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FOXD1 promotes breast cancer proliferation and chemotherapeutic drug resistance by targeting p27

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ABSTRACT

Forkhead transcription factors are essential for diverse processes in early embryonic development and organogenesis. As a member of the forkhead family, FOXD1 is required during kidney development and its inactivation results in failure of nephron progenitor cells. However, the role of FOXD1 in carcinogenesis and progression is still limited. Here, we reported that FOXD1 is a potential oncogene in breast cancer. We found that FOXD1 is up-regulated in breast cancer tissues. Depletion of FOXD1 expression decreases the ability of cell proliferation and chemoresistance in MDA-MB-231 cells, whereas overexpression of FOXD1 increases the ability of cell proliferation and chemoresistance in MCF-7 cells. Furthermore, we observed that FOXD1 induces G1 to S phase transition by targeting p27 expression. Our results suggest that FOXD1 may be a potential therapy target for patients with breast cancer.

1. Introduction

Breast cancer is the most common tumor in women around the world. Although significant advances have been made in chemotherapy, drug resistance is one of the major hurdles to overcome for the successful treatment and leads to poor outcome for the patients [1]. The underlying mechanisms of the acquisition of resistance to chemotherapeutic agents are still poorly understood. At present, the main hypotheses, genetic and epigenetic, have been proposed to explain the basis of cancer drug resistance [2,3]. This clearly suggests that drug resistance, whether intrinsic or acquired over time, constitutes a major hurdle to overcome for the successful treatment of breast cancer.

Forkhead box transcription factors, characterized by a common 100 amino acid winged-helix DNA-binding domain, have important roles in cell growth, survival, differentiation, longevity, migration, metabolism and immunity [4–7]. The mutation or dysregulation of FOX genes is often associated with a variety of cancers [8]. FOXM1 is expressed in glioma and pancreatic cancer cells and promotes angiogenesis by transactivating VEGF expression [9–11]. FOXQ1 is overexpressed in colorectal cancer and enhances tumorigenicity and tumor growth [12]. Furthermore, FOXM1, FOXQ1 and FOXC1 promote epithelial-mesenchymal transition (EMT) and increased cell proliferation, migration and invasion [13–16]. FOXO1/3/4 act as tumor suppressors in prostate cancer and leukemia [17,18]. Thus, the elucidation of FOX gene function will likely identify new strategies for the treatment of cancer.

As a member of the forkhead family, FOXD1 is required for kidney development [19] and is restricted to cortical interstitial cells, which give rise to glomerular mesangial cells and the interstitium of the mature kidney in the developing mouse kidney [20]. Furthermore, FOXD1 promotes nephron progenitor differentiation by repressing decorin in the embryonic kidney [21]. In addition, FOXD1 is a strong indicator of successful progression of the gene expression programme in cell reprogramming [22]. However, the role of FOXD1 in carcinogenesis and progression is still limited.

In the present study, we revealed the role of FOXD1 in breast tumorigenesis and progression. We found that FOXD1 expression is up-regulated in breast cancer tissues. Functional analysis indicated depletion of FOXD1 expression decreases the ability of cell proliferation and chemoresistance in MDA-MB-231 cells, whereas overexpression of FOXD1 increases the ability of cell proliferation and chemoresistance in MCF-7 cells. Furthermore, we observed that FOXD1 induces G1 to S phase transition by targeting p27 expression. Our results suggest that FOXD1 may be a potential therapy target for patients with breast cancer.

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2. Materials and methods

2.1. Cell culture and human samples

The human breast cancer cell line MCF-7, T47D, MDA-MB-231 and MDA-MB-435, and the normal human breast cell line MCF10A were purchased from American Type culture Collection (ATCC) and maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

Breast cancer specimens were obtained from the First Affiliated Hospital of the General Hospital of CPLA. This study was approved by the Institutional Review Board of the First Affiliated Hospital of the General Hospital of CPLA and written consent was obtained from all participants. All tumors were from patients with a newly diagnosed breast cancer who had received no therapy before sample collection. After radical prostatectomy, tissues were flash-frozen in liquid nitrogen and stored at −80 °C.

