heatmap illustrating the results of chi-squared tests examining associations between PWAR6 expression and multiple clinicopathological variables (n = 150 patients); (h) Forest plots of univariate and multivariate Cox regression analyses for overall survival (OS) in the FUSCC cohort 3 (n = 150 patients). (i) Comparison of PWAR6 expression between high- and low-myCAF-score groups, based on GSE39582 data (n = 133 patients). (j) Kaplan–Meier survival analysis comparing PWAR6-high versus PWAR6-low patients in the FUSCC cohort 3 (n = 150). Data are shown as mean  $\pm$  s.e.m. Statistical tests included one-way ANOVA for panels (a–d) and (i), the chi-squared test for panel g), Cox regression for panel (h), and the log-rank test for panel (j). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.

Next, samples were gathered from five paired normal intestinal tissues, ten cases of non-metastatic CRC, and ten CRLM patients (FUSCC cohort 2). Exosomes were isolated from these tissues, and PWAR6 expression was quantified by qPCR. Concurrently, chromogenic in situ hybridization (CISH) was utilized to visualize and compare PWAR6 levels within the corresponding intestinal tissues. Both analytical approaches consistently showed the highest PWAR6 expression in patients with CRLM, intermediate levels in those with non-metastatic CRC, and the lowest expression in enteritis cases (**Figurers 3d and 3e**). Representative abdominal MRI or CT images from these patients are displayed in **Figure 3f**.

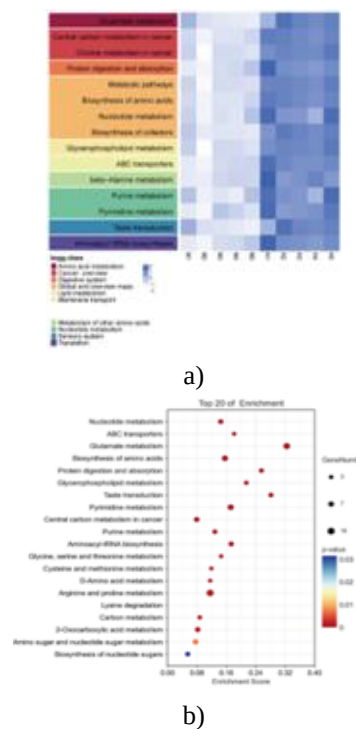
Thereafter, the clinical and prognostic importance of PWAR6 was evaluated in a distinct FUSCC cohort of 150 paraffin-embedded CRC specimens spanning stages I-IV, preserved at  $-80^{\circ}\text{C}$  (FUSCC cohort 3). Kaplan–Meier analysis of the FUSCC cohort 3 revealed a pronounced difference in patient survival by PWAR6 status. Individuals with low PWAR6 expression demonstrated significantly better overall survival (OS) than those with high expression (**Figure 3j**). Associations between PWAR6 expression and major

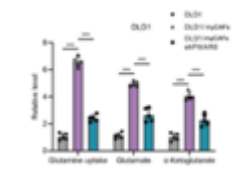
clinicopathological parameters were assessed using the chi-squared test. Significant correlations were identified with distant metastasis (M stage), patient survival status, perineural invasion, vascular invasion, age, and tumor diameter (**Figure 3g**). Univariate Cox regression analysis indicated that M stage and PWAR6 expression were significantly linked to prognosis ( $P < 0.05$ ). Subsequent multivariate Cox regression confirmed high PWAR6 expression as an independent prognostic indicator of reduced OS (**Figure 3h**).

Overall, this study demonstrates that increased PWAR6 expression, as assessed by <sup>68</sup>Ga-FAPI-PET imaging and immunohistochemistry, is associated with poorer disease-free and overall survival among CRC patients. Furthermore, elevated PWAR6 levels correlate strongly with greater CAF infiltration and more unfavorable clinicopathological characteristics in colorectal cancer.

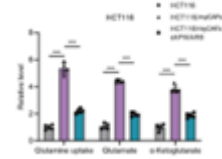
### CAF-derived exo-PWAR6 enhances glutamine uptake in CRC cells through upregulation of the amino acid transporter SLC38A2

Metabolomic analysis of exosomes previously isolated from myCAFs and NFs uncovered significant differences in glutamine metabolic pathways between myCAFs originating from liver metastatic lesions and NFs from normal hepatic tissue (**Figures 4a and 4b**).

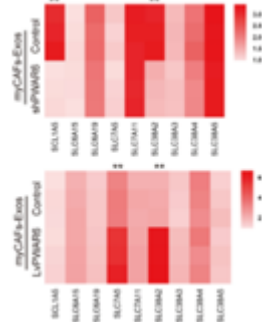




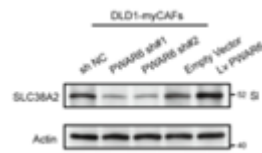
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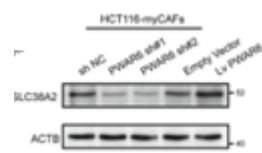
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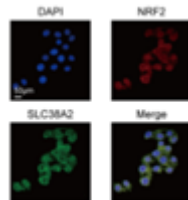
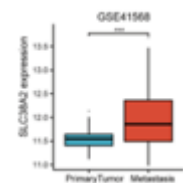
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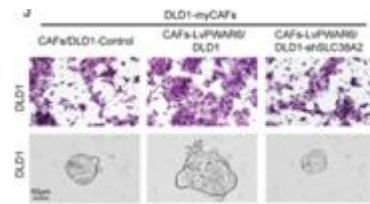
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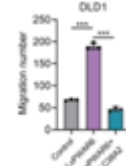
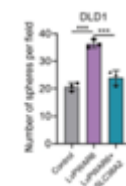
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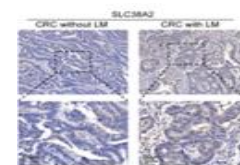
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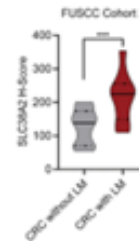
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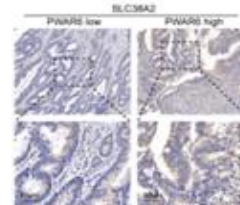
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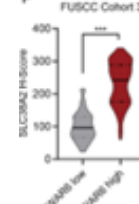
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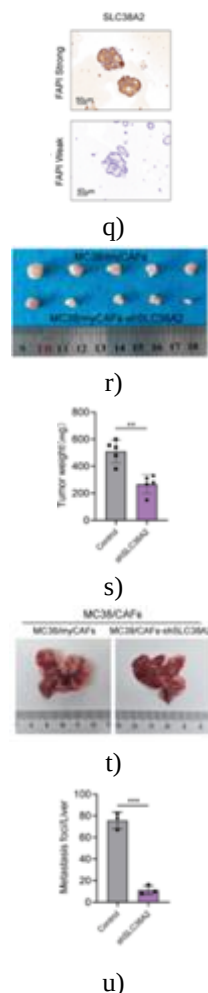
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**Figure 4.** myCAF-derived exo-PWAR6 enhances the glutamine uptake of CRC cells via upregulation of amino acid transporters SLC38A2

(a, b) Metabolomics profiling of exosomes released by myCAFs from CRLM tissues versus paired NFs ( $n = 5$  patients), (c, d) Measurement of glutamine uptake along with intracellular glutamate and  $\alpha$ -ketoglutarate concentrations in CRC cells after co-culture with PWAR6-silenced myCAFs ( $n = 5$ , representative of three biological replicates), (e) Transcriptional levels of glutamine transporters determined by qRT-PCR in CRC cells exposed to exosomes from myCAFs transfected with shPWAR6 or LvPWAR6 ( $n = 3$ , representative of three biological replicates), (f, g) Protein expression of SLC38A2 assessed by western blotting in DLD1 and HCT116 cells following treatment with myCAF-derived exosomes ( $n = 3$ , representative of three biological replicates), (h) Elevated SLC38A2 expression in metastatic tumors relative to primary CRC tumors according to the GSE41568 dataset ( $n = 133$  patients), (i)

