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Original Article

USP14 regulates cell cycle progression through deubiquitinating CDK1 in breast cancer

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Abstract

Abnormal proliferation and cell cycle perturbation are the main hallmarks of breast cancer. Cyclin-dependent kinase 1 (CDK1) is one of the key kinases for cell transition from the G2 phase to M phase during the cell cycle progression. However, little is known about the degradation mechanisms of CDK1. USP14 (ubiquitin-specific processing protease 14) is an important proteasome-associated deubiquitinase that is critical for proteome homeostasis and plays a crucial role in the initiation and development of cancer. In this study, we find that USP14 shows high expression in breast cancer cells and results in the abnormal proliferation of cancer cells. Furthermore, we examine cell cycle distribution by flow cytometry and find that inhibition of USP14 causes cell cycle arrest in G2/M phase. As CDK1 is the key kinase in G2/M phase, we detect the interaction between USP14 and CDK1 and the effect of USP14 on the deubiquitination of CDK1. The results reveal that USP14 interacts with CDK1 and stabilizes CDK1 by deubiquitinating K48-linked ubiquitination. In conclusion, our findings reveal an indispensable role of USP14 in regulating cell cycle progression by stabilizing CDK1 in breast cancer, suggesting that USP14 may be used as a potential therapeutic target in breast cancer therapy.

Key words breast cancer, USP14, CDK1, deubiquitination

Introduction

As one of the most frequent malignancies in women globally, breast cancer results in the majority of cancer-related death [1]. Based on the expression of human epidermal growth factor receptor 2 (HER2), progestogen receptor (PR), and estrogen receptor (ER) status, breast cancers are generally classified into five main subtypes: HER2-enriched (non-luminal), luminal B-like HER2$^+$, luminal B-like HER2$^-$, triple-negative, and luminal A-like [2]. At present, comprehensive treatments such as surgery, radiotherapy, chemotherapy, endocrine therapy, and molecular targeted therapy have significantly improved the therapeutic efficacy and survival of patients with breast cancer. However, the efficacy of targeted therapies is still very limited, especially for triple-negative breast cancer (TNBC) which makes up 15%–20% of breast cancers. Because of the lack of effective therapeutic targets, TNBC has a poor prognosis and a high rate of recurrence. Therefore, targeted therapy for breast cancer is a current challenge and an urgent need.

Ubiquitin-mediated degradation is one of the main processes for protein degradation and strictly controls the quality and quantity of intracellular proteins [3]. As an important member of the ubiquitin–proteasome system, deubiquitinase functions as an antagonist to E3 ubiquitin ligase. The human genome sequence encodes more than 100 known DUBs, and the major DUB family is the ubiquitin-specific processing protease (USP) family [4]. Among those putative USPs, USP14 can shorten the anchoring time of ubiquitin conjugates with the proteasome and stabilize the substrate protein by inducing deubiquitination of targeted proteins [5]. Until now, USP14 has been thought to be involved in the initiation and progression of different types of cancers. For example, USP14 is related to the malignant transformation of hepatocytes and promotes hepatocellular carcinoma development by increasing cell proliferation, altering the cell cycle and reducing apoptosis [6]. Screening for

genetic abnormalities in ovarian cancer cell lines pointed to the important role of USP14 in ovarian carcinogenesis [7]. Another study revealed that high expression of USP14 is correlated with melanoma development and a poorer survival rate in metastatic melanoma patients. Additionally, inhibition of USP14 instantly results in the accumulation of ubiquitinated chaperones or proteins that trigger caspase-independent cell death [8]. Recently, Liu *et al.* [9] identified 42 potential substrates of USP14, including CDK1 protein, indicating that USP14 might directly regulate cell cycle progression through CDK1.

Abnormal proliferation and cell cycle perturbation are the main hallmarks of cancer [10]. Eukaryotic cell proliferation depends on the cell division cycle, which is composed of four major phases: two gap phases (G1 and G2), the DNA replication phase (S phase) and the mitosis phase (M phase) [11]. The CDK1/Cyclin B1 complex is the key kinase for cells entering the mitosis phase of the cell cycle [12]. The substrates of CDK1–CyclinB1 complexes include a series of substrates necessary for centrosome separation, nuclear membrane rupture, mitotic spindle assembly and chromatin condensation. At present, dysregulation of CDK1 is found to be closely related to tumor development. CDK1 is highly expressed in prostate cancer, lung cancer, gastric carcinoma and cholangiocarcinoma [13–16]. Further research revealed that CDK1 interacts directly with FOXO1 and that overexpression of CDK1 specifically inhibits the transcription activity of FOXO1 through phosphorylation on serine 249 in PCa cells [17]. Under radiation conditions, CDK1 could relocate to the mitochondria and enhance SIRT3 enzymatic activity via Thr150/Ser159 phosphorylation to reduce the acetylation level of mitochondrial proteins [18].

In our previous study, we revealed that the deubiquitinating enzyme USP14 could regulate the expression of cyclin B1 [19]. However, the underlying mechanism of USP14 in influencing cell cycle progression is still not well understood. In this study, we found that USP14 was highly expressed in breast cancer cells and promoted the proliferation of breast cancer cells. Through flow cytometry, we demonstrated that *USP14* knockdown arrested the cell cycle at the G2/M phase. Further studies demonstrated that USP14 interacted with CDK1 and stabilized CDK1 through deubiquitination. These results reveal that USP14 regulates cell cycle progression through deubiquitinating and stabilizing CDK1 in breast cancer cells, indicating the potential role of USP14 as a therapeutic target in breast cancer.