2.2. Plasmids, siRNA and transfection

The human FOXD1 cDNA was cloned into the pcDNA-3.1 vector (Invitrogen). The FOXD1 siRNA and non-targeting siRNA were purchased from Santa-Cruz Biotechnology. Approximately 1×10^5 cells were plated in 6-well dishes before transfection and grown without antibiotics. siRNA at a final concentration of 50 nM or 4 μg plasmid were transfected into cells using Oligofectamine reagent (Invitrogen) according to the protocol. Cells were analyzed 24–48 h after transfection by RT-qPCR or Western blot analysis.

2.3. Real-time quantitative reverse transcription PCR

Total RNA was extracted with TRIZOL reagent according to the manufacturer's instructions. 2 μg of total RNA was converted to first-strand cDNA using SuperScript II Reverse Transcriptase (Invitrogen). The RT-qPCR analysis was performed using the Fast SYBR Green Master Mix System (Invitrogen) according to the manufacturer's instructions. The PCR reaction mixture, thermocycling conditions were 10 min at 95 °C, followed by 40 cycles of denaturation (95 °C, 15 s), annealing (60 °C, 30 s), extension (72 °C, 30 s). The targeted gene relative quantification was given by the CT values, and the CT value of GAPDH was subtracted to obtain ΔCT. The relative mRNA expression level of targeted genes was determined as $2^{-\Delta CT}$. The experiment was performed in triplicate and the data are presented as mean ± SD.

2.4. Western blot

A quantity of 50 μg per tissue or cell lysates was electrophoresed on a 10% SDS–PAGE gels and transferred to PVDF membrane (Millipore, Danvers, MA, USA). The membranes were then incubated with primary antibody and the HRP-conjugated second antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The same membranes were stripped and blotted with an anti-GAPDH antibody (Sigma–Aldrich, St. Louis, MO, USA) and used as loading controls. The probe proteins were detected using the enhanced chemiluminescence system according to the instructions of the manufacturer (Pierce Biotechnology, Rockford, IL, USA).

2.5. Proliferation assay

MTT and soft agar colony formation were used to observe and compare cell proliferation. For MTT assay, 2×10^3 cells in 100 μl culture medium were plated into a well of 96-well plates. After transfection and culturing cell for an appropriate time, 10 μl of 5 mg/ml MTT was added into each well and cultured for 4 h. Then, the cell culture medium was replaced by 100 μl of dimethyl sulfoxide. Thirty minutes after dimethyl sulfoxide addition, the plates were placed on a microplate autoreader (Thermo Scientific, Rockford, IL, USA). Optical density was read at 490 nm wavelength and cell growth curves were determined according to the optical density value.

For colony formation assay, 1×10^3 cells were seed in six-well plate. Three weeks after plating, cells were stained by 5 mg/ml MTT (Sigma–Aldrich) to visualize colonies.

2.6. Chromatin immunoprecipitation assay

ChIP was carried out using kit from Upstate Biotechnology according to manufacturer's protocol. The putative binding site of p27 was amplified with the following primers: 5′-ACATCTGCAG GCAACCCAGG-3′ and 5′-AGATGCAAGTCTCCTAACAC-3′ (site 1), and 5′-CCGTTTGGCTAGTTTGTTTG-3′ and 5′-TTGGCTGGTCGCGT GACTGC-3′ (site 2). The PCR products were resolved electrophoretically on a 2% agarose gel.