Representative immunofluorescence images showing NRF2 and SLC38A2 staining ( $n = 3$ , representative of three biological replicates). (j–l) Effects of SLC38A2 knockdown on DLD1 cell invasion and stemness evaluated using Transwell and sphere formation assays. Scale bars = 100  $\mu$ m (upper) and 50  $\mu$ m (lower) (representative images shown; data from three biological replicates), (m, n) SLC38A2 expression levels and typical immunohistochemical staining patterns in samples from FUSCC cohort 2. Scale bars = 50  $\mu$ m (representative images shown;  $n = 25$  patients), (o, p) SLC38A2 expression and corresponding immunohistochemical images in FUSCC cohort 3. Scale bars = 50  $\mu$ m (representative images shown;  $n = 150$  patients), (q) Immunohistochemical detection of SLC38A2 in PDOs generated from patients exhibiting different 68Ga-FAPI-PET SUVmax values. Scale bars = 50  $\mu$ m (representative images shown;  $n = 15$  patients), (r, s) In vivo subcutaneous tumor growth is significantly suppressed by SLC38A2 knockdown ( $n = 5$  mice per group), (t, u) Liver metastatic burden is notably diminished following SLC38A2 knockdown ( $n = 3$  mice per group). Data are expressed as mean  $\pm$  s.e.m. Statistical tests: one-way ANOVA for panels (c–e) and (j–l); two-tailed Student's  $t$ -test for panels (h) and (m–u). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Glutamine metabolism comprises a network of interconnected biochemical reactions with important roles in cellular physiology. In cancer cells, exogenous glutamine is predominantly taken up via members of the glutamine transporter family. Following uptake, glutamine is converted to glutamate, which is further metabolized to  $\alpha$ -ketoglutarate ( $\alpha$ -KG) and subsequently feeds into the tricarboxylic acid (TCA) cycle.

Encouragingly, our experiments demonstrated markedly increased glutamine uptake along with elevated intracellular glutamate and  $\alpha$ -ketoglutarate concentrations in CRC cells co-cultured with myCAFs. These increases were effectively abolished when PWAR6 was silenced in the CAFs (**Figures 4c and 4d**). These observations suggest that myCAF-derived PWAR6 actively modulates glutamine metabolism, thereby contributing to the reprogrammed metabolic profile and aggressive behavior of CRC.

We therefore hypothesized that PWAR6 may regulate glutamine influx by modulating the expression of amino acid transporters. Solute carrier (SLC) family transporters play central roles in glutamine transport. To test this hypothesis, qRT-PCR was performed to profile

the mRNA expression of major glutamine transporters in DLD1 and HCT116 cells. Notably, only SLC38A2 displayed significant downregulation or upregulation following treatment with exosomes derived from shPWAR6- or LvPWAR6-expressing myCAFs, respectively (**Figure 4e**).

Western blot analysis confirmed that PWAR6 knockdown reduced SLC38A2 protein levels, while PWAR6 overexpression increased SLC38A2 expression in DLD1 and HCT116 cells exposed to myCAF-derived exosomes (**Figures 4f and 4g**). Consistent with these findings, analysis of the GSE41568 dataset showed significantly higher SLC38A2 expression in metastatic CRC lesions compared with primary tumors (**Figure 4h**). Immunofluorescence staining further revealed nuclear co-localization of NRF2 and SLC38A2 in DLD1 cells (**Figure 4i**). Importantly, the enhancement of glutamine uptake, cell migration, and stemness induced by PWAR6 overexpression was partially reversed by concurrent SLC38A2 knockdown (**Figures 4j–4l**).

We next evaluated the clinical relevance of SLC38A2 using our FUSCC patient cohorts. In FUSCC cohort 1, higher <sup>68</sup>Ga-FAPI-PET SUVmax values were associated with increased SLC38A2 expression (**Figure 4q**). In FUSCC cohort 2, CRLM tissues exhibited significantly elevated SLC38A2 levels compared with non-metastatic cases (**Figures 4m and 4n**). Similarly, in FUSCC cohort 3, CRC specimens with high PWAR6 expression showed concurrent upregulation of SLC38A2 (**Figures 4o and 4p**). Analysis of the TCGA database revealed a positive correlation between SLC38A2 and PWAR6 expression.

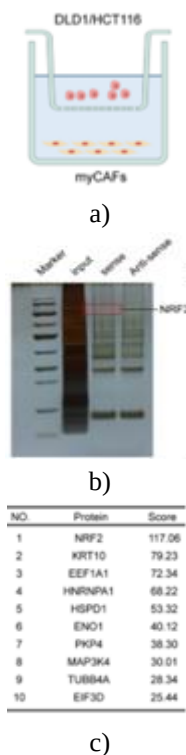
Furthermore, SLC38A2 knockdown in the previously established C57BL/6 mouse models led to a significant reduction in both subcutaneous tumor volume and liver metastatic burden (**Figures 4r–4u**).

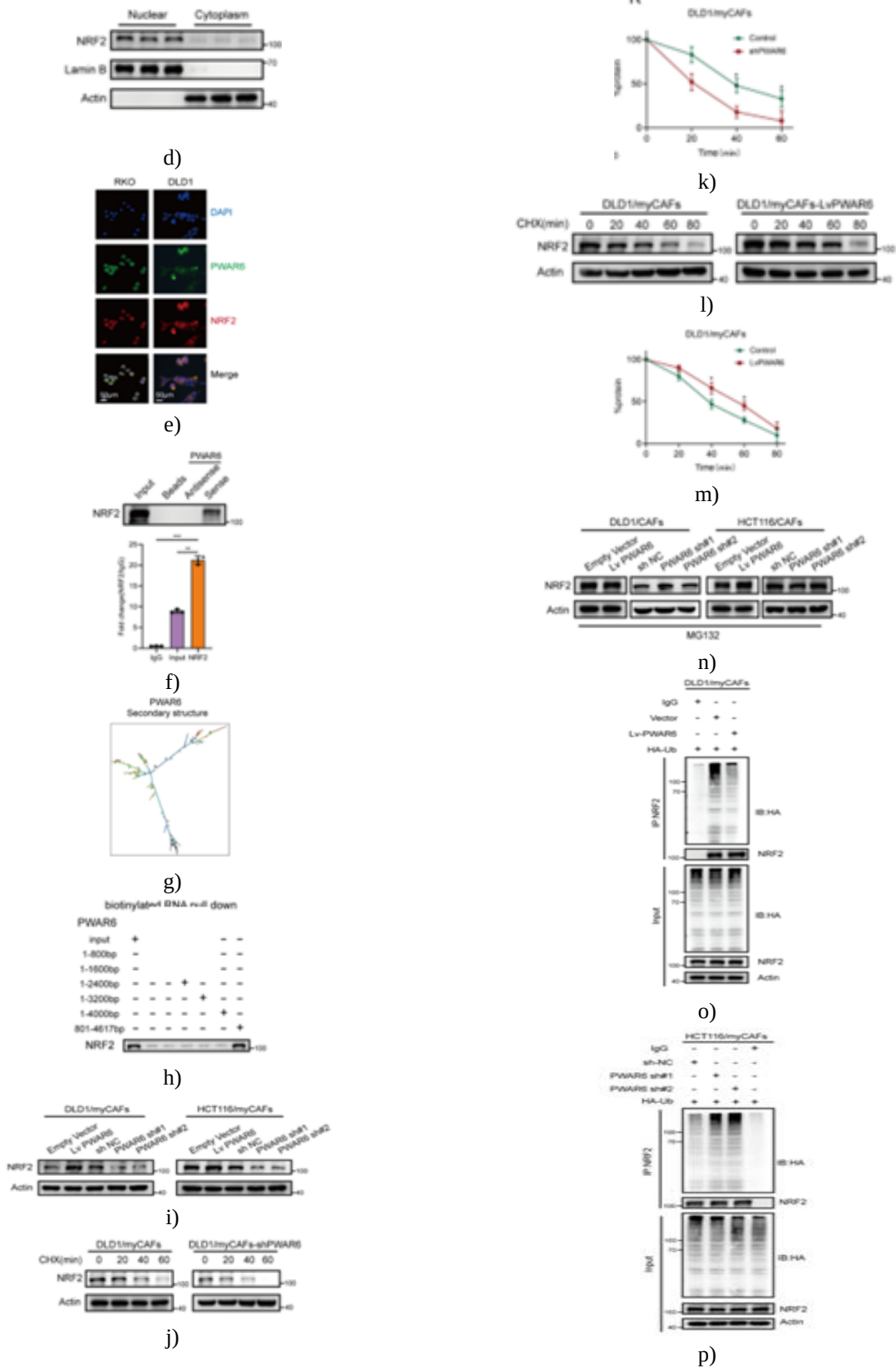
Taken together, these results demonstrate that PWAR6 delivered via myCAF-derived exosomes promotes glutamine uptake in CRC cells by upregulating the amino acid transporter SLC38A2. Both in vitro and in vivo experiments confirm that PWAR6-mediated upregulation of SLC38A2 drives CRC cell metastasis.

