Supplementary Information for:

Cancer-associated fibroblasts exosomes promote prostate cancer metastasis through miR-500a-3p/FBXW7/HSF1 axis under hypoxic microenvironment

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Supplementary Materials and Methods

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Supplementary Materials and Methods

Cell line and cell culture

The human prostate cancer cell line PC-3 and C4-2, prostate stromal cells WPMY-1, and 293T cells, were obtained from Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. PC-3, C4-2, and WPMY-1 were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco, Melbourne, Australia) and 1% penicillin -streptomycin (Gibco). CAFs and NFs cells were cultured in Dulbecco's Modified Eagle Medium F12-K (Gibco) supplemented with 10%FBS and 1%PS. 293T cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10%FBS and 1%PS. All cell lines had recently been tested for mycoplasma contamination and were maintained within a humidified environment containing 5% CO2 at a temperature of 37°C. For the induction of a hypoxic environment, the cells were cultured within a Forma Series II Water Jacket incubator featuring an atmosphere comprising 1% O2, 5% CO2, and 94% N2 (Thermo Scientific).

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was executed in accordance with previously established protocols[12]. Tissues were meticulously embedded in paraffin, and IHC was conducted employing the following antibodies: anti-HIF-1α antibody (1:400, Proteintech, Cat No. 20960-1-AP), anti-FBXW7 antibody (1:400,

Proteintech, Cat No. 28424-1-AP), anti-HSF1 antibody (1:600, Proteintech, Cat No. 16107-1-AP), and anti-P504S antibody (1:200, Proteintech, Cat No. 15918-1-AP). A negative control was included in the immunostaining procedure. Each sample was subjected to a random selection of three representative areas under a magnification of 20x to determine the Integral Optical Density (IOD) using image pro plus software.

Western blotting (WB)

Proteins were isolated from cells or exosomes with RIPA buffer containing 1mM PMSF. Subsequently, proteins underwent electrophoresis on a 10% SDS-PAGE gel and were subsequently transferred onto a PVDF membrane. Next, the membrane was blocked with 5% BSA for 1 hours at room temperature and was incubated overnight at 4°C with primary antibodies against the following antigens: HIF-1α (1:2000, proteintech), α-SMA (1:1000,ab7817), GAPDH (1:2000, ab181602), CD63 (1:1000, ab134045), Alix (1:1000, ab275377,), TSG101 (1:1000, ab125011), FBXW7 (1:2000, proteintech), HSF1(1:1000, ab52757). The membrane was then incubated with HRP (horseradish peroxidase)-linked secondary antibodies against rabbit at a dilution of 1:6000. Next, enhanced chemiluminescence (ECL) western blotting substrate was used for visualization and detection.

Immunofluorescence (IF) assay

CAF and NF cells were incubated in 24-well cell culture plate at a concentration of 6×104/well. After blocked and permeabilization, cells were incubated with primary antibodies against α-SMA (ab7817) overnight at 4°C. Then, cells were incubated with secondary antibody conjugated with fluorescent Alexa Fluor ® 488(ab150077). Next, cell nuclei were stained with DAPI for 15 minutes and cells were captured using a fluorescence microscope.

Cell proliferation assay

PC-3 and C4-2 cells were plated onto 96-well culture plate at a concentration of 3000/well. Following various treatments, 10μl of CCK8 reagent was added to each well, and the cells were then incubated at 37°C for 2 hours. Subsequently, the absorbance of the cells was measured using a microplate reader at a wavelength of 450 nm.

Apoptosis analysis

Cells were harvested and washed three times, and then treated with annexin V-FITC and PI Detection Kit (BD Biosciences, NJ, USA) according to the manufacturer's instructions. Apoptotic cells were fractioned by flow cytometry (BD FACSCanto™ II, NJ, USA).

