Original Article
Overexpression of CBX2 in breast cancer promotes tumor progression through the PI3K/AKT signaling pathway

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Abstract: Breast cancer is the second leading cause of cancer-related death among women worldwide. Emerging evidence suggests that chromobox homolog 2 (CBX2) is overexpressed in breast cancer and plays an essential role in tumor progression. However, its expression and functional roles in breast cancer development and progression require further exploration. Here, we evaluated CBX2 expression in breast cancer using mRNA expression data from the TCGA database; CBX2 expression was upregulated in breast cancer. Furthermore, upregulated CBX2 expression was significantly associated with poorer overall survival (OS) and progression-free survival (PFS) of breast cancer patients. Immunohistochemical analysis of CBX2 expression in a tissue microarray (TMA) cohort yielded concordant results. Univariate and multivariate analyses showed that elevated CBX2 expression was significantly and independently associated with poorer OS of patients in this TMA cohort. Additionally, we performed *in vitro* functional assays to evaluate the proliferation, migration, and invasion abilities of breast cancer cell lines wherein CBX2 was knocked down using short hairpin RNA (shRNA). CBX2 silencing inhibited cell proliferation, migration, and invasion *in vitro*. Furthermore, knockdown of CBX2 markedly reduced breast tumorigenesis in xenograft mouse models. Functional and pathway enrichment analyses indicated a positive correlation between high CBX2 expression and activation of the PI3K/AKT pathway, which were further confirmed by western blot and immunohistochemical analyses of mouse tumors. Our findings indicate that CBX2 is a potential prognostic biomarker and therapeutic target for breast cancer.

Keywords: CBX2, breast cancer, prognostic biomarker, PI3K/AKT pathway

Introduction

Worldwide cancer estimates indicate that about 2.1 million women were newly diagnosed with breast cancer in 2018, and that breast cancer was responsible for almost 1 in 4 cancer-related deaths among women [1]. In spite of advances in early diagnosis of the disease and adoption of improved therapeutic strategies, prognosis of breast cancer is often still poor, mainly due to aggressive biologic behavior and delayed diagnoses [2-4]. Even when diagnosed at an early stage, tumor recurrence and metastasis after surgery are persistent clinical problems in many breast cancer patients [5, 6]. Thus, there is an unmet clinical need to unravel molecular mechanisms underlying breast tumorigenesis and disease progression, and to identify novel prognostic/

predictive biomarkers and therapeutic targets for improving breast cancer outcomes.

Chromobox (CBX) member proteins, including CBX1-8 proteins, are essential regulators of gene expression and developmental programs [7, 8]. Growing evidence suggests that aberrant expression of CBX family members plays key roles in different cancers [9]. Studies have reported that CBX6 is significantly underexpressed in glioblastoma [10]. CBX7 is overexpressed in gastric and prostate cancers [11, 12], but downregulated in thyroid cancer, lung cancer, and colon cancer [13-15]. A recent study found that the expression of CBX4 was inversely correlated with the expression of Runx2. Importantly, the combination of overexpressed CBX4 and under-expressed Runx2 was strongly associated with overall survival (OS) in

colorectal carcinoma [16]. Based on *in silico* analysis of online datasets, Liang Y. *et al.* reported that some CBX member proteins are overexpressed in breast cancer [17]. However, the expression and roles of CBX2 in breast cancer remain unclear.

The PI3K/AKT pathway plays a central role in regulating key biological processes in several human cancers wherein its dysregulation is implicated both in tumorigenesis and tumor progression [18-21]. A growing tide of evidence also shows that the PI3K/AKT pathway contributes to breast tumorigenesis and progression of breast cancer. Previous studies have reported that many oncogenes, such as TPX2, MEG3, and HOXA1 *el at.* regulate PI3K/AKT signaling pathway in breast cancer [22]. However, the molecular mechanisms through which the PI3K/AKT pathway drives breast cancer progression remain largely to be elucidated.

In our study, we evaluated the expression levels of CBX2 mRNA and protein in breast cancer. CBX2 was remarkably overexpressed in breast cancer tissues, and upregulated CBX2 expression was strongly associated with poor prognosis in breast cancer. Additionally, CBX2 silencing attenuated proliferation and invasiveness of breast cancer cells *in vitro* and impaired tumorigenesis in *in vivo* models. Moreover, results from our *in silico* bioinformatics analyses combined with validation experiments suggest that CBX2 plays a pivotal role in regulating the cell cycle and DNA replication, and that the PI3K/AKT signaling pathway functions downstream of CBX2 in breast cancer tumorigenesis and/or progression. Our findings uncover a crucial role for CBX2 in regulating the proliferation and invasion of breast cancer cells via the PI3K/AKT pathway and suggest that elevated CBX2 may serve as a novel prognostic biomarker and promising therapeutic target for breast cancer.

Materials and methods

Clinical samples

Formalin-fixed paraffin-embedded breast tumor resection samples (n=160) and breast non-tumor tissues (n=60) were used for the construction of tissue microarrays. Breast cancer tissues were derived from patients consec-utively diagnosed with breast cancer between 2009 and 2013, for whom tissue samples and detailed clinicopathologic data were available. The clinicopathological features of the patients in our study cohort are presented in **Table 1**. All aspects of this study were reviewed and approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University. All patients were officially informed about the use of specimens and signed the written informed consent.

The cancer genome atlas (TCGA) dataset analysis

The mRNA expression and clinicopathologic data for a total of 1079 breast cancer tumor samples and 104 non-tumor breast tissue samples was downloaded from TCGA (http://tcga-data.nci.nih.gov/). Furthermore, we analyzed the correlation between CBX2 mRNA expression and clinical characteristics of the breast cancer patients.

