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CXCR4 promotes tumor stemness maintenance and CDK4/6 inhibitors resistance in ER-positive breast cancer

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Abstract

Background CDK4/6 inhibitors have significantly improved the survival of patients with HR-positive/HER2-negative breast cancer, becoming a first-line treatment option. However, the development of resistance to these inhibitors is inevitable. To address this challenge, novel strategies are required to overcome resistance, necessitating a deeper understanding of its mechanisms. Recent research has identified several dysregulated genes in CDK4/6 inhibitors-resistant breast cancer, but the underlying mechanism is complex due to tumor heterogeneity and warrants further investigation.

Methods RNA sequencing and KEGG pathway analysis was carried out to identify the mainly dysregulated genes in CDK4/6 inhibitors-resistant breast cancer cells. The effects of CXCR4 knockdown and overexpression via siRNAs and plasmids transfection were examined by mammosphere formation, RT-qPCR, flow cytometry, MTT and colony formation assays. The regulation mechanisms were analyzed by RT-qPCR, western blotting and immunofluorescence experiments. Mouse xenografts were used to analyze the role of CXCR4 in regulation palbociclib sensitivity in vivo. Additionally, we collected the clinical samples and performed immunohistochemistry to analyze the clinical significance of CXCR4.

Results In our study, we focused on cancer stem cells, a critical contributor to cancer metastasis and therapy resistance, and detected an upregulation of stemness in our established palbociclib-resistant ER-positive breast cancer cells. Additionally, our research pinpointed CXCR4 as a pivotal gene responsible for maintaining cancer stemness and promoting palbociclib resistance. Mechanistically, CXCR4 activates the WNT5A/β-catenin signaling pathway by enhancing the expression of WNT5A and β-catenin, facilitating the nuclear translocation of β-catenin protein. Targeting CXCR4 using siRNAs or small molecular inhibitors effectively reduces cancer stemness and reverses palbociclib resistance both in vitro and in vivo. Clinical sample analysis further underscores the overactivation of the CXCR4/WNT5A/β-catenin axis in palbociclib-resistant breast cancer, suggesting CXCR4 as a potential biomarker for predicting resistance to CDK4/6 inhibitors.

Conclusions Collectively, our study demonstrates that CXCR4 overexpression plays a vital role in maintaining breast cancer stemness and promoting resistance to CDK4/6 inhibitors through the activation of the WNT5A/β-catenin

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pathway. Targeting CXCR4 may offer a promising therapeutic approach for advanced CDK4/6 inhibitor-resistant ER-positive breast cancer.

Background

Estrogen receptor (ER)-positive breast cancer is the most commonly molecular subtype, accounting for 60–70% total patients with breast cancer [1]. Endocrine therapy is the standard therapeutics for these patients and most of them benefit from it [1, 2]. Nevertheless, around one third of patients with ER-positive breast cancer develop resistance to endocrine therapy and relapse with 5-years tamoxifen or aromatase inhibitors treatment [1, 3]. CDK4/6 and Cyclin D1 form a complex and inactivate Rb by phosphorylating it, Rb phosphorylation activates E2F1 and promotes the transcription of target genes, governing G1/S cell cycle transition [4, 5]. It has been proved that dysregulation of the cell cycle promotes endocrine resistance in ER-positive breast cancer [6–8]. Small molecular inhibitors targeting Cyclin-dependent kinase 4 and 6 (CDK4/6) have emerged as a promising approach to impede cell cycle progression [9, 10]. Three of these inhibitors-palbociclib, ribociclib and abemaciclib have received FDA approval for use in combination with endocrine therapy and significantly improved the progression-free survival (PFS) and overall survival (OS) in patients with advanced breast cancer [11–15].

However, a variety of patients with advanced breast cancer inevitably develop resistance to CDK4/6 inhibitors sooner or later [16]. It has been reported that approximately 30% of patients relapsed within 2-years treatment of CDK4/6 inhibitors, and in the PALOMA-2 trial more than 70% of patients progressed during treatment with palbociclib plus letrozole for 40 months [17]. Recently, many researches had delved into the molecular underpinnings of CDK4/6 inhibitors resistance, unveiling a multitude of biological processes and signaling pathways implicated in its development. Notably, aberrant FGFR signaling has been observed in CDK4/6 inhibitors-resistant breast cancer cells, with FGFR ctDNA amplification associated with shortened PFS in patients enrolled in the MONALEESA-2 clinical trial receiving ribociclib treatment [18]. Additionally, acquired CDK6 amplification was observed in breast cancer cells resistant to abemaciclib [19]. RNA sequencing analysis and phospho-proteomics profiling revealed the activation of MAPK signaling in CDK4/6 inhibitors-resistant cells, rendering these cells sensitive to MEK inhibitors [20]. Patients undergoing combination treatment with ribociclib and letrozole who developed acquired resistance, have exhibited RB and PTEN loss [21]. It follows that the complexity of CDK4/6 inhibitors resistance mechanisms arises from tumor heterogeneity, necessitating urgent and comprehensive exploration of these intricate processes.

Cancer stem cells (CSCs) constitute a subpopulation characterized by both phenotypic and functional heterogeneity [22]. Increasing evidence underscores the pivotal role played by CSCs in driving therapeutic resistance and metastasis, ultimately leading to cancer recurrence and mortality across multiple cancer types [23, 24]. Genetic and epigenetic regulation controls the biological behavior of tumor cell, including CSCs. Various signaling pathways, such as WNT, NOTCH and HIF, orchestrate the activation of stem cell-like phenotypes, endowing resistance to chemotherapy and radiotherapy in diverse cancers [25, 26]. Additionally, epigenetic regulation processes such as DNA methylation and acetylation significantly influence the acquisition of stem cell state, making them attractive targets for perturbing this state in cancers [27, 28]. Therefore, CSCs emerge as promising candidates for potential therapeutic interventions in the context of advanced therapy-resistant cancers.

CXCR4, a receptor for chemokine CXCL12 (also known as stromal-derived growth factor-1, SDF-1), plays a pivotal role in tumor growth, angiogenesis and metastatic dissemination of various cancers. The interaction of CXCR4 expressed in cancer cells and CXCL12 expressed in stromal cells format cross talk within the tumor microenvironment, anchoring tumor cells to metastatic sites [29–31]. While the CXCL12-CXCR4 axis has been recognized for its involvement in cancer metastasis, recent studies have revealed its participation in multiple processes within breast cancer, including endocrine resistance [32]. An autocrine loop has been identified, wherein the activation of estrogen receptors by CXCL12-CXCR4 dictates ER-dependent gene expression and promotes the growth of breast cancer cells [33]. In non-small cell lung cancer, CXCR4 has been deemed functionally crucial for maintaining stemness and conferring chemoresistance [34]. Until now, there is no research to uncover the role of CXCL12-CXCR4 axis in CDK4/6 inhibitors resistance in ER-positive breast cancer.

In this work, we conducted an unbiasedly screening of differentially expressed genes between parental and palbociclib-resistant breast cancer cells, pinpointing CXCR4 as a crucial mediator of CDK4/6 inhibitors resistance. Our research unequivocally demonstrated the significance of the CXCR4/WNT5A/β-catenin axis in CDK4/6

inhibitors resistance, highlighting CXCR4 as a potential novel therapeutic target for CDK4/6 inhibitors-resistant breast cancer.

Methods

Cell culture

MCF-7 and T47D breast cancer cells were purchased from the American Type Culture Collection (ATCC). MCF-7 cells were grown in DMEM medium (Gibco, USA) with 10% FBS (Hyclone, USA), while T47D cells were cultured in RPMI-1640 medium (Gibco, USA) plus 10% FBS (Hyclone, USA). Palbociclib-resistant MCF-7 and T47D cells have been established at our laboratory previously [35] and were cultured in DMEM or RPMI-1640 medium with 10%FBS plus 6 μM palbociclib.

SiRNAs and plasmids transfection

SiRNAs to CXCR4 were designed and synthesized by GenePhrama (China). The sequence of siCXCR4-1: sense (5′–3′) CCGACUUCAUCUUUGCCAATT, antisense (5′–3′) UUGGCAAAGAUGAAGUCGGTT; siCXCR4-2: sense (5′–3′) GGGACUAUGACUCCA UGAATT, antisense (5′–3′) UUCAUGGAGUCAUAG UCCCTT. For siRNA transfection, Cells were plated at a density of 30–40% in 6-well plates and cultured for 12 h. For transfection, 3.75 μl lipo3000 (L3000008, Invitrogen) and 5 μl siRNAs were mixed with 125 μl optimal medium (Gibco,USA) separately and incubated for 5 min, then were mixed together and incubated for another 20 min. The 250 μl mixture was adding to cultured cells with 1.75 ml fresh medium containing 10%FBS, then the medium was changed after 24 h. RT-qPCR and western blotting were performed as described below to verify whether CXCR4 was efficiently knocked down.

