Emerging Combination Strategy: FANCI Inhibition Induces PARP1 Redistribution to Enhance Efficacy of PARP Inhibitors in Breast Cancer

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Research Article

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Abstract

Background

While PARP inhibitors have made advancements in the treatment of breast cancer, challenges such as chemotherapy resistance and limited application persist. FANCI, a DNA repair protein associated with breast cancer development, represents a potential target for novel combination therapeutic strategies. However, the role of FANCI in breast cancer and its impact on the efficacy of PARP inhibitors require further investigation.

Methods

In this study, we analyzed FANCI expression in breast cancer tissues and cell lines, and its correlation with clinical parameters and patient prognosis. Lentiviral vectors were utilized and functional assays were performed to evaluate the effects of FANCI modulation on breast cancer cell growth and metastasis. Co-immunoprecipitation assays and protein interaction analysis were conducted to identify the interaction between FANCI and PARP1 and determine the specific binding region. The functionality and nuclear distribution of PARP1 were assessed upon FANCI modulation. Finally, the sensitivity of breast cancer cells to the PARP inhibitor talazoparib upon FANCI knockdown was evaluated in vitro and in vivo.

Results

Our findings demonstrated that FANCI was overexpressed in breast cancer and associated with poor prognosis. FANCI significantly promoted breast cancer cell proliferation both in vitro and in vivo. We identified the interaction between FANCI and PARP1, specifically at the FANCI HD2 binding site. FANCI inhibition led to reduced nuclear localization of PARP1 and decreased PARP1 activity. Importantly, combination treatment with FANCI knockdown and talazoparib significantly inhibited cancer growth in vitro and in vivo. Additionally, we found that the CDK4/6 inhibitor palbociclib, which effectively suppresses FANCI protein expression, exhibited a robust synergistic effect with talazoparib both in vitro and in vivo.

Conclusion

FANCI is a novel therapeutic target for breast cancer. Inhibition of FANCI regulates PARP1 redistribution and activity, making cells more responsive to PARP inhibitors. This combination therapeutic strategy shows potential in enhancing the effectiveness of PARP inhibitors for breast cancer treatment, regardless of BRCA mutations.

1. Background
Breast cancer has emerged as the most prevalent malignant tumor in 138 countries, surpassing lung cancer [1]. It is also the leading cause of cancer-related deaths among women, with incidence rates (11.7%) and mortality rates (6.9%) on the rise [1]. Projections indicate that by 2040, breast cancer incidence and mortality will peak in Asia, particularly in China [2, 3]. These statistics underscore breast cancer as a major global public health concern [4].

In recent years, translational and clinical research has witnessed remarkable advancements with the introduction of PARP (polyADP-ribose polymerase) inhibitors [5]. PARP inhibitors exert their effect by inhibiting the enzymatic function of PARP and enhancing its trapping on DNA. This trapping of PARP on DNA accumulates DNA single-strand breaks in cancer cells, which subsequently progress into double-strand breaks (DSBs) during cell division [6, 7]. As a result, PARP inhibitors can specifically target cells with compromised homologous recombination (HR) repair, ultimately inducing cell death [8, 9].

Yet, several challenges persist in the field. Firstly, chemotherapy resistance, which contributes to the high mortality rate among patients [10], is also observed with PARP inhibitors [8, 11, 12]. PARP inhibitor resistance arises through various mechanisms, including the restoration of HR repair and stabilization of DSBs [11–13]. Thus, understanding the mechanisms underlying drug resistance and developing innovative strategies to overcome it are pivotal in improving treatment outcomes for breast cancer patients. Secondly, while these targeted therapies have demonstrated efficacy in specific patient populations, such as those with BRCA (Breast cancer susceptibility protein) mutations [14, 15], their application remains limited. Nevertheless, emerging data indicate that PARP inhibitors may hold promise for treating breast cancers that lack BRCA1/2 mutations [5, 16–18]. This could potentially be attributed to alternative molecular defects in HR repair [19–21]. Innovative combination therapeutic strategies that target HR repair disruption in cancer cells and exploit the vulnerabilities of PARP inhibitors have shown promising outcomes in clinical trials across various cancer types, including breast cancer [5, 7, 22].

In line with these goals, our study explores a novel combination therapeutic strategy involving the inhibition of Fanconi Anemia Complementation Group I (FANCI) to enhance the efficacy of PARP inhibitors. FANCI is a well-acknowledged protein that plays an essential role in HR repair in cancer cells [23, 24], suggesting its potential impact on PARP inhibitors’ effectiveness. It is also among the 628 potential targets identified by Behan et al. through genome-scale CRISPR-Cas9 screens in 324 human cancer cell lines derived from 30 different types of cancer for cancer drug therapy [25]. The existing literature strongly supports the association between breast cancer and DNA repair deficiency mediated by the Fanconi anemia (FA)/BRCA pathway [26–28], which FANCI belongs to. In addition, frequent germline mutations in FANCI have been reported among breast cancer patients, suggesting its potential inclusion in predisposition screening in our investigation, we specifically identified the therapeutic potential of targeting FANCI in the context of breast cancer [29].

In this study, we observed elevated expression of FANCI in breast cancer, which correlated with a poor prognosis. It could increase breast cancer cell proliferation and metastasis. We also identified the interaction between PARP1 and FANCI, specifically at the F5 binding site corresponding to the FANCI
helical domain 2 (HD2). Inhibition of FANCI had significant effects on the nuclear localization and protein function of PARP1. Importantly, our experiments demonstrated that breast cancer cells, even in the absence of BRCA mutations, became sensitized to talazoparib, a PARP inhibitor. These findings hold considerable significance since FANCI, like BRCA1/2, belongs to the FA/BRCA pathway and actively participates in crucial DNA repair processes. We propose that inhibiting FANCI may induce a “BRCA-mutated-like” state in breast cancer cells, rendering them more responsive to PARP inhibitors. Notably, our study identified that the cyclin-dependent kinases 4 and 6 (CDK4/6) inhibitor palbociclib could increase the sensitivity of breast cancer cells to talazoparib by inhibiting FANCI in both in vitro and in vivo experiments.

