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Rack1 promotes breast cancer stemness properties and tumorigenesis through the E2F1-SOX2 axis

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Abstract

Background Breast cancer remains the most prevalent malignancy and the leading cause of cancer-related mortality among women worldwide. The primary factors contributing to the deterioration and death of patients with breast cancer are metastasis, recurrence, and drug resistance. These phenomena are closely related to the presence of breast cancer stem cells; however, the exact mechanisms regulating stemness remain to be elucidated. Rack1 (Receptor for Activated C Kinase 1), a well-known versatile scaffold protein, has been implicated in tumorigenesis and progression in numerous cancer types; however, its specific role in breast cancer stemness remains to be elucidated.

Methods Using bioinformatic and immunohistochemical approaches, we validated that the expression level of Rack1 is associated with cancer stemness and affects the prognosis of patients. Through a series of experimental methods including mammosphere formation assay, flow cytometry, qPCR, Western blotting, and CHX assays, we validated at the molecular and cellular levels the mechanism by which Rack1 influences cancer stemness via the E2F1/SOX2 axis. Furthermore, by designing and utilizing lentiviral constructs to establish xenograft tumor models in mice, we further confirmed in vivo the impact of the Rack1/E2F1/SOX2 axis on the tumorigenic capacity of breast cancer cells.

Results Our findings indicate that Rack1 plays a critical role in preserving the stemness characteristics of breast cancer cells. Mechanistically, the observed effects of Rack1 are achieved through the modulation of SOX2 expression, a master transcription factor that regulates cancer cell stemness and maintenance. We further demonstrate that Rack1 increases the stability of the E2F1 protein by inhibiting its ubiquitination and subsequent proteasome-mediated degradation, which in turn transcriptionally upregulates SOX2, thereby maintaining breast cancer cell stemness and tumorigenesis.

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Conclusion This study thus unveils a novel mechanism through which Rack1 executes its oncogenic function. This study also demonstrates that targeting the Rack1-E2F-SOX2 axis may be a potential strategy to inhibit breast cancer development and progression.

Keywords Breast cancer, Rack1, Stemness, E2F1/SOX2 axis, Ubiquitination

Introduction

According to the latest global cancer statistics in 2024, breast cancer is one of the most common types of cancer worldwide. Despite the spread of early screening and diagnosis and advances in treatment in recent years, breast cancer remains one of the leading causes of cancer-related death in women. Traditional therapeutic approaches, while effective in eliminating the majority of tumor cells, are often ineffective against a subpopulation of cells with stem-like properties, including self-renewal, differentiation, and chemoresistance, termed cancer stem cells (CSCs) [1]. On the one hand, the self-renewal capacity of CSCs can expand their population; on the other hand, their differentiation capacity can generate various heterogeneous cancer cell types to promote tumorigenesis and progression [2]. In addition, chemoresistance of CSCs is a major cause of treatment failure in breast cancer, which is closely related to cancer relapse or progression and ultimately endangers patients' lives. Thus, the strong tumorigenicity and chemoresistance of BCSCs is a major obstacle to the effective clinical treatment of breast cancer [3]. To date, although methods to isolate and characterize BCSCs are well established, the mechanisms that regulate the maintenance of BCSC stemness and their tumorigenic potential remain largely unknown. Therefore, there is an urgent need to elucidate the relevant regulatory mechanisms in order to find therapeutic approaches that specifically target BCSC. The receptor for activated C kinase 1 (Rack1) is a member of the tryptophan-aspartate repeat (WDR) family of proteins [4], which is a pivotal signaling hub that interacts with an variety of signaling molecules, including kinases, phosphatases, G proteins, and apoptosis-related molecules [5]. As a versatile scalfold protein, the functional effects of Rack1 on the chaperone proteins to which it binds can be categorized into four groups: facilitation of chaperone translocation, modification of chaperone activity, alteration of intermolecular interactions (enhancing binding affinity or facilitating dissociation), and regulation of the stability of bound proteins [6]. Notably, aberrant Rack1 expression has been documented in multiple malignancies, including lung adenocarcinoma, hepatocellular carcinoma, breast cancer and oral squamous cell carcinoma, and is positively associated with adverse patient outcomes [7, 8]. For instance, elevated Rack1 expression has been demonstrated to be closely associated with lymph node metastasis and has been identified as an independent prognostic factor for overall survival of lung adenocarcinoma patients [9]. In addition, Rack1-mediated ANXA2 phosphorylation has been shown to promote cholangiocarcinoma cell metastasis and poor prognosis [10]. Rack1 has also been shown to inhibit CSNK2B ubiquitination degradation, thereby promoting meningioma progression [11]. Furthermore, evidence suggests that Rack1 overexpression can serve as a valuable prognostic indicator in cases of melanoma [12]. The tumor-promoting activity of Rack1 is related to its ability to regulate cell cycle, proliferation, invasion, metastasis, angiogenesis, and chemoresistance. In our previous study, we demonstrated that Rack1 accelerates breast cancer progression by activating the WNT/β-catenin pathway to promote cell proliferation, mediating the phosphorylation of Anxa2 to enhance cell invasion, and upregulating the activity of P-glycoprotein to promote chemoresistance. However, the role of Rack1 in the carcinogenesis of breast cancer is not yet fully elucidated. Recently, several studies have indicated that Rack1 plays a critical role in maintaining the stemness properties of adult stem cells, such as neural stem cells, skeletal muscle progenitor cells and epidermal progenitor cells [13–15]. Therefore, whether Rack1 regulates the stemness properties of cancer stem cells, especially BCSC, is a topic of great interest.

In this study, we delineate the function of Rack1 in the modulation of BCSC properties and explore the underlying mechanisms. Our findings indicated that Rack1 is indispensable for the preservation of the stemness properties in breast cancer. We provide evidence that Rack1 increases the stability of the E2F1 protein, which in turn transcriptionally upregulates SOX2, thereby maintaining breast cancer cell stemness and tumorigenesis. Collectively, our findings point to a novel signaling cascade involving the Rack1-E2F1-SOX2 axis as a driver of breast tumorigenesis.

Materials and methods

Cell culture

Human breast cancer cell lines MDA-MB-231, MCF7, mouse breast cancer cell line 4T1, and HEK-293T cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231, MCF7, and 4T1 cells were cultured in RPMI-1640 medium (Hyclone, South Logan, UT, USA). HEK-293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), and all culture media were incubated under standard conditions of 5% carbon dioxide (CO2)

and 37 °C, supplemented with 10% fetal bovine serum (FBS) sourced from HyClone (Logan, UT, USA).

Colony formation assay

Cells were seeded in 6-well plates at a density of 500 cells per well and cultivated for a period of 10 to 14 days to allow for colony formation. Following two washes with phosphate-buffered saline (PBS), the cells were fixed using methanol, stained with 0.1% crystal violet, and colonies comprising over 50 cells were counted and analyzed statistically.