Materials and Methods

Reagents

MG132 was purchased from BioVision (Waltham, USA). IU1 was purchased from APE × BIO (Houston, USA). Crystal violet, cycloheximide (CHX), chloroquine (CQ), DMSO and other analytical grade chemical reagents were obtained from Sigma (St Louis, USA). The mouse monoclonal anti-β-actin, anti-His and anti-Flag antibodies were obtained from Proteintech (Rosemont, USA). The rabbit polyclonal anti-USP14, anti-CDK1 and anti-UB antibodies were obtained from Proteintech. Rabbit anti-K48-linked polyubiquitin antibody was obtained from Cell Signaling Technology (Danvers, USA). Horse radish peroxidase-labeled goat anti-mouse or anti-rabbit IgG (H + L) secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, USA). The pcDNA3.1-His-USP14 plasmid was ordered from TSINGKE (Beijing, China).

Gene overexpression and knockdown

For transfection to overexpress certain genes, cells were transiently transfected using the certain plasmids using the SuperFectin DNA Transfection Reagent kit (Pufei, Shanghai, China). Then, the relative transient transfection efficiency in cells was measured by western blot assay using appropriate antibodies.

For transfection to knock down the *USP14* gene, cells were transiently transfected with certain RNAi nucleotides using the SuperFectin siRNA Transfection Reagent (Pufei). Then, the relative transient transfection efficiency in cells was measured by western blot analysis using the relevant antibodies. *USP14* knockdown was performed using two individual Stealth Select RNAi duplexes. The Stealth RNAi negative control duplex (Cat No. 12935100; Life Technologies, Carlsbad, USA) was used as a control. The siRNA sequences were as follows: USP14 siRNA1: 5′-CCUUAGAGAUUUG UUUGAUUCCAUG-3′; USP14 siRNA2: 5′-GGAAGCAAUAGAGGAUG AUUCUGUU-3′.

Cell culture

MCF-10A cell basal medium (Procell, Wuhan, China) was used to culture the normal breast cells (MCF-10A). Dulbecco's modified Eagle's medium (DMEM; Solarbio, Beijing, China) containing 10% fetal bovine serum (ExCell Bio, Shanghai, China) was used to culture the human breast cancer cell lines (BT549, MDA-MB-468, SKBR3, MDA-MB-231 and MCF7) and 293T cells. All cells were obtained from American Type Culture Collection (Manassas, USA) and incubated in a 5% CO_2 incubator at 37°C.

MTT assay

Cells were seeded in 96-well plates at a density of 3000 cells per well in 200 μL DMEM supplemented with 10% FBS. The cell culture medium was replaced every 2 days. At certain time points, cells were treated with 20 μL MTT reagent (5 mg/mL; Sigma, St Louis, USA) and incubated for 4 h in the dark. When the purple precipitate was clearly visible, 100 μL DMSO was added to each well. Then, the relative absorbance was measured at 495 nm. Each experiment was conducted in triplicate.

Crystal violet staining assay

Cells were seeded in 24-well plates at a density of 5000 cells per well in 500 μL DMEM containing 10% FBS. The cell culture medium was replaced every 2 days. At certain time points, cells were fixed with 4% formaldehyde for 30 min, and then stained with 0.1% crystal violet, followed by extraction with 10% acetic acid. Then, the relative absorbance was measured at 595 nm. Each experiment was conducted in triplicate.

Colony formation assay

Cells were seeded in 6-well plates at a density of 500 cells per well in 1 mL DMEM containing 10% FBS. The cell culture medium was replaced every 2 days. Ten days later, after fixation with 4% formaldehyde for 30 min, the cells were stained with 0.1% crystal violet. Images were obtained under a microscope with a digital camera, and the number of colonies was counted.

Saturation density assay

Cells were seeded in 12-well plates at a density of 10^5 cells per well in 500 μL DMEM containing 10% FBS. The cell culture medium was replaced every 2 days. Six days later, the number of cells was

counted. Each experiment was conducted in triplicate.

RT-qPCR

TRIzol reagent (Invitrogen, Carlsbad, USA) was used to extract total RNA from cells and the PrimeScript RT reagent kit with gDNA Eraser (Takara, Dalian, China) was used to synthesize cDNA. A SYBR Green Premix Ex Taq II kit (Takara) was used to perform qPCR experiments on the ABI Vii 7 Real-Time PCR System (Applied Biosystems, Foster City, USA). All primers were validated by universal cDNA standards and listed in Table 1. The relative mRNA expression levels of target genes were calculated by analysing the comparative Ct values. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. Measurements were conducted in triplicate.

Immunoprecipitation and western blot analysis

For immunoprecipitation, NP-40 lysis buffer supplemented with PMSF (Amresco, Houston, USA) was used to lyse cells for 30 min at 4°C. Then, the cell lysates were centrifuged at 12,000 *g* for 20 min at 4°C and the supernatants were collected. The supernatants were mixed with certain antibodies and protein G agarose beads (Roche, Shanghai, China) pre-washed with NP-40 buffer at least three times, and incubated overnight at 4°C. Then, the agarose beads was washed with lysis buffer, mixed with loading buffer, boiled for 10 min, and subject to western blot analysis.

For the western blot analysis, the protein samples were separated by 10% SDS-PAGE, and transferred to PVDF membranes (Millipore, Billerica, USA). Then, the membranes were blocked with 5% skim milk for one hour at room temperature and incubated with the appropriate primary antibodies overnight. The next day, TBST was used to wash the membranes at least three times, and HRP-conjugated secondary antibody was used to incubate with the membranes for one hour at room temperature. Protein bands were visualized using the pro-light HRP chemiluminescent kit (TIANGEN, Beijing, China) and analysed with a digital gel image analysis system (Tanon, Shanghai, China), using β-actin as the loading control.

GST affinity isolation

The GST-CDK1 protein was purchased from Sino Biological (Beijing, China). For the GST pull-down assay, glutathione-agarose (Millipore, Billerica, USA) was washed with PBS at least three times. Then, NP-40 lysis buffer supplemented with PMSF was used to lyse cells for 30 min at 4°C. After centrifugation at 12,000 *g* for 15 min, the supernatants were transferred to fresh tubes, and GST-CDK1 protein together with glutathione-agarose was added and incubated at 4°C overnight. After that, the beads were washed with PBS at least three times and boiled with loading buffer. The bound proteins were detected by western blot analysis.