To obtain CXCR4 overexpression plasmids, the sequence of CXCR4 mRNA was cloned into a pCDH-puro-vector (pCDH-puro-CXCR4). For plasmids transfection, cells were planted at a density of 40%–50% into 6-well plates. The indicated plasmids (1 μg/well) were mixed with P3000(10 μl/well, Thermo Scientific, L3000008) in 125 μl optimal medium and incubated for 5 min before adding to another 125 μl optimal medium with Lipo3000 transfection reagent (3.75 μl/well, Thermo Scientific, L3000008). The mixture was then incubated for 20 min at room temperature before adding to the cultured cells. After 48 h, the cells were harvested for subsequent analysis.

Colony formation and MTT assay

For colony formation assay, cells were plated in 6-wells plate(1000/well) and cultured in medium with 10% FBS, and treated with palbociclib or AMD3100 for 2 weeks, then were fixed by 4% paraformaldehyde and stained with crystal violet for colony number count.

For MTT analysis, 5 mg/ml MTT solution (3580MG250, Biofrox) was prepared and added into the cells cultured in 96-well plates with a ratio of 1:10. After incubation at 37 °C for 4 h, the absorbance was measured at a wavelength of 490 nm by a microplate spectrophotometer.

RNA extraction and RT-qPCR

Cultured cells were washed twice by PBS buffer, then total RNA was extracted by RNA separation and purification kit (ES-RN001, YISHAN BIOTECHNOLOGY) according to the manufacturer's instructions. RNA concentration was detected by NanoDrop Spectrophotometer (Thermo Scientific, USA). RNA was reversed transcribed to cDNA using PrimeScript RT Master Mix (RR036A, Takara) followed the description. Then the cDNA was subjected to qPCR analysis using TB Green Premix Ex Taq II (RR820A, Takara) in Real Time-PCR system (LC480, Roche). Primer sequences: CXCR4 Forward (5′–3′): ACTACACCGAGGAAATGGGCT; Reverse (5′–3′): CCCACAATGCCAGTTAAGAAGA), CXCL12 Forward (5′–3′):ATTCTCAACACTCCAAAC TGTGC; Reverse: (5′–3′): ACTTTAGCTTCGGGTCAA TGC, WNT5A Forward (5′–3′): ATTCTTGGTGGT CGCTAGGTA, Reverse (5′–3′): CGCCTTCTCCGA TGTACTGC, CTNNB1 Forward (5′–3′): AGCTTCCAG ACACGCTATCAT; Reverse (5′–3′): CGGTACAAC GAGCTGTTTCTAC; WNT5B Forward (5′–3′): CAT GGCCTACATAGGGGAGG, Reverse (5′–3′): CTG TGCTGCAATTCCACCG, WNT16 Forward (5′–3′): AGTATGGCATGTGGTTCAGCA; Reverse (5′–3′): GCGGCAGTCTACTGACATCAA, WNT8B Forward (5′–3′): CCGACACCTTTCGCTCCATC; Reverse (5′–3′): CAGCCCTAGCGTTTTGTTCTC, WNT10A Forward (5′–3′): AGATCGCCATCCACGAATGC; Reverse (5′–3′): ATCTTGTTGCGAGTCTCCAGG, WNT11 Forward (5′–3′): GACCTCAAGACCCGATACCTG; Reverse (5′–3′): TAGACGAGTTCCGAGTCCTTC, GAPDH Forward (5′–3′): CTGGGCTACACTGAGCAC C; Reverse (5′–3′): AAGTGGTCGTTGAGGGCAATG.

Western blotting

Cells were plated in 6-well plate and with given treatment in advance. Then cultured cells were washed twice by cold PBS buffer before lysed in 70ul RIPA lysis buffer plus cocktail and phosphatase inhibitors (78,442, Thermo Scientific) on ice for 30 min. Supernatant of the lysates were isolated by centrifuging at maximum speed for 30 min and the concentration of total protein was measured by BCA kit (23,227, thermo scientific). Before immunoblotting analysis, the quantified supernatant was

boiled with 4xloading buffer for 5 min at 98 °C. After SDS-PAGE electrophoresis for around 1 h, the protein was transferred onto a PVDF membrane and was closed in 5% BSA for 2 h, then incubated with primary antibodies overnight at 4 °C. The primary antibodies of CXCR4 (ab124824, Abcam), CXCL12 (17,402, proteintech), WNT5A (2392, CST), β-catenin (51,067, proteintech) were purchased and were diluted into 5%BSA at a ratio of 1:1000. Next day, HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (7076/7074, CST) were added and incubated for 1 h at room temperature, then signals were detected by enhanced chemiluminescence (34,095, Pierce) and were visualized by CCD camera.

Immunofluorescence

Cells were plated in confocal dishes(5×10^4/dish) and cultured in the medium plus 10%FBS for 12 h, then changed the medium with 10 μM AMD3100 in the absence of serum and cultured for 3 h, added 100 ng/ml CXCL12 and grown for another 24 h. These treated cells were washed 3 times by PBS buffer, and then were fixed by 4% paraformaldehyde for 20 min at room temperature, permeabilized in PBS containing 0.05% Triton X-100 for 3 min on ice, and closed in 5%BSA for 30 min at room temperature. Then cells were incubated overnight at 4 °C with primary antibodies against CXCR4 and β-catenin, which were diluted into 5%BSA at a ratio of 1:1000. Next day, Alexa Fluor secondary antibodies were added and incubated for 1 h at room temperature. Finally, DAPI was added and stained for 10 min before taking images by confocal microscopy (LSM800, Zeiss).

Immunohistochemistry

Paraffin-embedded tissue sections were deparaffinized before antigen retrieval by boiling in 0.01 M citrate buffer (pH 6.0) for 30 min. 3% hydrogen peroxide in PBS was added for 15 min to block endogenous catalase activity. Furthermore, 5% BSA in PBS was used to close nonspecific binding for 30-min incubation at room temperature. Tissues were then incubated with anti-CXCR4 (ab124824, Abcam) antibody at 4 °C overnight. Next day, tissues were incubated with HRP-conjugated secondary antibody and stained with DAB (GSK500710, Gene Tech) before taking images by microscopy (Nikon, Japan). The IHC staining scores were determined as we previously described.

Mammosphere formation assay

Cultured cells were isolated into signal cell dilution by 0.4% trypsin, and washed twice by PBS buffer to remove FBS absolutely. Then cells were plated in 24-wells ultra-low adhesion plate (1000/well) in the spheroid medium, which containing of BSA, B27, insulin, FGF and Penicillin–Streptomycin in DMEM/F12 medium. To avoid cell gathering, blow it with a pipette twice a day, and spheroids grown up around 1 week later. Microscopy (Nikon, Japan) were used to take images of spheroid and the formation rate was calculated for quantitative analysis.

Flow cytometry

ALDH activity was assayed by ALDEFLUOR kit (01700, Stem Cell Technologies), following the manufacturer's instructions. In brief, spheroid cells were dissociated into single cells by 0.4% trypsin, washed twice with PBS, suspended in 500 µl ALDEFLUOR assay buffer, then 5 µl ALDH substrate was added to each tube. What's more, 5 µl DEAB (diethylaminobenzaldehyde, a specific ALDH inhibitor) was added to preformed as a negative control. After an incubation at 37 °C for 45 min, cells were washed twice and re-suspended in ALDEFLUOR assay buffer, then flow cytometer (BD, USA) was used to analyze the proportion of ALDH positive cells.

Animal experiment

All the animal studies were approved by Sun Yat-sen University laboratory animal care and use committee. Four weeks old femal BABL/c nude mice, provided by Vital River Laboratory, were housed under a specific pathogen-free condition of 12 h light/12 h dark cycle in a temperature- and humidity-controlled cage and were fed ad libitum. palbociclib-resistant MCF-7 cells (1×10^7 per mouse) were inoculated to mammary fat pad of BABL/c nude mice after 17β-Estrogen pellets (0.72 mg, 60-day release, innovative research of America) were implanted subcutaneously for a week. For CDK4/6 inhibitors treatment groups, palbociclib (100 mg/kg) was given by gavage every day. And for AMD3100 treatment groups, AMD3100 (5 mg/kg) was given via intraperitoneal injection every 3 days. 6 weeks later, all mice were euthanized in a humane manner, all tumors were collected and the tumor volumes were calculated. Then the tumors were embedded by paraffin and subjected to IHC staining.

Statistics and reproducibility

Statistical analyses were performed using R (version 4.4.0) or excel software. Unless otherwise noted, results were expressed as means ± SD. The p values were calculated by Student's t-test for two group and one-way ANOVAs for multiple groups comparison.