2.7. Luciferase assay

The p27 promoter (−2000 to +1) was amplified by from genomic DNA of MDA-MB-231 cells and the fragment was cloned into the luciferase reporter plasmids pGL3-basic vector (Promega, Madison, WI, USA). 5×10^4 cells per well in 24-well plates were cultured without antibiotics overnight and then transfected the p27 promoter into the cells with or without pcDNA3.1-FOXD1/FOXD1 siRNAs. After 24 h, cells were washed with phosphate-buffered saline (PBS), subjected to lysis, and their luciferase activities measured by using a dual luciferase assay kit (Promega). The results were normalized against Renella luciferase. All transfections were performed in triplicate.

2.8. Fluorescence activated cell sorting analysis

Cells were washed once with PBS and then resuspended in the wash buffer. Combinations of fluorochrome-conjugated CD24 and CD44 (BD Biosciences) at concentrations recommended by the manufacturer and incubated at 4 °C in dark for 30 min. The labeled cells were washed in the wash buffer, then fixed in PBS containing 1% paraformaldehyde, and then analyzed using a BD FACS Canto II flow cytometer (BD Biosciences).

2.9. Statistical analysis

Data were displayed by mean ± SD of three independent experiments and student's two-sided *t*-test was used to compare data between two groups. One-way ANOVA test was used to compare data between three groups. All calculations were performed with the SPSS for Windows statistical software package (SPSS Inc., Chicago, IL, USA). The level of significance was set to $P < 0.01$.

3. Results

3.1. FOXD1 is overexpressed in human breast cancer

We first determined the FOXD1mRNA expression in human primary breast cancer and the paired adjacent normal breast tissues by RT-qPCR ($n = 30$) and Western blot ($n = 10$) analyses (Fig. 1A and B).The results showed that both mRNA and protein expression of FOXD1 were up-regulation in breast cancer compared with normal breast tissues. Furthermore, the data from Finak's breast cancer cohort showed that the FOXD1 expression is highly enriched in

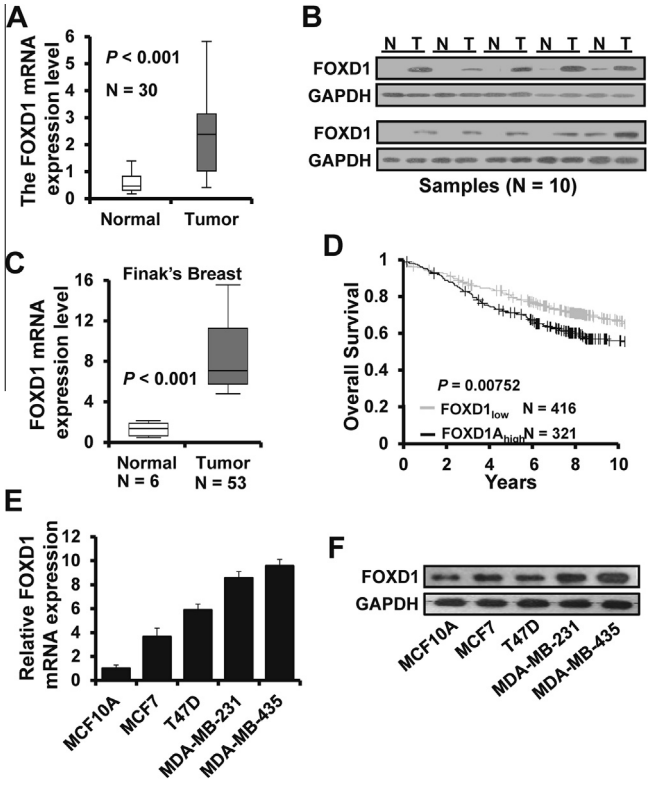

Fig. 1. FOXD1is overexpressed in breast cancer. (A and B) FOXD1 expression levels in human breast cancer and the paired adjacent normal breast specimens by RT-qPCR (A) and Western blot (B). (C) FOXD1 expression in Finak's breast cancer cohort. (D) Kaplan–Meier analysis of patients with different FOXD1 expression. (E and F) FOXD1 expression levels in breast cancer cell lines and the normal human breast cell line by RT-qPCR (E) and Western blot (F).