2. Material and methods

Clinical samples and public datasets

Breast cancer tissues and adjacent normal tissues were procured from Nanjing Medical University First Affiliated Hospital. The inclusion criteria for patient samples included those who had not received neoadjuvant therapy. Following resection, the collected samples were promptly frozen in liquid nitrogen to preserve their molecular integrity. Ethical considerations were given utmost importance, and all patients involved in this study provided written informed consent. The study protocol was approved by the Ethics Committee of Nanjing Medical University First Affiliated Hospital. Public datasets were obtained from Oncomine (https://www.oncomine.org/) [30], UALCAN (https://ualcan.path.uab.edu/index.html) [31], bc-GenExMiner 4.9 (http://bcgenex.ico.unicancer.fr/BC-GEM/GEM-Accueil.php?s=1) [32], Kaplan–Meier plotter (http://kmplot.com/analysis/) [33, 34], Search Tool for the Retrieval of Interacting Genes (STRING, https://string-db.org) [35], Comparative Toxicogenomics Database (CTD, http://ctdbase.org) [36], Genomics of Drug Sensitivity in Cancer (GDSC, https://www.cancerrxgene.org/) [37], SWISS-MODEL (https://swissmodel.expasy.org/) [38] and The Cancer Genome Atlas (TCGA) [39] for analysis.

Cell culture

Human breast cancer cell lines (MCF-7, ZR-75-1, BT474, MDA-MB-231, MDA-MB-453, and T-47D) and a normal mammary epithelial cell line (MCF-10A) were procured from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The SUM-1315 cell line was generously provided by Stephen Ethier (University of Michigan, Ann Arbor, MI, USA). The HEK293T cell line was maintained and preserved by our laboratory. MCF-7, ZR-75-1, BT474, MDA-MB-231, MDA-MB-453, SUM1315, and HEK293T cells were cultured in DMEM (Gibco, USA), while T-47D cells were cultured in RPMI 1640 (Gibco, USA). The culture media for all cell lines contained 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Cell viability assay and determination of drug synergy

Cell viability assays were performed using the Cell Counting Kit-8 (CCK-8) (Vazyme, China) following the manufacturer’s instructions. Briefly, 2000 cells were seeded into each well of a 96-well plate, with 100 µL
of DMEM supplemented with 10% FBS. At the specified time points, the medium was replaced with 100 µL of DMEM containing CCK-8 (90 µL DMEM and 10 µL CCK-8), and the cells were incubated for 2 h. The absorbance was measured spectrophotometrically at 450 nm.

To evaluate the synergistic effect of the two drugs, the combination index (CI) was determined using the app SiCoDEA [40]. The CI provides a quantitative measure of drug synergy, and it is calculated using Loewe additivity model and the Log-logistic [01] method as the drug-response curve model [41].

**RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA from cells or tissues was isolated using Trizol reagent (Takara, Japan). Complementary DNA (cDNA) was synthesized using the HiScript Q RT SuperMix (Vazyme, China). Subsequently, qRT-PCR was conducted using the AceQ qPCR SYBR Green Master Mix (Vazyme, China). β-actin were employed as endogenous controls for the relative expression of mRNA, respectively. The specific primer sequences utilized in this study are listed in Supplementary Table S1. The relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method, normalizing to the appropriate control and calculating the fold change.

**Western blot analysis**

The total protein from breast cancer cells was extracted using RIPA buffer and separated by 10% SDS-PAGE. Subsequently, the proteins were transferred onto a PVDF membrane (Bio-Rad, USA). The membranes were blocked using 5% skimmed milk powder and then incubated with primary antibodies against FANCI (1:2000, Proteintech, China, 20789-1-AP), PARP1 (1:2000, Proteintech, China, 13371-1-AP), Histone H3 (1:2000, Proteintech, China, 17168-1-AP), γH2AX (1:2000, Proteintech, China, 10856-1-AP), GAPDH (1:1000, Proteintech, China, 10856-1-AP), β-actin (1:1000, Proteintech, China, 20536-1-AP), pADPr (1:200, SANTA CRUZ, USA, sc-56198), and FLAG-Tag (1:1000, Beyotime, China, AF0036) at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies (1:5000) (Cell Signaling Technology, USA, 7074P2) at room temperature for 2 h. Finally, the protein bands were visualized using ImmobilobTM Western Chemiluminescent HRP Substrate (Millipore, USA).

**Lentiviral infection and plasmid transfection**

Commercially available lentiviral vectors containing FANCI-coding and short hairpin RNA (shRNA)-targeting FANCI sequence (FANCI, FANCI-sh1, and FANCI-sh2) were obtained from NOVOBIO (Shanghai, China) to achieve up- or down-regulation of FANCI in breast cancer cells. Scrambled lentiviral constructs were employed as negative controls (shNC and vector) to assess the baseline response. All vectors were verified through DNA sequencing. When the breast cancer cell lines SUM-1315 and ZR-75-1 reached approximately 30 to 40% confluence, they were infected with FANCI-sh1, FANCI-sh2, shNC, FANCI, and the vector at an appropriate multiplicity of infection. Stable cell lines were established by treating them with 5 µg/ml Blasticidin (InvivoGen, USA, ant-bl-1) for 1 week. The expression of FANCI in the cells was evaluated by qRT-PCR and Western blot analysis. shRNA sequences used in this study are listed in Supplementary Table S2.
The FLAG-FANCI fragments vectors were obtained from Yormbio (Shanghai, China). Based on the protein structure provided by SWISS-MODEL (https://swissmodel.expasy.org/), we generated seven fragmented DNA sequences encoding truncated domains of FANCI, derived from its full-length sequence (NM 001113378.2). These truncated DNA fragments, tagged with the FLAG epitope, were then subcloned into vectors to create the FLAG-FANCI fragments expression plasmids. Supplementary Table S3 provides details about the domain and length of each truncated fragment.