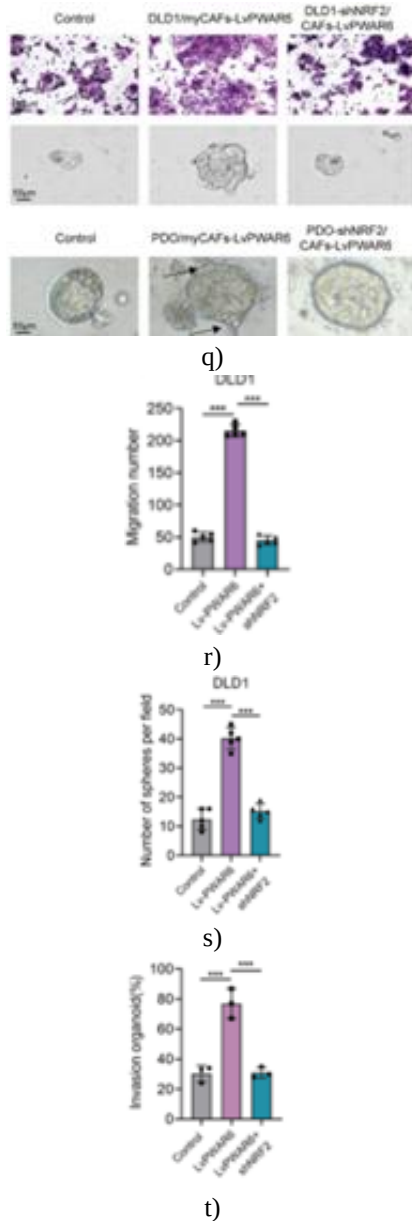
#### *PWAR6 blocks NRF2 ubiquitination and degradation*

To uncover the molecular pathway by which myCAF-derived PWAR6 influences glutamine metabolism and promotes CRC metastasis, DLD1 cells were initially co-cultured with myCAFs (**Figure 5a**). Biotin-labeled sense and antisense PWAR6 RNA probes were synthesized.

RNA pulldown experiments were conducted to identify associated proteins in CRC cells (**Figure 5b**). The enriched proteins were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by mass spectrometry. After filtering nonspecific interactions, NRF2 was identified as a key protein interacting with PWAR6 (**Figure 5c**). Nuclear-cytoplasmic fractionation experiments confirmed that NRF2 is primarily localized in the nucleus (**Figure 5d**). FISH imaging further demonstrated co-localization of PWAR6 and NRF2 inside cells (**Figure 5e**). The physical interaction was validated through RNA pulldown followed by western blotting with anti-NRF2 antibody, which showed specific enrichment in the sense PWAR6 lane (**Figure 5f**), (upper panel). Reciprocal RNA immunoprecipitation (RIP) using an anti-NRF2 antibody, followed by PCR, also detected PWAR6 (**Figure 5f**), (lower panel). The predicted secondary structure of PWAR6 was generated using Vienna RNA (<http://rna.tbi.univie.ac.at/>) (**Figure 5g**). To determine the binding domain, various truncated PWAR6 constructs based on this structure were tested by RNA pulldown and western blot. These experiments mapped the NRF2-interacting region to nucleotides 4000–4617 bp of PWAR6 (**Figure 5h**).







**Figure 5.** PWAR6 blocks NRF2 ubiquitination and degradation

(a) Setup of CRC cell and myCAF co-culture system before pulldown assays, (b, c) Silver-stained SDS-PAGE showing proteins pulled down by sense versus antisense PWAR6 probes. Bands with differential intensity were selected for mass spectrometry identification, (d) Subcellular fractionation indicating predominant nuclear localization of NRF2 ( $n = 3$ , representative of three biological replicates), (e) Representative immunofluorescence images demonstrating co-localization of NRF2 and PWAR6 in DLD1 and RKO cells. Scale bars = 50  $\mu\text{m}$  ( $n = 3$ , representative of three

biological replicates), (f) Confirmation of PWAR6-NRF2 interaction by western blot after pulldown (upper panel) and by RIP-PCR (lower panel) ( $n = 3$ , representative of three biological replicates), (g) Secondary structure prediction of PWAR6 using the centroid method (<http://rna.tbi.univie.ac.at/>), (h) Identification of specific PWAR6 truncation fragments that bind NRF2 in DLD1 cell lysates ( $n = 3$ , representative of three biological replicates), (i) Western blot analysis of NRF2 protein abundance in DLD1 and HCT116 cells after incubation with myCAF-derived exosomes ( $n = 3$ , representative of three biological replicates), (j–m) Cycloheximide (CHX, 50  $\mu\text{g/mL}$ ) chase assays to determine NRF2 protein stability upon PWAR6 overexpression (j, k) or knockdown (l, m) ( $n = 3$ , representative of three biological replicates), (n) Effect of proteasome inhibitor MG132 (10  $\mu\text{M}$ ) on NRF2 levels in DLD1 and HCT116 cells treated with myCAF exosomes from overexpression or knockdown conditions ( $n = 3$ , representative of three biological replicates). (o, p) Co-immunoprecipitation assays evaluating NRF2 ubiquitination levels in the presence or absence of PWAR6 overexpression (o) or knockdown (p) after MG132 and HA-Ub treatment ( $n = 3$ , representative of three biological replicates), and (q–t) Functional rescue experiments following NRF2 knockdown: Transwell invasion assay in DLD1 cells (upper panel and R, scale bars = 100  $\mu\text{m}$ ), sphere formation assay for stemness (middle panel and S), and 3D PDO invasion assay (lower panel and T, scale bars = 50  $\mu\text{m}$ ) (representative images shown; data from biological replicates). Data are shown as mean  $\pm$  s.e.m. Statistical analysis by one-way ANOVA for panels (f), (k), (m), and (q–t). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Given the demonstrated interaction between PWAR6 and NRF2 in CRC cells, we examined whether PWAR6 regulates NRF2 expression. PWAR6 did not affect NRF2 transcript levels. In contrast, PWAR6 knockdown led to a pronounced reduction in NRF2 protein abundance. At the same time, PWAR6 overexpression resulted in a substantial increase (Figure 5i). Protein stability was then evaluated by treating CRC cells with cycloheximide (CHX, 50  $\mu\text{g/mL}$ ) for different time intervals. PWAR6 depletion shortened the half-life of NRF2 and accelerated its turnover (Figures 5j and 5k), whereas PWAR6 overexpression extended NRF2 protein stability (Figures 5l and 5m). Application of the proteasome inhibitor MG132 led to NRF2 accumulation despite PWAR6 silencing, indicating that PWAR6

controls NRF2 abundance post-translationally in a proteasome-dependent manner (**Figure 5n**).