breast cancer tissues (Fig. 1C). The Gene expression-based Outcome for Breast cancer Online (GOBO; http://co.bmc.lu.se/gobo), a tool for prognostic validation of genes, was used to analyze a pooled breast cancer data set generated on Affymetrix U133A microarrays. The results showed that the patients with FOXD1 high expression had a lower disease-free survival compared to the patients with FOXD1 low expression (Fig. 1D). Next, we determined the FOXD1 expression levels in breast cancer cell lines (MCF-7, T47D, MDA-MB-231 and MDA-MB-435) and normal human breast cell line (MCF10A) by RT-qPCR and Western blot. The results showed that higher expression of FOXD1 mRNA and protein was evident in MCF-7, T47D, MDA-MB-231 and MDA-MB-435 cells than MCF10A cells (Fig. 1E and F). Together, these results show that FOXD1 is overexpressed in breast cancer.

3.2. FOXD1 promotes breast cancer cell proliferation in vitro

To determine the effect of FOXD1 depletion on the proliferation of breast cells, we used two specific siRNAs targeting FOXD1, and both of them could efficiently reduce FOXD1 expression in MDA-MB-231 cells by RT-qPCR and Western blot (Fig. 2A). By MTT assay, we found that transfection of siRNAs resulted in a decreased rate of cell proliferation (Fig. 2B). Subsequently, we investigated whether FOXD1 is involved in the tumorigenic properties of breast cancer cells. We found that depletion of FOXD1 significantly reduced the ability of MDA-MB-231 cells to form colonies in soft agar (Fig. 2C).

To determine the consequences of overexpression of FOXD1 in breast cancer cells, we overexpressed pcDNA3.1-FOXD1 in MCF-7 cells. RT-qPCR and Western blot analysis showed that FOXD1 was overexpressed in pcDNA3.1-FOXD1-transfected MCF-7 cells than the control cells (Fig. 2D). The ability of cell proliferation was also increased in FOXD1-overexpressed MCF-7 cells by MTT and colony formation assays (Fig. 2E and F). Collectively, these