Plasmid construction, stable cell line generation and siRNAs

Our group previously constructed lentiviral shRNAs targeting Rack1, with one targeting the coding region (shRack1-#1) and the other the non-coding region (shRack1-#2), using a standard three-plasmid system. MDA-MB-231, MCF7, or 4T1 cells were then infected with the virus, and 1 μg/mL puromycin was used to generate stable Rack1 knockdown cells. The small interfering RNAs (siRNAs) targeting E2F1, Rack1, SOX2 and negative control were purchased from RIBOBIO (Guangzhou, China). The sequences of the siRNAs were shown in Table S1. The plasmids pGL3-SOX2-Promoter, pCDH-CMV-E2F1-Puro, pCDH-CMV-SOX2-Puro were purchased from Suzhou Xunhong Company. Transfections of siRNAs and plasmids were performed using Lipofectamine RNAiMAX and Lipofectamine 3000 reagents (Thermo Fisher Scientific, CA, USA), respectively, according to the manufacturer's instructions.

Western blot analysis, protein stability and ubiquitination assay

Western blot assay was conducted as outlined previously [16]. Protein concentrations were determined using NanoDrop, and 150–200 μg of protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE). The membrane was incubated with the following primary antibodies: Rack1 (Santa, sc-17754, 1:1000), SOX2 (Proteintech, 11064-1-AP, 1:1000), E2F1 (Santa, sc-251, 1:1000), HA tag (Abcam, ab137838, 1:1000), ubiquitin (Proteintech, 10201-2-AP, 1:1000), β-actin (Sigma, A1978, 1:5000), AKT (CST, 2965, 1:1000), p-AKT-473 (CST, 4060, 1:1000). To assess protein stability, cells were treated with the protein synthesis inhibitor Cycloheximide (CHX; 20 μM, Sigma Aldrich, R750107), the proteasome inhibitor MG132 (10 μM, Sigma Aldrich, R750107), or the autophagy inhibitor CQ (200 nM, Sigma Aldrich, C6628) for the indicated times, respectively, and total protein was subjected to immunoblotting analysis. For the ubiquitination assay, HA-tagged ubiquitin (HA-UB) was transfected into control or Rack1 knockdown breast cancer cells, and then the cells were treated with 10 μM MG132 for 6 h. Protein samples were then collected and the ubiquitination status of E2F1 was measured.

qRT-PCR

Total RNA was isolated from cell lysates using TRIzol. This RNA was then turned into cDNA using HiScript II QRT SuperMix. qRT-PCR was performed with AceQ qPCR SYBR Green, normalizing gene expression to β-actin. The process was repeated three times, and primer sequences are in Supplementary Table 1.

Dual-luciferase reporter assay

The dual-luciferase reporter assay was performed using a commercial kit (Vazyme, DL101-01, China) following the manufacturer's instructions. In brief, cells were plated in triplicate in 24-well plates. The next day, 250 ng of pGL3-SOX2-promoter, 250 ng of pLKO.1-shRack1-#1 or pCDH-E2F1-puro and 2 ng of Renilla luciferase vector were co-transfected into the cells, and cell lysates were collected after 48 h, and measured by a luminometer. The assay was repeated three times.

Immunohistochemical assay

Immunohistochemical assay was performed as previously described. In brief, human tumor tissue sections were first subjected to a standard protocol of deparaffinization, rehydration, and antigen retrieval, followed by incubation with pre-diluted primary antibodies at 4 ℃ overnight. The next day, the sections were incubated with secondary antibodies and then stained with a DAB horseradish peroxidase color development kit, followed by nuclear counterstaining with hematoxylin.

Flow cytometry analysis

In brief, CD44-FITC and CD24-PE antibodies, PE Mouse IgG2a, κ isotype Control, FITC Mouse IgG2a, κ isotype Control (BD Pharmingen, San Diego, CA, USA) were used to fractionate the CD24 – CD44 + population. To obtain single cell suspensions, the cultured cells were enzymatically dissociated using 0.05% trypsin/EDTA. Subsequently, 1×10^6 cells were resuspended in 200 μL of phosphate-buffered saline (PBS) supplemented with 1% fetal bovine serum (FBS). This suspension was then incubated with the above antibodies for a duration of 30 min. Flow cytometry analysis was employed to identify and quantify the CD24 – CD44 + cell population using the CytoFLEX system (Beckman Coulter, USA). Data were analyzed using CytExpert.

Mammosphere formation assay

Cells were plated at a low density of 1000 viable cells/mL in ultralow attachment 6-well plates (Coring, USA) and incubated in RPMI-1640 medium supplemented with 2% B27 (Invitrogen, USA) 20 ng/mL epidermal growth factor

(PeproTech, USA), 20 ng/mL basic fibroblast growth factor (PeproTech, USA) for 7–10 days at 5% CO2, 37 ℃ to allow the cells to form spheroids. The spheroids were photographed and analyzed under an inverted light microscope.

Cell viability assay

Human breast cancer cell lines MDA-MB-231 (3000 cells) and MCF7 (5000 cells) were seeded into 96-well plates and incubated for 24 h. Subsequently, various concentrations of epirubicin were administered, followed by an additional 72 h of incubation. Cell viability was assessed utilizing the CCK-8 reagent, with measurements conducted in triplicate to ensure accuracy. Data were analyzed with GraphPad Prism 8.0.

Animal studies and human tissue samples

Four- to five-week-old female BALB/c mice were purchased from Beijing Huafukang Bio-technology Co., Ltd. The mice were randomly divided into 8 groups (7 mice per group). Different numbers of control or Rack1 knockdown 4T1 cells (5×10^3, 1×10^4, 2×10^4, and 5×10^4 cells) were injected into the mammary fat pads of mice in each group, respectively. The diameters of the tumors were measured every other day. Tumor volume (mm3) was calculated using the formula: volume = length $\times$width$^2\times0.5$. After the mice were euthanized on day 36, the tumor masses were excised and photographed. All experimental procedures were approved by and conducted in accordance with the Animal Ethics and Welfare Committee of Tianjin Medical University Cancer Institute and Hospital. In addition, with the informed consent of the patients, human tissue samples of 31 breast cancer cases were obtained from Tianjin Medical University Cancer Institute and Hospital. The study followed the ethical guidelines of the 1975 Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital.

Bioinformatics analysis.

The expression levels of the Rack1 were assessed in breast cancer tissues versus normal tissues utilizing the UALCAN online platform (https://ualcan.path.uab.edu). Subsequently, the effect of Rack1 on cell viability was analyzed using the data from the Project Score database (https://score.depmap.sanger.ac.uk). Additionally, we employed the Kaplan-Meier Plotter database (https://kmplot.com/analysis) to analyze the association between Rack1 expression and prognosis in breast cancer. For RNA-Seq data, the processed differentially expression data were subjected to Gene Set Enrichment Analysis (GSEA) using clusterProfiler R package. The GSEA was also performed to analyze the pathways correlated to Rack1 expression using TCGA (The Cancer Genome Atlas) data. In brief, the mRNA expression data were downloaded from the TCGA data portal (https://portal.gdc.cancer.gov) and converted to TPM (Transcripts Per Million). The Pearson's correlation coefficients were calculated and subjected to clusterProfiler R package for the GSEA analysis. The gene sets used in the analysis were obtained from the MSigDB database (https://www.gsea-msigdb.org/gsea/msigdb).

Statistical analysis

Statistical analyses were conducted using the SPSS16.0 software. Comparisons between groups were analyzed by the t-test, one-way ANOVA. Overall survival curves were plotted according to the Kaplan–Meier method with the log-rank test applied for comparison. Survival was measured from the day of surgery. Variables with values of $P<0.05$ by univariate analysis were used in subsequent multivariate analysis based on the Cox proportional hazards model. Differences were considered statistically significant at $P<0.05$.