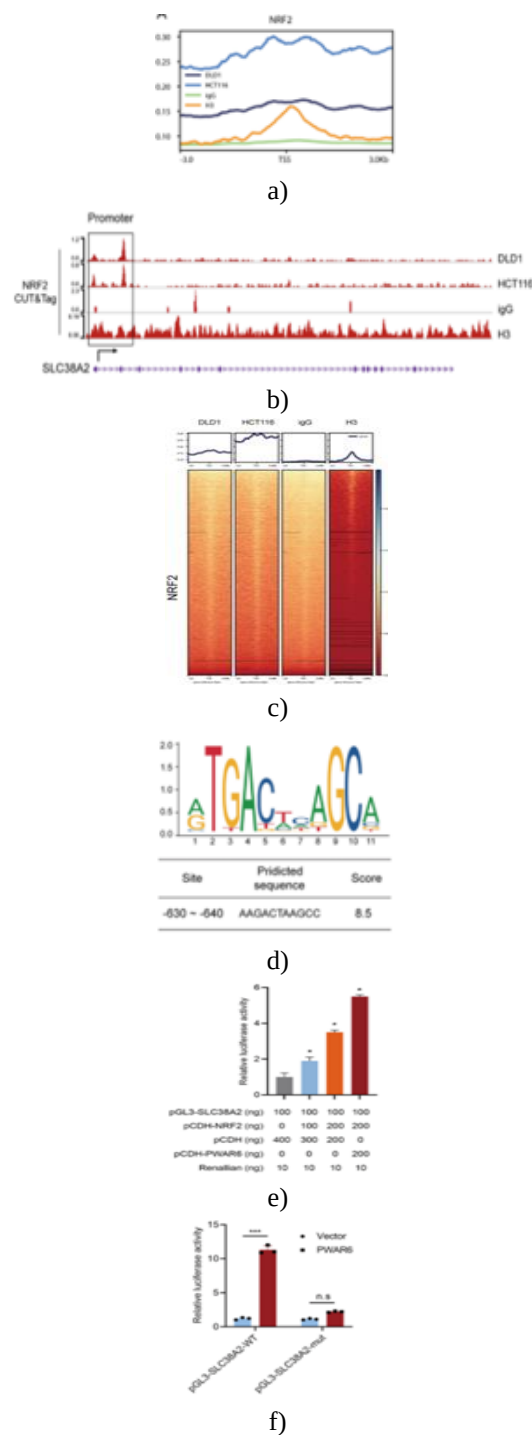
Ubiquitination assays by western blotting showed that PWAR6 overexpression markedly suppressed NRF2 polyubiquitination (**Figure 5o**). Conversely, PWAR6 knockdown enhanced NRF2 ubiquitination with no alteration in total input protein (**Figure 5p**). These findings indicate that PWAR6 prevents proteasomal degradation of NRF2 in CRC cells. Subsequent functional studies addressed cell migration, stemness properties, and glutamine uptake. NRF2 levels were modulated in combination with PWAR6 alteration to test mediation. Successful manipulation of NRF2 in the context of PWAR6 changes was confirmed by western blotting. Transwell invasion assays revealed that PWAR6 promoted CRC cell motility, an effect that was abrogated upon NRF2 silencing (**Figure 5q**) (upper panel), (**Figure 5r**). Sphere-forming capacity was augmented by PWAR6 overexpression and reversed by NRF2 knockdown (**Figure 5q**) (middle panel). Parallel changes occurred in stemness markers CD133 and LGR5. In 3D PDO invasion assays, organoids co-cultured with PWAR6-overexpressing CAFs developed prominent invasive fronts into the matrix. This phenotype was prevented by NRF2 depletion (**Figure 5q**), (lower panel), and (**Figure 5t**). Glutamine uptake was also reduced following PWAR6 knockdown but was rescued by NRF2 restoration. In summary, PWAR6 drives migration, stemness, and glutamine metabolism in CRC cells by stabilizing NRF2.

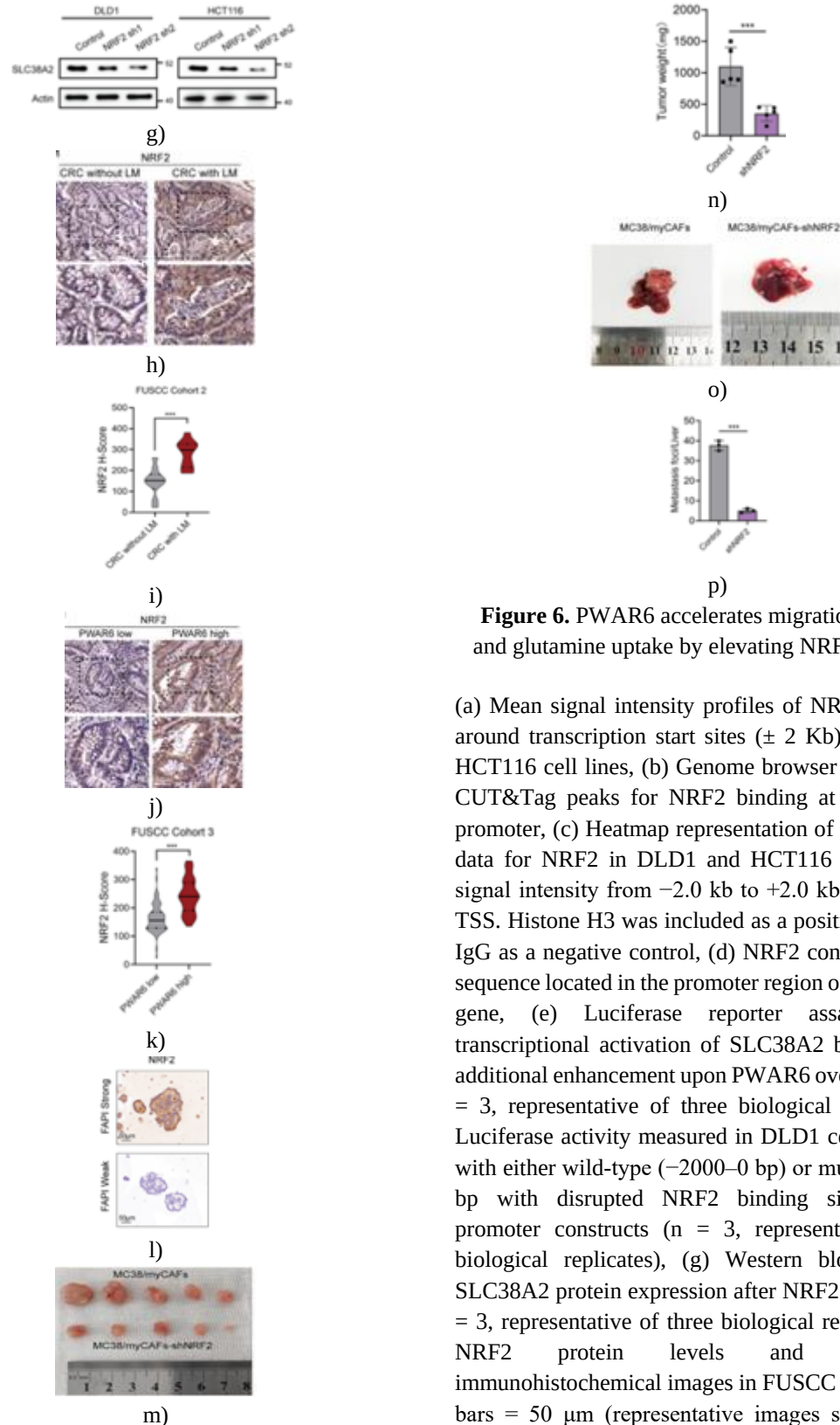
To delineate the mechanisms underlying PWAR6-driven CRC progression, we investigated its interaction with NRF2. Immunoprecipitation with an anti-NRF2 antibody, followed by mass spectrometry, identified Keap1 as a prominent interacting partner, consistent with its known function in the ubiquitin–proteasome degradation pathway. Keap1 typically promotes NRF2 ubiquitination and proteasomal turnover under homeostatic conditions through the Cul3 E3 ligase complex, thereby suppressing NRF2 activity.