Table 1. The sequences of primers used in qRT-PCR

Name	Sequence
USP14	Forward: 5′-CCAGAAGAACCCTCAGCCAAA-3′
	Reverse: 5′-CTCAAGGCACCTGCATACCT-3′
CDK1	Forward: 5′-GGAAATTGAGCGGAGAGCGA-3′
	Reverse: 5′-TGGCTACCACTTGACCTGTAG-3′
GAPDH	Forward: 5′-GGCTGTTGTCATACTTCTCATGG-3′
	Reverse: 5′-GGAGCGAGATCCCTCCAAAAT-3′

Cell cycle analysis

For cell cycle progression analysis, cells were seeded in 6-well plates. After different treatments, cells were harvested and washed with PBS at least three times. The cells were fixed with 4 mL cold 70% alcohol for at least 2 h, and then resuspended in 400 μL guava cell cycle reagent (Millipore). After being washed with PBS, the cells were incubated for at least 15 min at 37°C and analysed with a BD FACS Jazz™ Cell Sorter (BD, Franklin Lakes, USA).

Statistical analysis

Each experiment was conducted in triplicate. All data are expressed as the mean ± SD. Student's *t*-test was used to conduct statistical evaluation. One-way analysis of variance followed by Dunnett's test was used to compare intergroup differences. A P value less than 0.05 was considered statistically significant.

Results

USP14 promotes the proliferation of breast cancer cells

To determine the major functions of USP14 in breast cancer, we first detected the expression level of USP14 in different breast cancer cells. As shown in Figure 1A, the protein level of USP14 was markedly higher in breast cancer cell lines than in normal mammary epithelial cells (MCF-10A), suggesting a potential role for USP14 in the progression of breast cancer. Abnormal cell proliferation is one of the main hallmarks of cancer. Thus, we detected the effects of USP14 on proliferation in breast cancer cells (MDA-MB-231 and MDA-MB-468) in which the USP14 protein level was higher. In proliferation assay, as shown in Figure 1B,C, overexpression of USP14 significantly increased the proliferation rate, while knockdown of *USP14* showed the opposite effect. The protein expression level of USP14 was measured by western blot analysis (Supplementary Figure S1A,B). To further validate the above results, after knockdown or overexpression of USP14, the corresponding cell proliferation was checked at the different time points. Figure 1D,E showed that the protein level of USP14 affected the proliferation and colony formation of breast cancer cells. The MTT assay was also performed and further demonstrated that USP14 plays a crucial role in cell proliferation (Supplementary Figure S2A, B). Then, in MDA-MB-231 cells, following treatment with IU1, which is a specific inhibitor of USP14, similar inhibitory effects on cell proliferation were observed (Figure 1F). All these results demonstrated that USP14 is crucial and promotes proliferation in breast cancer cells.

USP14 inhibition arrests the cell cycle at G2/M phase

Then, we studied the potential function and mechanism by which USP14 regulates the proliferation in breast cancer cells. Generally, cell cycle is crucial for the abnormal proliferation of cancer cells; thus, we studied the role of USP14 in cell cycle progression. MDA-MB-231 cells were transiently transfected with CTL siRNA or USP14 siRNAs. After propidium iodide staining, cell cycle progression was analysed by flow cytometry. Knockdown of *USP14* obviously upregulated the proportion of cells in G2/M phase to 24.15% (14.75% in cells transfected with CTL siRNA) and decreased the proportion of cells in G0/G1 phase to 67.41% (76.78% in cells transfected with CTL siRNA) (Figure 2A,B). Similar effects on cell cycle progression were also observed following treatment with IU1 (Figure 2C,D). All these results suggested that USP14 was essential for cell cycle progression, and genetic or pharmacological inhibition of USP14 arrested the cell cycle at the G2/M phase. CDK1 is the key