Tissue microarray (TMA) construction and immunohistochemical (IHC) staining of the Zhengzhou University (ZZU) cohort

Human paraffin-embedded breast cancer TMA slides containing 160 paraffin-embedded breast cancer tissues and 60 non-tumor breast tissues were constructed using 1.5-mm diameter cores. In brief, deparaffinization, rehydration, and antigen retrieval of the tissue slides were performed according to established protocols. Subsequently, TMA slides were blocked with 5% bovine serum albumin (BSA) (Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature. Sections were incubated with antibody against CBX2 (dilution 1:100, Proteintech Group, Wuhan, China) overnight at 4°C and then probed with biotinylated goat anti-rabbit secondary antibody (AmyJet Scientific, Wuhan, China). Enzyme-labelled streptavidin was then added to the TMA slides, and the slides were incubated according to the manufacturer's protocol. The TMA slides were then placed in the chromogenic substrate (prepared using the SignalStain® DAB Substrate Kit from CST, USA) until the desired reaction was achieved. Slides were then counterstained with hematoxylin and eosin, dehydrated, and mounted. The presence of CBX2 and its abundance (signal intensity) were evaluated semi-quantitatively as follows: scores of 1+, 2+, and 3+ represented low CBX2

Table 1. Correlation of clinicopathological features with CBX2 expression in breast cancer ZZU TMA cohort

Variables	Clinicopathological features	Total (n=160)	CBX2 expression level		P-value
			Low (n, %)	High (n, %)	
Age (years)	≤50	95	44 (63.7)	51 (56.0)	0.324
	>50	65	25 (36.2)	40 (43.9)	
Her-2 status	Negative	124	54 (61.1)	70 (66.7)	0.840
	Positive	36	15 (38.9)	21 (33.3)	
PR status	Negative	98	39 (56.5)	59 (64.8)	0.285
	Positive	62	30 (43.4)	32 (35.1)	
ER status	Negative	95	49 (62.0)	46 (56.7)	0.500
	Positive	65	30 (37.9)	35 (43.2)	
TNBC status	TNBC	64	29 (42.0)	35 (38.4)	0.648
	Non-TNBC	96	40 (57.9)	56 (61.5)	
Tumor size	<20 mm	49	24 (34.7)	25 (27.4)	0.320
	≥20 mm	111	45 (65.2)	66 (72.5)	
Involved lymph node	Negative	58	32 (46.3)	26 (28.5)	0.020
	Positive	102	37 (53.6)	65 (71.4)	
Differentiation grade	Well & Moderate	98	49 (71.0)	49 (53.8)	0.027
	Poor	62	20 (28.9)	42 (46.1)	
TNM stage	Stage I and II	86	50 (72.4)	36 (39.5)	0.000
	Stage III and IV	74	19 (27.5)	55 (60.4)	

expression, while scores of 4+ and 5+ represented high CBX2 expression for statistical analyses.

Cell culture

The breast cancer cell lines MDA-MB-231 and MCF-7 (Cell Bank of the Chinese Academy of Science, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Solarbio, Beijing, China) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), at 37°C under a 5% CO_2 atmosphere.

Western blotting

Protein isolation and western blotting was performed as previously described [23, 24]. Briefly, equal amounts of protein lysates were resolved using 12% SDS-PAGE gels (Invitrogen) and then transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). After blocking for 1 hour, membranes were probed with specific antibodies at 4°C for 12 hours and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 hours at room temperature. Signal quantification and analysis were performed using an Odyssey

infrared imaging system (LI-COR Biosciences). Primary antibodies used in this study were: anti-CBX2 rabbit monoclonal antibody (Proteintech Group) used at 1:2000 dilution; anti-β-actin monoclonal mouse antibody (Proteintech Group) used at a dilution of 1:5000.

Short hairpin RNA (shRNA) construction and transfection of cell lines

Stable transfectants harboring targeting CBX2 (sh-CBX2) or negative control shRNA (sh-NC) were generated in MDA-MB-231 and MCF-7 breast cancer cells via lentiviral transduction of a plasmid (synthesized by GenePharma, Shanghai, China) carrying the corresponding shRNA sequence and the gene encoding firefly luciferase. Stably transfected cell lines were selected over a period of 4 weeks using puromycin (100 μg/ml).

Cell proliferation assay

Stably transfected MDA-MB-231 and MCF-7 cells were seeded into 96-well plates in triplicates. Cell viability was determined using the Cell Counting Kit-8 (CCK-8) (Dojindo; Kumamoto, Japan) 24 hours after the cells were seeded.

Absorbance was read at 450 nm using a spectrophotometer (Molecular Devices, USA).

To evaluate the effects of CBX2 knockdown on DNA replication in proliferating cells, stably transfected MDA-MB-231 and MCF-7 breast cancer cells harboring CBX2 knockdown (sh-CBX2) or negative control shRNA (sh-NC) were incubated in DMEM containing 50 μM 5-ethynyl-2'-deoxyuridine (EdU; RiboBio, Guangzhou, China) in triplicate. DNA was stained using Hoechst 33342 stain and cells were visualized using an inverted fluorescence microscope (Olympus, Tokyo, Japan). For each EdU experiment, five random fields were imaged at 100× magnification. Images were processed and analyzed using the ImageJ software. The percentage of EdU-positive cells among the total number of cells in each field was determined.

Transwell cell migration and invasion assay

For the transwell cell migration and invasion assays, 1×10^4 indicated cells were resuspended in serum-free medium and then seeded into the upper chamber of a transwell system (Costar, Corning, NY, USA). Medium containing 10% FBS was placed into the bottom chamber and served as the chemo-attractant. After incubation for 24 hours, the transwell insert was removed, and the side of the insert facing the upper compartment was carefully cleansed with a cotton swab to remove culture medium and cells that had not migrated through the insert. Cells that had migrated through the filter pores to the underside of the insert were fixed, stained with 0.1% crystal violet, and counted using a microscope (Olympus).

Tumor xenograft experiments

BALB/c nude mice (4 weeks old, male) were obtained from Vital River Laboratory Technology (Beijing, China). MCF-7 cells (1×10^7 cells) stably transfected with sh-CBX2 or sh-NC were inoculated subcutaneously into the lower flank of the mice. Tumor growth was measured every week using non-invasive bioluminescent imaging. After 5 weeks, mice were sacrificed. Tumors were weighed, and tissue specimens were harvested for IHC staining.

Statistical analysis

All statistical analyses were performed by using SPSS 23.0 and GraphPad Prism 7 software. χ^2 tests or Fisher's exact tests were used to analyze the relationship between CBX2 expression and outcomes of breast cancer patients. Kaplan-Meier survival analyses and log-rank tests were conducted for overall survival (OS) and progression-free survival (PFS) analyses. Student's t-tests were used for comparisons between two groups. At least three independent experiments were repeated. Data are presented as means ± SD and P<0.05 was considered to be statistically significant.