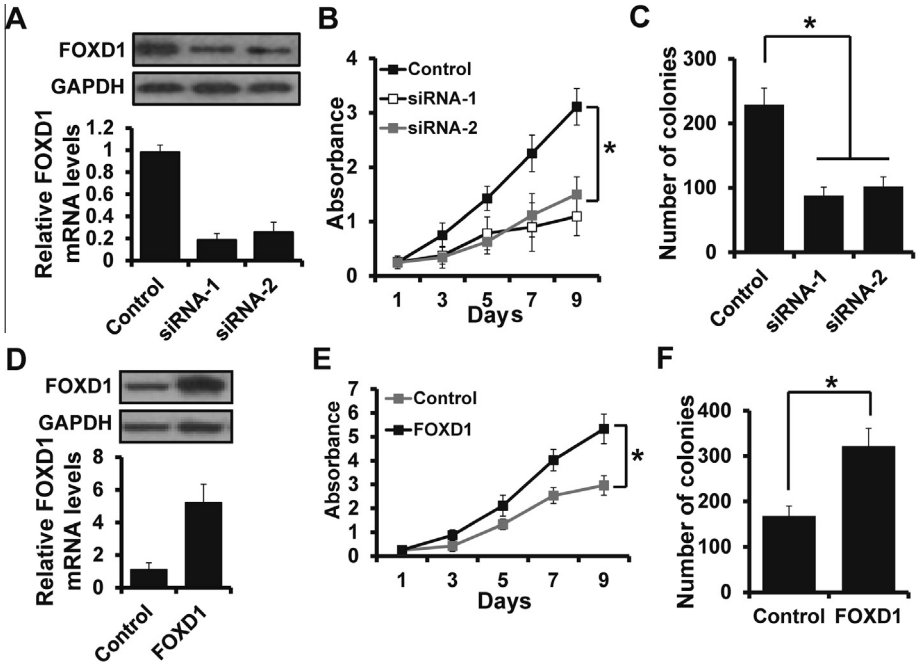

Fig. 2. FOXD1 promotes breast cancer cell proliferation *in vitro*. (A) FOXD1 expression in MDA-MB-231 cells transfected with FOXD1and control siRNAs by RT-qPCR (lower) and Western blot (upper). (B) MTT analysis of FOXD1 siRNAs-transfected and control cells. (C) Colony formation analysis of FOXD1 siRNAs-transfected and control cells. (D) FOXD1 expression in MCF-7 cells transfected with pcDAN3.1-FOXD1 and vector control by RT-qPCR (lower) and Western blot (upper). (E) MTT analysis of FOXD1-transfected and control cells. (F) Colony formation analysis of FOXD1-transfected and control cells. Data are represented as mean + SD of three independent experiments. $^{*}P < 0.01$, compared with the control.

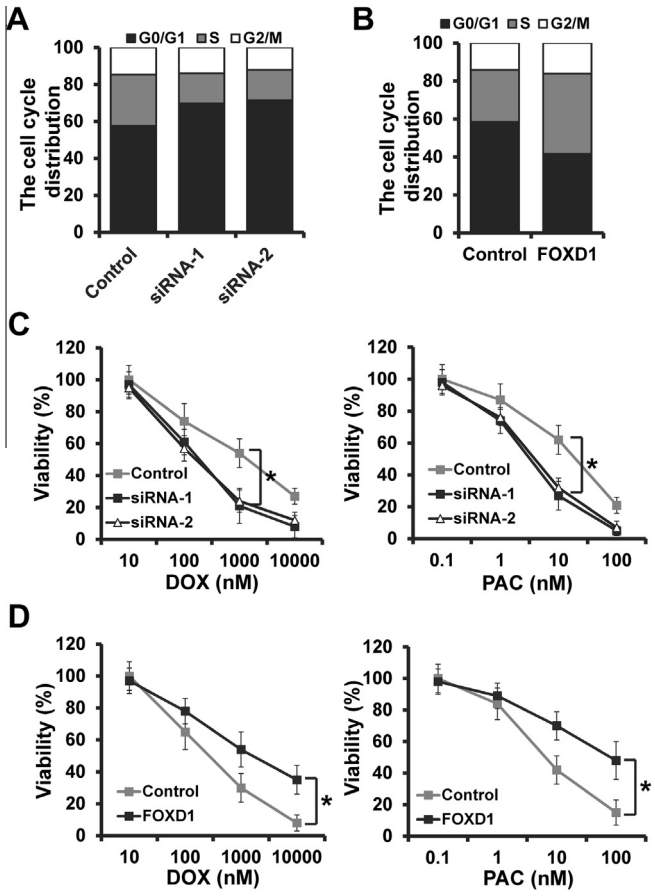

Fig. 3. FOXD1enhances the chemoresistance of breast cancer cells by induction of G1 to S phase. (A) The cell cycle distribution of FOXD1-depleted and control MDA-MB-231 cells. (B) The cell cycle distribution of FOXD1-overexpressed and control MCF-7 cells. (C) The chemosensitivity of FOXD1-depleted and control MDA-MB-231 cells by MTT. (D) The chemosensitivity of FOXD1-transfected and control MCF-7 cells by MTT. Data are represented as mean + SD of three independent experiments. $^{*}P < 0.01$, compared with the control.

results indicate that FOXD1 promotes breast cell proliferation *in vitro*.