**Cell proliferation assay**

Cell proliferation assays were performed using the Cell Counting Kit-8 (CCK-8) (Vazyme, China) according to the manufacturer’s instructions. Briefly, 2000 cells were seeded into each well of a 96-well plate, with 100 µL of DMEM supplemented with 10% FBS. At the designated time points, the medium was replaced with 100 µL of DMEM containing CCK-8 (90 µL DMEM and 10 µL CCK-8), and the cells were incubated for 2 h. The absorbance was measured spectrophotometrically at 450 nm.

**Colony formation assay**

For the colony formation assay, 500 cells were seeded in a six-well plate and cultured in DMEM medium supplemented with 10% FBS for approximately 2 weeks. Proliferating colonies were fixed with methanol and stained with 1% crystal violet (Beyotime, China). The number of colonies was quantified by visual observation of single-cell proliferation.

**EdU incorporation assay**

Cell proliferation was assessed using the 5-ethynyl-2’-deoxyuridine (EdU) assay (Beyotime, China). The incorporation of EdU into newly synthesized DNA provides a specific and quantitative measurement of cell proliferation. Breast cancer cells were seeded in 96-well plates at a density of $2 \times 10^4$ cells per well and cultured in DMEM medium supplemented with 10% FBS for 24 h. Following that, the cells were incubated with 50 µM EdU at 37°C for 2 h. Subsequently, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with 1× Apollo® reaction cocktail for 30 min. Finally, the cell nuclei were counterstained with DAPI, and the cells were visualized under a fluorescence microscope.

**Transwell assay**

For the Transwell assay, a total of $3 \times 10^4$ cells were suspended in 100 µL of serum-free medium and added to the upper compartment of each chamber. The lower chamber was filled with 600 µL of medium containing 20% fetal bovine serum. Transwell plates (Corning, USA) were placed in a humidified incubator with 95% air and 5% CO$_2$ at 37°C for 48 h to allow cell migration. After incubation, the chambers were carefully washed with PBS, fixed with formaldehyde, and stained with 0.5% crystal violet (Beyotime, China). The migrated cells were then imaged and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) [42].
Cell wound-healing assay

The cell wound-healing assay was employed to assess cell migration. Breast cancer cells were seeded in 6-well plates and cultured until they reached confluence. Subsequently, linear scratch wounds were generated using a 200 µL sterile pipette tip. After washing the cells three times with PBS, images were acquired using an inverted fluorescence microscope at 0 and 48 h (Olympus, Japan).

Immunofluorescent staining

For immunofluorescent staining, adherent breast cancer cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 1% bovine serum albumin (BSA) in PBS. The cells were then incubated with primary antibodies against γH2AX (2 µg, Proteintech, China, 10856-1-AP), FANCI (2 µg, Proteintech, China, 20789-1-AP), or PARP1 (2 µg, Proteintech, China, 13371-1-AP) in 1% BSA at 4°C overnight. Afterward, the cells were washed three times with PBS for 5 min each and incubated with the appropriate secondary antibody (Beyotime, China, A0516/A0468). DNA staining was performed using Gold Antifade Mountant with DAPI (Invitrogen, USA, P36931). Immunofluorescence imaging was carried out using a Leica Stellaris STED confocal laser scanning microscope (Leica Microsystems, Buffalo Grove, USA). The data was analyzed by Leica Application Suite X.

Animal experiment

All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Nanjing Medical University. In different stages of the study, we injected 1×10^6 wild-type SUM-1315 cells or cells expressing shNC or FANCI-sh1 vectors into the mammary fat pads of 4-week-old female BALB/c nude mice for the in vivo xenograft model. The mice were housed in cages with five animals each, maintained at a room temperature of 23°C ± 1°C, and provided with ad libitum access to standard rodent diet and water. Tumor volumes were monitored every 4 days by measuring the length and width of the tumors using calipers. The tumor volume was calculated using the formula: (length) × (width)^2/2. A total of three animal experiments were conducted at different stages.

In the first animal experiment, fourteen female BALB/c nude mice were randomly divided into two groups (seven mice each group): shNC group and FANCI-sh1 group, without any further treatment. This experiment aimed to elucidate the impact of FANCI on the proliferation of breast cancer cells in in vivo animal models.

In the second animal experiment, fifty-six mice were divided into four treatment groups using random assignment: shNC (control group), shNC + talazoparib (talazoparib group), FANCI-sh1 + sterile PBS (FANCI knockdown group), and FANCI-sh1 + talazoparib (combination treatment group). Each group was further divided into two subgroups (seven mice each subgroup): one receiving immediate treatment after cell implantation and the other receiving delayed treatment initiated when the tumors reached an average volume of 15 mm^3. Talazoparib was administered at a dosage of 0.33 mg/kg daily through
intraperitoneal injection. This experiment aimed to investigate the effect of FANCI on the therapeutic efficacy of PARP inhibitors in *in vivo* animal models, as well as to compare the outcomes between early treatment and delayed treatment strategies.

In the third experiment, a total of twenty-eight mice were injected with SUM-1315 wild-type cells and subsequently divided into four groups (seven mice per group) using random assignment. The groups included: Control (treated with sterile PBS), talazoparib (0.33 mg/kg/day, administered intraperitoneally), palbociclib (100mg/kg/day, administered orally), and talazoparib + palbociclib (combination treatment). Each group received the respective treatment once daily at the indicated dosage. The experiment was ceased after twenty-seven days, and tumors were harvested and imaged. Euthanasia of the mice and tumor collection were performed upon reaching the humane endpoint.