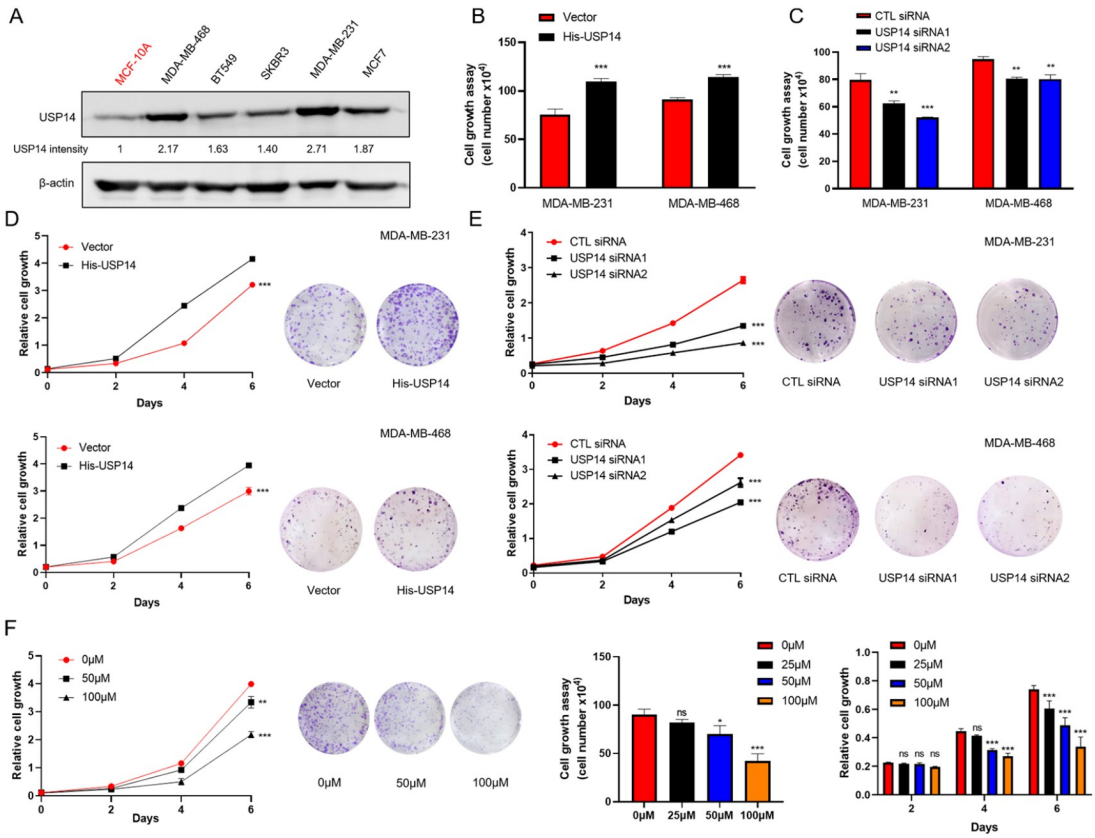

Figure 1. USP14 promotes the proliferation of breast cancer cells (A) The protein levels of USP14 in normal mammary epithelial cells (MCF-10A) and five breast cancer cell lines (BT549, MDA-MB-468, SKBR3, MDA-MB-231 and MCF7) were detected by western blot analysis. β-Actin served as an internal control. (B,C) MDA-MB-231 and MDA-MB-468 cells were cultured for 6 days following transient transfection with control vector or pcDNA3.1-His-USP14 (His-USP14) plasmid (B) and with control (CTL) siRNAs or siRNAs specifically targeting USP14 (USP14 siRNAs) (C). Then, trypsin-digested cells were counted and analysed. $^{**}P < 0.01$, $^{***}P < 0.001$. (D,E) MDA-MB-231 and MDA-MB-468 cells were transiently transfected with control vector or His-USP14 plasmid (D) or transiently transfected with CTL siRNA or USP14 siRNAs (E). At certain time points, the cells were fixed with formaldehyde, stained and extracted with acetic acid. Then, the relative absorbance was measured at 595 nm. $^{***}P < 0.001$ (left). The cells were transiently transfected as above. Ten days later, images of colony formation were obtained by crystal violet staining and photographed (right). (F) MDA-MB-231 cells treated with various concentrations of IU1 (0, 25, 50 and 100 μM) were subject to the above cell growth assays. Data are presented as the mean ± SD from three independent experiments.$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns: not significant.

regulator of the G2 to M phase transition. As shown in Figure 3A, the protein expression trends of CDK1 and USP14 was similar in breast cancer cells. Overexpression of USP14 obviously increased the protein level of CDK1 (Figure 3B). In contrast, the protein level of CDK1 was decreased when *USP14* was knocked down or inhibited by IU1 (Figure 3C,D). Therefore, these results suggested that USP14 might affect the G2/M phase transition by regulating the protein expression of CDK1 in breast cancer cells.

USP14 stabilizes CDK1 protein in breast cancer

The above results showed that USP14 regulated the protein level of CDK1, which contributed to the G2/M phase transition. DUBs can regulate substrate proteins at different levels, such as the interaction between two proteins or post-translation modification. They can also bind to specific transcription factors or histone-associated proteins to regulate transcription [20]. Thus, we examined whether USP14 affects the transcription level of *CDK1*. We used real-time PCR to measure the mRNA level of *CDK1*. Figure 4A,B showed that the transcription levels of CDK1 were not affected or were only slightly affected when USP14 was overexpressed or inhibited in MDA-MB-231 and MDA-MB-468 cells. These results suggested that USP14 might regulate the expression of CDK1 by posttranslation modifications. We further investigated whether USP14, as a deubiquitinase, could regulate the protein stability of CDK1. Under the condition of USP14 overexpression, inhibition or silencing, we investigated the protein degradation rate of CDK1 in the presence of CHX. Figure 4C showed that the degradation rate of CDK1 was decreased when USP14 was overexpressed, while cotreatment with CHX and IU1 or USP14 siRNA resulted in a faster reduction in the levels of endogenous CDK1 protein, suggesting that USP14 is essential for the stability of CDK1 protein. MG132 is a proteasomal inhibitor, and chloroquine (CQ) is a lysosomal inhibitor. Figure 4D showed that the decreased protein expression of CDK1 could be recovered by MG132 but not by CQ. Figure 4E showed that MG132 reversed the level of CDK1 degradation caused by USP14 inhibition. These results revealed that CDK1 protein was degraded by the proteasomal degradation pathway and that USP14 stabilized CDK1 protein.