Results

Elevated Rack1 is strongly associated with poor breast cancer prognosis and tumorigenesis

We have previously shown that Rack1 promotes breast cancer progression, to further investigate the function of Rack1 in accelerating breast cancer malignancy, we queried the CPTAC-based UALCAN database, which includes data from 125 breast cancer patients. First, we found that Rack1 protein expression was significantly increased in breast cancer tissues compared with normal tissues (Fig. 1A). Additionally, we evaluated the effect of Rack1 on cell viability in in vitro culture using the Project Score database. Our results revealed that the ablation of Rack1 exerted a profound impact on the health status and cell viability of breast cancer cell lines (Fig. 1B). In addition, utilizing the Kaplan-Meier Plotter tool, we found that breast cancer patients with higher Rack1 expression in the TCGA had a worse clinical outcome (Fig. 1C). In addition, GSEA analysis was performed using TCGA (The Cancer Genome Atlas) data to analyze the pathways associated with RACK1 co-expressed genes. The results showed that RACK1 co-expressed genes were significant enriched in pathways such as stemness, Wnt/beta-catenin, and Notch (Fig. 1D-E). In light of the intimate connection between pathways such as Notch and WNT and the modulation of tumor stemness, the aforementioned findings indicate that elevated Rack1 is markedly associated with unfavorable prognosis and tumorigenesis in breast cancer patients.

Suppression of Rack1 undermines the stemness of breast cancer cells

Next, we generated stable Rack1 knockdown cell lines by infecting three different breast cancer cells with

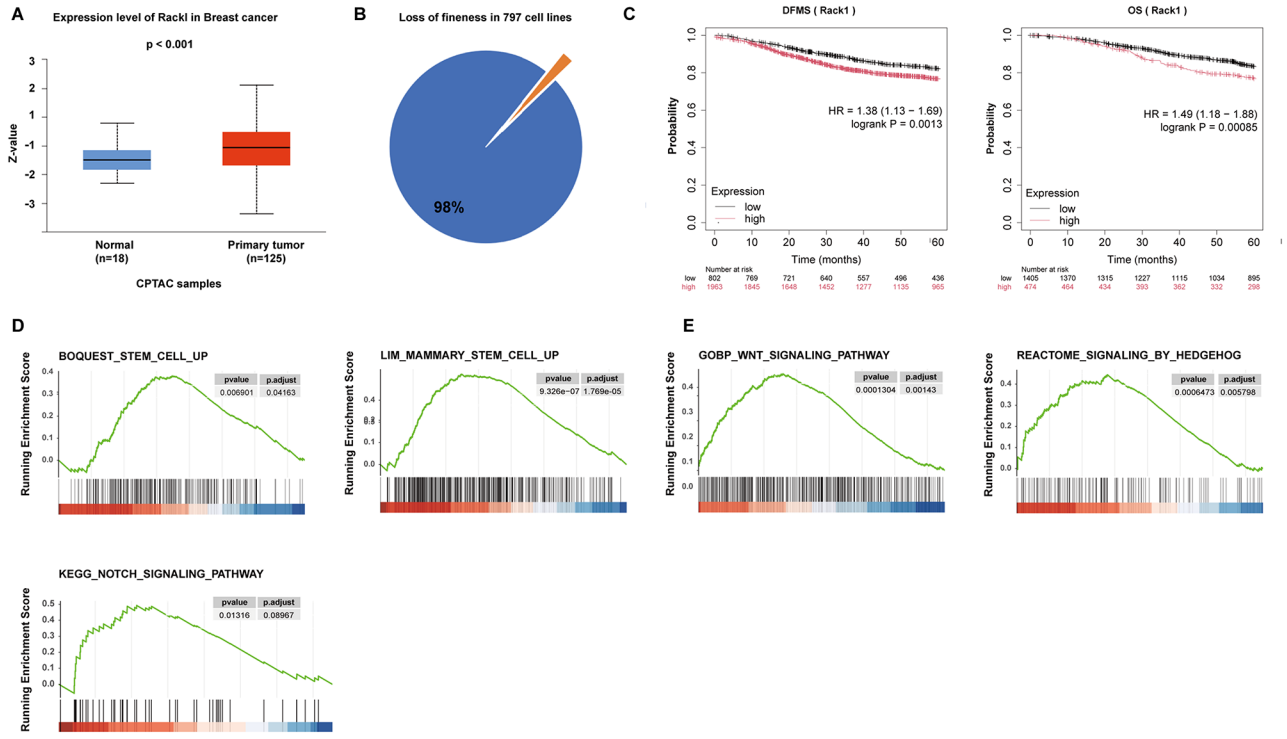

Fig. 1 Elevated Rack1 is strongly associated with poor breast cancer prognosis and tumorigenesis (**A**) Analysis of the UALCAN database indicated that Rack1 protein expression levels were significantly higher in breast cancer tissues than in normal tissues. (**B**) Analysis of the Project Score database revealed that Rack1 is a core fitness gene that affects the health and cell viability of the majority of breast cancer cell lines. (**C**) Kaplan–Meier survival curve analysis showed that breast cancer patients with high Rack1 expression had a poorer prognosis. (**D**-**E**) The GSEA analysis showed that the Rack1 co-expressed genes were significantly enriched in the stemness, Wnt, hedgehog, Notch pathways based on the TCGA database

lentivirus expressing shRNAs specifically targeting Rack1 (Fig. 2A). To evaluate the effect of Rack1 on cell proliferation, we conducted a colony formation assay. As depicted in Fig. 2B, both the size and number of colonies were significantly reduced in the Rack1-silenced group relative to the control group. In light of the established definition of BCSCs as those with high CD44 and low CD24 expression, we confirmed by flow cytometry analysis that the proportion of BCSCs ($CD44^{bright}CD24^{dim}$) was significantly decreased after Rack1 knockdown compared to control cells (Fig. 2C). Conventional treatments not only have limited killing effect on CSCs, but also promote the generation of drug-resistant CSCs [17], Cancer stem cells are also thought to be mediators of chemoresistance. So, we next investigated the effect of Rack1 knockdown on chemoresistance in breast cancer cells. As shown in Fig. 2D, silencing of Rack1 increased the sensitivity of both MCF7 and MDA-MB-231 cells to EPI treatment. Consistently, the diameter and number of tumor spheroids were also remarkably reduced in Rack1 knockdown cells compared with control cells. (Fig. 2E). Finally, we assessed the effect of Rack1 knockdown on tumorigenesis in vivo by injecting BALB/c mice with varying amounts of control or Rack1-stable knockdown 4T1 cells. The results demonstrated that Rack1-silenced breast cancer cells exhibited a diminished capacity to induce tumorigenesis in BALB/c mice (Fig. 2F). Taken together, these findings suggest that Rack1 may contribute to tumor progression by modulating the stemness properties of breast cancer cells.