Results

Tumor stemness is elevated in palbociclib-resistant breast cancer

CDK4/6 inhibitors-resistant breast cancer cell lines were established as described before [35]. Cancer stem cells

have been widely recognized as key contributors to cancer metastasis and therapy resistance. However, their role in CDK4/6 inhibitors resistance remains largely unknown. To explore whether cancer stem cells are involved in CDK4/6 inhibitors resistance in ER-positive breast cancer, we conducted mammosphere formation, RT-qPCR and flow cytometry assay in MCF-7 and T47D parental (MCF7-Pa and T47D-Pa) and palbociclib resistance (MCF7-palR and T47D-palR) cells. Mammosphere formation assay revealed a significantly higher proportion of mammosphere formation in MCF7-palR and T47D-palR compared to their parental cells (Fig. 1a, b). Markers such as ALDH, Oct-4, Sox2, and Nanog are well-established indicators of both normal and malignant mammary stem cells [36, 37]. RT-qPCR analysis showed the expression of stemness-associated genes Oct-4, Sox2 and Nanog was much higher in MCF7-palR and T47D-palR than their parental cells (Fig. 1c, d). Consistent with the results of mammosphere formation and RT-qPCR assay, flow cytometry showed a larger proportion of ALDH-positive cells in MCF7-palR and T47D-palR than their parental cells (Fig. 1e, f). These results suggest

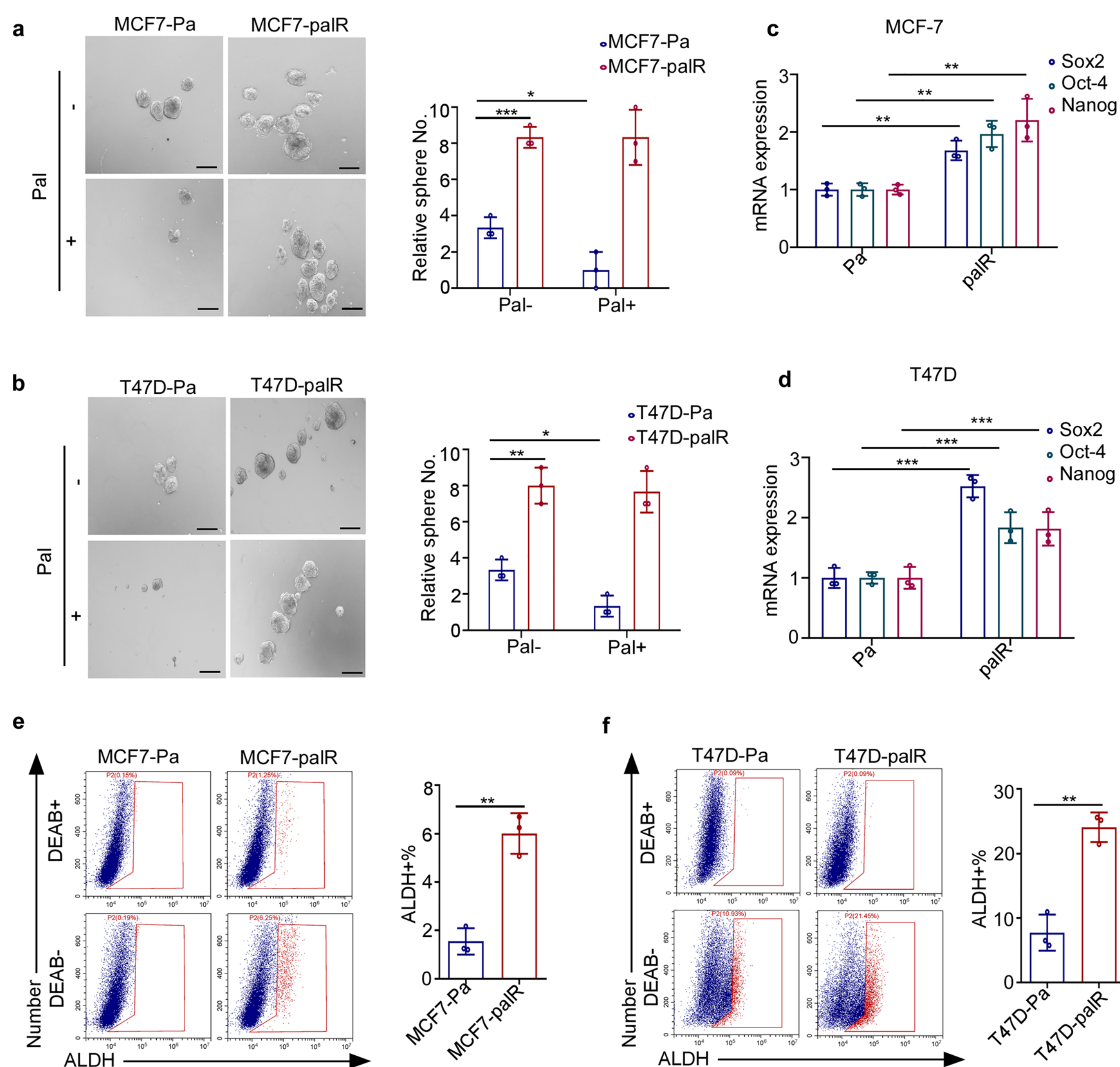

Fig. 1 Tumor stemness is elevated in palbociclib-resistant breast cancer. **a**–**f** Parental and palbociclib-resistance MCF-7 and T47D cells were cultured in spheroids medium with or without palbociclib for 1 week. **a** and **b** Mammosphere formation assay. Representative images of at the left panel and statistical analysis at the right panel. Scale bars represent 100 μm. **c** and **d** RT-qPCR showing the expression of stem cell markers Oct-4, Sox2 and Nanog. **e** and **f** Flow cytometry showing the proportion of ALDH positive cells. Representative images at the left panel and statistical analysis at the right panel. For **a**–**f**, n = 3 biologically independent experiments, means ± s.d. were shown, the *p* values were calculated by Student's t-test, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

that tumor stem cells are enriched in palbociclib-resistant breast cancer, underscoring the pivotal role of tumor stemness maintenance in the development of CDK4/6 inhibitors resistance.

Identification of CXCR4 as a crucial gene mediating palbociclib resistance in breast cancer

To delve further into the exploration of alternative mechanisms of CDK4/6 inhibitors resistance, total RNA of MCF7-Pa and MCF7-palR cells was isolated and subjected to RNA sequencing as described before [35]. Our analysis included a focused examination of the differential genes associated with "Pathways in cancer" via KEGG pathway analysis (Fig. S1a). Then we used hierarchical clustering analysis to elucidate the dysregulated genes involved in "Pathways in cancer" (Fold change > 2, p value < 0.05) (Fig. 2a). Notably, our scrutiny revealed a significant increase in the mRNA levels of CXCR4 and its ligand CXCL12 in MCF7-palR compared to MCF7-Pa cells. CXCR4, a pivotal chemokine receptor involved in the intricate communication between tumor cells and their microenvironment, has previously been implicated in the regulation of metastasis and resistance to both chemotherapy and endocrine therapy. Enhanced CXCL12 secretion and autocrine/paracrine activation of CXCR4 had been reported to promote the proliferation of ER-positive breast cancer cells [33]. However, its role in regulating CDK4/6 inhibitor resistance has remained largely unexplored. So, we focused on CXCR4 to unravel its role and the involved mechanisms underlying CDK4/6 inhibitors resistance.

We conducted RT-qPCR assay and found a higher mRNA level of CXCR4 and CXCL12 in MCF7-palR and T47D-palR than their parental cells (Fig. 2b). Consistently, western blotting showed an increased protein level of CXCR4 and CXCL12 in MCF7-palR and T47D-palR than their parental cells (Fig. 2c). To further ascertain the clinical relevance of CXCR4 in breast cancer, we extended our analysis to examine CXCR4 expression in both normal breast tissues and breast carcinoma using METABRIC and TCGA datasets. Our findings demonstrated a significant increase in CXCR4 expression in breast cancer when compared to normal controls (Fig. S1b, c). Furthermore, Kaplan–Meier survival analysis showed that higher expression of CXCR4 predicted shorter relapse free survival (RFS), specifically in patients with ER-positive breast cancer (Fig. S1d). These results underscore the upregulation of CXCR4 in palbociclib-resistant breast cancer cells and its association with poor prognosis in patients with ER-positive breast cancer.

To investigate whether the heightened CXCR4 expression contributes to CDK4/6 inhibitors resistance, CXCR4 was silenced using specific siRNAs and then MTT and colony formation assay were carried out in palbociclib-resistant MCF-7 and T47D cells. MTT assays demonstrated that CXCR4 knockdown effectively restored palbociclib sensitivity in MCF7-palR and T47D-palR cells (Figs. 2d and S2a). Colony formation assays revealed that knocking down CXCR4 had no impact on colony formation in MCF7-palR and T47D-palR cells. However, when palbociclib treatment followed CXCR4 knockdown, a potent inhibition of colony formation was observed (Figs. 2e and S2b). On the other hand, MTT and colony formation assays were carried out in CXCR4-overexpressed MCF-7 and T47D cells. MTT assays demonstrated that CXCR4-overexpressed cells were resistant to palbociclib, compared to the control MCF-7 and T47D cells (Figs. 2f and S2c). Colony formation assay showed that CXCR4 overexpression increased the colony formation and could not be inhibited by palbociclib treatment (Figs. 2g and S2d). These findings suggest that CXCR4 plays a pivotal role in mediating CDK4/6 inhibitors resistance in ER-positive breast cancer.