Results

High expression of CBX2 is associated with poor prognosis of breast cancer patients in the TCGA dataset

To evaluate how CBX2 expression in breast cancer compares to its expression level in breast non-tumor tissues, we analyzed microarray data from the TCGA dataset. Our results showed that CBX2 mRNA expression was higher in breast cancer tissues compared to normal tissues (**Figure 1A**). CBX2 was particularly upregulated in the Basal-like and Her2-enriched breast cancer subtypes (**Figure 1B**). We then analyzed CBX2 expression based on the ER/PR status of breast tumors in the TCGA dataset and found that CBX2 mRNA expression was elevated in ER- and PR-negative breast cancer tissues (**Figure 1C** and **1D**). The expression of CBX2 was much higher in late TNM stages (III-IV stages) than in TNM stage I-II samples (**Figure 1E**). Additionally, we uncovered a significant positive correlation between the expression of CBX2 and Ki-67 (**Figure 1F**). The OS and PFS of breast cancer patients with high CBX2 expression were markedly worse than those of patients with low CBX2 expression (**Figure 1G** and **1H**). Taken together, these findings suggest that CBX2 mRNA is significantly upregulated in breast cancer tissues compared to non-tumor tissues, and CBX2 overexpression is associated with unfavorable prognosis of breast cancer patients.

CBX2 is overexpressed in breast cancer tissues and is associated with poorer prognosis of patients in the ZZU cohort

We next analyzed CBX2 protein expression in breast cancer and tumor-adjacent normal tissues in our ZZU cohort TMA via IHC staining/scoring (**Figure 2A**). CBX2 expression was markedly elevated in breast cancer tissues in
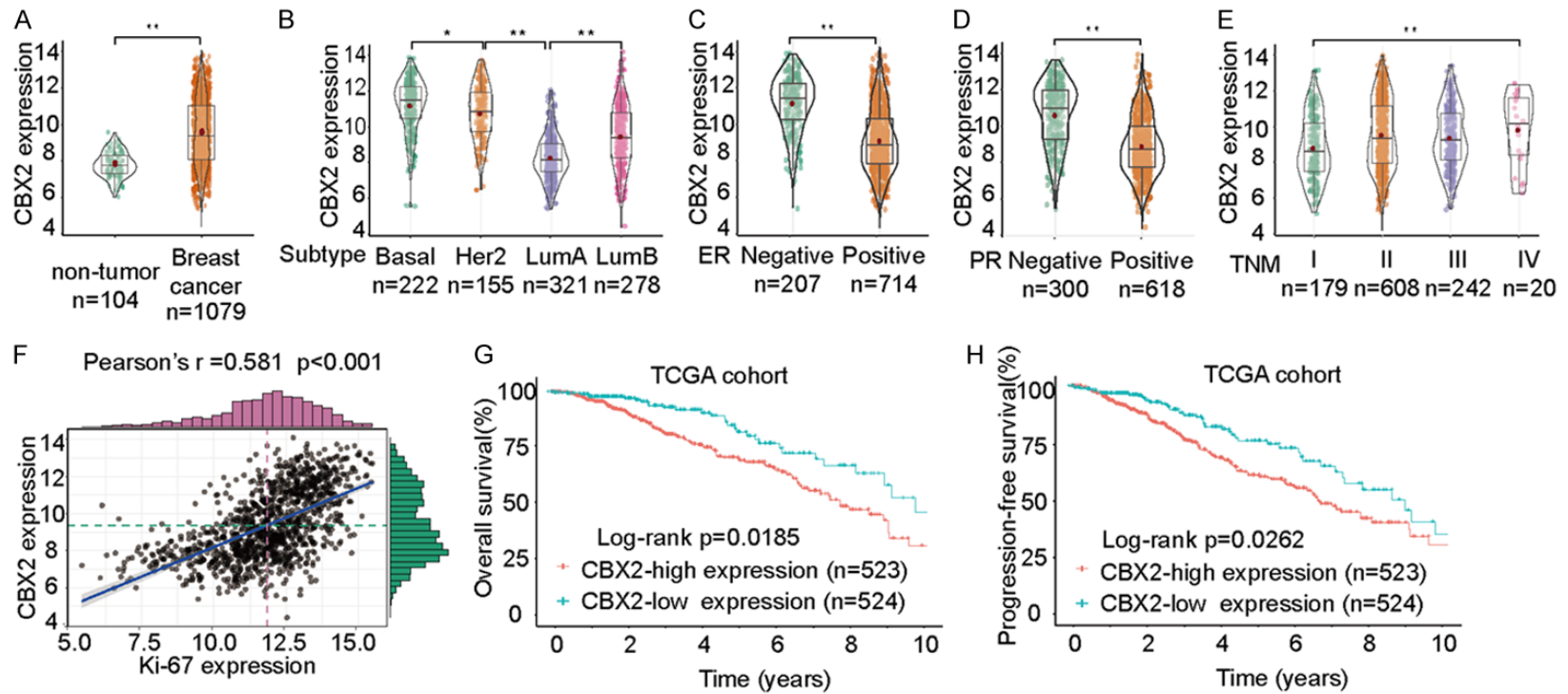
Figure 1. CBX2 is upregulated in breast tumors and CBX2 expression is associated with prognosis of breast cancer patients in TCGA dataset. A. Comparison of CBX2 mRNA expression between breast cancers and normal tissues in the TCGA dataset. B. Comparison of CBX2 mRNA expression in breast cancers of different molecular subtypes. CBX2 was upregulated in Basal-like and Her2-enriched subtypes compared to LumA and LumB subtypes. C, D. Elevated CBX2 mRNA expression was observed in ER- and PR-negative breast cancer tissues. E. CBX2 mRNA expression in breast tumor tissues according to TNM stage. F. Analysis of the correlation between CBX2 expression and Ki-67 expression. G, H. Kaplan-Meier survival curves showing stratification of breast cancer patients in TCGA dataset based on CBX2 expression (red: high CBX2 expression; green: low CBX2 expression). Breast cancer patients with high CBX2 expression had markedly shorter OS and PFS time than those with low CBX2 expression. *P<0.05, **P<0.01.
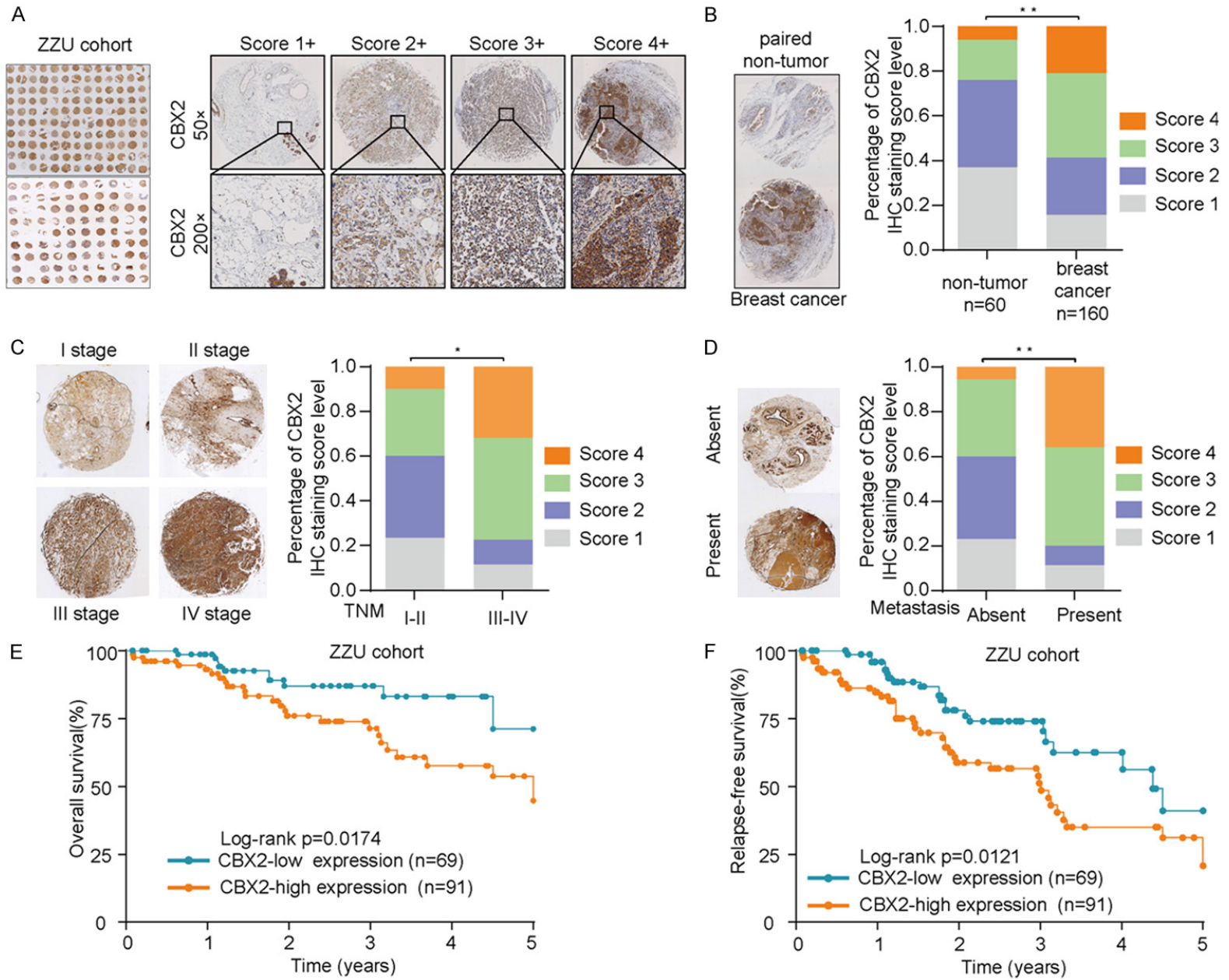
Figure 2. CBX2 is overexpressed in breast cancer tissues and high CBX2 expression is associated with poorer overall survival. A. Representative micrographs showing CBX2 immunohistochemical staining patterns with different staining scores in breast cancer tissues in the ZZU TMA. B. Representative images showing CBX2 staining in breast cancer tissues and paired non-tumor samples in the ZZU TMA (left) and comparison of score distributions between breast cancer tissues and paired non-tumor samples in the ZZU TMA (right). CBX2 was overexpressed in breast cancer tissues compared with non-tumor breast tissues. C. Representative images showing CBX2 staining in tumor samples from patients with different TNM classifications (left) and comparison of score distributions based on TNM stage (right). CBX2 expression was notably high in breast cancer patients with advanced TNM stage. D. Representative images showing CBX2 staining in samples from patients with or without distant metastasis at presentation (left) and comparison of score distributions based on presence/absence of metastases (right). E, F. Kaplan-Meier survival curves showing stratification of patients in the ZZU TMA cohort based on CBX2 expression (red: high CBX2 expression; green: low CBX2 expression). Breast cancer patients with high CBX2 expression had markedly shorter OS and RFS time than those with low CBX2 expression. *P<0.05, **P<0.01.