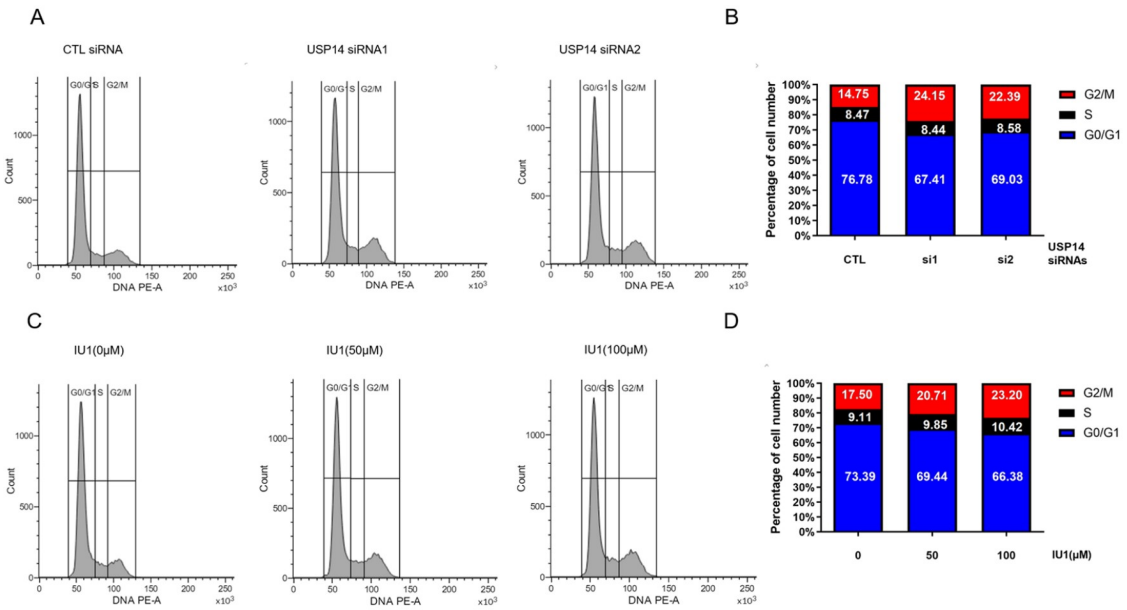

Figure 2. USP14 inhibition arrests the cell cycle at G2/M phase (A,B) MDA-MB-231 cells were transiently transfected with CTL siRNA or USP14-specific siRNAs. The cells were resuspended, stained and incubated with cell cycle reagent. Then, cell cycle progression was analysed by flow cytometry. The proportion of cells in each stage was counted. (C,D) MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS and treated with various concentrations of IU1 (0, 50 and 100 μM). Then, cell cycle progression was analysed by flow cytometry. The proportion of cells in each stage was counted.

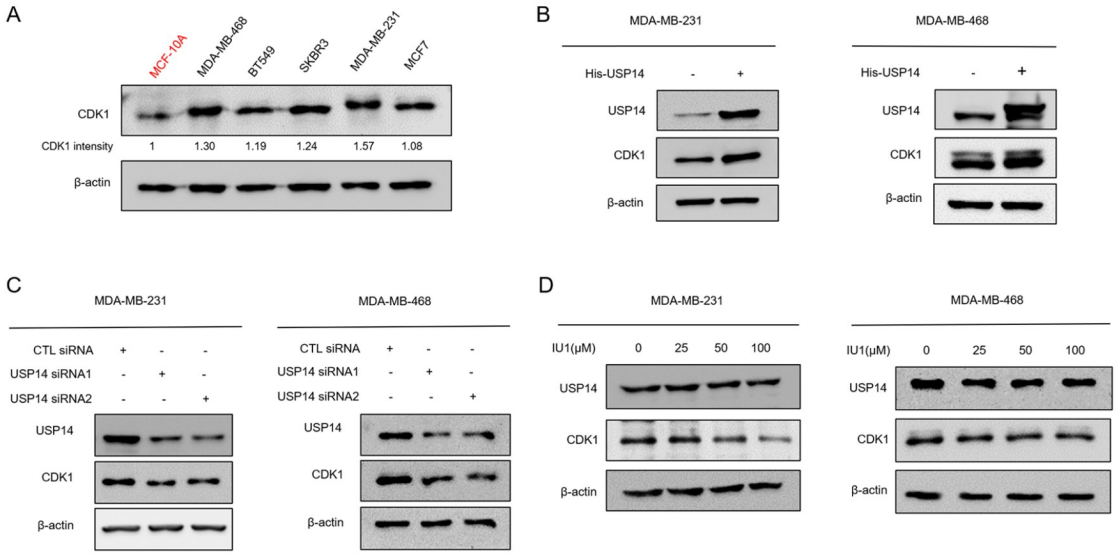

Figure 3. USP14 regulates the protein expression of CDK1 in breast cancer (A)The protein levels of CDK1 in normal mammary epithelial cells (MCF-10A) and five breast cancer cell lines (BT549, MDA-MB-468, SKBR3, MDA-MB-231 and MCF7) were detected by western blot analysis. β-Actin served as an internal control. (B–D) MDA-MB-231 cells were transiently transfected with control vector or His-USP14 plasmid (B), transiently transfected with CTL siRNA or USP14-specific siRNAs (C) or treated with different concentrations of IU1 (0, 25, 50 and 100 μM) (D). Two days later, the cells were lysed, and protein expressions were detected by western blot analysis.

USP14 deubiquitinates the K48-linked ubiquitin chain of the CDK1 protein

To determine whether USP14 could interact with CDK1, we performed co-immunoprecipitation and GST pull-down experiments and found that USP14 could interact with CDK1 (Figure 5A,B). Overexpression of USP14 reduced the ubiquitination level of endogenous CDK1, while either genetic or pharmacological inhibition of USP14 increased the ubiquitination level of CDK1 (Figure 5C, D and Supplementary Figure S3A). Intracellular proteins attached by K48-linked ubiquitin chains usually tend to undergo proteasome-mediated degradation [21]. To further elucidate the function of USP14 in CDK1 deubiquitination and protein stabilization, we further repeated the above experiment by using an antibody specific for K48-linked ubiquitination. Figure 6A,B and Supplementary Figure S3B showed that the reduced ubiquitination of CDK1 regulated by USP14 might be K48-linked. To further validate the