CXCR4 is necessary for tumor stemness maintenance in palbociclib-resistant breast cancer

Building upon the founding that cancer stem cells were enriched in CDK4/6 inhibitors-resistant breast cancer,

(See figure on next page.)

Fig. 2 Identification of CXCR4 as a crucial gene mediating palbociclib resistance in breast cancer. **a** Heatmap showing the differential expression genes related to pathways in cancer, determined by RNA-sequencing in palbociclib-resistance than parental MCF-7 cells. (Fold change > 2, p value < 0.05). The p values were determined by negative binomial generalized linear models. **b** RT-qPCR showing the mRNA level of CXCR4 and CXCL12 in parental and palbociclib-resistance MCF-7 and T47D cells. **c** Western blotting showing the protein expression of CXCR4 and CXCL12 in parental and palbociclib-resistance MCF-7 and T47D cells. GAPDH as a loading control. Representative images of three biologically independent experiments were shown. **d**–**e** Palbociclib-resistance MCF-7 cells were transfected with NC or one of the two siRNAs targeting CXCR4 and then were treated with palbociclib for 5 days (**d**) or 2 weeks (**e**). MTT assay (**d**) and Colony formation assay (**e**) showing the cell viability with different concentration of palbociclib treatment in control and CXCR4 knocked-down cells. **f**–**g** MCF-7 cells were transfected with plasmid vector carried with CXCR4 sequence and then were treated with palbociclib for 5 days (**f**) or 2 weeks (**g**). MTT assay (**f**) and Colony formation assay (**g**) showing the cell viability with different concentration of palbociclib treatment in control and CXCR4-overexpressed cells. For **e**, **g**, representative images of at the left panel and statistical analysis at the right panel. For **b**–**g**, n = 3 biologically independent experiments, the p values were calculated by Student's t-test for two group comparison and one-way ANOVAs for multiple groups, ***$p < 0.001$, **$p < 0.01$

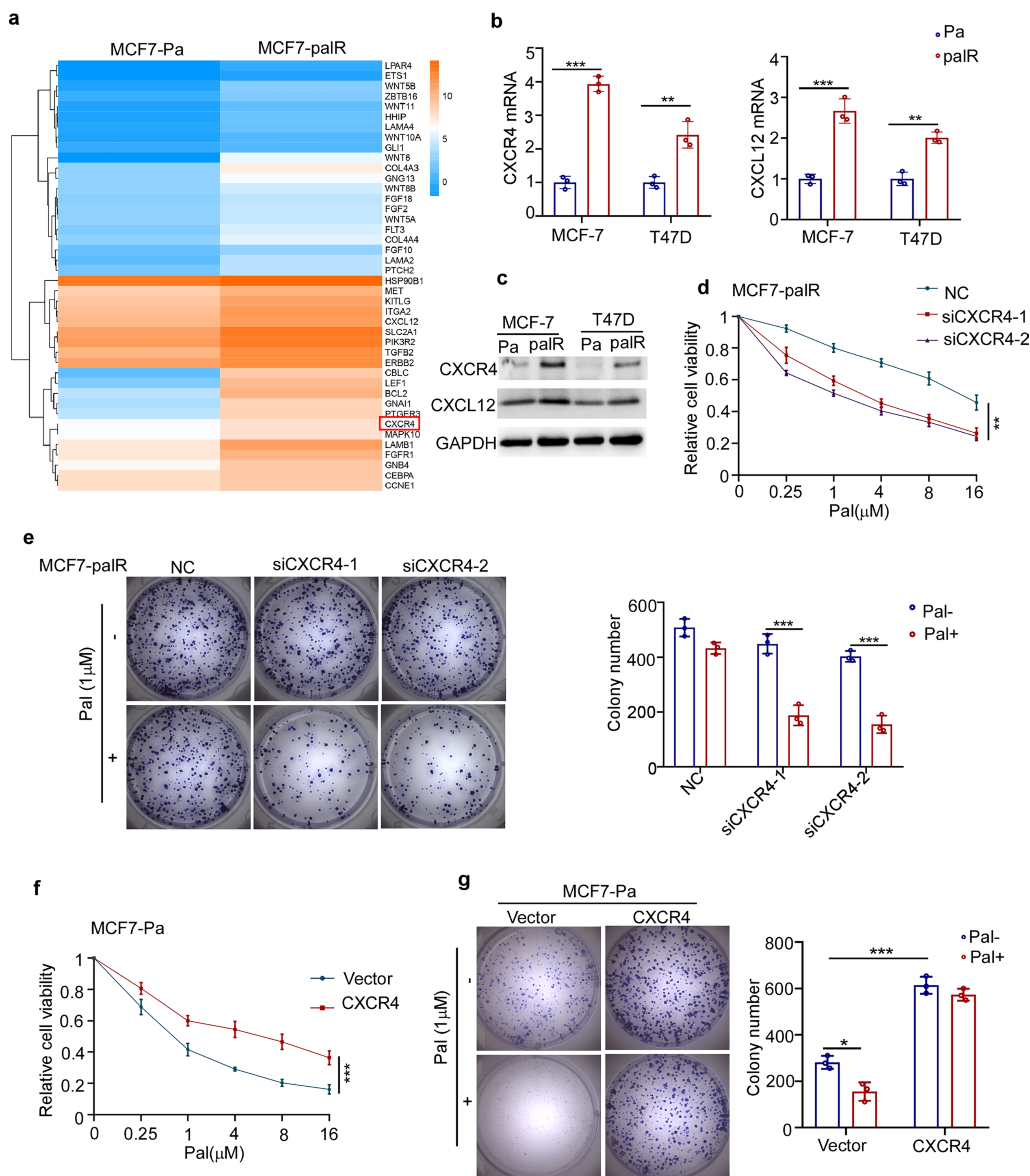

Fig. 2 (See legend on previous page.)

we next explored the role of CXCR4 in tumor stemness maintenance in palbociclib-resistant cells. AMD3100 is a highly selective CXCR4 receptor antagonist to potently inhibit the binding of CXCL12 to CXCR4. MCF7-palR and T47D-palR cells were treated with AMD3100 and then were subjected to mammosphere formation assay. The proportion of mammosphere formation was effectively decreased by AMD3100 in MCF7-palR and T47D-palR cells, and could be further reduced by palbociclib treatment (Figs. 3a and S3a). RT-qPCR assay revealed a