Cell migration and invasion assays

PCa cells were cultured in six-well plates at a concentration of 1.5×105 cell per well. Each well received one of the following treatments: CAFs CM, exosomes(40μg/ml), or transfected with FBXW7 plasmid. Subsequently, migratory and invasive capacities of cells were evaluated using scratch and transwell assays, respectively. In the scratch assay, cells were captured by a light microscope at the time of 0h, 24h and 48h. For the transwell assay, 4×104 cells in serum-free RPMI 1640 were seeded into the upper chamber with 50μl Matrigel (Corning, New York, USA). The lower chamber contained RPMI 1640 supplemented with 30% FBS. After 24 or 48 hours, the cells migrated through the membrane were fixed with 4% paraformaldehyde for 15 minutes and then stained with crystal violet for another 15 minutes. Photographs were acquired using a microscope, and cell counts were quantified using ImageJ software.

miRNA-sequencing (miRNA-seq)

Total RNA was extracted and purified from CAFs exosomes using miRNeasy Serum/Plasma Advanced Kit (Qiagen, cat. No. 217204) according to the manufacturer’s protocol. A total amount of 1ng-500ng RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using QIAseq miRNA Library Kit (Qiagen, Frederick, MD) following manufacturer’s recommendations and index codes were added to attribute sequences to each sample.

GO and KEGG pathway enrichment analysis

Gene Ontology (GO) enrichment analysis of the target genes of differentially expressed miRNAs was implemented by the topGO R packages. KEGG[12] is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-levelinformation, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). We used KOBAS[13] software to test the statistical enrichment of differential expression genes in KEGG pathways.

Real-time quantitative PCR

Cellular RNA was extracted using the Trizol reagent. Serum RNA from patients was extracted and purified using miRNeasy Serum/Plasma Advanced Kit (XP BioMed, China). mRNA expression levels were normalized to GAPDH expression, while miRNA levels were normalized to U6 expression. Relative RNA expression was calculated employing the delta/delta CT method. Specific primers sequences can be found in Supplementary Table S1.