Co-localization of NRF2 and Keap1 was confirmed by immunofluorescence. Co-immunoprecipitation in DLD1 cells expressing Flag-Keap1 and Myc-NRF2 further validated their direct binding.

Since PWAR6 inhibits NRF2 ubiquitination while Keap1 facilitates it, we examined potential crosstalk between them. PWAR6 silencing upregulated Keap1 expression, whereas PWAR6 overexpression downregulated Keap1 (**Figures 6g and 6h**). These

results point to an antagonistic relationship between PWAR6 and Keap1. To confirm Keap1's involvement, its expression was altered, and NRF2 ubiquitination was assessed. Keap1 overexpression increased NRF2 ubiquitination, while its knockdown decreased ubiquitination.





**Figure 6.** PWAR6 accelerates migration, stemness and glutamine uptake by elevating NRF2 expression

(a) Mean signal intensity profiles of NRF2 enrichment around transcription start sites ( $\pm 2$  Kb) in DLD1 and HCT116 cell lines, (b) Genome browser view (IGV) of CUT&Tag peaks for NRF2 binding at the SLC38A2 promoter, (c) Heatmap representation of CUT&Tag-seq data for NRF2 in DLD1 and HCT116 cells, showing signal intensity from  $-2.0$  kb to  $+2.0$  kb relative to the TSS. Histone H3 was included as a positive control and IgG as a negative control, (d) NRF2 consensus binding sequence located in the promoter region of the SLC38A2 gene, (e) Luciferase reporter assays verifying transcriptional activation of SLC38A2 by NRF2, with additional enhancement upon PWAR6 overexpression ( $n = 3$ , representative of three biological replicates), (f) Luciferase activity measured in DLD1 cells transfected with either wild-type ( $-2000-0$  bp) or mutant ( $-2000-0$  bp with disrupted NRF2 binding site) SLC38A2 promoter constructs ( $n = 3$ , representative of three biological replicates), (g) Western blot analysis of SLC38A2 protein expression after NRF2 knockdown ( $n = 3$ , representative of three biological replicates), (h, i) NRF2 protein levels and representative immunohistochemical images in FUSCC cohort 2. Scale bars =  $50 \mu\text{m}$  (representative images shown;  $n = 25$  patients), (j, k) NRF2 protein levels and representative immunohistochemical images in FUSCC cohort 3. Scale

bars = 50  $\mu$ m (representative images shown; n = 150 patients), (l) Immunohistochemical detection of NRF2 in PDOs from patients stratified by 68Ga-FAPI-PET SUVmax values. Scale bars = 50  $\mu$ m (representative images shown; n = 15 patients), (m, n) NRF2 knockdown significantly inhibits subcutaneous tumor growth (n = 5 mice per group), and (o, p) NRF2 knockdown substantially decreases liver metastatic load (n = 3 mice per group). Data are presented as mean  $\pm$  s.e.m. Statistical analysis: one-way ANOVA for panel (e); two-tailed Student's t-test for panels (f) and (h–p). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.

To gain deeper insight into their functional relationship, DLD1 cells expressing Lv-PWAR6 were transfected with Flag-tagged Keap1 or Keap1-targeted siRNA. These experiments showed that PWAR6 could partially counteract Keap1-mediated NRF2 suppression. Quantitative co-immunoprecipitation studies indicated that PWAR6 interferes with Keap1-mediated post-translational ubiquitination of NRF2. In the presence of the proteasome inhibitor MG132, PWAR6 overexpression weakened the physical association between Keap1 and NRF2. Furthermore, depletion of Keap1 increased glutamine uptake in CRC cells.

In summary, the data confirm that Keap1 is a major driver of NRF2 ubiquitination and proteasomal degradation, whereas PWAR6 exerts a protective effect by shielding NRF2 from Keap1-dependent ubiquitination and subsequent proteasomal degradation.

#### *NRF2 enhances glutamine uptake in colorectal cancer cells through transcriptional control of SLC38A2*

Owing to the co-localization of NRF2 and SLC38A2, we postulated that NRF2 acts as a transcription factor by interacting with the SLC38A2 promoter. To examine this possibility, CUT&Tag experiments were performed using an anti-NRF2 antibody in DLD1 and HCT116 cell lines.

The genomic sequences adjacent to the CUT&Tag-seq peaks showed strong evolutionary conservation and were often located within promoter regions. The Integrative Genomics Viewer (IGV) tracks NRF2 occupancy at the proximal promoter of SLC38A2 (**Figures 6a and 6b**). In addition, the heat map showed prominent enrichment of these peaks at transcription start sites (TSSs) (**Figure 6c**).

CUT&Tag data identified a principal binding site within the GLUD1 promoter (–1000 to 100 bp), located specifically between –630 bp and –640 bp (**Figure 6d**).

Complementary luciferase reporter assays confirmed that NRF2 increases SLC38A2 expression via regulation of its transcriptional activity (**Figure 6f**). Of note, forced expression of PWAR6 produced an additional increase in SLC38A2 transcriptional activity (**Figure 6e**).

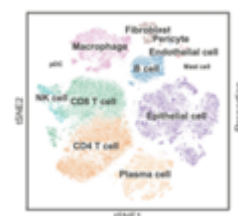
Western blotting further showed that NRF2 depletion led to a substantial reduction in SLC38A2 protein abundance (**Figure 6g**).

The clinical implications of NRF2 were then assessed. Analysis of FUSCC cohort 1 revealed a positive correlation between elevated 68Ga-FAPI-PET CT SUVmax values and higher NRF2 expression levels, with lower SUVmax readings associated with decreased NRF2 (**Figure 6l**). In the FUSCC cohort 2, patients with liver metastasis (LM) displayed markedly higher NRF2 expression than those without LM (**Figures 6h and 6i**). Within the FUSCC cohort 3, higher PWAR6 expression in CRC tissues was directly associated with increased NRF2, whereas diminished PWAR6 levels coincided with lower NRF2 (**Figures 6j and 6k**).