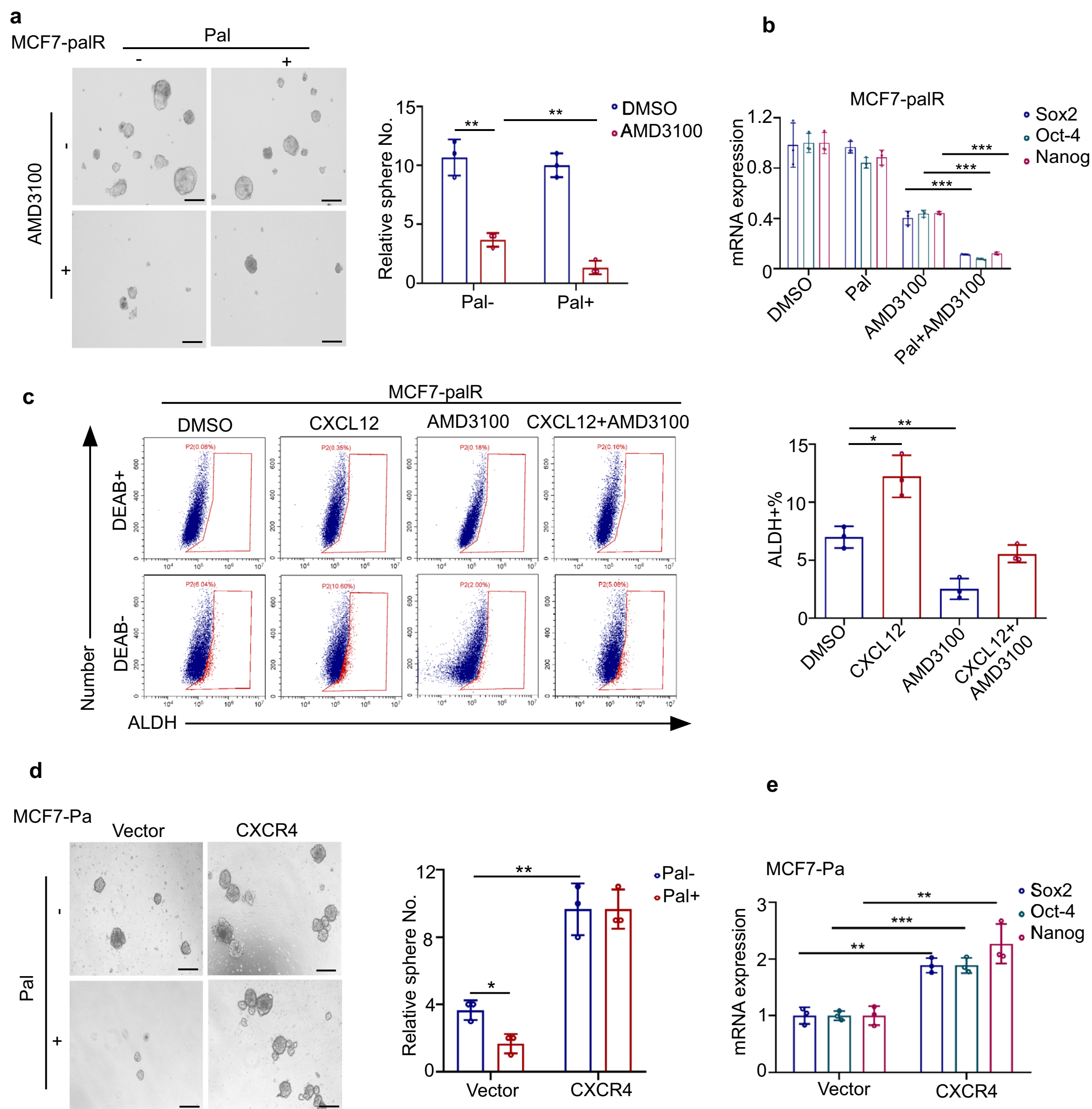

Fig. 3 CXCR4 is necessary for tumor stemness maintenance in palbociclib-resistant breast cancer. **a**, **b** Palbociclib-resistance MCF-7 cells were cultured in spheroids medium and with palbociclib and AMD3100 monotherapy or combination treatment for 1 week. **a** Mammosphere formation assay. Representative images at the left panel and statistical analysis at the right panel. Scale bars represent 100 μm. **b** RT-qPCR assay showing the expression of stem cell markers Oct-4, Sox2 and Nanog. **c** Palbociclib-resistance MCF-7 (**c**) cells were cultured in spheroids medium with CXCL12 and (or) AMD3100 treatment for 1 week. Flow cytometry showing the proportion of ALDH positive cells. Representative images at the left panel and statistical analysis at the right panel. **d**, **e** MCF-7 cells were transfected with plasmid vector carried with CXCR4 sequence and then were treated with palbociclib for 1 week. **d** Mammosphere formation assay. Representative images at the left panel and statistical analysis at the right panel. Scale bars represent 100 μm. **e** RT-qPCR assay showing the expression of stem cell markers Oct-4, Sox2 and Nanog. For **a**–**e**, n = 3 biologically independent experiments, means ± s.d. were shown, the *p* values were calculated by Student's t-test for two group comparison and one-way ANOVAs for multiple groups, ****p* < 0.001, ***p* < 0.01, **p* < 0.05

notable decrease in the expression of stemness-associated genes Oct-4, Sox2, and Nanog in MCF7-palR and T47D-palR cells treated with AMD3100. Interestingly, this reduction could be further intensified by subsequent palbociclib treatment (Figs. 3b and S3b) Consistently, flow cytometry results indicated a significant decrease in the proportion of ALDH-positive cells in MCF7-palR and T47D-palR cells following AMD3100 treatment. Notably, the introduction of CXCL12 led to an increase in the proportion of ALDH-positive cells, and can be effectively

counteracted by CXCR4 inhibition (Figs. 3c and S3c). On the other hand, we performed mammosphere formation and RT-qPCR assay in CXCR4-overexpressed MCF-7 and T47D cells, and the results demonstrated that CXCR4 overexpression increase the proportion of mammosphere formation (Figs. 3d and S3d) and the expression of the stemness-associated genes (Figs. 3e and S3e). These results suggest that CXCR4 promote stemness maintenance in palbociclib-resistant breast cancer.

CXCR4 enhances β-catenin nuclear translocation and activates WNT5A/β-catenin pathway in palbociclib-resistant breast cancer

RNA-seq analysis revealed significantly higher expression levels of a series of WNT genes (WNT5A, WNT5B, WNT16, WNT8B, WNT10A, WNT11) in MCF7-palR compared to MCF7-Pa cells (Fig. 2a). CXCR4 has been widely reported to regulate cancer metastasis by the canonical WNT/β-catenin signaling pathway [38–41], but its potential role in activating the alternative WNT signaling pathway and promoting CDK4/6 inhibitors resistance in breast cancer remains unknown. To address this, we carried out RT-qPCR assay to detect the expression of WNT5A, WNT5B, WNT16, WNT8B, WNT10A, WNT11 in both parental and palbociclib-resistant breast cancer cells. The result demonstrated a significant upregulation mRNA level of WNT5A rather than other WNT genes in MCF7-palR and T47D-palR compared to their respective parental cells (Figs. 4a and S4a, b). Additionally, western blotting confirmed increased WNT5A protein levels in MCF7-palR and T47D-palR relative to their parental cells (Fig. 4b, c). These results suggested that WNT5A, rather than other WNT genes, may play a crucial role in conferring palbociclib resistance in breast cancer. Furthermore, western blotting revealed higher protein level of β-catenin in palbociclib-resistant cells than their parental ones (Fig. 4b, c), while the mRNA level remained similarly (Fig. S4c). So, we focused on WNT5A/β-catenin axis and explore its involvement in CDK4/6 inhibitors resistance.

To determine whether increased CXCR4 expression contributes to the activation of WNT5A/β-catenin pathway, we utilized siRNAs to knock down CXCR4 in MCF7-palR and T47D-palR cells. RT-qPCR and western blotting assay showed that silencing of CXCR4 reduced the expression of WNT5A and β-catenin (Figs. 4d, g and S4d, e). On the other hand, CXCR4 was overexpressed in MCF-7 and T47D cells (Fig. S4f), RT-qPCR and western blotting assays were carried out, and the result showed an upregulation of WNT5A and β-catenin level when CXCR4 was overexpressed (Figs. 4h, i and S4g). Nuclear translocation of β-catenin is a recognized hallmark of WNT/β-catenin signaling pathway activation [42–44]. To evaluate the distribution of β-catenin between the nucleus and cytoplasm, we conducted nuclear and cytoplasm fractionation followed by western blotting assay. The result showed a higher level of nuclear β-catenin protein in MCF7-palR and T47D-palR cells compared to their parental cells, indicating the activation of the WNT5A/β-catenin signaling pathway in palbociclib-resistant breast cancer (Fig. 4j, k). Knockdown of CXCR4 in palbociclib-resistant cells significantly decreased the level of nuclear β-catenin (Figs. 4l and S4h). Immunofluorescence assay also showed that β-catenin was higher stained in nucleus of palbociclib-resistant cells and was decreased by silencing of CXCR4 (Fig. 4m). Importantly, nuclear and cytoplasm fractionation assay also showed that nuclear β-catenin protein is higher in CXCR4-overexpressed MCF7-Pa and T47D-Pa cells, compared to the control parental cells (Figs. 4n and S4i). Collectively, these data suggest that CXCR4 promotes β-catenin

(See figure on next page.)

Fig. 4 CXCR4 enhances β-catenin nuclear translocation and activates WNT5A/β-catenin pathway in palbociclib-resistant breast cancer. **a** RT-qPCR showing the mRNA level of WNT5A in parental and palbociclib-resistance MCF-7 and T47D cells. **b**, **c** Western blotting showing the protein level of WNT5A and β-catenin in parental and palbociclib-resistance MCF-7 (**b**) and T47D(**c**) cells. **d**–**g** Palbociclib-resistance MCF-7 and T47D cells were transfected with NC or one of the two CXCR4 siRNAs to knock down the expression of CXCR4. RT-qPCR (**d**, **e**) showing the mRNA level of CXCR4 and WNT5A and Western blotting (**f**, **g**) showing the protein level of CXCR4, WNT5A and β-catenin. **h**, **i** Western blotting showing the protein level of CXCR4, WNT5A and β-catenin in control and CXCR4-overexpressed MCF7-Pa (**h**) and T47D-Pa (**i**) cells. **j**, **k** The nuclear and cytoplasmic protein was extracted and then was subjected to western blotting analysis, the results showing the levels of nuclear and cytoplasmic β-catenin in parental and palbociclib-resistance MCF-7 (**j**) and T47D (**k**) cells. **l** Palbociclib-resistance MCF-7 cells were transfected with NC or one of the two CXCR4 siRNAs to knock down the expression of CXCR4, western blotting showing the levels of nuclear and cytoplasmic β-catenin in control and CXCR4 knocked down palbociclib-resistance MCF-7 cells. **m** Immunofluorescence assay showing the expression and the nuclear and cytoplasmic distribution of β-catenin and CXCR4 in parental and palbociclib-resistance MCF-7 cells and CXCR4 knocked down MCF7-palR cells. **n** Western blotting showing the levels of nuclear and cytoplasmic β-catenin in control and CXCR4-ovexpressed MCF7-Pa cells. For **a**, **d**, **e**, n = 3 biologically independent experiments, the *p* values were calculated by Student's t-test for two group comparison and one-way ANOVAs for multiple groups, ***$p < 0.001$, **$p < 0.01$. For **b**, **c**, **f**, **g**, **h**, **i**, GAPDH as a loading control. For **j**–**l**, **n**, GAPDH and Lamin B as cytoplasmic or nuclear loading control, respectively. For **b**, **c**, **f**–**n**, representative images of three biologically independent experiments were shown