Dual-luciferase reporter assay

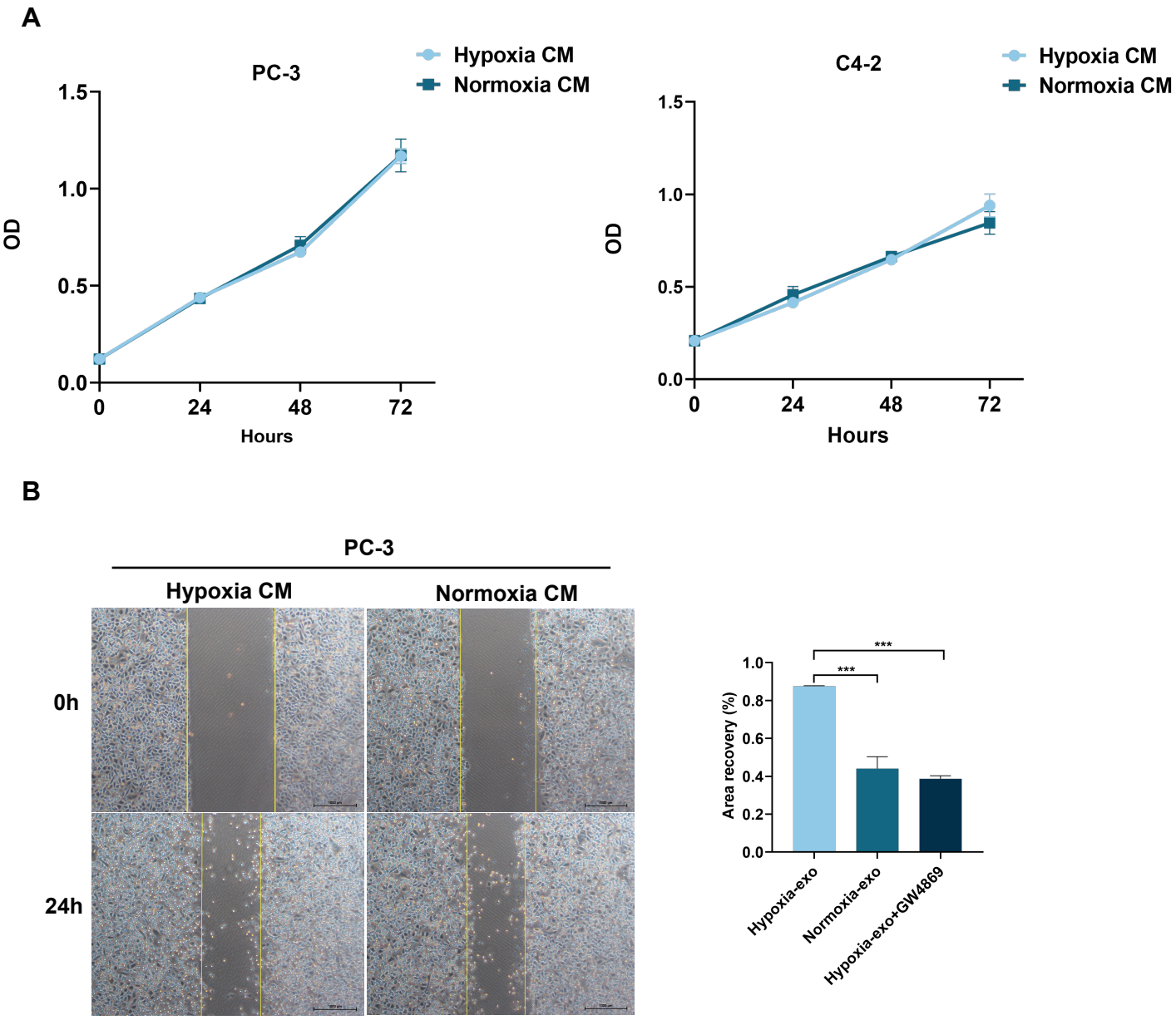
Wild-type (WT) or mutant (MUT) 3'UTR of FBXW7 containing miR-500a-3p binding sites were cloned into GP-miRGLO vector. 293T cells were seeded in

6-well plates (3.5×10^5 per well). The cells were harvested 24h after transfection, and Firefly/Renilla luciferase activity was determined using a dual-luciferase reporter assay kit according to the manufacturer's instructions (Promega, Wisconsin, USA).