**Immunoprecipitation and co-immunoprecipitation assays**

Breast cancer cells were washed three times with cold PBS and then homogenized in IP lysis buffer (Beyotime, China) supplemented with protease and phosphatase inhibitor cocktails (Beyotime, China). To remove non-specific binding, total protein lysates were precleared by adding 1.0 µg of the appropriate control IgG (normal mouse or rabbit IgG, depending on the host species of the primary antibody) along with 20 µl of resuspended Protein A/G agarose beads (SANTA CRUZ, USA, SC-2003). Discard the pellet after 30 min incubation at 4°C. The precleared lysate was then used for each immunoprecipitation (IP) using the specific antibody of interest, and Protein A/G agarose beads were added to the cells, followed by overnight incubation at 4°C. The precipitated proteins were subsequently washed and analyzed by western blot. The mass spectrometry analysis was conducted by BGI Genomics (Shenzhen, China). For FLAG-tag protein IP Assay, cell extracts were immunoprecipitated with Anti-FLAG Agarose Gel (Beyotime, China, P2202S-4) at 4°C overnight. The immunoprecipitates were thoroughly washed with lysis buffer five times, eluted with SDS loading buffer, boiled at 99°C for 5 min, and then immunoblotted with the indicated antibodies. The samples were incubated with primary antibodies against FANCI (2µg, Proteintech, China, 20789-1-AP), PARP1 (2µg, Proteintech, China, 13371-1-AP), normal rabbit IgG (2µg, Beyotime, China, A7016).

**Statistical Analysis**

Statistical analysis was conducted using SPSS 24.0 and GraphPad Prism 8.0. The experimental data are presented as mean ± standard deviation (SD). Group comparisons were assessed using Student’s t-test or ANOVA as appropriate. A significance level of P < 0.05 was considered statistically significant.

3. Results

**FANCI is overexpressed and predicts poor prognosis in breast cancer**

Firstly, the data analysis with ONCOMINE indicated upregulated expression of FANCI in 17 cancer types, particularly in breast cancer (Fig. 1A). Furthermore, compared to normal tissues, higher FANCI expression
was observed in breast cancer tissues by analysis of TCGA samples (Fig. 1B). Also, FANCI expression showed significant correlations with clinical parameters such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), with particularly elevated expression in basal-like breast cancer subtype (Fig. S1).

The qRT-PCR measurement of FANCI expression in 30 paired breast cancer tissues and adjacent normal tissues demonstrated that 93.3% (n = 28) of patients exhibited higher FANCI expression in breast cancer tissues compared to matched normal tissues (Fig. 1C). Consistently, qRT-PCR analysis of 7 breast cancer cell lines and the MCF-10A normal human mammary epithelial cell line revealed elevated FANCI expression in breast cancer cell lines (Fig. 1F). Western blot assay further confirmed increased FANCI protein expression in breast cancer tissues and cell lines (Fig. 1D and E).

Utilizing the Kaplan-Meier plot analysis, we found that breast cancer patients with high FANCI expression exhibited a tendency towards poor relapse-free survival (RFS), post-progression survival (PPS), distant metastasis-free survival (DMFS), and overall survival (OS) (Fig. 1G, H, I, and J). These findings highlight the potential prognostic significance of FANCI expression in breast cancer patients.

**FANCI promotes breast cancer cell growth in vitro and in vivo**

Lentiviral vectors containing shRNA-targeting FANCI (FANCI-sh1 and FANCI-sh2) and FANCI-coding sequence (FANCI) were transfected into the cells to achieve knockdown and overexpression of FANCI, respectively. Western blot assay and qRT-PCR demonstrated reduced expression and overexpression of FANCI in the SUM-1315 and ZR-75-1 cell lines (Fig. 2A-B, Fig. S2A-C). Both the SUM-1315 and ZR-75-1 cell lines are non-BRCA mutant cell lines. CCK-8 assays manifested that knockdown of FANCI significantly suppressed cell proliferation in both SUM-1315 and ZR-75-1 cell lines (Fig. 2C). Similarly, colony formation assays revealed a notable inhibitory effect of FANCI knockdown on cell proliferation (Fig. 2D). Moreover, EdU incorporation assays showed a significant reduction in the number of EdU-positive cells following FANCI knockdown, indicating decreased DNA synthesis and cell proliferation (Fig. 2E-F).

Conversely, overexpression of FANCI was shown to promote the proliferation ability of breast cancer cells (Fig. S2D-J). This finding further supports the role of FANCI in enhancing breast cancer cell growth. In the in vivo experiment, the tumors in the FANCI-sh1 group exhibited a noticeably reduced growth rate and smaller size compared to the shNC group (Fig. 2G). The growth of tumors derived from FANCI-sh1 cells was significantly slower than that of the control group (Fig. 2I), and the average tumor weight was significantly lower 27 days after injection (Fig. 2H).

**FANCI accelerates breast cancer cell migration in vitro and in vivo**

Given that metastasis is a critical characteristic of breast cancer, we investigated the impact of FANCI on breast cancer cell migration. In the wound healing assay, the migratory capacity of breast cancer cells significantly decreased upon knockdown of FANCI (Fig. 3D-G). Additionally, transwell assays were performed to evaluate the effect of FANCI on breast cancer cell metastasis. Remarkably, a notable reduction in the number of migrated cells was observed following FANCI knockdown in transwell assays.
Conversely, overexpression of FANCI enhanced the migration abilities of breast cancer cells (Fig. S3A-G).

**FANCI modulates the nuclear distribution and functionality of its interacting protein, PARP1**

Given FANCI's role in DNA repair and its propensity for protein-protein interactions (PPI) [43], we performed an immunoprecipitation assay to identify its potential binding partners. FANCI antibodies were used to immunoprecipitate SUM-1315 and ZR-75-1 cell lines, and subsequent mass spectrometry analysis identified 129 candidates after excluding non-specific binding (Fig. 4A). Interestingly, based on Qscore rankings, which indicate confidence levels [44], we discovered that PARP1 exhibited a high potential for interaction with FANCI (Fig. S4A). With the objective of exploring FANCI as a potential drug target for breast cancer, our subsequent study focused on the interaction between FANCI and PARP1 to expand the application of PARP inhibitors in breast cancer.