3.3. FOXD1 enhances resistance to chemotherapy in breast cancer cells

We next investigated the effect of FOXD1 expression on the breast cancer cell cycle distribution. The results showed that FOXD1 siRNAs caused cell arrest at G1 phase in MDA-MB-231 cells (Fig. 3A). Moreover, the percentage of cells at S phase was significantly increased in FOXD1-transfected MCF-7 cells than that of control cells (Fig. 3B). Increasing evidence indicates that cancer chemosensitivity is associated with the alternation of cell cycle distribution. We next examine the effect of FOXD1 on the breast cancer chemosensitivity. We observed that the number of viable cells was significantly reduced in FOXD1-depleted MDA-MB-231 cells (Fig. 3C), whereas the number of viable cells was significantly enhanced in FOXD1-overexpressed MCF-7 cells after treatment with DOX and PAC than that of control cells (Fig. 3D). Together, these results indicated FOXD1 enhances the chemoresistance of breast cancer cells by induction of G1 to S transition.

3.4. FOXD1 transcriptionally suppresses p27 expression

p27 is considered to be a tumor suppressor by inhibition of Cyclin E/CDK2 activity [23]. We identified two FOXD1-binding sites on the p27 promoter (Fig. 4A). To determine whether p27 could be a direct transcriptional target of FOXD1, we performed chromatin immunoprecipitation assays with MDA-MB-231 cells. We found that endogenous FOXD1 protein bound to both FOXD1-binding sites of the p27 promoter in MDA-MB-231 cells (Fig. 4B). To further investigate the role of FOXD1 in regulating p27 transcription, we explored whether FOXD1 regulates p27 promoter activity. The p27 promoter luciferase construct pGL-p27 was transfected into MDA-MB-231 and MCF-7 cells with FOXD1 siRNAs or pcDNA3.1-FOXD1. The luciferase activity was higher in FOXD1-depleted MDA-MB-231 cells, whereas it was lower in FOXD1-overexpressed MCF-7 cells than that of control cells (Fig. 4C). Furthermore, the p27 expression is up-regulated in FOXD1-depleted MDAMB-231 cells and the p27 expression is down-regulated in FOXD1-overexpressed MCF-7 cells than that of control cells by Western blot (Fig. 4D). The FOXD1 expression is negatively related to the p27 expression by RT-qPCR in 30 cases of breast cancer specimens (Fig. 4E). Together, these results indicated that FOXD1 transcriptionally suppresses p27 expression.

4. Discussion

Forkhead box (FOX) proteins, an evolutionarily conserved family of transcriptional regulators, mediate a wide spectrum of biological processes, such as metabolism, differentiation, proliferation, apoptosis, and migration [8,24], and participate in the onset and progression of tumors [25]. Previous studies shows FOXD1 is related to development [19,20], cell differentiation [21], cell reprogramming [22]. However, the role of FOXD1 in tumorigenesis and progression is still unknown. In the current study, we demonstrated that FOXD1 is upregulated in breast cancer tissues and cell lines. In addition, we found that depletion of FOXD1 inhibits breast cancer cell proliferation and leads to cell cycle arrest at G1 phase, whereas ectopic expression of FOXD1 promotes cell proliferation and induces G1 to S transition, suggesting that FOXD1 as a potential oncogene during the breast cancer proliferation by inducing cell cycle progression.

Cyclin-dependent kinase inhibitor ($p27^{kip1}$) is an enzyme inhibitor that in human is encoded by *CDKN1B* gene. It encodes a protein which belongs to the Cip/Kip family of cyclin dependent kinase (CDK) inhibitor proteins. The encoded protein binds to and prevents the activation of Cyclin E/CDK2 or Cyclin/CDK4 complexes, and thus controls the cell cycle progression at G1 phases. During carcinogenesis and progression, p27 is inactivated through multiple mechanisms. Including impaired synthesis, increased protein degradation and mislocalization [23]. p27 inhibited Cyclin-CDK complexes from phosphorylating histone H1. Furthermore, overexpression of p27 was found to prevent CDK activation and entry into the S phase of the cell cycle [26,27]. In the present study, we found that FOXD1 binds to p27 promoter region and suppresses p27 expression. The FOXD1 is closely related to the p27 expression in breast cancer specimens. Furthermore, FOXD1 promotes the cell cycle progression by inducing G1 to S transition, suggesting that FOXD1 induces G1 to S transition by down-regulation of p27 expression. In addition, absence or cytoplasmic localization of p27 has been linked to drug resistance in many in vitro cellular models [28,29]. In this study, we observed that the number of viable cells was significantly higher in FOXD1-overexpressed breast cancer cells compared to the control cells after treatment with doxorubicin or paclitaxel, suggesting that FOXD1 enhances resistance to chemotherapy by suppression of p27 in breast cancer cells.