Rack1 knockdown inhibits SOX2 expression in breast cancer cells

Tumor stemness plays a pivotal role in tumorigenesis. To explore the potential mechanism of Rack1 on stemness in breast cancer cells, we examined the expression levels of key markers associated with stemness, (i.e., SOX2, NANOG, POU5F1 (also known as OCT4), and KLF4) in Rack1-silenced cells [18, 19]. Our qPCR results showed a notable reduction of SOX2 and POU5F1 transcript levels upon Rack1 knockdown in both MDA-MB-231 and MCF7 breast cancer cell lines (Fig. 3A). At the protein level, Western blot analysis corroborated a significant decrease in SOX2 expression in Rack1-knockdown cells, while POU5F1 protein levels remained unaltered. Based on these findings, we postulated that Rack1 specifically affects SOX2 expression in breast cancer, but not other stemness genes (Fig. 3B). Similarly, the transcriptional activity of SOX2 was assessed by a dual luciferase reporter gene assay and found to be markedly suppressed

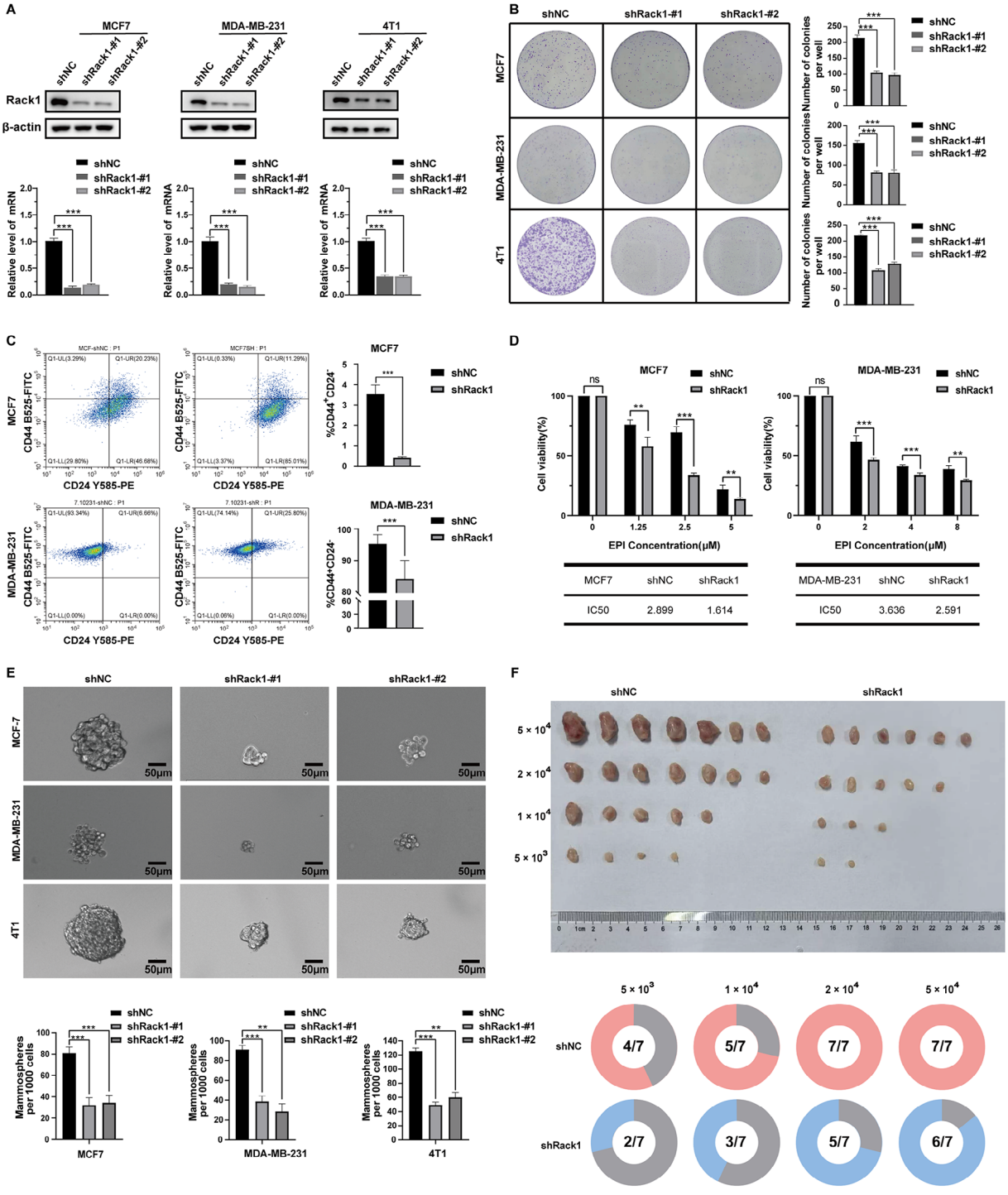

Fig. 2 Suppression of Rack1 undermines the stemness of breast cancer cells. (**A**) The mRNA and Protein expression levels of Rack1 in control and three breast cancer cell lines with stable knockdown of Rack1 were determined by qRT-PCR and Western Blotting assays. (**B**) Representative results showed that breast cancer cells with Rack1 knockdown had significantly reduced colony forming ability compared to control groups. (**C**) Flow cytometric assays was used to determine the percentage of CD44 + CD24- cells in control or Rack1 knockdown MCF7 or MDA-MB-231 cells. (**D**) The control and Rack1-silenced cells were exposed to EPI at various concentrations for 48 h, and the viability and half-maximal inhibitory concentration (IC50) values of EPI in the indicated groups were determined using the CCK8 assay. (**E**) The sphere-forming ability was detected in shNC or shRack1 breast cancer cells. (**F**) In vivo limited dilution assay was used to detect the effect of Rack1 on breast cancer tumorigenesis. Tumors dissected from allograft mice (top), and corresponding estimated frequencies were shown (bottom). The data presented are expressed as mean ± SD, with statistical significance indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$