Table 2. Analysis of associations between clinicopathological features and clinical outcomes of patients in the breast cancer TMA cohort

Clinicopathological features	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Univariate and multivariate analyses of overall survival in breast cancer patients (n=160)						
Age (>50 vs <50)	1.120	0.719–1.439	0.692			
Her-2 status (Negative vs positive)	0.953	0.498-1.042	0.549			
PR status (Negative vs positive)	1.503	0.621-2.482	0.732			
ER status (Negative vs positive)	1.388	0.939-2.181	0.529			
TNBC status (Negative vs positive)	1.063	0.729-1.875	0.495			
Tumor size (>20 mm vs <20 mm)	1.249	1.029-2.054	0.260			
Involved lymph node (Negative vs positive)	2.160	1.584-4.827	0.028	2.307	1.784-2.981	0.024
Differentiation grade (Well & Moderate vs Poor)	2.279	1.651-3.742	0.014	1.625	1.244-2.129	0.079
TNM stage (I & II vs III & IV)	2.679	1.951-4.841	0.000	2.532	1.986-3.821	0.002
CBX2 expression level	2.104	1.751-3.926	0.003	1.909	1.604-3.921	0.021
Univariate and multivariate analyses of relapse-free survival in breast cancer patients (n=160)						
Age (>50 vs <50)	1.232	0.778–2.212	0.761			
Her-2 status (Negative vs positive)	1.146	0.942-1.567	0.659			
PR status (Negative vs positive)	1.728	0.821-3.011	0.842			
ER status (Negative vs positive)	1.607	0.939-2.905	0.651			
TNBC status (Negative vs positive)	1.163	0.910-1.754	0.545			
Tumor size (>20 mm vs <20 mm)	1.327	0.729-2.154	0.286			
Involved lymph node (Negative vs positive)	2.592	1.781-4.211	0.034	2.730	2.112-3.908	0.025
Differentiation grade (Well & Moderate vs Poor)	2.734	1.957-4.609	0.017	1.472	1.104-2.894	0.129
TNM stage (I & II vs III & IV)	3.214	2.011-4.942	0.000	3.021	2.809-4.391	0.006
CBX2 expression level	2.524	1.902-3.947	0.004	2.919	2.201-3.783	0.021