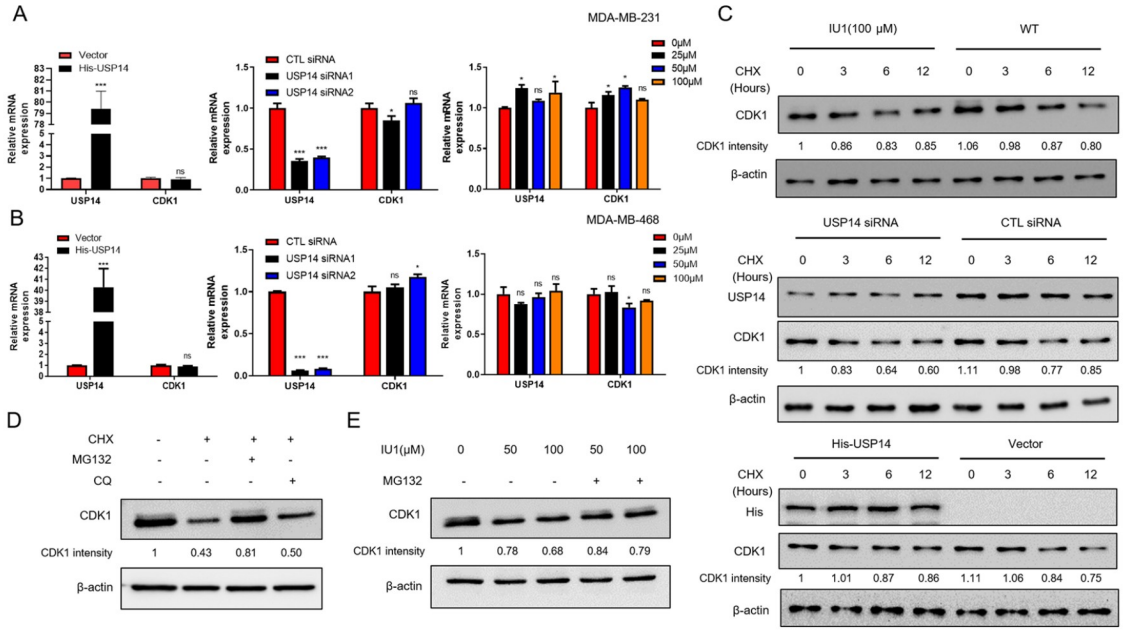

Figure 4. USP14 stabilizes CDK1 protein in breast cancer (A,B) MDA-MB-231 and MDA-MB-468 cells were transiently transfected with control vector or His-USP14 plasmid or transiently transfected with CTL siRNA or USP14-specific siRNAs or treated with different concentrations of IU1 (0, 25, 50 and 100 μM), and cDNA was extracted for real-time PCR. The mRNA expression levels of USP14 and CDK1 were measured. $*P<0.05$, $***P<0.001$. ns: not significant. (C) MDA-MB-231 cells were treated with 25 μg/mL cycloheximide (CHX) for various time intervals and were transiently transfected with His-USP14 plasmid or USP14-specific siRNA or treated with 100 μM IU1. CDK1 protein expression was measured by western blot analysis, and CDK1 protein expression relative to β-actin level was quantified. (D) MDA-MB-231 cells were treated with 25 μg/mL CHX alone or with 25 μg/mL CHX plus 20 μM MG132 or with 25 μg/mL CHX plus 20 μM chloroquine (CQ) for 6 h. CDK1 protein expression was measured by western blot analysis. CDK1 protein expression relative to β-actin level was quantified. (E) MDA-MB-231 cells were treated with different concentrations of IU1 (0, 50 and 100 μM) plus 20 μM MG132 for 12 h. CDK1 protein expression was measured by western blot analysis. CDK1 protein expression relative to β-actin level was quantified.

above results, USP14 was co-expressed along with one of four different mutant isoforms of Ub, including Ub carrying a single lysine residue at position 48 (K48), Ub in which K48 was mutated to arginine (K48R) but all other lysine residues were intact, Ub carrying a single lysine residue at position 63 (K63), and Ub in which K63 was mutated to arginine (K63R) but all other lysine residues were intact. As shown in Figure 6C, overexpression of USP14 reduced the ubiquitination of CDK1 when cotransfected with K48-Ub but not with K63-Ub. We next used K48R and K63R plasmids to detect the altered ubiquitination level of CDK1. When cotransfected with the K48R mutant, the reduced ubiquitination level of CDK1 regulated by USP14 overexpression was not observed. However, the reduced ubiquitination level of CDK1 could still be found when co-transfected with the K63R mutant plasmid, indicating that USP14 hydrolyzed the K48-linked ubiquitination of CDK1 (Figure 6D). All these results suggested that USP14 regulated CDK1 protein expression by deubiquitinating the K48-linked ubiquitin chain.

CDK1 overexpression recovers the reduced proliferation rate and cell cycle arrest regulated by *USP14* knockdown

To further elucidate whether the decreased proliferation of breast cancer cells caused by USP14 knockdown is caused by the decreased protein expression of CDK1, we co-transfected MDA-MB-231 cells with Flag-CDK1 plasmid and USP14 siRNA. Then, we repeated the above cell proliferation experiments. The USP14 and CDK1 expression levels were checked by western blot analysis (Figure 7A). We found that the decreased proliferation in breast cancer cells caused by *USP14* knockdown was recovered when the expression of CDK1 was rescued (Figure 7B,C). Next, we investigated the effects of recovered CDK1 expression on cell cycle progression, which was arrested in G2/M phase after *USP14* knockdown. Figure 7D,E showed that overexpression of CDK1 could rescue the arrest of cell cycle progression resulted from *USP14* knockdown. All the above results revealed that USP14 regulated the proliferation in breast cancer cells by affecting the ubiquitination level of CDK1.

Discussion

Until now, only a few studies have been carried out on the role of USP14 in breast cancer, and its underlying molecular mechanism is not well understood. Recent studies have shown that USP14 plays a regulatory role by stabilizing specific substrates. For example, USP14 overexpression could promote the accumulation of liver triglyceride accumulation via stabilization of fatty acid synthase (FASN) in obese mice, resulting in hepatosteatosis, insulin resistance and hyperglycemia [9]. In prostate cancer, USP14 binds to androgen receptor (AR), and USP14 inhibition promotes the ubiquitination of AR and accelerates its degradation, thus inhibiting the proliferation of prostate cancer cells and arresting the cell cycle in G0/G1 phase [22]. In breast cancer, inhibition of USP14 was also found to cause G0/G1 arrest, and this effect is closely associated