Co-immunoprecipitation (co-IP) assay and Western blot analysis supported the specificity of the physical interaction between FANCI and PARP1 (Fig. 4D). Furthermore, breast cancer samples in TCGA exhibited a positive correlation in the expression of FANCI and PARP1 (R = 0.58, p-value < 0.01) (Fig. 4C and S4B). To gain insights into the functional implications of FANCI and its interactors, we conducted functional analysis using the KEGG and GO databases (Fig. S4C-D). Employing the STRING database, we predicted a high potential connection between FANCI and PARP1 (Fig. 4B). Notably, these two proteins share similar physiological functions. PARP inhibitors primarily exert their effects through synthetic lethality with BRCA1/2, which are part of the FA complementation group that includes FANCI [45].

With a confirmed protein interaction between FANCI and PARP1, our next objective was to identify their specific binding region. To achieve this, a series of plasmids containing different-length fragments of FANCI was constructed, each tagged with FLAG (Fig. 4E). Overexpression of these seven plasmids in HEK293T cells was followed by immunoprecipitation, which demonstrated that only the FANCI F5 fragment (555-803aa) (Fig. 4F) interacted with endogenous PARP1, while the remaining truncated fragments failed to bind.

Moreover, we examined FANCI's regulation of PARP1 protein expression. Surprisingly, knockdown of FANCI did not result in significant changes in the protein and mRNA expression of PARP1 (Fig. 5A-D). However, upon FANCI knockdown, the activity of PARP1, measured by pADPr protein expression, notably decreased [46]. This led us to investigate FANCI's impact on the nuclear-cytoplasmic distribution of PARP1, given PARP1's primary nuclear function [47].

Western blot analysis revealed a dramatic reduction in the nuclear localization of PARP1 following FANCI knockdown (Fig. 5E), while no significant change was observed in the total protein level of PARP1. Immunofluorescence double staining and confocal microscopy analysis confirmed these results, demonstrating a highly colocalization of FANCI and PARP1 signals, and a decrease in the distribution of PARP1 within the nucleus upon FANCI knockdown (Fig. 5F-I). Therefore, we conclude that the alteration in
PARP1 functionality mediated by FANCI is attributed to the regulation of its nuclear-cytoplasmic distribution rather than changes in the total protein level.

**FANCI inhibition enhances the sensitivity of breast cancer cells to talazoparib**

Based on our findings, we propose that FANCI can regulate PARP1 functionality, potentially further impacting the effectiveness of PARP inhibitors. To explore this, we screened the GDSC database for FANCI-affected PARP inhibitors. Analysis revealed a notable increase in the IC50 value of the PARP inhibitor talazoparib (TALZENNA, Pfizer) in breast cancer cells harboring the cnaPANCAN411 mutation compared to the wild-type (P values = 0.050520) (Fig. 6A and S5A). Specifically, the cnaPANCAN411 mutation event contains the expression mutation of FANCI in the GDSC database. Remarkably, under CCK-8 analysis, the proliferation after FANCI knockdown decreased significantly compared to the control group under talazoparib treatment at 400 nM (Fig. 6B-C). Additionally, we analyzed the formation of γH2AX foci in cells treated with talazoparib (400 nM/48 h) through Western blotting (Fig. 6D) and immunofluorescence staining under fluorescence microscopy (Fig. 6E-H, S5B-C). Following FANCI knockdown, more γH2AX foci were observed under talazoparib treatment compared to the control group, indicating enhanced DNA damage after treatment [48].

Subsequently, we assessed the efficacy of PARP inhibition and FANCI knockdown *in vivo*. Mice were divided into four treatment groups: shNC (control group), shNC + talazoparib (talazoparib group), FANCI-sh1 + sterile PBS (FANCI knockdown group), and FANCI-sh1 + talazoparib (combination treatment group). Each group was further divided into two subgroups: one receiving immediate treatment after cell implantation and the other receiving delayed treatment initiated when the tumors reached an average volume of 15 mm³.

To our surprise, talazoparib treatment particularly inhibited tumor growth in the early treatment scenario (P < 0.05; Fig. 6I-M). In the early treatment group, tumors from FANCI knockdown and combination treatment groups displayed reductions of 65% and 93%, respectively, in weight compared to the control group. Compared with the talazoparib group, the combination group showed an 84% reduction in tumor weight in the early treatment setting. Moreover, the addition of PARP inhibition to FANCI knockdown further reduced tumor weight by approximately 79% in the early treatment group (Fig. 6K). These *in vivo* results align with the *in vitro* data, indicating that FANCI knockdown could inhibit the progression of breast cancer cells, and the addition of a PARP inhibitor enhances this effect. Based on these findings, we suggest that FANCI inhibition leads to decreased nuclear distribution of PARP1, who physiologically modifies various nuclear proteins [49–52], resulting in reduced PARP1 function. This enhances the sensitivity of breast cancer cells to PARP inhibitors, including talazoparib.

**Palbociclib increases the sensitivity of breast cancer cells to talazoparib by FANCI inhibition**

Our study so far highlights the significance of FANCI in determining breast cancer cells sensitivity to talazoparib. With the primary aim of broadening breast cancer treatment strategies, we investigated the potential of PARP inhibitors to enhance existing therapeutic approaches.
We initially searched the CTD database to identify potential drugs capable of modulating FANCI expression. Our search revealed that the CDK4/6 inhibitor palbociclib (IBRANCE, Pfizer) could decrease FANCI expression [53]. To validate the effectiveness of the combination strategy using talazoparib and palbociclib, we first extracted protein samples from SUM-1315 and ZR-75-1 wild-type cells after 48 h of co-culture with 5 µM palbociclib. We observed a reduction in FANCI protein expression following palbociclib treatment in breast cancer cell lines (Fig. 7A). Subsequently, we conducted CCK-8 experiments to determine the proliferation curves of SUM-1315 (Fig. 7B) and ZR-75-1 (Fig. 7C) under talazoparib and palbociclib treatment when used alone and in combination. Additionally, we calculated the combination index (CI) using the Loewe additivity model and the log-logistic (log-logistic [01]) method as the drug-response curve model using the app SiCoDEA. The combination of talazoparib with palbociclib exhibited significant synergistic effects in the SUM-1315 cell line. Moreover, this synergistic effect was most pronounced at specific concentrations: talazoparib (500 nM) and palbociclib (5 µM) (Fig. 7D and S6A). Finally, we validated the effects of the combination therapy on nude mice and obtained consistent results (Fig. 7E-G).