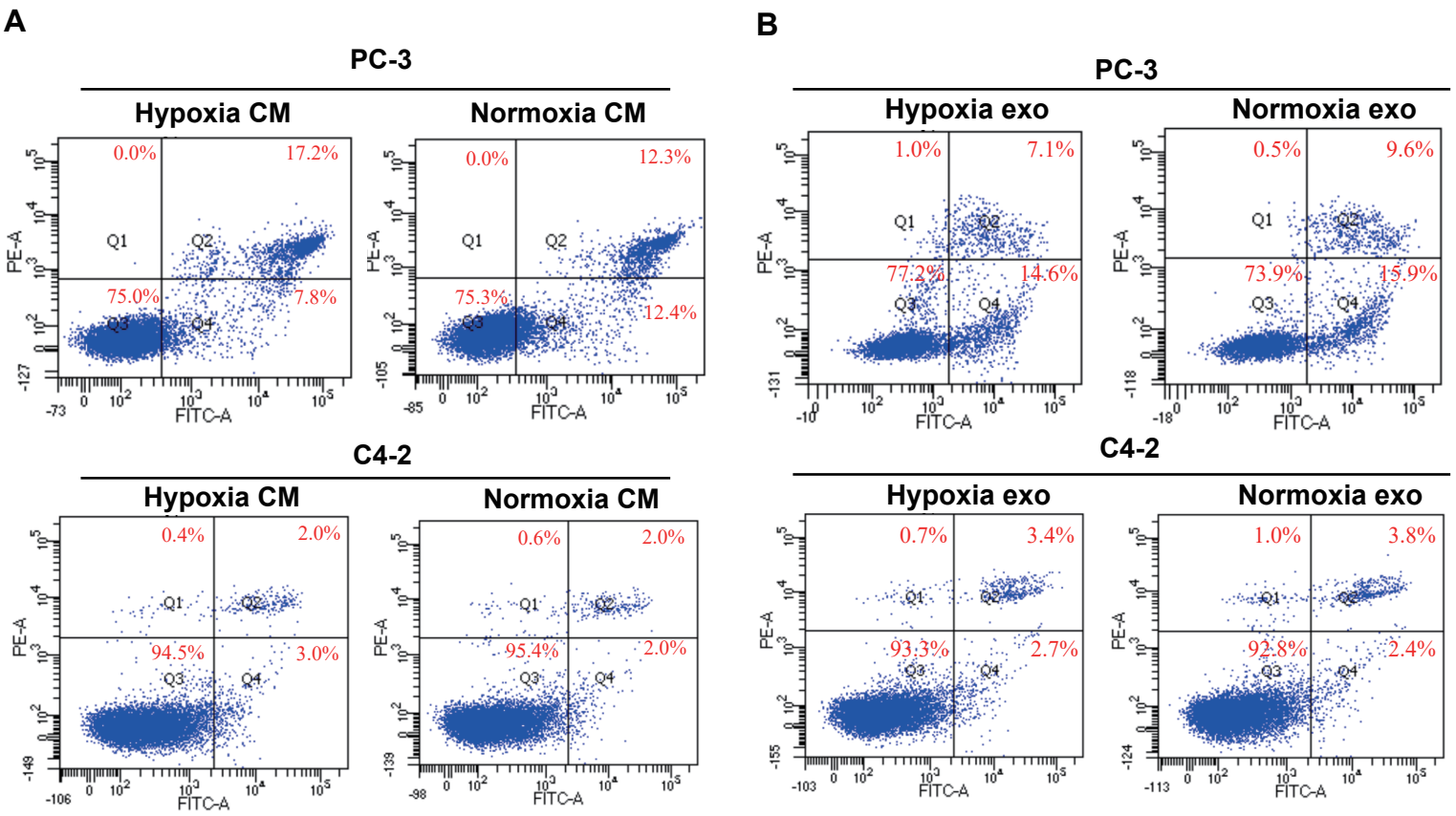
miRNA and plasmid transfection

miR-500a-3p mimics and inhibitors were generated by GenePharma (Suzhou, China). The sequence of mimics and inhibitor are shown in Supplementary Table S2. The infection method was performed in accordance with the manufacturer's instruction. When the cell confluence reached at 60%, the cells were transfected by using GP-transfectmate (GenePharma). Cells were harvest after transfection 24h for PCR and 48h for western blotting.