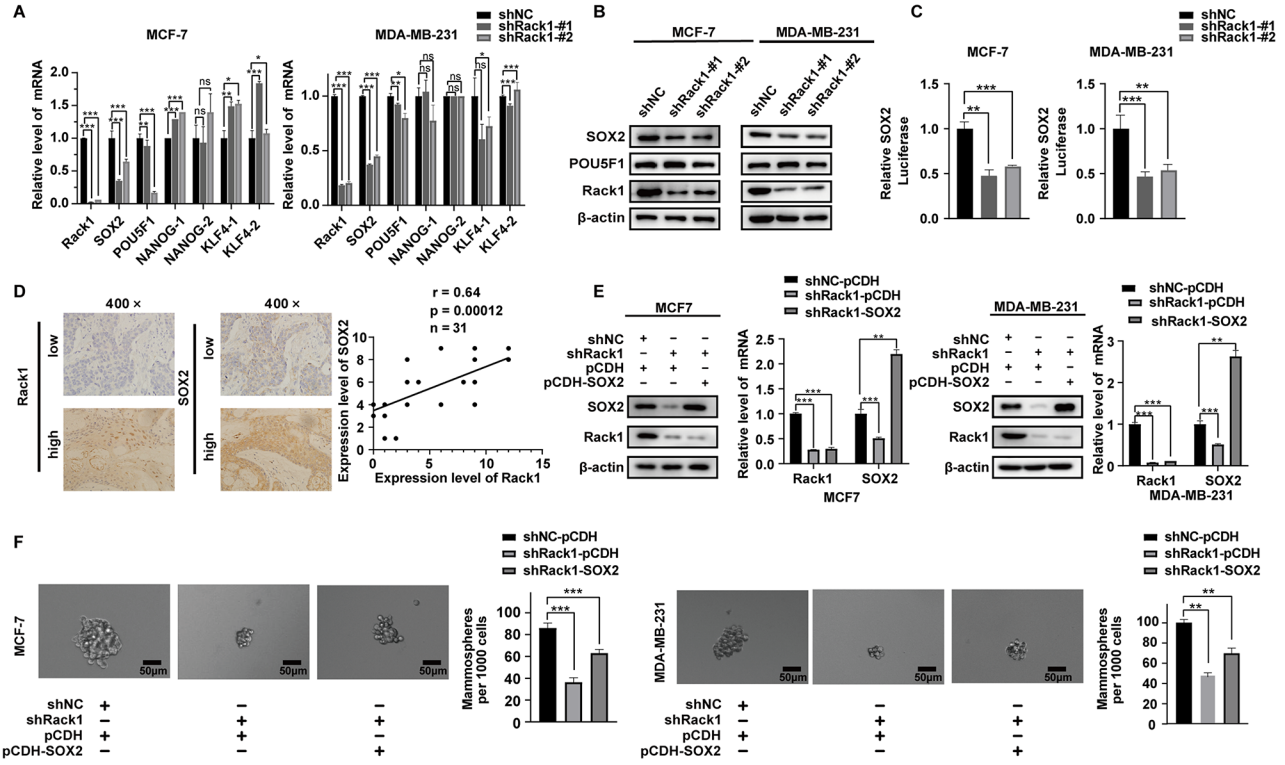

Fig. 3 Rack1 knockdown inhibits SOX2 expression in breast cancer cells. (**A**-**B**) qRT-PCR and Western blot were performed to determine the mRNA (**A**) and protein (**B**) expression levels of Rack1 and stemness-related genes in control or Rack1 knockdown MCF7 and MDA-MB-231 cells. (**C**) The dual-luciferase reporter assay was used to determine the transcriptional activity of SOX2 in control and Rack1 knockdown breast cancer cells. (**D**) Correlation analysis of Rack1 and SOX2 expression in 31 cases of breast cancer tissue. (**E**) Western blotting and qRT-PCR analysis of the expression levels of Rack1 and SOX2 in control, Rack1-silenced breast cancer cells and after exogenous overexpression of SOX2 in Rack1-silenced cells. (**F**) Effect of SOX2 overexpression on the sphere-forming ability in Rack1-silenced breast cancer cells. All data are shown as the number of mammospheres per 1000 cells. The data presented are expressed as mean ± SD, with statistical significance indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$

in Rack1-knockdown breast cancer cells (Fig. 3C). In addition, we examine the expression patterns of Rack1 and SOX2 in 31 breast cancer tissues by immunohistochemical analysis. Statistical analysis unveiled a significant positive correlation between Rack1 and SOX2 expression ($R = 0.64$; $P = 0.012$), further implicating their interplay in breast cancer (Fig. 3D). Moreover, to further confirm the function of SOX2 in Rack1-mediated breast cancer cell stemness, we forced exogenous overexpression of SOX2 in Rack1-silenced cells (Fig. 3E). As shown in Fig. 3F, overexpression of SOX2 restored the spheroid formation capacity in Rack1-silenced MCF7 and MDA-MB-231 cells. Taken together, these findings suggest that Rack1 is a modulator of breast cancer stemness that exerts its influence by regulating SOX2 expression.

E2F1 regulates SOX2 expression and stem cell properties in breast cancer

It has been documented that transcription factors (TFs) such as GLI2, FOXO1, and E2F1 can regulate SOX2 expression and thus affect tumor stemness [19]. To explore the mechanism by which Rack1 affects SOX2 expression in breast cancer cells, we further treated breast cancer cells with GLI2 and FOXO1 inhibitors and found that inhibition of GLI2 and FOXO1 protein expression did not significantly alter SOX2 levels (Fig. 4A). In contrast, knockdown of E2F1 could lead to a marked decrease in SOX2 protein expression in both breast cancer cell lines (Fig. 4B). Moreover, a dual luciferase reporter assay showed that silencing E2F1 expression decreased the transcriptional activity of SOX2 in both MCF7 and MDA-MB-231 cell lines (Fig. 4C). Consistently, using plate colony and spheroid formation assays, we demonstrated that E2F1 knockdown impaired the colony formation and spheroid formation abilities in two types of breast cancer cells (Fig. 4D-E). Collectively, these findings suggest that E2F1 promotes breast cancer stemness by modulating SOX2 expression.

Rack1 controls breast cancer stemness through the E2F1-SOX2 axle in BCCs

To further elucidate the role of E2F1 in regulating stemness and SOX2 expression in Rack1-silenced breast cancer cells, we next investigate the effect of Rack1 knockdown on E2F1 and SOX2 expression in breast cancer cells. Western blot analysis confirmed that Rack1