4. Discussion

PARP inhibitors have demonstrated effectiveness in specific breast cancer patient groups, but their usage is currently limited. In this study, we proposed a novel approach to enhance the efficacy of PARP inhibitors in breast cancer by targeting FANCI. Our findings revealed increased FANCI expression in breast cancer, which correlated with unfavorable prognosis, enhanced cell proliferation, and metastasis. We uncovered the interaction between FANCI and PARP1 and demonstrated that inhibiting FANCI could influence the nuclear localization and function of PARP1. Importantly, we observed that breast cancer cells without BRCA mutations became more responsive to the PARP inhibitor talazoparib upon FANCI inhibition. Furthermore, our study revealed that palbociclib, a CDK4/6 inhibitor, could heighten the sensitivity of breast cancer cells to talazoparib by inhibiting FANCI expression.

Tumor cells exploit DNA repair to survive genetic damage caused by various factors, including replicative stress and chemotherapies. PARP1, known for its role in DNA repair and promoting cancer cell proliferation [54–56], has gained attention with the development of PARP inhibitors. The emergence of PARP inhibitors as a distinct therapeutic approach has brought hope to a significant number of breast cancer patients who are resistant to frontline chemotherapy [7].

However, PARP inhibitors face two main limitations: common resistance and restricted use. Resistance often arises from HR repair restoration and DSBs stabilization, reducing inhibitor effectiveness [11–13]. Additionally, PARP inhibitors are primarily used in BRCA1/2 mutation patients, limiting benefits to a subset of breast cancer patients [15].

Fortunately, emerging reports suggest potential solutions. Unexpectedly positive outcomes have been observed when administering PARP inhibitors to non-BRCA-mutated advanced cancer patients resistant to frontline therapies [57–59]. These findings suggest that PARP inhibitors possess additional therapeutic
mechanisms beyond synergistic effects in BRCA1/2-mutated cells, which could potentially be attributed to alternative abnormalities in HR repair [60–62], warranting further exploration. Also, clinical trials have demonstrated promising outcomes for novel combination therapeutic strategy that aim to disrupt HR repair and exploit the vulnerabilities of PARP inhibitors in various cancer types, including breast cancer [5, 22, 62].

In conclusion, for improving the drawbacks of PARP inhibitors and benefiting more breast cancer patients, an ideal target for combination therapy should possess three key characteristics. Firstly, this target should be directly implicated in breast cancer development, with its modulation resulting in suppressed proliferation and decreased malignancy [55, 56]. Secondly, this target should interact with PARP proteins, improving the effectiveness of PARP inhibitors through novel mechanisms [58]. In the optimal scenario, it should modulate HR repair, thereby improving the effectiveness of PARP inhibitors by enhancing the recognized mechanism [61, 62].

Firstly, to identify a target protein highly associated with breast cancer development, we conducted a comprehensive analysis of 628 potential targets identified from genome-scale CRISPR-Cas9 screens in 324 human cancer cell lines [25]. This analysis leads to the identification of a potential therapeutic candidate known as FANCI. FANCI, a DNA repair protein belonging to the FA complementation group, plays a critical role in repairing DNA DSBs through HR and is primarily associated with maintaining genomic stability [63, 64], suggesting its potential impact on PARP inhibitors’ effectiveness. To further examine the involvement of FANCI in breast cancer, we performed open database analysis, WB analysis, and qRT-PCR, which demonstrated FANCI overexpression in breast cancer cells and its correlation with poor prognosis. In addition, functional assays were employed to investigate its association with cell proliferation and metastasis. Indeed, our results showed elevated expression of FANCI could promote breast cancer cell proliferation and metastasis, revealing the novel role of FANCI and underscoring its potential as a therapeutic target in breast cancer.

More importantly, using co-IP and FLAG-tag protein IP assays, we successfully identified the interaction between FANCI and PARP1, specifically at the FANCI HD2 binding site. Our immunofluorescence staining and confocal microscopy analysis revealed notable effects of inhibiting FANCI on the nuclear localization and protein function of PARP1. Building upon these encouraging results, we inquired into the combined impact of inhibiting FANCI and PARP inhibitors. Our in vivo xenograft experiment demonstrated increased sensitivity of breast cancer cells to the PARP inhibitor talazoparib, even in the absence of BRCA mutations. FANCI knockdown and talazoparib treatment significantly reduced tumor weight. Based on these discoveries, our findings indicate that suppressing FANCI leads to a decrease in the nuclear distribution of PARP1, which is known to play a physiological role in modifying various nuclear proteins [49–52]. As a consequence, PARP1 function is reduced, enhancing the sensitivity of breast cancer cells to PARP inhibitors, including talazoparib. Additionally, our investigation revealed that the CDK4/6 inhibitor palbociclib could reduce FANCI expression. Palbociclib is an oral inhibitor that selectively targets CDK4/6 [65, 66]. It has shown promising efficacy in inhibiting the growth of estrogen receptor positive breast cancer cells, overcoming endocrine resistance, and synergizing with antiestrogens [67–70]. Ongoing
research investigates its synergistic potential with various tumor therapeutics [71] and its impact on the tumor immune microenvironment [72–74]. Notably, recent studies have demonstrated the therapeutic synergy of palbociclib with olaparib in triple-negative breast cancer [75] and in castration-resistant prostate cancer and neuroendocrine prostate cancer [76]. Considering these findings and our own results, we hypothesize that palbociclib can enhance the sensitivity of breast cancer cells to talazoparib by modulating FANCI. Our consistent in vitro and in vivo experiments demonstrated that palbociclib effectively increased the sensitivity of breast cancer cells to talazoparib by inhibiting FANCI. These findings underscore the significant role of FANCI as a therapeutic target and its potential to enhance the efficacy of PARP inhibitor-based treatments in breast cancer.