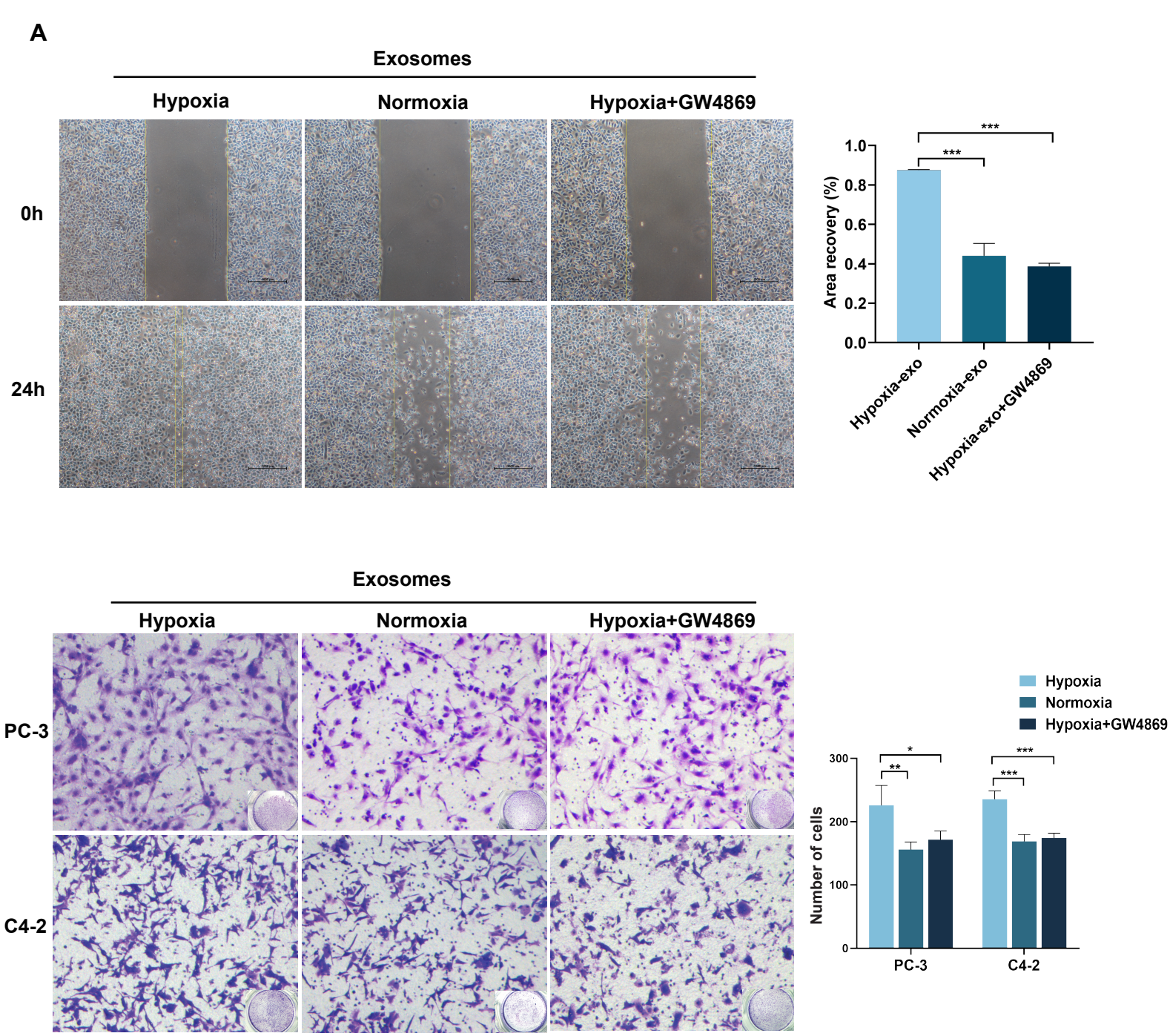
Plasmid vectors and appropriate cDNA sequence fragments were purchased from GenePharma. All plasmid sequences were confirmed by DNA sequencing. Similarly, plasmid vectors were transfected to PC-3 and C4-2 cells to overexpress FBXW7 by using GP-transfectmate.