comparison with adjacent normal tissues (**Figure 2B**). Additionally, our results indicated that CBX2 expression was significantly increased in breast cancer patients with advanced TNM stages (III-IV stages) (**Figure 2C**) and in those who presented with distant metastasis (**Figure 2D**). Furthermore, Kaplan-Meier analysis revealed that breast cancer patients whose tumors showed low expression of CBX2 had a notably longer OS and relapse-free survival (RFS) time compared to patients whose tumors showed an above-threshold expression

of CBX2 (**Figure 2E** and **2F**). Details pertaining to relationships between CBX2 expression and clinicopathological characteristics of breast cancer patients in the ZZU TMA are shown in **Table 1**. Upregulated CBX2 expression was significantly correlated with breast cancer TNM stage, lymph node involvement, and differentiation grade (**Table 1**). Furthermore, univariate and multivariate analyses showed that advanced TNM stages, lymph node involvement, and high CBX2 expression were independent prognostic factors for OS and RFS (**Table 2**).

Collectively, our data suggest that CBX2 expression is significantly upregulated in breast cancer and upregulated CBX2 expression is associated with poor clinical outcomes in breast cancer.

CBX2 silencing suppresses breast cancer cell proliferation and invasion in vitro

To better understand how upregulated expression of CBX2 impacts the proliferation and invasion of breast cancer cells, we examined the effects of lentivirus-mediated CBX2 knockdown in breast cancer cell lines. The efficiency of CBX2 knockdown in breast cancer cells was confirmed by western blot analysis (**Figure 3A**). To assess the role of CBX2 in the proliferation of breast cancer cells, we performed the CCK-8 assay. Our results showed that CBX2 silencing significantly reduced proliferation of MDA-MB-231 and MCF-7 cells (**Figure 3B**). Consistent with a reduction in proliferation, our EDU assay suggested that CBX2 knockdown decreased the growth of breast cancer cells (**Figure 3C**). Our transwell migration and invasion assays indicated that the migration and invasion abilities of breast cancer cells were remarkably impaired by CBX2 knockdown (**Figure 3D** and **3E**). These results provided evidence that CBX2 likely functions as an oncogene by promoting proliferation and invasion in breast cancer cells.

Downregulation of CBX2 inhibits breast tumorigenesis in vivo

To gain insights into the impact of CBX2 silencing on breast tumorigenesis *in vivo*, sh-NC and sh-CBX2 cells were injected into the flanks of nude mice and tumor growth was monitored over time. Tumor growth in the sh-CBX2 group was dramatically reduced compared to that in the sh-NC group (**Figure 4A-C**). Tumor weight was also significantly decreased in the sh-CBX2 group (**Figure 4D**) compared to the sh-NC group. Moreover, IHC analysis of the expression of CBX2 and Ki-67 in mouse tumor tissues revealed that the expression of both CBX2 and Ki-67 proteins was markedly suppressed in the sh-CBX2 group (**Figure 4E**). Collectively, our findings demonstrated that CBX2 silencing inhibits breast tumorigenesis; thus, CBX2 can serve as a potential target for breast cancer therapy.

CBX2 may play essential roles in regulating cell cycle and DNA replication, and the PI3K/ AKT pathway functions downstream of CBX2

To further our understanding of the downstream molecular mechanisms by which CBX2 drives breast tumorigenesis and/or progression, we performed clusterProfile GO and KEGG analyses using gene expression data from the TCGA dataset. These bioinformatics analyses showed that cell cycle and DNA replication pathways were markedly enriched in CBX2-high expression samples (**Figure 5A** and **5B**), suggesting that CBX2 may contribute to breast cancer development/progression by regulating cell cycle and DNA replication. Moreover, we found a striking positive correlation between the expression of CBX2 and the activation of the PI3K/AKT signaling pathway based on Gene Set Enrichment Analysis (GSEA) of the TCGA data (**Figure 5C**). There was a highly significant positive correlation between the expression of CBX2 and the expressions of PIK3CA, PIK3CD, and AKT (**Figure 5D-F**). To corroborate the findings from our *in silico* analyses, we examined the expressions of p-mTOR, p-PI3K, and p-AKT in tumor tissues derived from our xenograft models by western blotting and IHC. As shown in **Figure 5G** and **5H**, the expressions of p-mTOR, p-PI3K, and p-AKT were significantly decreased in the sh-CBX2 group compared to the levels observed in the sh-NC group. Taken together, these findings indicated that (a) CBX2 potentially drives breast tumorigenesis and/or progression by regulating the cell cycle and DNA replication in breast cancer cells, and (b) the PI3K/AKT signaling pathway functions downstream of CBX2.