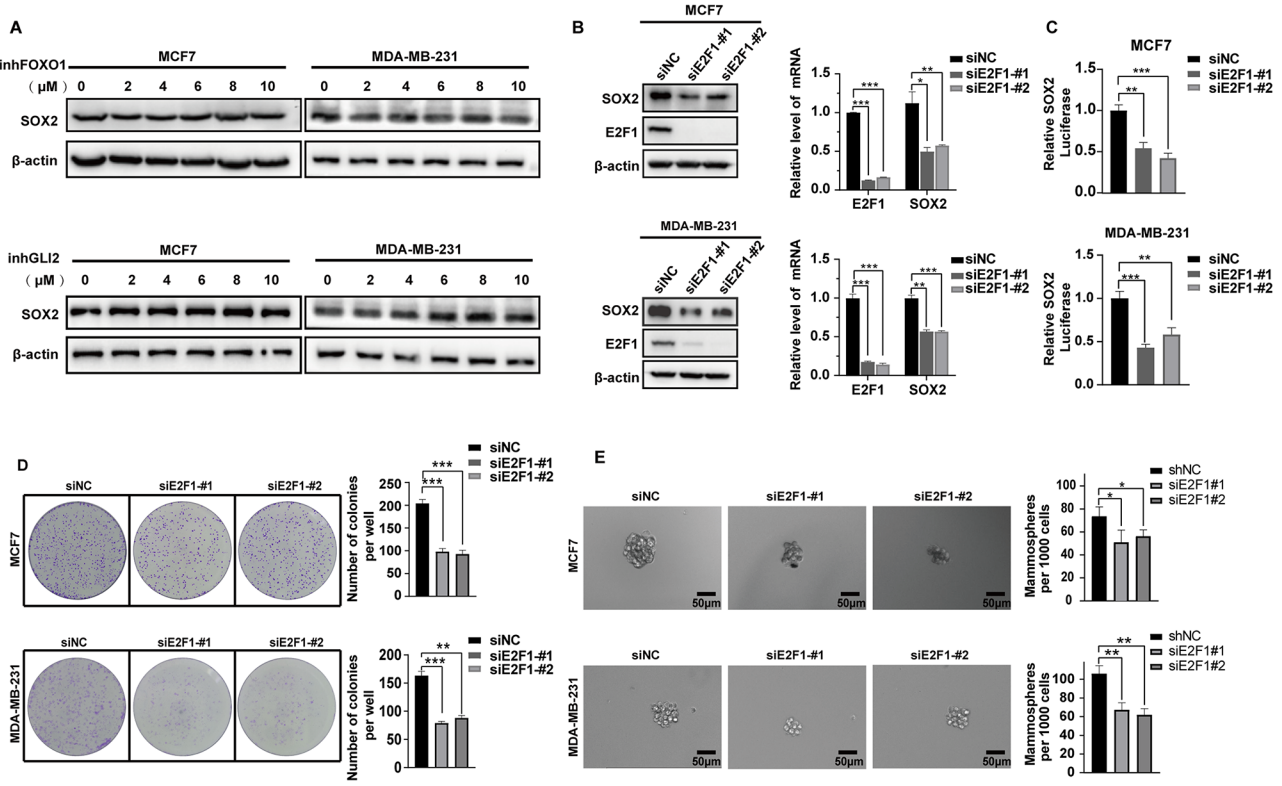

Fig. 4 E2F1 regulates SOX2 expression and stem cell properties in breast cancer. (**A**) Western blotting analysis of the expression of SOX2 protein levels in breast cancer cells after 48-hour of treatment with varying concentrations of FOXO1 or GLI2 inhibitors. (**B**) qRT-PCR and Western blotting were used to investigate the silencing efficiency of E2F1 in breast cancer cells and the effect of E2F1 silencing on the expression level of SOX2. (**C**) The dual-luciferase reporter assay was used to determine the transcriptional activity of SOX2 in MCF7 and MDA-MB-231 cells transfected with siNC or siE2F1. (**D**-**E**) The effect of E2F1 on the stemness function of breast cancer cells was verified by colony formation assay (**D**) and sphere formation assay (**E**), and the statistical results are presented in bar graphs. All data are shown as the number of mammospheres per 1000 cells. The data presented are expressed as mean ± SD, with statistical significance indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$

silencing substantially reduced the expression of E2F1 and SOX2 proteins in breast cancer cells (Fig. 5A). Moreover, we also overexpressed E2F1 in Rack1-silenced breast cancer cells. Notably, E2F1 overexpression rescued SOX2 expression in two Rack1-knockdown cells (Fig. 5B). Likewise, E2F1 overexpression in Rack1 knockdown cells also restored the transcriptional activity of SOX2 (Fig. 5C). Furthermore, E2F1 re-expression also rescued the impairedsphere formation capacity caused by Rack1 depletion, as evidenced by the increase in sphere size and number in two breast cancer cells (Fig. 5D-E). Collectively, these results demonstrate that Rack1 enhances breast cancer stemness by maintaining the E2F1/SOX2 axis.

Rack1 enhances E2F1 stability by inhibiting its ubiquitination

To delineate the underlying mechanism by which Rack1 regulates E2F1 expression, we first examined the mRNA level of E2F1 in control and Rack1-silenced cells, and found that there was no significant alteration in the mRNA level of E2F1 between the two groups (Fig. 6A). To investigate whether Rack1 regulates E2F1 expression at the post-transcriptional level, we treated the aforementioned cells with cycloheximide (CHX) to block de novo protein synthesis and measured the half-life of E2F1. As shown in Fig. 6B, the degradation rate of E2F1 was significantly elevated in Rack1-silenced breast cancer cells. These data indicate that Rack1 depletion contributes to the acceleration of E2F1 protein instability. To further investigate how Rack1 regulates E2F1 protein stability, two breast cancer cells were incubated with CHX (10μM), CHX plus the autophagy-lysosome inhibitor Chloroquine (CQ, 50μM), or the proteasome inhibitor MG132 (10 μM) for the indicated times, and then E2F1 protein levels were measured. As shown in Fig. 6C, MG132 slowed the rate of E2F1 protein degradation in both breast cancer cells lines, whereas CQ did not exhibit a similar effect. These data suggest that Rack1 regulates E2F1 protein expression through the ubiquitin-proteasome system (UPS). In addition, MG132 (but not CQ) nearly reversed the decrease in E2F1 protein levels caused by Rack1 knockdown in both breast cancer cells, further demonstrating that Rack1 enhances E2F1 stability

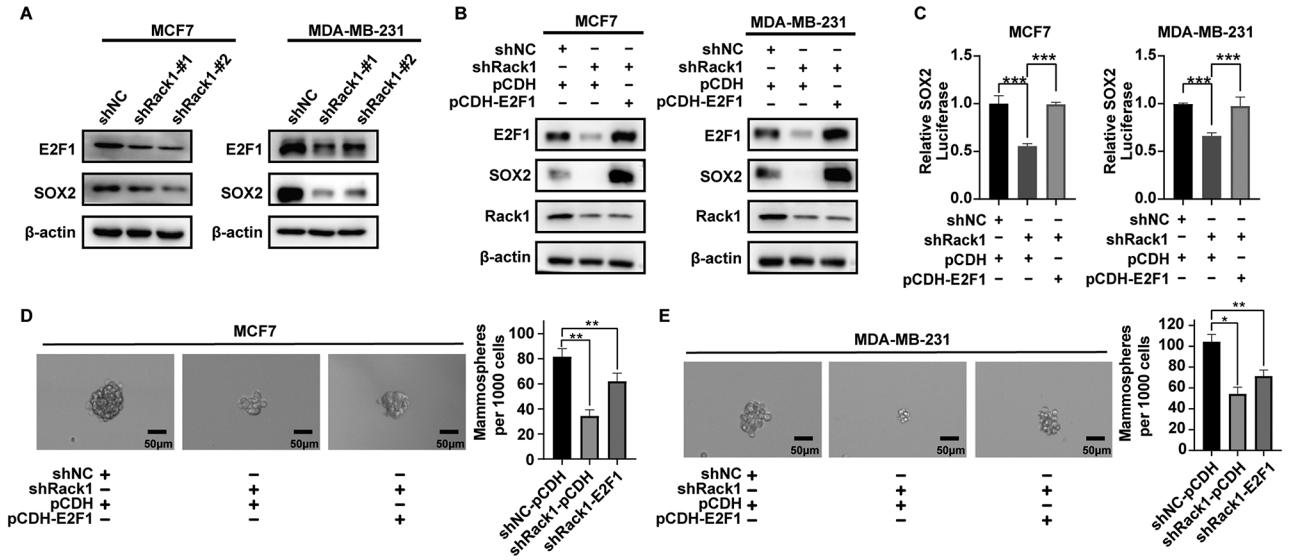

Fig. 5 Rack1 controls breast cancer stemness through the E2F1-SOX2 Axle in BCCs. (**A**) Expression levels of SOX2 and E2F1 proteins in control and Rack1 knockdown MCF7 and MDA-MB-231 cells. (**B**) The effect of E2F1 overexpression on SOX2 protein levels in Rack1-silenced breast cancer cells was analyzed by Western blotting. (**C**) The effect of E2F1 overexpression on the transcriptional activity SOX2 in Rack1-silenced breast cancer cells was analyzed by dual-luciferase assay. (**D**-**E**) The effect of E2F1 overexpression on the sphere-forming ability of Rack1-silenced breast cancer cells