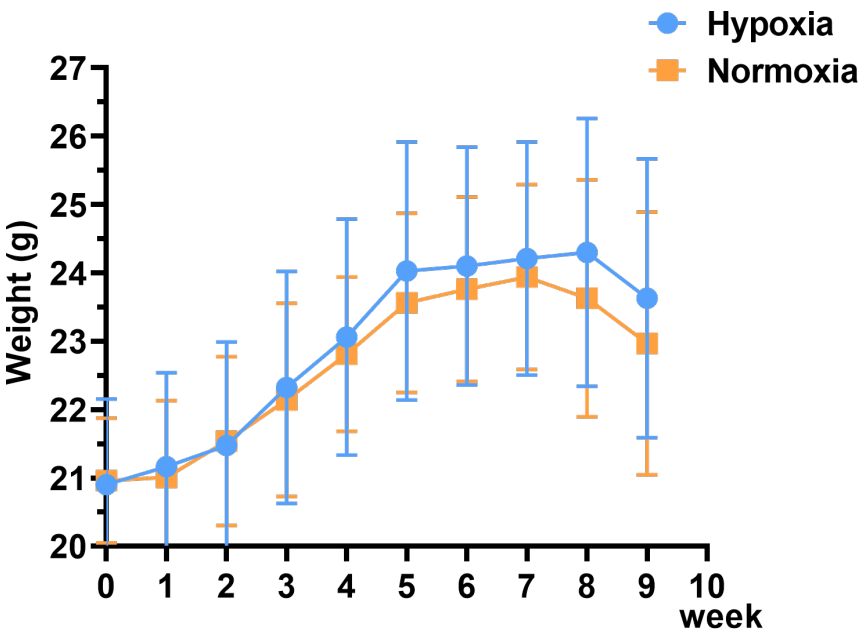
Supplementary Figure 1. Minimal impact of WPMY-1 CM on PCa cells. (A) CCK8 assay revealed that WPMY-1 CM exhibited negligible influence on the proliferation capacity of both PC-3 and C4-2 cells. (B) Cell scratch assays revealed that WPMY-1 CM exhibited negligible influence on the migration ability of PC-3.



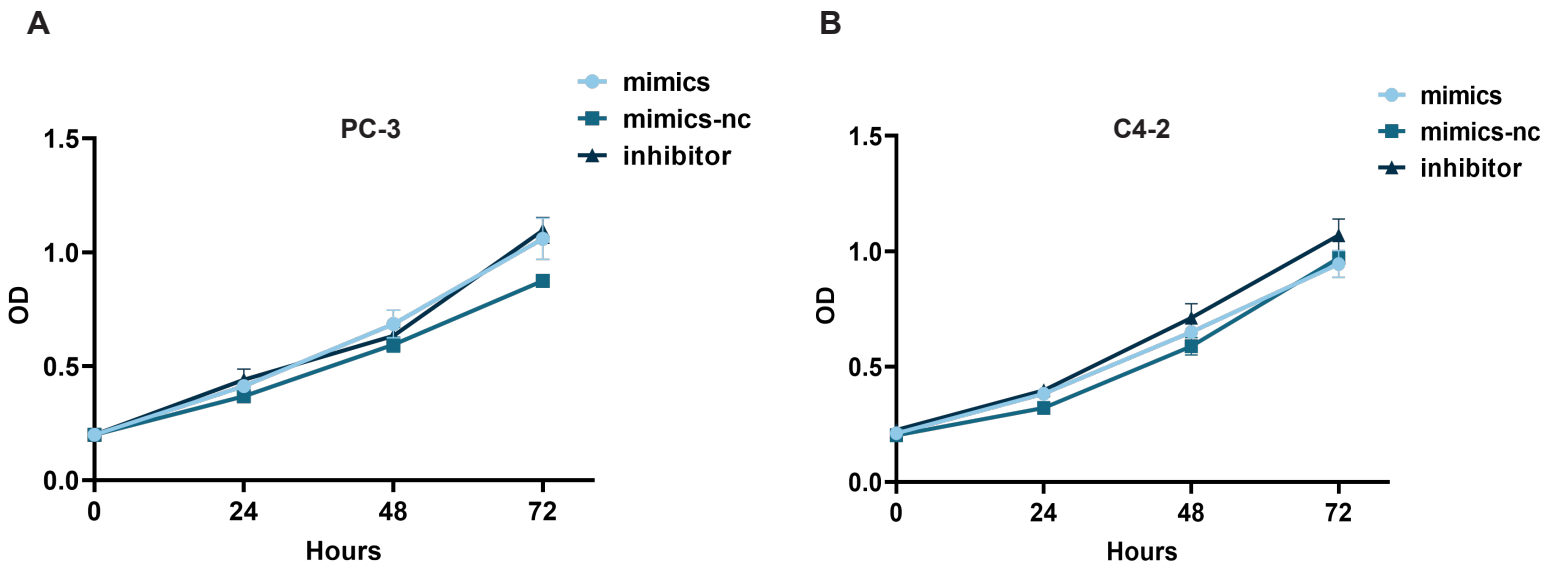
Supplementary Figure 2. Impact of CAFs CM and exosomes on apoptosis in PCa cells. (A) The apoptotic potential of PC-3 and C4-2 cells remained largely unaffected upon exposure to CM originating from CAFs. (B) Exosomes derived from CAFs exhibited a modest apoptotic suppressive effect on PC-3 cells, while exerting a minimal influence on the apoptosis of C4-2 cells.