Discussion

Breast cancer continues to be one of the most commonly diagnosed malignancies among women worldwide [25]. Advanced breast cancer with metastasis remains the most common cause of breast cancer-associated death [26]. Therefore, there is an urgent need to better understand the molecular underpinnings of breast tumorigenesis and progression, to identify new biomarkers to enable the personalization of breast cancer management, and to find novel and effective therapeutic targets for breast cancer treatment.
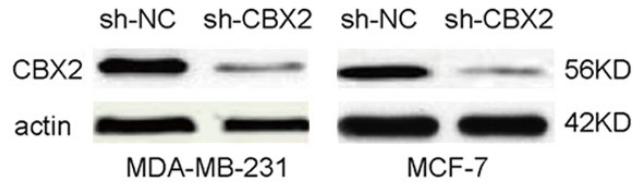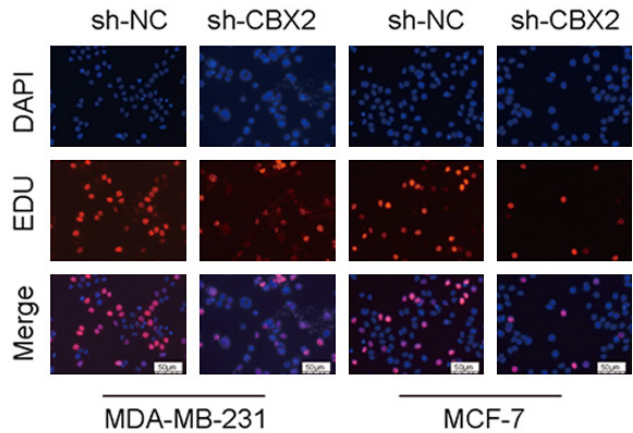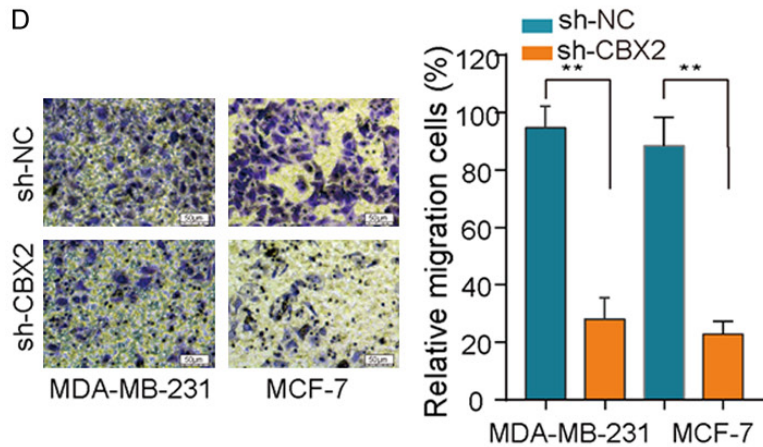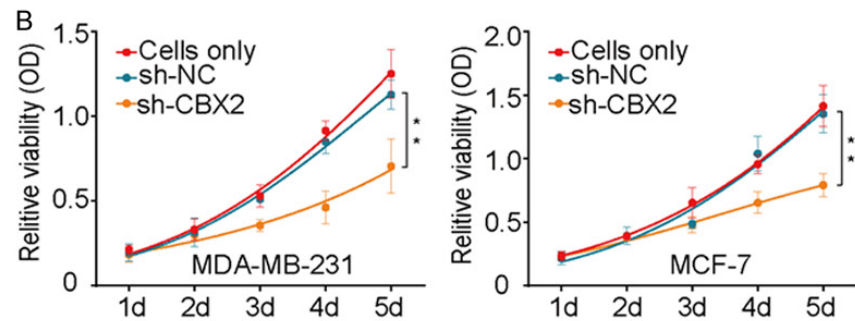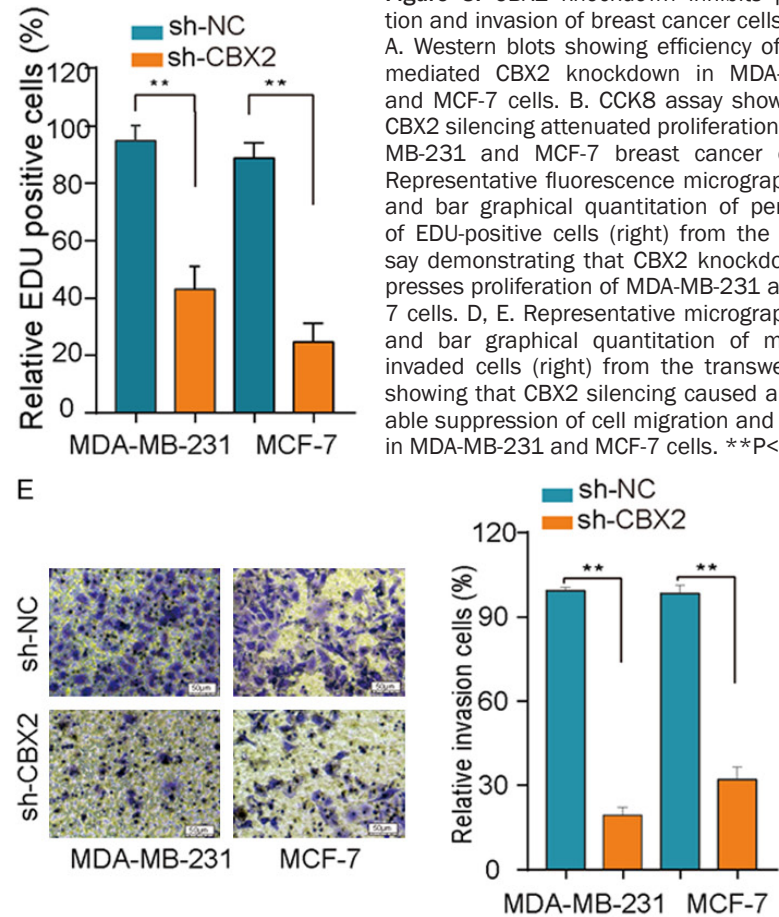
Figure 3. CBX2 knockdown inhibits proliferation and invasion of breast cancer cells *in vitro*. A. Western blots showing efficiency of shRNA-mediated CBX2 knockdown in MDA-MB-231 and MCF-7 cells. B. CCK8 assay showing that CBX2 silencing attenuated proliferation of MDA-MB-231 and MCF-7 breast cancer cells. C. Representative fluorescence micrographs (left) and bar graphical quantitation of percentage of EDU-positive cells (right) from the EDU assay demonstrating that CBX2 knockdown suppresses proliferation of MDA-MB-231 and MCF-7 cells. D, E. Representative micrographs (left) and bar graphical quantitation of migrated/invaded cells (right) from the transwell assay showing that CBX2 silencing caused a remarkable suppression of cell migration and invasion in MDA-MB-231 and MCF-7 cells. **P<0.01.
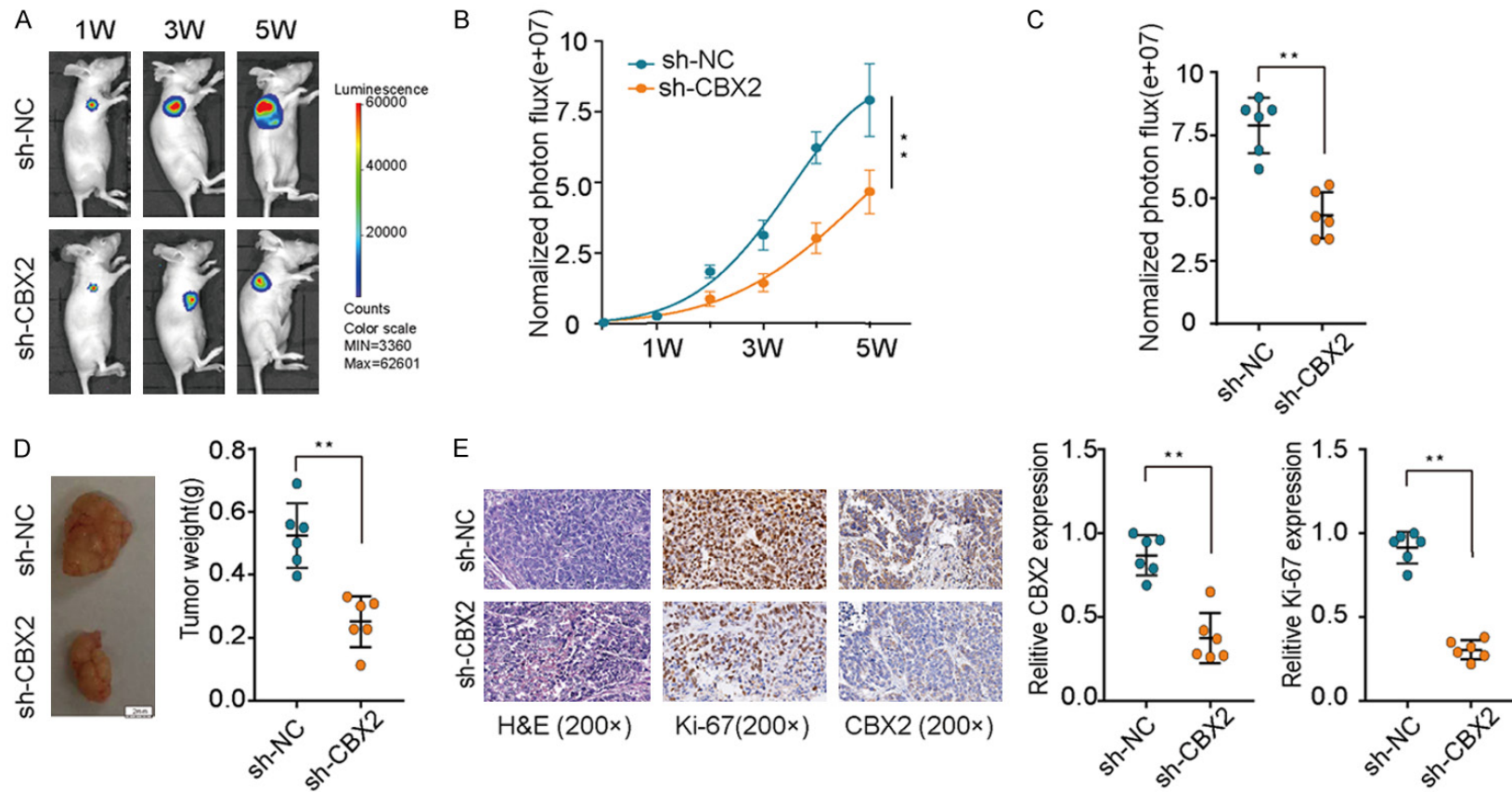