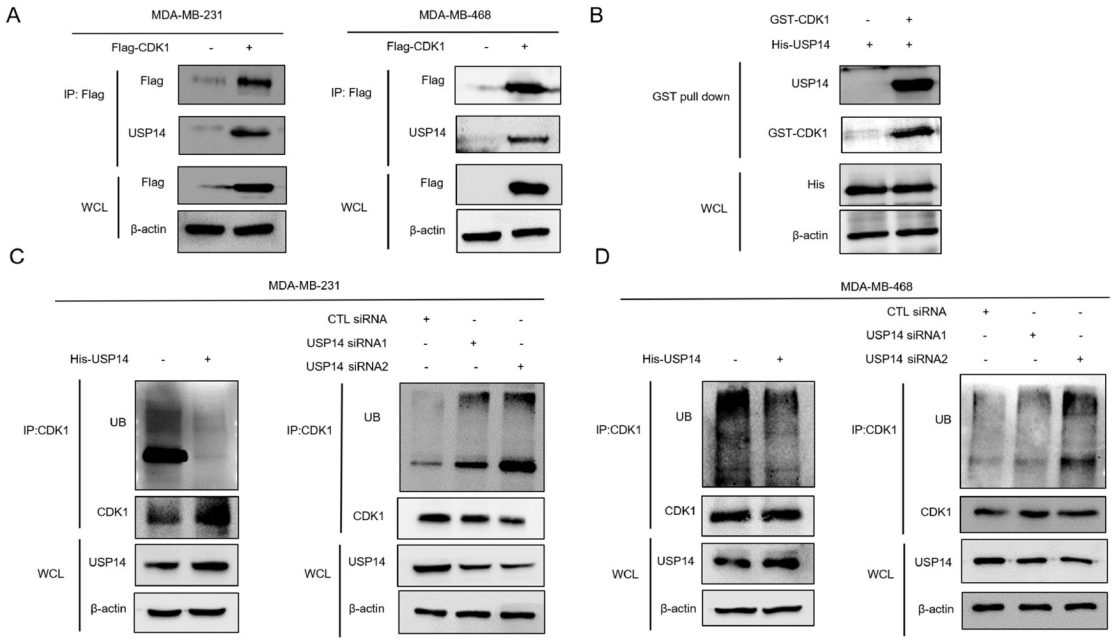

Figure 5. USP14 interacts with CDK1 and regulates the ubiquitination of CDK1 (A) MDA-MB-231 and MDA-MB-468 cells were transiently transfected with or without Flag-CDK1 plasmid. Then, the cells were lysed for immunoprecipitation by using an anti-Flag antibody and blotted with certain antibodies. (B) GST-CDK1 was incubated with extracts from His-USP14-transfected 293T cells. Western blot analysis was performed to detect the indicated proteins using anti-USP14 and anti-GST antibodies. (C,D) MDA-MB-231 and MDA-MB-468 cells were transiently transfected with control vector or His-USP14 plasmid and with CTL siRNA or USP14-specific siRNAs. Then, the cells were lysed for immunoprecipitation by using an anti-CDK1 antibody. Ubiquitination was measured by using the indicated antibody for ubiquitin.

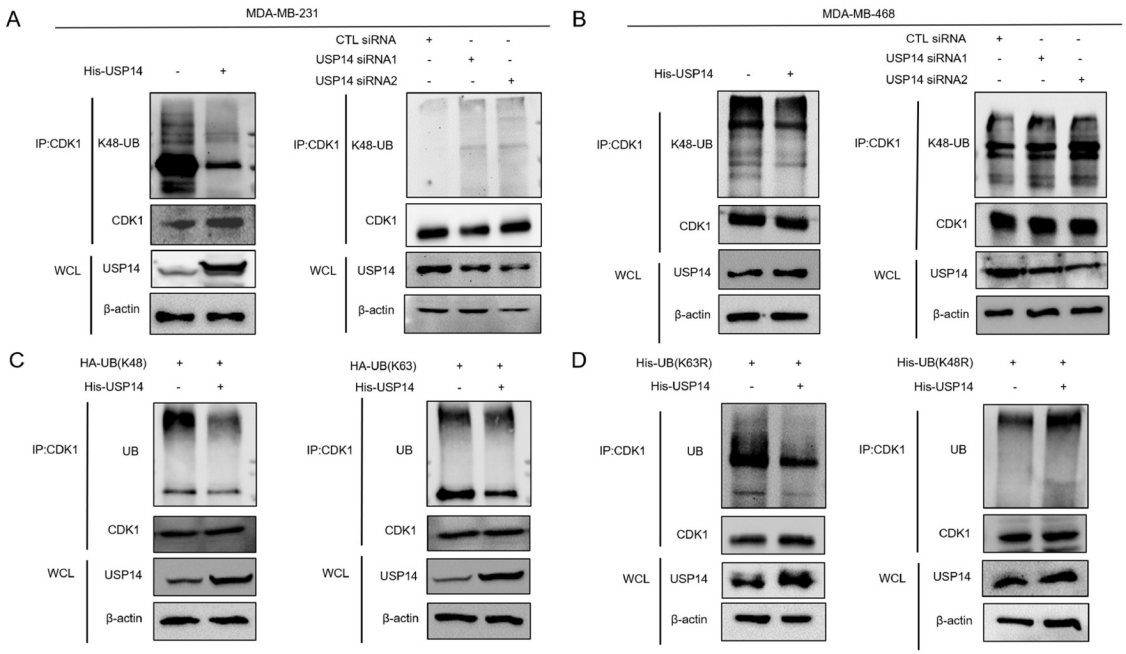

Figure 6. USP14 deubiquitinates the K48-linked ubiquitin chain of CDK1 (A,B) MDA-MB-231 (A) and MDA-MB-468 (B) cells were transiently transfected with control vector or His-USP14 plasmid and with CTL siRNA or USP14-specific siRNAs. Then, the cells were lysed for immunoprecipitation by using an anti-CDK1 antibody. K48-linked ubiquitination was measured by using the indicated antibody for ubiquitin K48 linkage. (C) MDA-MB-231 cells were co-transfected with plasmids encoding HA-UB(K48) or HA-UB(K63) and with or without the His-USP14 plasmid. Then, the cells were lysed for immunoprecipitation using an anti-CDK1 antibody. Ubiquitination was measured by using the indicated antibody for ubiquitin. (D) MDA-MB-231 cells were co-transfected with plasmids encoding His-UB(K63R) or His-UB(K48R) with or without the His-USP14 plasmid. The lysates were immunoprecipitated using anti-CDK1 antibody. Ubiquitination was measured by using the indicated antibody for ubiquitin.