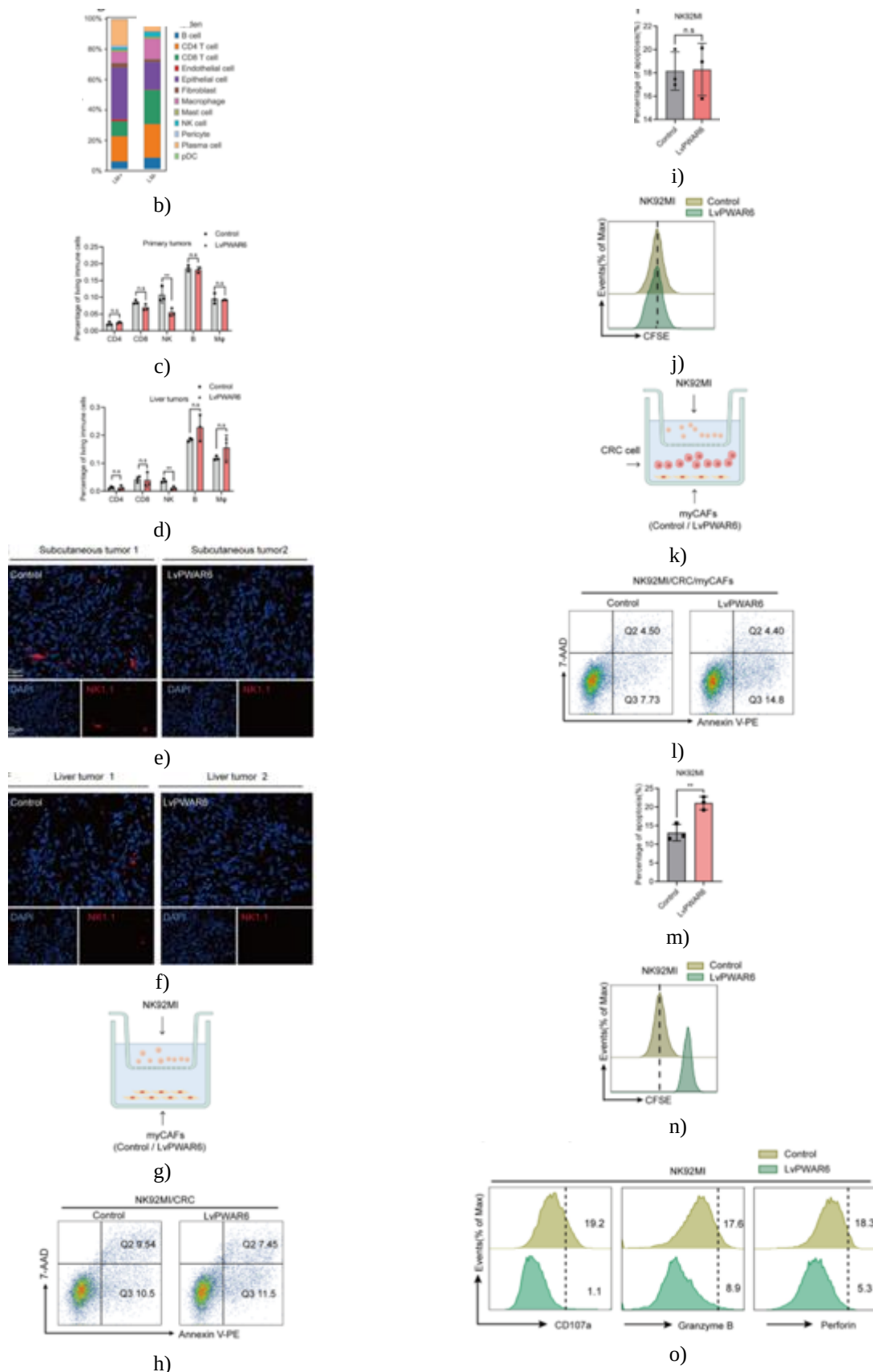
Thereafter, myCAFs were co-cultured with MC38 cells (control and shNRF2 variants), and both subcutaneous xenograft and liver metastasis models were generated in C57BL/6 mice following established protocols (**Figures 6m–6p**). These experiments demonstrated that NRF2 silencing in MC38 cells resulted in significant inhibition of subcutaneous tumor growth and liver metastasis.

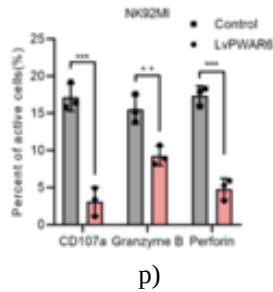
#### *PWAR6-driven glutamine shortage in NK cells contributes to tumor immune escape*

In addition to the myCAF-tumor cell interactions outlined earlier, we examined whether PWAR6 influences the tumor immune microenvironment (TIME). Through focused cluster analysis of immune cell populations in single-cell sequencing datasets (GSE16655 and GSE178318), we observed that colorectal liver metastasis (CRLM) was linked to substantially lower infiltration levels of NK cells, CD4+ T cells, CD8+ T cells, B cells, and macrophages when compared with CRC patients lacking liver metastasis (**Figures 7a and 7b**).



a)





**Figure 7.** SLC38A2-dependent glutamine depletion in NK cells enhances tumor cell migration and immune evasion

(a, b) Immune cell cluster analysis from single-cell data (GSE16655 and GSE178318;  $n = 18$  patients), (c, d) Proportions of immune cell subsets present in liver tumors of LvPWAR6 mice ( $n = 3$  technical replicates shown, representative of three independent biological replicates), (e) Typical immunohistochemical staining for NK1.1 in subcutaneous tumor sections (scale bars = 20  $\mu\text{m}$ ;  $n = 3$ , representative of three biological replicates), (f) Typical immunohistochemical staining for NK1.1 in liver metastatic tumors (scale bars = 20  $\mu\text{m}$ ;  $n = 3$ , representative of three biological replicates), (g) Diagram illustrating the co-culture setup of NK92MI cells with myCAFs (NK92MI cells seeded in the upper transwell chamber and myCAFs in the lower chamber), (h, i) Flow cytometry plots and corresponding quantification of apoptotic NK92MI cells following co-culture with myCAFs (representative data shown; results from three biological replicates), (j) Flow cytometry evaluation of proliferative activity in NK92MI cells within the myCAF co-culture system ( $n = 3$ , representative of three biological replicates), (k) Schematic of the triple co-culture arrangement including NK92MI cells, CRC cells, and myCAFs (NK92MI in the upper chamber; CRC cells plus myCAFs in the lower chamber), (l, m) Flow cytometry illustrations and statistical evaluation of NK92MI apoptosis rates in the triple co-culture system (representative images provided; data from three biological replicates), (n) Flow cytometric assessment of NK92MI cell proliferation in the triple co-culture environment ( $n = 3$ , representative of three biological replicates), and (o, p) Flow cytometry results and quantitative bar graphs depicting surface CD107a as well as intracellular Granzyme B and Perforin levels in NK92MI cells (representative plots shown; data from three biological replicates). All data are expressed as mean  $\pm$  s.e.m. For panels (c, d), (h–j),

and (l–p), statistical significance was determined by two-tailed Student's  $t$ -test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Following the establishment of subcutaneous and liver metastasis models in C57BL/6 mice using a PWAR6 overexpression plasmid according to prior protocols, flow cytometry was used to determine the effects of PWAR6 on the tumor immune microenvironment (TIME). The analysis showed a marked decline in NK cell populations, while changes in CD4 $^{+}$  T cells, CD8 $^{+}$  T cells, and macrophage levels remained comparatively minor (**Figures 7c and 7d**). Immunofluorescence staining corroborated that PWAR6 overexpression strongly diminished NK cell presence within both subcutaneous tumors and liver metastases (**Figures 7e and 7f**). Based on these observations, further research concentrated on delineating the specific contributions of NK cells.

To validate the *in vivo* findings, we conducted a series of *in vitro* co-culture assays. When NK92MI cells were cultured together with myCAFs engineered to overexpress PWAR6, no considerable alteration in NK92MI cell behavior was detected (**Figures 7g–7j**). Parallel experiments using human NK92MI cells and myCAFs yielded equivalent results. By contrast, incorporation of CRC cells into the triple co-culture system (NK92MI cells, CRC cells, and myCAFs) led to pronounced suppression of NK cell function and elevated apoptosis rates upon PWAR6 overexpression in myCAFs (**Figures 7k–7n**). Comparable effects were noted with primary human NK cells. These patterns imply that exosomal PWAR6 secreted by myCAFs inhibits NK cell function indirectly via crosstalk with CRC cells.

It is well established that glutamine is a critical nutrient supporting NK cell effector functions. Consistent with our earlier data, we proposed that heightened PWAR6 levels in CRC cells promote avid glutamine consumption, thereby limiting glutamine availability for neighboring NK cells in the tumor microenvironment and attenuating their activity. Supplementation of the co-culture medium with additional glutamine successfully counteracted the functional decline in NK cells, even under conditions of PWAR6 overexpression in myCAFs. This rescue experiment underscores that PWAR6 primarily disrupts NK cell competence by amplifying glutamine competition between malignant cells and NK cells.