In the context of cancer treatment, PARP inhibitors have been effective in a subset of non-BRCA-mutated patients who have developed resistance to frontline therapies [77]. However, the mechanisms behind this benefit remain unclear. Our experiments suggest a possible explanation: FANCI, highly expressed in wild-type breast cancer cells, increases the nuclear localization of PARP1 and enhances HR repair capacity. This leads to increased cancer cell proliferation and reduced susceptibility to DNA damage, thereby compromising the effectiveness of PARP inhibitors. Notably, FANCI, like BRCA1/2, belongs to the FA/BRCA pathway and actively participates in crucial HR repair processes. We hypothesize that inhibiting FANCI could induce a state resembling “BRCA-mutated-like” characteristics in breast cancer cells. This alteration weakens the functionality of PARP1 and renders the cells more sensitive and responsive to the effects of PARP inhibitors. Consequently, the dual benefits of inhibiting FANCI, namely suppressing a protein associated with breast cancer development and poor prognosis, and increasing the sensitivity to PARP inhibitors, hold promising implications for improving breast cancer treatment outcomes (Fig. 7H).

Our investigation of FANCI also offers valuable insights and lays the foundation for future research in breast cancer drug therapy. Specifically, targeted “FANCI inhibitors” could be designed to address PARP inhibitor resistance in specific scenarios, utilizing the binding site we identified between FANCI and PARP1. Additionally, our findings warrant additional research to elucidate the mechanisms by which FANCI modulates the nuclear-cytoplasmic localization of PARP1. The F5 fragment of FANCI, corresponding to the FANCI HD2, plays a critical role in DNA repair processes and interacts with other proteins involved in regulating DNA repair-related signal transduction and cell cycle control [78]. It is possible that FANCI, upon binding to PARP1, modulates PARP1’s ubiquitination levels through DNA repair-related signal transduction, thereby regulating its nuclear transport (Fig. 7H). Moreover, investigating the role of BRCA1/2 in the FANCI-PARP1 interaction would be a valuable direction for future research.

5. Conclusion

In summary, our study addressed challenges associated with PARP inhibitors, expanded their potential applications, and provided insights into overcoming resistance. We introduced an alternative combination strategy for breast cancer treatment and revealed novel insights into the role of FANCI in breast cancer and its interaction with PARP1. By demonstrating the impact of FANCI inhibition on talazoparib response, we presented new possibilities for utilizing PARP inhibitors and overcoming resistance. These findings
not only contributed to the understanding of breast cancer pathogenesis but also identified a promising target for the diagnosis and treatment of breast cancer.

6. Abbreviations

FANCI: Fanconi Anemia Complementation Group I; FA: Fanconi Anemia; FANCI HD 2: FANCI helical domain 2; STRING: Search Tool for the Retrieval of Interacting Genes; CTD: Comparative Toxicogenomics Database; GDSC: Genomics of Drug Sensitivity in Cancer; TCGA: The Cancer Genome Atlas; ATCC: American Type Culture Collection; CCK-8 Cell Counting Kit-8; EdU: 5-ethynyl-2’-deoxyuridine; co-IP: co-immunoprecipitation; SD: standard deviation; TNBC: Triple-negative breast cancer; ER: Estrogen receptor; PR: Progesterone receptor; HER2: Human epidermal growth factor receptor 2; PARP: Poly-(ADP)-ribose polymerase; PARPi: PARP inhibitors; CDK: Cyclin-dependent kinase; HR: Homologous recombination; DSBs: Double-stranded breaks; CI: Combination index; qRT-PCR: Quantitative reverse transcription PCR; WB: Western blot; IP: Immunoprecipitation; shRNA: Short hairpin RNA; OE: Overexpression; KD: Knockdown; BRCA: Breast cancer susceptibility protein.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Animal Management Committee of Nanjing Medical University. All procedures involving human participants in this study were approved by the Research Ethics Committee of Nanjing Medical University First Affiliated Hospital. All patients included in the present study signed informed consent.

Consent for publication

All authors provided their consent to publish the study.

Availability of data and materials

Please contact the corresponding author (Qiang Ding, dingqiang@njmu.edu.cn) for data requests.

Competing interests

The authors declare no competing interest.

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Authors’ contributions

YZ H, JF W and QD designed the experiments. YZ H and MY S carried out the experiments and acquired the data. YZ H, PW X and RX X analyzed and interpreted the data. MY C and ZW W analyzed the TCGA dataset. JY Z, YH L and PY were responsible for clinical sample collection. JF W and QD conceived of the study, designed experiments, helped interpret data, and revised the draft manuscript. All authors reviewed and approved the final manuscript.

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Author details

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Figures
FANCI overexpression and poor prognosis prediction in breast cancer. **A** Comparison of FANCI expression in 20 common cancers and paired normal tissues using the Oncomine database. Red and blue bars represent the number of datasets with statistically significant (P < 0.05) increased and decreased FANCI levels, respectively. **B** FANCI mRNA expression analysis based on TCGA dataset. **C** mRNA expression levels of FANCI in 30 pairs of tissues and adjacent normal tissues. **D-E** Protein expression of FANCI in breast cancer cell lines and tissues. **F** Comparison of FANCI mRNA expression in MCF-10A and breast cancer cell lines. **G-J** Kaplan-Meier survival analysis demonstrating the correlation between FANCI expression and clinical outcomes in breast cancer patients. High FANCI expression is associated with poor relapse-free survival (RFS), post-progression survival (PPS), distant metastasis-free survival (DMFS), and overall survival (OS) based on TCGA dataset. (*p < 0.05, **p < 0.01, ***p < 0.001. Data are presented as mean ± SD).
Figure 2