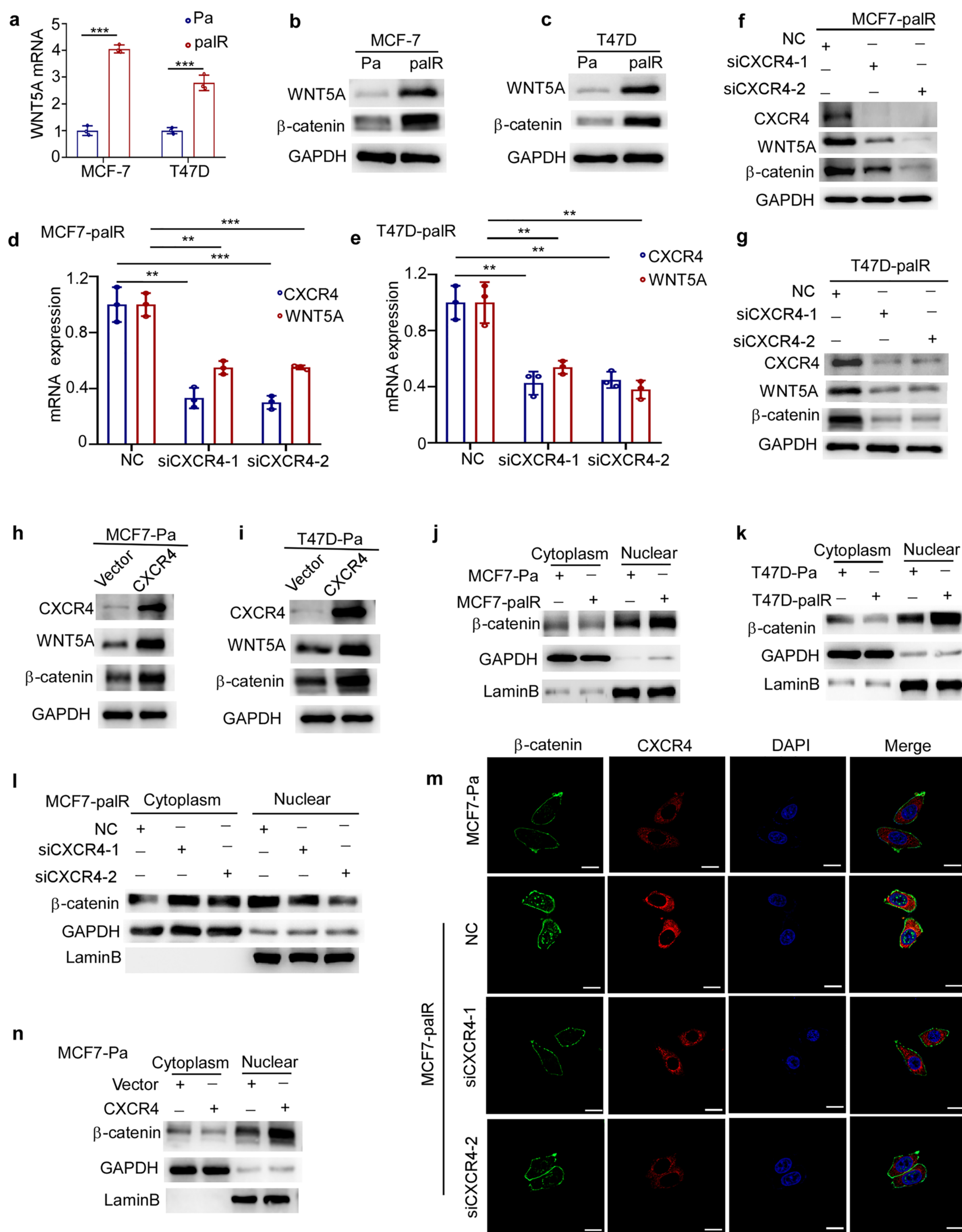

Fig. 4 (See legend on previous page.)

nuclear translocation and activates WNT5A/β-catenin pathway in palbociclib-resistant breast cancer.

CXCR4 inhibitors reverse palbociclib resistance in breast cancer cells

To further elucidate the role of CXCR4 inhibition in the treatment of palbociclib-resistant breast cancer, colony formation assay was carried out, and the result showed

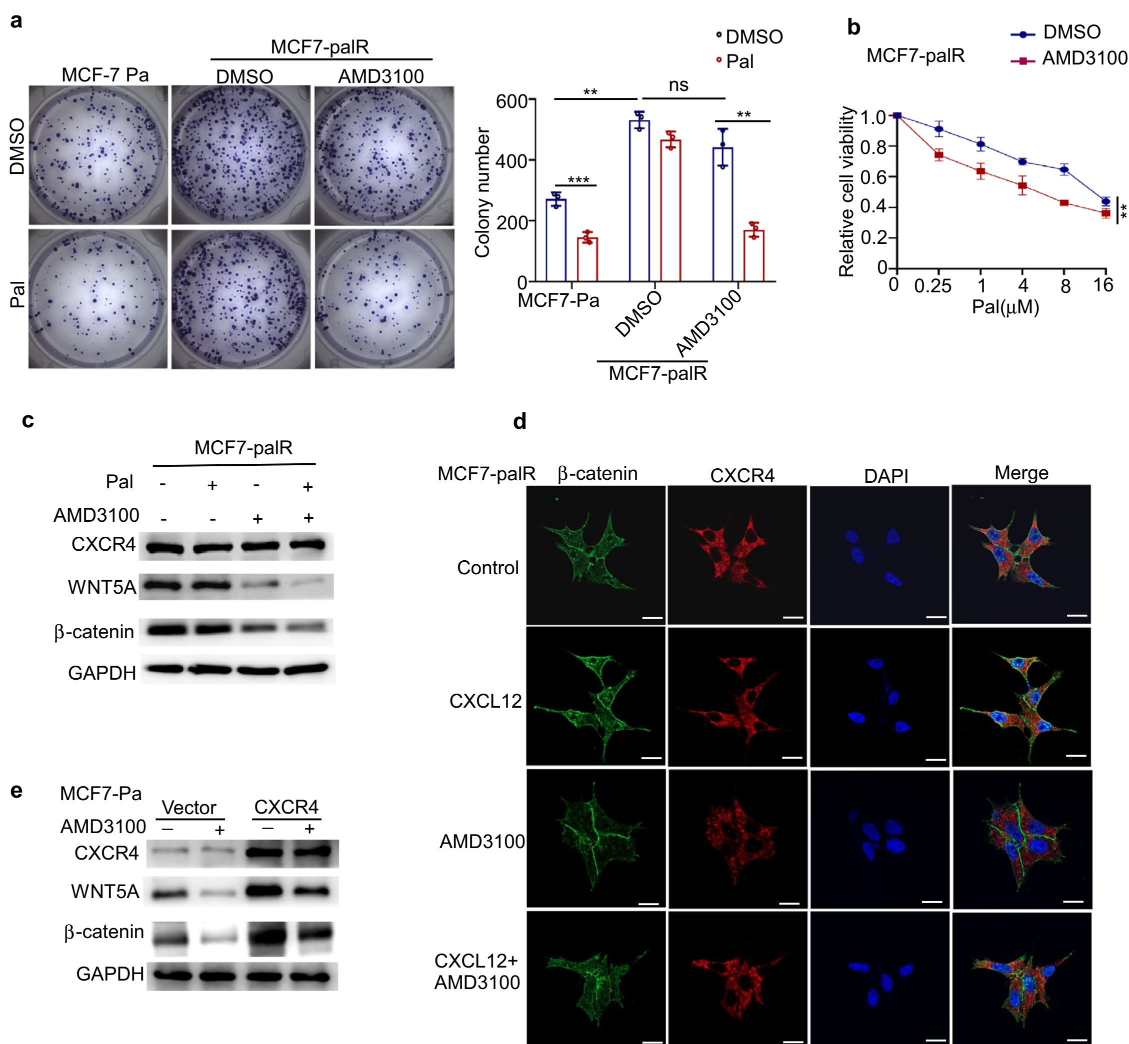

Fig. 5 CXCR4 inhibitors reverse palbociclib resistance in breast cancer cells. **a** Colony formation assay showing the cell viability in parental and palbociclib-resistance MCF-7 cells treated with monotherapy or combination palbociclib (1 μM) and AMD3100 (1 μM). Representative images at the left panel and quantification analysis at the right panel. **b** MTT assay showing the cell viability of palbociclib-resistance MCF-7 cells treated with different concentration of palbociclib with or without AMD3100 (1 μM) for 5 days. **c** Western blotting showing the protein level of CXCR4, WNT5A and β-catenin in palbociclib-resistance MCF-7 cells with palbociclib (1 μM) or AMD3100 (1 μM) mono- or combination treatment. GAPDH as a loading control. **d** Immunofluorescence assay showing the nuclear and cytoplasmic distribution of β-catenin and CXCR4 in MCF7-palR cells with mono- or combination CXCL12 and AMD3100 treatment. **e** Western blotting showing the protein level of CXCR4, WNT5A and β-catenin in CXCR4-overexpressed MCF-7 cells treated with AMD3100 (1 μM). GAPDH as a loading control. For **a**, **b**, n = 3 biologically independent experiments, the *p* values were calculated by Student's t-test for two group comparison and one-way ANOVAs for multiple groups, ****p* < 0.001, ***p* < 0.01. For **c-e**, representative images of three biologically independent experiments were shown