Because PWAR6 regulates glutamine transport in CRC cells chiefly through SLC38A2, we hypothesized that SLC38A2 upregulation allows CRC cells to acquire glutamine at the expense of NK cells preferentially. To address this, SLC38A2-deficient CRC cells were generated and co-cultured with myCAFs and NK cells. Notably, SLC38A2 knockdown preserved NK92MI cell functionality despite PWAR6 overexpression in myCAFs. Importantly, Western blot analysis indicated that SLC38A2 expression in NK92 cells was unaffected by PWAR6 overexpression. These experiments demonstrate that PWAR6 exerts its influence on NK cells indirectly, via SLC38A2-mediated competitive glutamine uptake by tumor cells.

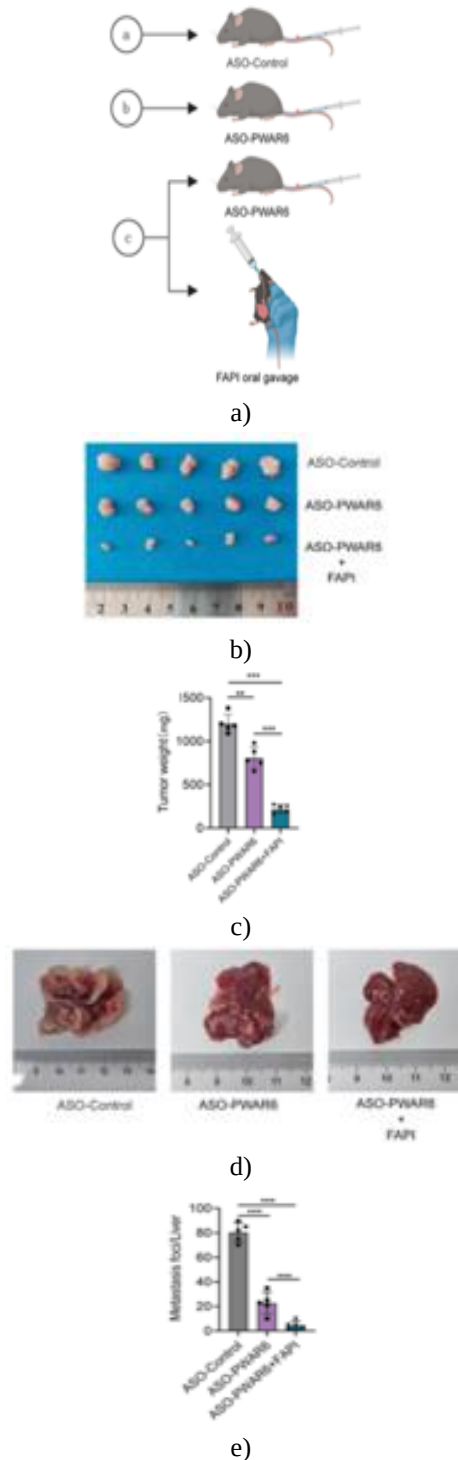
Additional assays explored potential changes in the antitumor cytotoxicity of NK92MI cells exposed to PWAR6-overexpressing myCAFs. PWAR6 overexpression produced negligible changes in NK cell receptor expression or CRC cell ligand levels. Nevertheless, isolation of NK92MI or human NK cells from the co-culture environment revealed substantial reductions in CD107a, perforin, and Granzyme B expression (**Figures 7o and 7p**). ELISA measurements confirmed decreased secretion of Granzyme B and perforin by NK cells under PWAR6-overexpressing conditions. Analogous reductions were observed in NK cells recovered from mouse subcutaneous tumors. Cytotoxicity assays further showed that the ability of NK92MI cells to lyse CRC targets at varying effector-to-target ratios was significantly compromised in the presence of PWAR6-overexpressing myCAFs, with partial restoration achieved through glutamine supplementation.

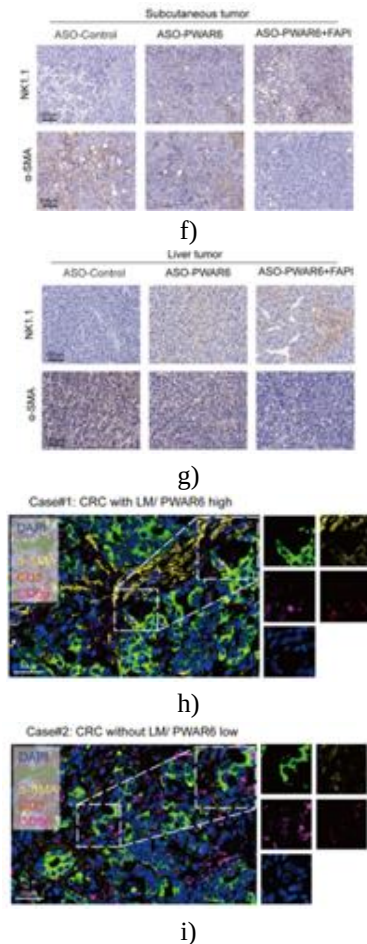
In summary, our data indicate that PWAR6 reprograms CRC cells to outcompete NK cells for glutamine within the tumor microenvironment. The resulting glutamine depletion in NK cells leads to profound functional impairment and diminished cytotoxic capacity, thereby enabling tumor progression and immune escape.

#### *PWAR6 represents a promising therapeutic target in colorectal cancer*

Given PWAR6's pivotal role in colorectal cancer (CRC) development, we evaluated its suitability as a therapeutic target. An antisense oligonucleotide (ASO) inhibitor targeting PWAR6 was developed and optimized for systemic in vivo delivery to effectively silence the target RNA. Subcutaneous tumor and liver metastasis models were first established in C57BL/6 mice using MC38 cells

following co-culture with cancer-associated fibroblasts, as detailed in previous methods. Tumor-bearing animals were then randomly assigned to three treatment arms: a control group, an ASO-PWAR6 monotherapy group, and a combination group receiving ASO-PWAR6 together with FAPI administered by oral gavage (**Figure 8a**).





**Figure 8.** PWAR6 constitutes a viable therapeutic target for colorectal cancer

(a) Schematic representation of the experimental design involving tail vein administration of an *in vivo*-optimized PWAR6 inhibitor and oral FAPI gavage in C57BL/6 mice, (b–e) Representative photographs of subcutaneous xenograft tumors, (b, c) and liver metastatic nodules, (d, e) harvested from mice in each treatment cohort. Animals bearing xenografts received either control antisense oligonucleotide (ASO-Ctrl), the optimized PWAR6 inhibitor (ASO-PWAR6), or the combination of ASO-PWAR6 plus FAPI gavage ( $n = 5$  mice per group), (f, g) Immunohistochemical staining for NK1.1 and  $\alpha$ -SMA in subcutaneous tumors and liver metastases across the different treatment groups. Scale bars = 100  $\mu$ m ( $n = 3$ , representative of three biological replicates), and (h, i) Immunohistochemical detection of pan-CK,  $\alpha$ -SMA, CD3, and CD56 in clinical CRC specimens from FUSCC Cohort 2. Scale bars = 50  $\mu$ m ( $n = 3$ , representative of three biological replicates). Data are expressed as mean  $\pm$  s.e.m. Statistical comparisons

for panels (b–e) were conducted using one-way ANOVA. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Tail vein delivery of the ASO-PWAR6 inhibitor led to a substantial reduction in both subcutaneous tumor volume and liver metastatic burden. Notably, combining ASO-PWAR6 administration with oral FAPI gavage resulted in synergistic suppression of tumor growth at both anatomical sites (**Figures 8b–8e**).

Immunohistochemical evaluation demonstrated a stepwise increase in NK1.1-positive cell infiltration from the control group to the ASO-PWAR6 group, and then to the combination therapy group, in both subcutaneous and metastatic liver lesions. In parallel,  $\alpha$ -SMA expression progressively decreased across the successive treatment conditions (**Figures 8f and 8g**).