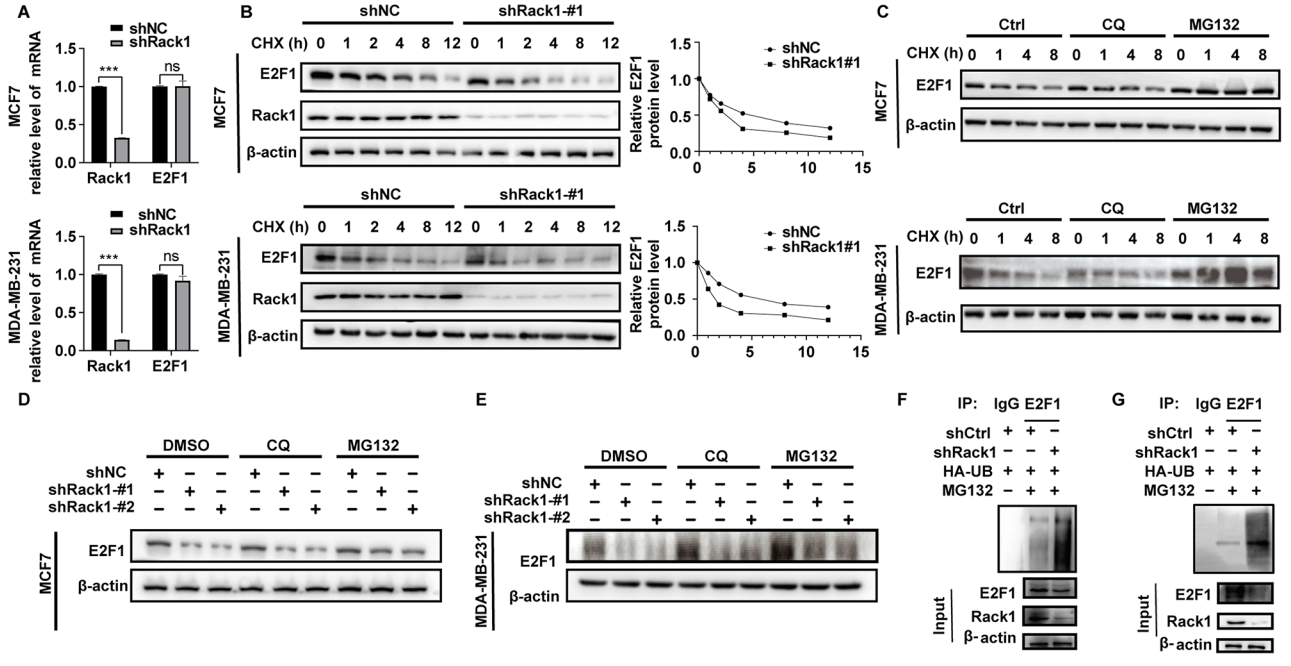

Fig. 6 Rack1 enhances E2F1 stability by inhibiting its ubiquitination. (**A**) Expression levels of E2F1 mRNA in control and Rack1 knockdown MCF7 and MDA-MB-231 cells. (**B**) Control and Rack1-silenced MCF7 and MDA-MB-231 cells were incubated with 20 μM cycloheximide (CHX) for the indicated times, and then the protein levels of E2F1 were determined by Western blotting. (**C**) MCF7 and MDA-MB-231 cells were incubated with CHX (20 μM) or CHX plus the autophagy inhibitor Chloroquine (CQ 200 nM), or MG132 (10 μM) for the indicated times, and then the protein levels of E2F1 were determined by Western blotting. (**D**-**E**) Control and Rack1-silenced MCF7 (**D**) and MDA-MB-231 (**E**) cells were incubated with CQ (200 nM) or MG132 (10 μM) for 6 h, then the protein levels of E2F1 were determined by Western blotting. (**F**-**G**) After transfection of control and Rack1-silenced breast cancer cells with HA-tagged ubiquitin (HA-UB), the cells were treated with MG132 for 6 h and the level of poly-ubiquitinated E2F1 was assessed

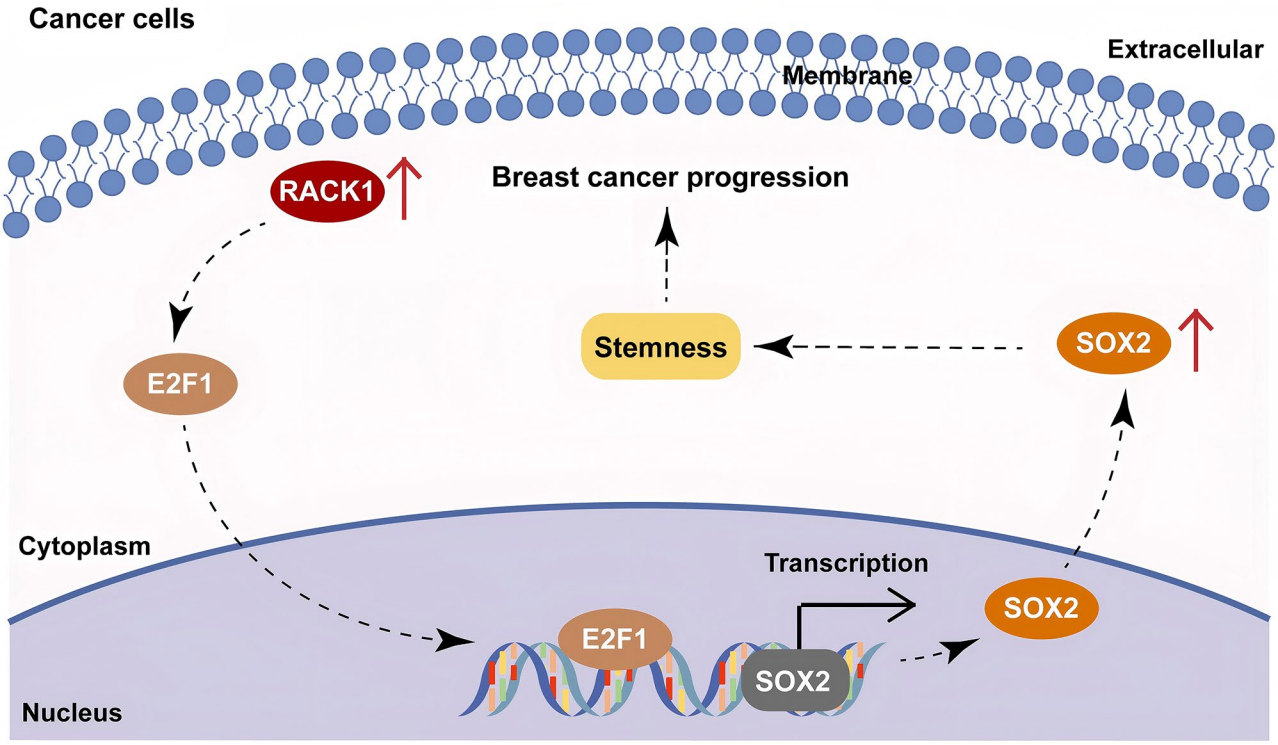

Fig. 7 Diagram of the mechanism. Rack1 enhances the stability of E2F1 through inhibition of the proteasome pathway, influencing the expression of the stemness factor SOX2, and ultimately promoting breast cancer stemness and chemoresistance

by inhibiting the UPS (Fig. 6D-E). Moreover, we validated that the poly-ubiquitination level of E2F1 was greatly elevated in MCF7 and MDA-MB-231 cells following Rack1 silencing (Fig. 6F-G). Taken together, these data suggest that Rack1 interferes with E2F1 degradation by compromising the UPS.