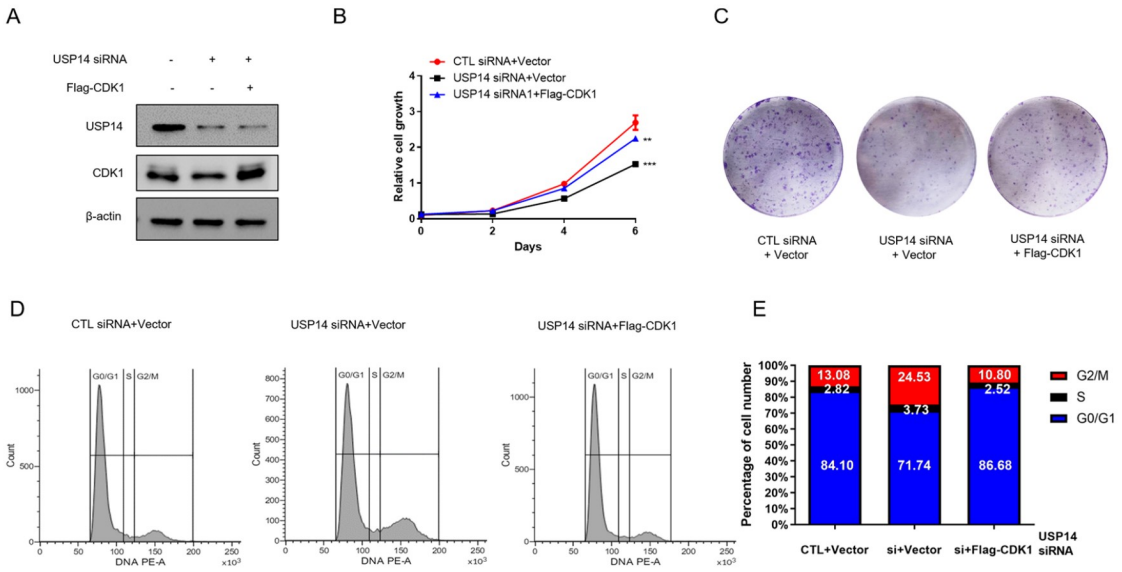

Figure 7. CDK1 overexpression recovers the reduced proliferation and cell cycle arrest induced by *USP14* knockdown (A) MDA-MB-231 cells were transiently co-transfected with USP14-specific siRNA and Flag-CDK1 plasmid. Two days later, the cells were lysed, and protein expression was detected by western blot analysis. (B) MDA-MB-231 cells were transiently co-transfected with USP14-specific siRNA and Flag-CDK1 plasmid. At certain time points, the cells were fixed with formaldehyde, stained and extracted with acetic acid. Then, the relative absorbance was measured at 595 nm. $^{**}P < 0.01$, $^{***}P < 0.001$. (C) MDA-MB-231 cells were transiently transfected as above. Ten days later, images of colony formation were obtained by crystal violet staining and photographed. (D,E) MDA-MB-231 cells were transiently transfected as above. The cells were resuspended, stained and incubated with cell cycle reagent. Then, cell cycle progression was analysed by flow cytometry. The proportion of cells in each stage was calculated.

with the expression of androgen receptor (AR) [22]. This result seems to contradict our results, which showed G2/M arrest when USP14 was inhibited. However, their study mainly used breast cancer cells with high AR expression. In breast cancer cells with low expression of AR, knockdown of *USP14* also arrested cells at G2/M phase except for the inhibition of G0/G1 phase [23]. In our study, we used MDA-MB-231 and MDA-MB-468 cells which expressed low level of AR, and inhibition of USP14 arrested the cell cycle at G2/M phase. This indicated that the effects of USP14 on cell cycle regulation might depend on the AR expression level.

Cell proliferation is a cyclic process of material accumulation and cell division. CDKs, a group of serine/threonine protein kinases, phosphorylate their corresponding substrates and drive cell cycle progression. Abnormal expression of CDK can cause an inordinate cell cycle process, leading to malignant proliferation and tumor formation. The activity of CDK1 depends on cyclin A/B, which is the key kinase that regulates cell cycle progression from G2 to M phase [24,25]. In breast cancer, miR424 suppresses cell proliferation and arrests cells in G2/M phase by negatively regulating CDK1 mRNA level, possibly through the Hippo pathway and the extracellular signalregulated kinase pathway [26]. For MYC-driven breast cancer, inactive CDK1 markedly reduces viability and promotes apoptosis. CDK1 inhibitors can induce apoptosis, which is related to the overexpression of the pro-apoptotic molecule BIM [27]. These results suggest that inhibiting CDK1 in breast cancer is an effective cancer treatment strategy. Until now, there have been few studies on the degradation mechanism of CDK1. The E3 ubiquitin ligase $SCF^{\beta\text{-}TRCP}$ ubiquitinates CDK1 and promotes degradation, indicating the important role of ubiquitination in protein stability. However, there is no relative report about CDK1 deubiquitination. In our study, USP14 was shown to interact with CDK1 and regulate its ubiquitination. Further study revealed that USP14 hydrolyzes the K48-linked ubiquitin chain on CDK1 and subsequently inhibits proteasome-mediated degradation of CDK1, consistent with a previous report that USP14 could shorten the anchoring process of ubiquitin substrates on the proteasome to inhibit proteasome-mediated deubiquitination. Therefore, our study first reported that USP14 is the key deubiquitinase for CDK1, which affects cell cycle progression by regulating the ubiquitination of CDK1.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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