We additionally examined CAF and NK cell markers in specimens from FUSCC Cohort 2. Tumor tissues from CRC patients displaying high liver metastasis and PWAR6 expression showed intense  $\alpha$ -SMA staining accompanied by relatively sparse CD3<sup>−</sup>/CD56<sup>+</sup> NK cell infiltration. Conversely, samples from patients with low liver metastasis and PWAR6 levels showed attenuated  $\alpha$ -SMA expression, along with increased CD3<sup>−</sup>/CD56<sup>+</sup> NK cells (**Figures 8h and 8i**).

Distant metastasis remains a major determinant of poor clinical outcomes in patients with colorectal cancer (CRC). Elucidating the mechanisms underlying CRC metastasis is essential for discovering new therapeutic targets and treatment approaches. Tumor cell stemness is a critical driver of CRC initiation and progression [33]. Our earlier investigations showed that diminishing stemness properties in patient-derived organoid (PDO) models effectively reduces the metastatic capacity of CRC. While intrinsic features of tumor cells are important, complex bidirectional interactions between cancer cells and the surrounding tumor microenvironment (TME) also exert substantial influence. Nevertheless, most prior studies have emphasized cell-autonomous alterations within tumor cells, frequently overlooking the multifaceted crosstalk between malignant cells and the TME.

myCAFs are among the predominant stromal populations in the TME and exert considerable control over tumor cell behavior and other microenvironmental elements. In this study, we provide the first evidence that co-culture of colorectal cancer cells with myCAFs augments their metastatic propensity, sphere-forming capacity, and expression of stemness-related markers.

Exosomes function as key vehicles for intercellular communication within the TME, enabling the exchange of cytokines, miRNAs, and lncRNAs among diverse cell populations. Emerging evidence indicates that lncRNAs released by CAFs contribute significantly to tumor dissemination and the acquisition of stem-like properties across multiple malignancies [34, 35]. In the present work, myCAFs were successfully isolated from colorectal liver metastases (CRLM). These myCAFs were shown to deliver lncRNA cargo via exosomes, thereby enhancing the migratory and metastatic abilities of CRC cells. Among the transferred transcripts, PWAR6 exhibited prominent upregulation in exosomes derived from myCAFs. Clinical analyses incorporating 68Ga-FAPI-PET/CT imaging and tissue microarray datasets further established associations between PWAR6 expression, myCAF abundance, and the occurrence of distant metastasis in CRC patients. Although the current investigation primarily addresses the extracellular role of exosomal PWAR6 secreted by myCAFs, it also prompts consideration of PWAR6's intracellular functions within myCAFs, including possible effects on exosome biogenesis and release. Fluorescence in situ hybridization (FISH) performed on CAFs indicated that PWAR6 is mainly localized in the cytoplasm, a distribution that may favor its loading into exosomes destined for secretion into the TME. The precise molecular processes governing this localization and packaging, however, require further exploration to fully delineate their contributions to cancer progression. Our experimental results provide robust support for PWAR6's role in promoting CRC metastasis, as evidenced by comprehensive *in vitro* and *in vivo* assessments. Mechanistically, PWAR6 appears to engage with NRF2 within CRC cells. NRF2, a master transcription factor, when aberrantly stabilized and activated, drives tumor proliferation and metabolic adaptation [36, 37]. Under normal conditions, NRF2 protein abundance is tightly controlled by the Keap1 complex, which promotes its ubiquitination and proteasomal degradation [38]. We therefore examined potential links between PWAR6 and the NRF2/Keap1 axis. Our data indicate that PWAR6 interferes with NRF2-Keap1 binding, thereby preventing NRF2 ubiquitination and subsequent degradation. Pulldown assays coupled with mass spectrometry excluded a direct physical interaction between PWAR6 and Keap1. We therefore propose that PWAR6 indirectly attenuates Keap1-mediated suppression by facilitating NRF2

release and nuclear translocation. This model aligns with reports showing that sustained NRF2 activation can induce hypermethylation of the KEAP1 promoter, reinforcing the observed regulatory loop [38].

Cancer cells frequently exhibit dysregulated uptake and utilization of key nutrients to sustain their accelerated growth. Such metabolic rewiring is governed by multiple pathways, including the Keap1/NRF2 cascade [39]. Within the TME, various cell types compete for limited resources, such as glucose and glutamine, underscoring the microenvironment's competitive dynamics. Nevertheless, detailed knowledge regarding nutrient exchange and competition among resident cells in the TME remains limited.

To address this gap, we conducted metabolomic profiling of paired myCAFs and normal fibroblasts (NFs). The analysis revealed heightened glutamate metabolism in CRLM specimens, a change that strongly correlated with PWAR6 and NRF2 expression. Further experiments demonstrated that altering PWAR6 levels significantly modulated SLC38A2 transcription. SLC38A2 functions as a major glutamine transporter, and its upregulation has been documented in breast and pancreatic cancers [40, 41], where it increases cellular dependence on glutamine and alanine. Enhanced glutamine metabolism has also been linked to the maintenance of cancer stemness [42, 43]. In our model, PWAR6 stabilizes NRF2 by shielding it from ubiquitin-dependent degradation. Stabilized NRF2 subsequently binds to the SLC38A2 promoter, driving its transcriptional activation. This cascade augments the ability of tumor cells to acquire glutamine from the TME, thereby bolstering both their metabolic fitness and stem-like characteristics.

Glutamine, an indispensable nutrient in the tumor microenvironment (TME), is indispensable not only for sustaining the rapid expansion of cancer cells but also for enabling the proper functioning of immune effector cells, including CD8<sup>+</sup> T cells, macrophages, and NK cells [44-46]. In the present study, we established that myCAF-derived PWAR6 augments glutamine scavenging by tumor cells from the TME, thereby limiting glutamine availability to surrounding immune populations. Both *in vivo* and *in vitro* investigations pinpointed NK cells as the immune subset most vulnerable to PWAR6-mediated glutamine scarcity. Although prior reports have documented that imbalances in amino acids, such as serine, can compromise NK cell killing capacity [47], the specific consequences of glutamine restriction on NK

cell performance remain incompletely understood. Using co-culture models, flow cytometry, and complementary analytical methods, we demonstrated that glutamine insufficiency in the TME attenuates NK cell cytotoxicity, thereby accelerating CRC metastasis. These observations reinforce the critical dependence of NK cell effector functions on glutamine supply and illuminate the intense competitive dynamics for this nutrient among different cellular constituents of the TME.

We further propose that PWAR6 is expressed at substantially higher levels in myCAFs relative to normal fibroblasts, a distinction that may underlie the metastasis-promoting attributes of the altered microenvironment. Such differential expression strengthens the rationale for therapeutic deployment of ASO-PWAR6, which could preferentially suppress myCAFs while sparing normal fibroblasts. This targeted approach holds promise for improving treatment effectiveness and reducing unintended toxicity in healthy tissues. These possibilities will be addressed in future investigations.

Among various RNA-targeting modalities for both solid and hematological malignancies, antisense oligonucleotides (ASOs) have emerged as one of the most clinically advanced platforms [48-50]. In this work, we developed specific ASO sequences directed against PWAR6. Initial therapeutic evaluation in murine models revealed that these ASOs effectively suppressed CRC metastatic dissemination and tumor growth. Nevertheless, additional studies are necessary to optimize delivery systems for greater precision and to confirm that the selected ASO sequences do not inadvertently influence the expression of unrelated endogenous RNAs.

## Conclusion

Collectively, our findings identify PWAR6 as a myCAF-derived lncRNA that promotes stemness, migratory capacity, and glutamine acquisition in CRC cells. At the molecular level, PWAR6 associates with NRF2, thereby disrupting its binding to Keap1 and blocking ubiquitin-dependent degradation. The resulting stabilization of NRF2 enables it to act as a transcriptional activator of SLC38A2, which in turn increases glutamine uptake by CRC cells from the TME. This process creates a glutamine-depleted microenvironment that induces NK cell apoptosis and impairs antitumor immunity.

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**Conflict of Interest:** None

**Financial Support:** None

**Ethics Statement:** None

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