Discussion

The most significant finding of our study is that Rack1 is a key positive regulator involved in modulating breast cancer stemness. Both in vivo and in vitro evidences demonstrate that Rack1 is indispensable for tumorigenicity, chemoresistance, spheroid formation, and the maintenance of CD44(high)/CD24(low) subpopulation of breast cancer cells. These findings signify that Rack1 plays a pivotal role in promoting tumor stem cell properties. Rack1 exerts this effect by regulating the expression of SOX2, a master transcription factor that governs cancer cell stemness and maintenance. Mechanistic investigations revealed that Rack1 increases the stability of E2F1 by inhibiting its ubiquitination and subsequent proteasome-mediated degradation, which in turn transcriptionally activates SOX2, thereby affecting the stemness of breast cancer cells (Fig. 7). In conclusion, the present findings suggest a novel mechanism by which Rack1 promotes breast cancer progression by driving cancer stem cell-like properties.

Deregulation of Rack1 has been implicated in the pathogenesis and progression of various tumors. Numerous studies have demonstrated that Rack1 can exert pro- or anti-tumorigenic functions depending on the tissue or cellular context [15, 20]. As a scaffolding protein, the function of Rack1 may be related to the proteins it binds [21]. In gastric cancer, Rack1 negatively regulates the Wnt signaling pathway by stabilizing the β-catenin destruction complex, thereby functioning as a tumor suppressor [22]. However, Rack1 exerts a tumor-promoting function in breast cancer by enhancing β-catenin stability through competitively inhibiting the binding of β-catenin to PSMD2, a key component of the proteasome [23]. Notably, the oncogenic function of Rack1 is not exclusive to breast cancer, as it has also been implicated in the development of various other types of cancers, including lung [24], liver [25], glioma [26], esophageal [27], oral [28], ovarian [29], and cervical [30]. Consistent with previous studies, our results support a tumor-promoting role for Rack1 in breast cancer, as evidenced by the fact that Rack1 depletion delays the tumorigenic potential of breast cancer cells in vivo in mice, and inhibits the proliferative activity of cells in vitro. In addition, analysis using the public TCGA-BRCA database showed that Rack1 expression was positively correlated with the expression of the stemness gene sets. These findings suggest the potential for Rack1 to modulate the stemness

of breast cancer cells. As expected, silencing of Rack1 reduced the ratio of CD44 (high) / CD24 (low) subpopulations and the spheroid-forming activity in breast cancer cells. These findings are consistent with a previous study showing that Rack1 promotes self-renewal of cancer stem cells in hepatocellular carcinoma [8]. Similarly, a recent study has shown that Rack1 is essential for the activation of skeletal muscle-resident adult stem cells (i.e., satellite cells) [14]. In addition, another study has reported that Rack1 is highly enriched in neural stem cells and inhibits the cellular senescence phenotype [13]. Collectively, these data suggest that the ability of Rack1 to maintain stemness potential is one mechanism for its oncogenic function in breast cancer.

The maintenance of tumor stemness is closely linked to the expression of stemness-associated transcription factors such as SOX2, OCT4, NANOG, KLF4, and c-Myc [19]. The functional regulatory role of Rack1 in breast cancer stemness prompted us to investigate the potential relationship between Rack1 expression and the aforementioned transcription factors. This study found that Rack1 specifically regulates SOX2 expression in breast cancer cells, thereby maintaining their stemness. Notably, the mechanisms by which Rack1 regulates breast cancer cell stemness are different from those that regulate other types of cell stemness. For instance, Rack1 promotes the self-renewal of human liver cancer stem cells by stabilizing Nanog [8]. Rack1 inhibits neural stem cell senescence by blocking the p21 signaling pathway [13].In addition, Rack1 prevents premature differentiation by repressing IRF6 expression in epidermal progenitor cells [15]. These findings suggest that the varied mechanisms through which Rack1 regulates stemness may be associated with its function as a scaffolding protein that exhibits distinct binding affinities for proteins across various cell types, thereby being cell type dependent [31]. Several studies have shown that SOX2 expression can be transcriptionally activated by E2F1, GLI2 and FOXO1 in various cell types. Here we found that E2F controls SOX2 expression and stemness properties in breast cancer cells. E2F1 is a well-known key transcription factor that regulates cell cycle transition [32, 33]. Recent studies have suggested a potential link between E2F and the regulation of stemness [34–36] and tumorigenic potential [37, 38] in various cancers. However, the precise mechanisms involved remain to be fully elucidated. Thus, our findings provide a novel explanation for the regulation of stemness by E2F1. Moreover, we provide evidence that Rack1 regulates E2F1 stability through the ubiquitin-proteasome system, thereby affecting SOX2 expression. To the best of our knowledge, this study is the first to show that Rack1 regulates the stability of E2F1 in breast cancer cells. Interestingly, Rack1 has been reported to regulate the stability of several key tumor-associated proteins, including HDAC1/2, c-Jun, Nanog and β-catenin [8, 23, 39–41], via the UPS. These data also suggest that the regulation of protein stability by Rack1 is a pivotal mechanism for its pro-tumorigenic effects. Taken together, our findings suggest that Rack1 can modulate the stemness of breast cancer cells through the E2F1-SOX2 axis.

In conclusion, our results demonstrate that Rack1 is highly expressed in breast cancer and is closely associated with patient survival. We uncover a novel role for Rack1 in the modulation of breast cancer stemness. Elevated Rack1 promotes the stability of E2F1, which in turn transcriptionally activates SOX2 expression to maintain the stemness and tumorigenesis of breast cancer cells. Thus, we uncover a novel mechanism by which Rack1 exerts its oncogenic function. This study also demonstrates that targeting the Rack1-E2F1-SOX2 axis may be a potential strategy to inhibit breast cancer development and progression.

Conclusions

In summary, our study indicates that Rack1 supports breast cancer stemness, which is coordinated with upregulation of the SOX2 axis Thus, this work provides a proof-of-concept for directly targeting breast cancer stem cells against breast cancer through inhibition of the Rack1-E2F-SOX2 axis.

Abbreviations

OS	Overall survival time
DFS	Disease-free survival
CSC	Cancer stem cell
IP	Immunoprecipitation
CHX	Cycloheximide
CQ	Chloroquine
GSEA	Gene Set Enrichment Analysis
TCGA	The Cancer Genome Atlas
qPCR	Quantitative polymerase chain reaction
NC	Negative control

Supplementary Information

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Supplementary Material 1: Supplementary Table S1. shRNA and siRNA sequences used in this study. Supplementary Table S2. Primers used for qRT-PCR in this study.

Supplementary Material 2

Acknowledgements

Not applicable.

Author contributions

Y.J., F.Z. conceptualized this idea. Y.J, L.Z., W.Z. performed the experiments. Y.J, L.Z., W.Z., S.C., H.Z., L.L., and H.G. analyzed and interpreted the data. Y.J. and F.Z. wrote the paper. Z.W., Y.C. assisted experiments. F.Z., R.N. coordinated and directed the project. All authors reviewed the manuscript.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
The study protocol was approved by the Animal Ethical and Welfare Committee of Tianjin Medical University Cancer Institute and Hospital.

Consent for publication
All participants provided written informed consent, and the study was conducted according to the Declaration of Helsinki principles.

Competing interests
The authors declare no competing interests.

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