Figure 4. CBX2 knockdown suppresses breast cancer tumorigenesis *in vivo*. A, B. Breast cancer cells stably transfected with sh-CBX2 or sh-NC were injected subcutaneously into nude mice (n=12). Images of tumors that formed in the nude mice were acquired using a live imaging system to detect the luciferase signal. C. The luciferase activity of the sh-CBX2 tumors was lower than that of the sh-NC group. D. Tumor weights in sh-CBX2 group were markedly lower than those of sh-NC group. E. Representative images of mouse (xenograft) tumor sections stained with hematoxylin and eosin and immunohistochemically stained for Ki-67 and CBX2. **P<0.01.
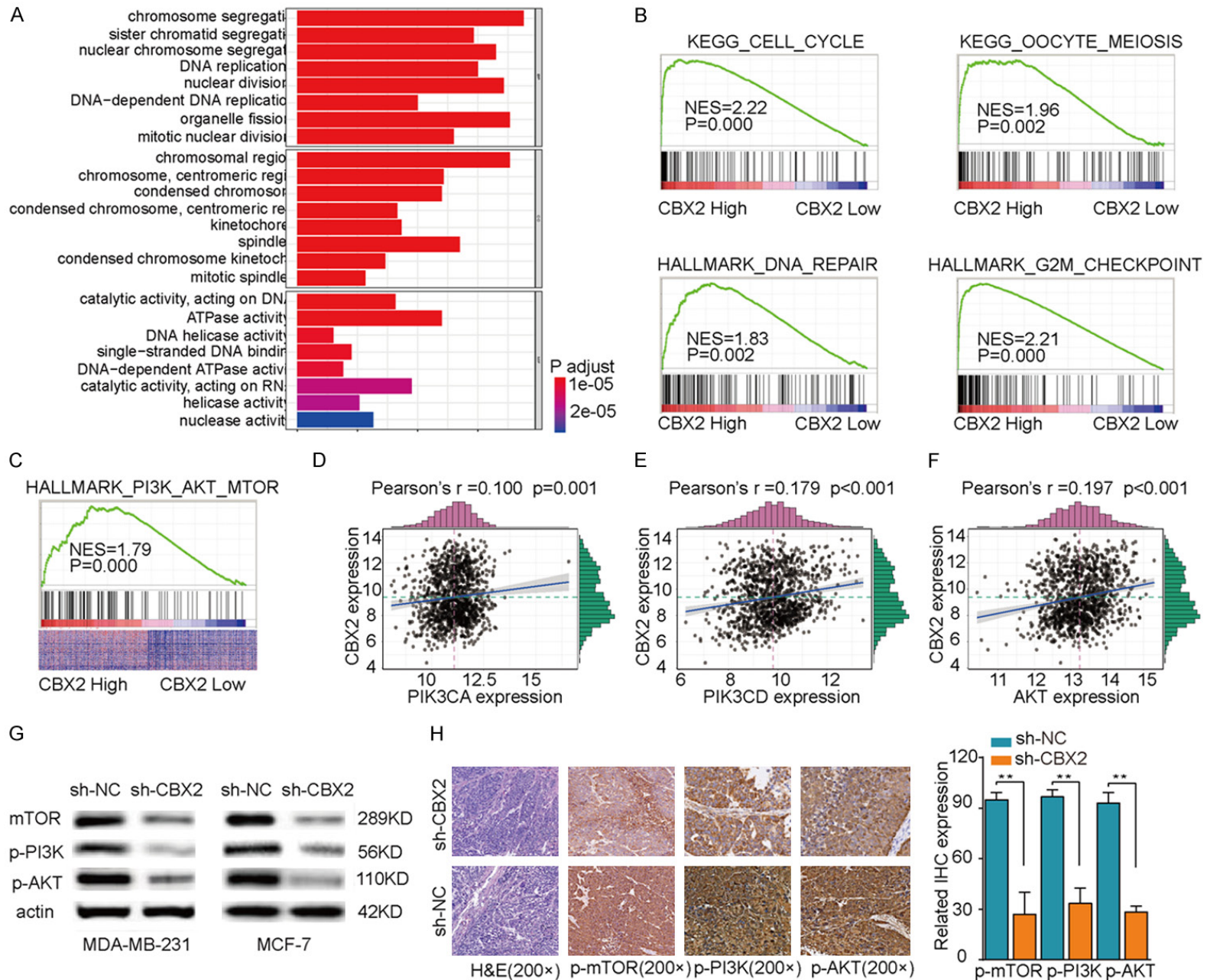
Figure 5. Functional and pathway enrichment analysis. A. Analysis of correlation between expressions of CBX2 and cell cycle-related genes. B. KEGG and GO enrichment analysis of correlation between differentially expressed CBX2 genes and cell cycle, as well as DNA replication pathway-related genes. C. GO enrichment analysis of correlation between differentially expressed CBX2 genes and PI3K/AKT pathway related genes. D-F. Analyses of correlations between expression of CBX2 and expressions of PIK3CA, PIK3CD, and AKT. G. Western blot analysis showing that the expressions of p-mTOR, p-PI3K, and p-AKT were significantly decreased in the sh-CBX2 group compared to the sh-NC group. H. Representative images of immunohistochemically stained mouse tumor sections (left) and bar graphical quantitation of IHC expression scores (right) showing that the expressions of p-mTOR, p-PI3K, and p-AKT were significantly decreased in the sh-CBX2 group compared to the sh-NC group. **P<0.01.