that AMD3100 monotherapy didn't impair colony formation in palbociclib-resistant cells, but significantly reduced by it when combined with palbociclib, suggesting that CXCR4 inhibitors can restore palbociclib sensitivity in MCF7-palR cells (Fig. 5a). In line with it, MTT assay also demonstrated that AMD3100 treatment rendered MCF7-palR cells sensitive to palbociclib again (Fig. 5b). Western blotting analysis showed that AMD3100 treatment reduced the expression of WNT5A and β-catenin, which was further diminished by palbociclib, while CXCR4 expression remained unchanged (Fig. 5c). Additionally, immunofluorescence assay showed that CXCL12 increased nuclear β-catenin protein level in MCF7-palR cells, and could be rescued by AMD3100 treatment (Fig. 5d). To confirm that AMD3100 reduces WNT5A and β-catenin levels by targeting CXCR4, we performed a rescue experiment. The result demonstrated that CXCR4 overexpression significantly elevated the

protein levels of WNT5A and β-catenin, which were nearly restored to the levels of negative control MCF7-Pa cells following AMD3100 treatment (Fig. 5e). These results further support the notion that CXCR4 inhibitors reduce β-catenin nuclear translocation, impair WNT5A/β-catenin signaling, and reverse palbociclib resistance in breast cancer cells.

CXCR4 inhibitors restore palbociclib sensitivity in palbociclib-resistant breast cancer in vivo

To further clarify the impact of CXCR4 inhibitors on treating palbociclib-resistant breast cancer in vivo, we inoculated MCF7-palR cells into the mammary fat pads of nude BALB/c mice. When the tumor volume reached approximately 100 mm^3, the tumor-bearing mice were randomly divided into four groups (six mice per group) and treated with control PBS, palbociclib alone, AMD3100 alone, or a combination of palbociclib and AMD3100 for six weeks. Tumor size was measured weekly, and tumor volumes were calculated using the formula: $volume = (length \times width^2)/2$. Consistent with the in vitro findings, AMD3100 or palbociclib monotherapy didn't effectively reduced tumor size but the combination treatment significantly shank the tumor volume of MCF7-palR xenografts, suggesting that AMD3100 restored the sensitivity to palbociclib in vivo (Fig. 6a). Meanwhile, the weight of the nude mice was also monitored using an electronic balance. The results indicated that neither the monotherapies nor the combination treatment significantly affected mouse weight (Figure S5a), suggesting that AMD3100 alone or in combination with palbociclib is well-tolerated. Immunohistochemistry assay showed the expression of Ki67 remained similar with AMD3100 or palbociclib monotherapy, but markedly reduced with the combination treatment, further indicating the restoration of palbociclib sensitivity by AMD3100 treatment. Moreover, the protein level of WNT5A and β-catenin was much lower in AMD3100 mono- or combination treatment group, suggesting that AMD3100 effectively inhibited the downstream WNT5A/β-catenin axis of CXCR4 in vivo (Fig. 6b). These results collectively demonstrate that CXCR4 inhibitors reverse palbociclib resistance and significantly halt tumor progression by suppressing the WNT5A/β-catenin signaling pathway in vivo.

CXCR4/WNT5A/β-catenin axis activation is associated with palbociclib resistance in patients with breast cancer

To elucidate the clinical significance of the above findings, we enrolled seven patients with advanced breast cancer who progressed with palbociclib treatment and collected paired naive and progressed tumor tissues of them. All samples were collected with signed informed consent according to the internal review and ethics boards of Sun Yat-Sen Memorial Hospital. Then we conducted immunohistochemistry to analyze the protein levels of CXCR4, WNT5A and β-catenin. The stain scores were calculated as previous described [45]. Compared to the tumor before palbociclib treatment, the stain scores of CXCR4, WNT5A and β-catenin of palbociclib-resistant tumor all were significantly higher (Fig. 7a, b). This result indicates that CXCR4/WNT5A/β-catenin axis activation is related to palbociclib resistance in patients with breast cancer.

Discussion

In this study, we demonstrate that tumor stemness upregulation is closely related to CDK4/6 inhibitors resistance and identify that CXCR4 is responsible for this phenotype maintenance by activating the downstream WNT5A/β-catenin pathway. Inhibiting CXCR4 has the potential to reduce stemness and reverse palbociclib resistance in CDK4/6 inhibitors-resistant breast cancer, both in vitro and in vivo.

CDK4/6 inhibitors significantly prolonged PFS and OS in patients with HR-positive/HER2-negative breast cancer, but as a target therapy, resistance to CDK4/6 inhibitors is inevitable, posing a substantial clinical dilemma. Previous research has elucidated that CDK4/6 inhibitors resistance primarily stems from mechanisms such as RB1 deletion, heightened activity of its targets (e.g. CDK6), overexpression or amplification of downstream components like CDK2 and Cyclin E1, or activation of alternative growth signals such as the FGFR pathway. In our prior work, we have delved into the mechanisms of endocrine resistance, uncovering the significant role of LncRNAs in conferring tamoxifen and palbociclib resistance by stabilizing cyclin proteins through post-translational regulation [45]. Our previously study also demonstrated that overexpressed Cyclin D1 and CDK4 proteins are responsible for the resistance to CDK4/6 inhibitors in breast cancer and can be reversed by PI3K/mTOR inhibitors [35]. However, the complexity of the underlying mechanisms behind CDK4/6 inhibitors resistance, influenced by tumor heterogeneity, prompted us to explore this phenomenon further in this study.

Stem cells was reported to be one of the most important reasons of cancer metastasis and therapy resistance [22, 26]. In our study, we observed an upregulation of stem cell-like properties in the established palbociclib-resistant ER-positive breast cancer cells. Importantly, we further identified that CXCR4 as a crucial gene responsible for this. CXCR4 inhibition by siRNAs or small-molecule inhibitors, AMD3100, significantly reduced the proportion of tumor stem cell and downregulated the expression of tumor stemness markers. Importantly,