Supplementary Figure3. Reversal of CAF hypoxic exosomes-induced enhancement in migration and invasion by GW4869. (A) Cell scratch assays revealed that exosomes derived from CAFs under hypoxic environment significantly promoted the migration ability of PC-3, while the administration of GW4869 (20μg/ml) effectively counteracted the promotional effect. (B) Cell transwell assay revealed that exosomes derived from CAFs under hypoxic environment significantly promoted the invasion ability of PC-3 and C4-2 cells, while the administration of GW4869 (20μg/ml) effectively counteracted this promotional effect.



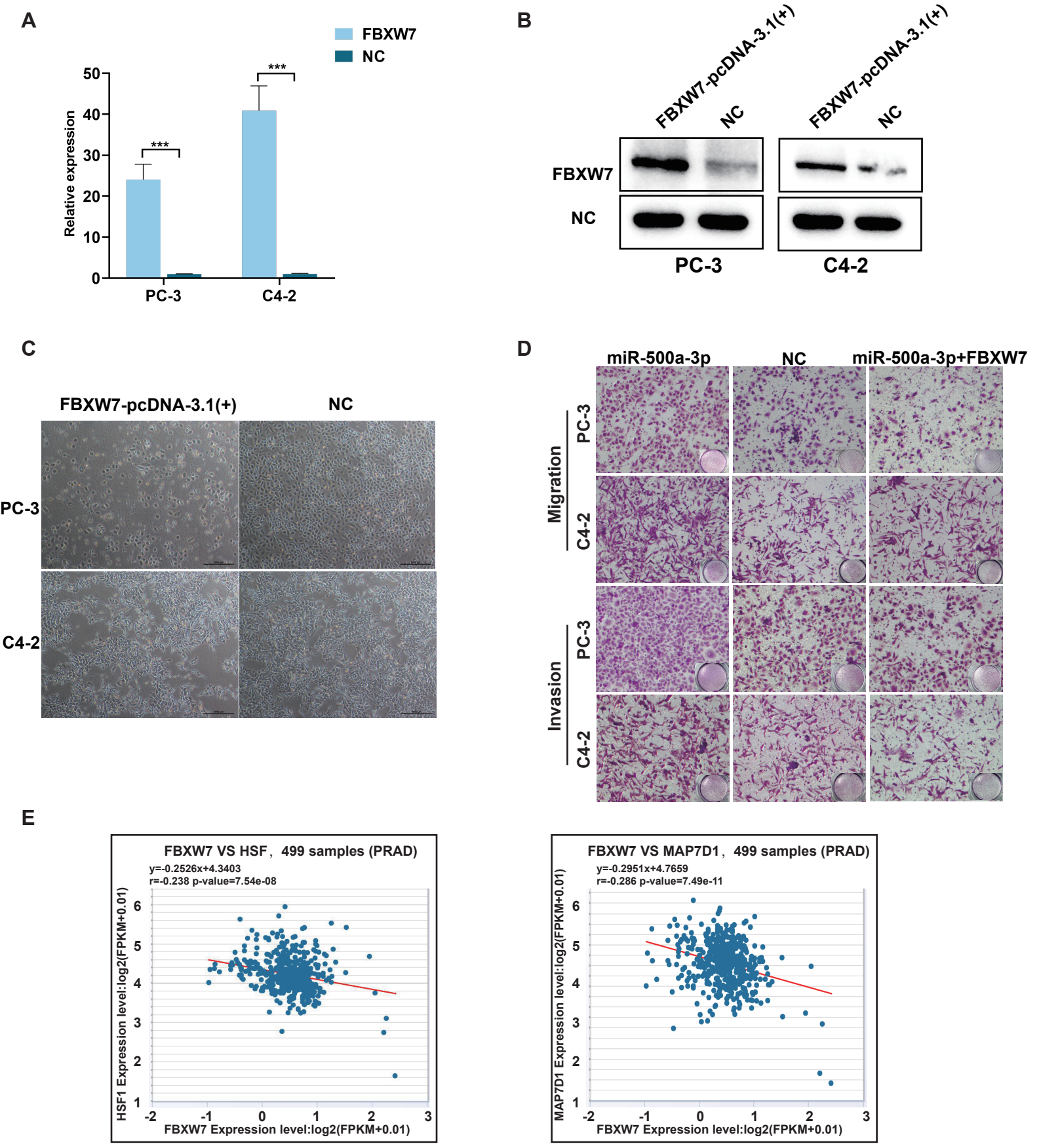
Supplementary Figure 4. Schematic representation of body weight changes in nude mice.