A growing body of evidence indicates that aberrant expression of CBX family members contributes to the progression of various types of cancer. For instance, Zeng J. *et al.* reported that CBX4 protein expression was upregulated in breast cancer and high expression of CBX4 was associated with increased metastasis, advanced TNM stage, and poor OS of breast cancer patients [27]. Zheng H. *et al.* reported that CBX6 protein expression was frequently elevated in hepatocellular carcinoma (HCC). In addition, elevated CBX6 expression was strongly associated with larger tumor sizes and poor OS of patients with HCC [28]. Concordant with these results, we found that CBX2 was significantly upregulated in breast cancer tissues both at the mRNA and protein levels. Additionally, upregulated CBX2 expression was associated with ER-negative and PR-negative status of breast tumors and with late TNM stages and metastasis. Moreover, the OS and PFS of patients with low expression of CBX2 were significantly better than those of patients with high expression of CBX2. The associations between CBX2 expression and clinical outcomes were upheld in both univariate and multivariate analyses, thereby indicating that CBX2 expression can serve as an independent prognostic factor. These results suggest that CBX2 has a critical role in breast cancer development and/or progression, and CBX2 might serve as a potential novel prognostic biomarker and potential therapeutic target for breast cancer.

Previous studies have identified CBX2 as a positive regulator of cell growth and metastasis in several cancer types [29-31]. However, the functional role of CBX2 in driving breast cancer progression remained undefined. We investigated the biological function of CBX2 in breast cancer and showed that knockdown of CBX2 inhibits proliferation of breast cancer cells *in vitro*. The migration and invasion abilities of breast cancer cells were also markedly impaired in CBX2 knockdown breast cancer cells *in vitro*. Moreover, we observed that tumorigenesis was suppressed in xenografts of breast cancer cells wherein CBX2 was silenced, indicating that CBX2 may function as an essential regulator of breast tumorigenesis and/or tumor growth. Consistent with our findings, one study showed that CBX4 silencing suppressed proliferation, colony formation, and migration abilities of colorectal cancer cells *in vitro*, and tumorigenicity *in vivo* [32]. Collectively, these finding suggest that CBX2 may play an oncogenic role in breast cancer, and silencing of CBX2 may be a potential therapeutic strategy for breast cancer.

Next, we explored the molecular mechanisms underlying CBX2's role in driving breast cancer development and/or progression. Through bioinformatics analysis based on the TCGA dataset, we found that CBX2 was involved in regulating cell cycle progression and DNA replication. Our GSEA results supported a model wherein CBX2 promoted signaling via the PI3K/AKT pathway. Results from our *in vitro* and *in vivo* experiments showed significant downregulation of PIK3CA, PIK3CD, and p-mTOR upon CBX2 silencing; these findings suggest that CBX2 directly regulates tumor growth via the PI3K/AKT pathway. In support of our results, deregulation of the PI3K/AKT pathway has been previously associated with cancer progression in multiple cancer types, including gallbladder cancer, colorectal cancer, endometrial carcinoma, and breast cancer [33-37]. Zhu M. *et al.* have reported that overexpression of the long non-coding RNA (lncRNA) MEG3 suppresses breast tumorigenesis via the PI3K/AKT pathway [38]. Altogether, these data powerfully indicate that CBX2 participates in breast cancer cell proliferation, migration, and invasion via regulation of the PI3K/AKT pathway.

Conclusions

Our findings provide significant insights into the progression of breast cancer. Our study has demonstrated that high expression of CBX2 is

associated with breast cancer development and poor prognosis, and that CBX2 regulates the PI3K/AKT pathway. Thus, CBX2 overexpression in breast cancer potentially drives breast cancer growth and invasion by enhancing signaling via the PI3K/AKT pathway. CBX2 merits further study as a potential therapeutic target for advanced breast cancer.

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Disclosure of conflict of interest

None.

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