FANCI-mediated enhancement of breast cancer cell proliferation. **A-B** Transfection efficiency of shNC and shRNA-targeting FANCI (FANCI-sh1 and FANCI-sh2) expression vectors in SUM-1315 cell line and ZR-75-1 cell line was determined using qRT-PCR and western blot analyses. **C** Effect of FANCI on breast cancer cell proliferation was investigated using the cck-8 assay. **D** Colony formation assay was performed to assess the proliferation ability of breast cancer cells following transfection. **E-F** EdU assay was utilized to
determine the effects of FANCI on breast cancer cell proliferation. G-I Orthotopic xenograft model comparing tumor growth in SUM-1315 cells transfected with FANCI-sh1 and shNC (negative control), as visualized by representative tumor images (G), endpoint tumor weights (H), and tumor growth curves (I). (Data presented as mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001).
FANCI-mediated enhancement of breast cancer cell migration. A-C Transwell assays illustrating the increased migration of breast cancer cells upon knockdown of FANCI in the SUM-1315 and ZR-75-1 cell lines. D-G Wound healing assays conducted to evaluate the impact of FANCI on cell motility at 0 and 48 hours in the ZR-75-1 (D-E) and SUM-1315 (F-G) cell lines. (Data are presented as mean ± SD. Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001).
Comprehensive analysis of FANCI interacting protein PARP1 using mass spectrometry. A Venn diagram showing proteins identified by immunoprecipitation and mass spectrometry in SUM-1315 and ZR-75-1 cell lines. B Protein-protein interaction (PPI) network of FANCI obtained from the STRING database. C Scatter plot and fitted curve depicting the correlation between FANCI and PARP1 expression levels in breast cancer patients from TCGA dataset. The region marked transparent blue represents the confidence interval (R=0.58, P<0.05). D Co-immunoprecipitation (co-IP) analysis of FANCI and PARP1 interaction by Western blotting. Lysates of FANCI and ZR-75-1 cells were immunoprecipitated with anti-FANCI antibody, and the presence of PARP1 protein was detected. Conversely, immunoprecipitation with anti-PARP1 antibody showed the presence of FANCI protein in the PARP1 complex. E Schematic representation of the different fragments of FANCI constructs. The lengths of the fragments are depicted to scale, and the FANCI-interacting domain on PARP1 is highlighted by the red bar (F5, 555-804aa). F Mapping of FANCI binding to PARP1. HEK293T cells were transfected with plasmids encoding various fragments of FLAG-FANCI, as indicated. The cells were then harvested for immunoprecipitation with FLAG beads, followed by immunoblotting with the indicated antibodies.
Figure 5

Functionality and nuclear localization of PARP1 governed by FANCI. 

A-B Impact of FANCI knockdown on the expression level and functionality of PARP1, as indicated by the protein expression of Poly (ADP-Ribose) Polymer (pADPr), assessed through western blot experiments in SUM-1315 (A) and ZR-75-1 (B) cell lines.

C-D Effect of FANCI knockdown on the mRNA expression level of PARP1 evaluated by qRT-PCR.

E Investigation of the influence of FANCI knockdown on the nuclear distribution of PARP1. Cytoplasmic
and nuclear proteins were extracted from SUM-1315 and ZR-75-1 FANCI knockdown cell lines and subjected to western blot analysis using anti-FANCI and PARP1 antibodies. GAPDH and Histone-H3 were used as loading controls. F-I Analysis of FANCI and PARP1 distribution through immunofluorescence double staining under confocal microscopy. The nucleus was stained with DAPI (blue). Quantification of nuclear aggregation of PARP1 in SUM-1315 (F) and ZR-75-1 (G) cell lines.

Figure 6

Enhanced sensitivity of breast cancer cells to talazoparib by FANCI inhibition. A Talazoparib IC50 values for breast cancer cells harboring the cnaPANCAN411 mutation compared to the wild-type identified by GDSC database. P values = 0.050520. B-C Cell proliferation curves of control and FANCI knockdown cell
lines (SUM-1315 and ZR-75-1) under talazoparib treatment. **Western blot analysis of γH2Ax expression in FANCI knockdown breast cancer cells treated with DMSO (control) or talazoparib for 48 hours.**

**E-H** Percentage of γH2AX fluorescence intensity as shown by immunofluorescence staining. Immunofluorescence staining with DAPI and γH2AX was performed on control group and FANCI knockdown cell lines (SUM-1315 and ZR-75-1) in different treatment groups. Compared to the control group, FANCI knockdown breast cancer cell lines exhibited increased γH2AX fluorescence intensity in response to talazoparib treatment. **I-M** Comparison of representative tumor images (I), tumor growth curves (J, L), and endpoint tumor weights (K, M) among different treatment groups. SUM-1315 cells stably expressing shNC or FANCI-sh1 were implanted into the mammary fat pads of 4-week-old female BALB/c nude mice (7 mice in each subgroup). Four groups of treatment were established: shNC (control group), shNC + talazoparib (talazoparib group), FANCI-sh1 + sterile PBS (FANCI knockdown group), and FANCI-sh1 + talazoparib (combination treatment group). Treatment was initiated either immediately after implantation (left) or when tumors reached an average volume of 15 mm$^3$ (right). (Data presented as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001.)
Figure 7

Synergistic enhancement of talazoparib sensitivity in breast cancer cells by FANCI inhibition with palbociclib. A. Downregulation of FANCI protein expression in wild-type SUM-1315 and ZR-75-1 cell lines after 48 hours of treatment with 5µM palbociclib, as observed by Western blot analysis. B-C. Cell growth curves of wild-type SUM-1315 and ZR-75-1 cells under different treatment conditions. D. Log CI values indicating the combination effect of talazoparib and palbociclib on SUM-1315 cell lines, measured at various drug concentrations for 72 hours. The size of the circle represents the strength of the CI value, where larger circles indicate higher CI values. E-G. Representative tumor images (E), tumor growth curves (F), and tumor weight graphs (G).
(F), and endpoint tumor weights (G) compared among different treatment groups in an *in vivo* animal tumor model. **H** Schematic presentation of proposed molecular mechanism underlying the interaction between FANCI and PARP1.

**Supplementary Files**

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