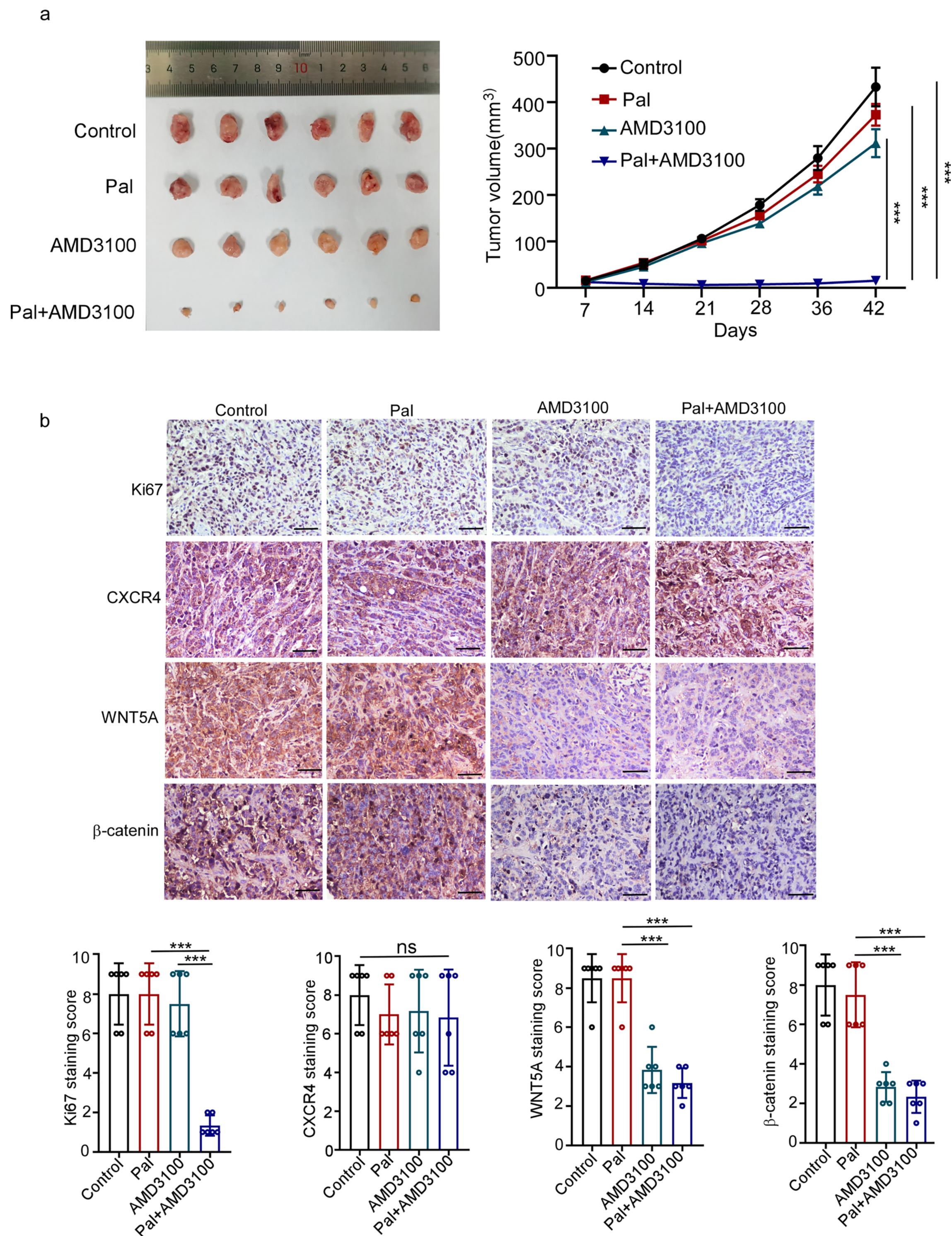

Fig. 6 CXCR4 inhibitors restore palbociclib sensitivity in palbociclib-resistant breast cancer in vivo. MCF7-palR cells were inoculated into the mammary fat pads of BALB/c mice. When tumors became palpable, mice were administrated with palbociclib (100 mg/kg, once a day) by gavage or AMD3100 (5 mg/kg, every 3 days) by intraperitoneal injection. **a** Tumor picture at the left panel and growth curve at the right panel. **b** IHC staining showing the expression of CXCR4, WNT5A, β-catenin and ki67. Representative images of IHC staining at the top panel and statistical analysis at the bottom panel. n = 6 mice per group, means ± s.d. were shown, and *p* values were determined by one-way ANOVAs, ****p* < 0.001 and ns for *p* > 0.05. Scale bars represent 50 μm

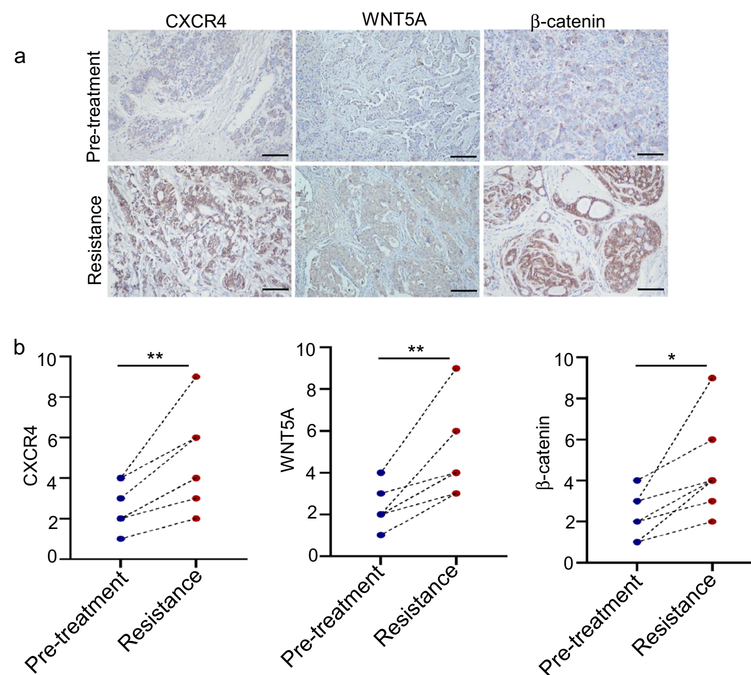

Fig. 7 CXCR4/WNT5A/β-catenin axis activation is associated with palbociclib resistance in patients with breast cancer. Patients with advanced breast cancer who progressed with palbociclib treatment were enrolled, and the paired naive and progressed tumor tissues were collected. Then immunohistochemistry was performed to analyze the protein expression of CXCR4, WNT5A and β-catenin. The stain scores were calculated and the expression difference of them between the before palbociclib treatment group and the palbociclib-resistant group was analyzed. Representative images showed in panel **a**, and quantification analysis showed in panel **b**. The *p* values were determined by paired Student's test, $^{**}p < 0.01$, $^{*}p < 0.05$. Scale bars represent 50 µm

CXCR4 inhibition led to the reversal of palbociclib resistance by attenuating tumor stemness. The CXCL12-CXCR4 signaling axis has primarily been recognized for its role in mediating communication between tumor cells and the microenvironment, thereby regulating cancer metastasis [29–31]. In this study, we firstly uncovered the role of CXCL12-CXCR4 axis in CDK4/6 inhibitors resistance by regulating tumor stemness in ER-positive breast cancer.

Our RNA sequencing analysis revealed a series of WNT genes were upregulated in palbociclib-resistance MCF-7 cells, such as WNT5A, WNT5B, WNT16, WNT8B, WNT10A and WNT11, prompting that WNT signal maybe important in acquired CDK4/6 inhibitors resistance. RT-qPCR and western blotting assay demonstrated that WNT5A/β-catenin is activated and could be impaired by CXCR4 inhibition. The WNT/β-catenin pathways play a critical role in controlling embryonic and organ development, as well as cancer progression [46]. Numerous studies have implicated that WNT/β-catenin signaling is also important in multiple biological characteristics in breast cancer, including immune regulation, stemness maintenance, and therapeutic resistance [46, 47]. Notably, prior research has demonstrated that β-catenin was activated in endocrine-resistant breast cancer, and combining β-catenin inhibitors with CDK4/6 inhibitors has shown synergistic effects [48] . Our current research has unveiled that CXCR4 inhibition leads to the downregulation of the WNT5A/β-catenin pathway, thereby restoring palbociclib sensitivity in resistant cells. These findings further underscore the significance of the WNT5A/β-catenin axis in breast cancer progression.

There are some limitations of this study. Firstly, the clinical sample analysis suggested that highly activation of CXCR4/ WNT5A/β-catenin axis in patients with palbociclib-resistant breast cancer, underscoring the clinical significance of this research. However, we have not been able to enroll a sufficient number of patients with paired native and palbociclib-resistant tumor samples, necessitating a larger patient cohort for a more comprehensive analysis. Additionally, our RNA sequencing analysis also revealed dysregulation of other genes, such as BCL2, MAPK10, GNB4, in palbociclib-resistant breast cancer cells, suggesting the need for further research to uncover additional potential mechanisms contributing to CDK4/6 inhibitors resistance.

Conclusions

This study demonstrates that CXCR4 is responsible for upregulating tumor stemness and contributes to CDK4/6 inhibitors resistance by activating the downstream WNT5A/β-catenin pathway. Inhibiting CXCR4 has the potential to reduce stemness and reverse palbociclib resistance in CDK4/6 inhibitors-resistant breast cancer, both in vitro and in vivo. These findings provide new insights into the mechanisms underlying CDK4/6 inhibitors resistance in ER-positive breast cancer and highlight the crucial role of CXCR4 in maintaining tumor stemness and promoting acquired palbociclib resistance. Targeting CXCR4 may offer a promising therapeutic approach for advanced CDK4/6 inhibitors-resistant breast cancer.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13058-025-01965-3.

Additional file 1

Additional file 2

Acknowledgements

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Author contributions

Q.S., Z.C and Y.L. conceived and designed the experiments. Q.S. and W.Y. performed most of the experiments; Y.O. and Z.C. collected the patient samples and performed clinical analysis. Q.S. and Z.C. wrote and completed the paper. All authors read and approved the final manuscript.

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Availability of data and materials

All data relevant to the study are included in the article or uploaded as supplementary information.

Declarations

Ethics approval and consent to participate

All clinical samples were collected with signed informed consent and ethics approval from Sun Yat-Sen Memorial Hospital.

Consent for publication

The content of this paper has not been submitted to any other scientific publications. All the authors have declared that no financial conflict of interest exists. All authors have approved the submission of this work for publication in Breast Cancer Research.

Competing interests

The authors declare no competing interests.

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