Supplementary Figure 5. The effect of miR-500a-3p mimics or inhibitor on the proliferation of PC-3 (A) and C4-2 (B) cells.

Gene	Target Score
PPP2R2C	98
ARHGAP29	96
NRG4	95
SOCS2	95
C11orf24	94
DAAM1	93
PRRC2B	93
TNRC6B	93
LIN54	93
PDE8A	92
FBXW7	90

Supplementary Figure 6. MiR-500a-3p target gene scores in the miRDB database.



Supplementary Figure 7. FBXW7 inhibited the metastasis of PCa through regulating the HSF1 expression. The transfection efficacy of the FBXW7 plasmid was successfully ascertained through both RT-qPCR (A) and western blotting (B). (C) The morphological alterations in PC-3 and C4-2 cells after FBXW7 overexpression under light microscopy. (D) FBXW7 overexpression effectively counteracted the promotive influence of miR-500a-3p. (E) Starbase database (https://starbase.sysu.edu.cn/) showed the relationship between FBXW7 and HSF1, along with MAP7D1.

Primer	Forward	Reverse
β-actin	GAGCGGGAAATCGTGCGTGACATT	GATGGAGTTGAAGGTAGTTTCGTG
FBXW7	CGACGCCGAATTACATCTGTC	CGTTGAAACTGGGGTTCTATCA
HSF1	CCGTGTCCTGTGGTTTGGTT	CTGTCTTGTCCGTCCATCCA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
miR-1306-5p	ACTATCCCACCTCCCCTGCA	
miR-500a-3p	AAGCACATGCACCTGGGCA	
miR-598-3p	ACGAGATCTACGTCATCGTTGTC	
miR-382-3p	GCACGTCCAATCATTCACGGA	
miR-376c-5p	AAGCGCCTGGTGGATATTCCT	
miR-296-5p	AATATGAGGGCCCCCCCTCA	
miR-2682-3p	CCATAAGTCGCCTCTTCAGCG	
miR-1908-3p	CTTAAGATATCCGGCCGCCG	
miR-1469	CTTAAGATATCTCGGCGCGGG	
miR-184	AAGTACTCTGGACGGAGAACTGA	

hsa-miR-500a-3p mimics sequence:

sense (5’-3’): AUGCACCUGGGCAAGGAUUCUG

anti-sense(5’-3’): GAAUCCUUGCCCAGGUGCAUUU

hsa-miR-500a-3p inhibitor sequence:

sense (5’-3’): CAGAAUCCUUGCCCAGGUGCAU