Altogether, these findings indicate that FOXD1 is a potential oncogene in breast cancer tumorigenesis. Furthermore, p27 is a transcriptional target of FOXD1 and may mediated FOXD1-induced cell cycle progression. Thus, FOXD1 may be a therapeutic target for breast cancer treatment.

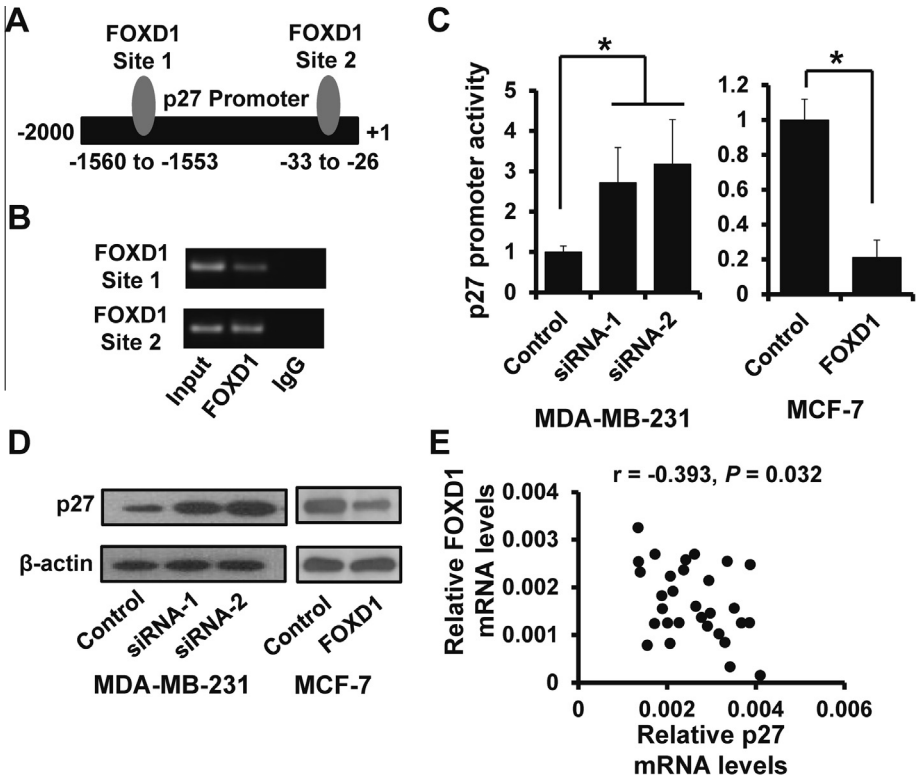

Fig. 4. The p27 as a transcriptional target of FOXD1. (A) Schematic representation ofFOXD1-binding site in p27promoter. (B) ChIP analysis of the human p27 promoter by anti-FOXD1 antibody in MDA-MB-231 cells. (C) Luciferase activity of p27 promoter in FOXD1-depleted MDA-MB-231 and FOXD1-overexpressed MCF-7 cells. (D) Western blot analysis of p27 expression in FOXD1-depleted MDA-MB-231 and FOXD1-overexpressed MCF-7 cells. (E) RT-qPCR analysis of FOXD1 and p27 mRNA expression in 30 cases of breast cancer specimens. Data are represented as mean + SD of three independent experiments. $^{*}P < 0.01$, compared with the control.

Conflict of interest

The authors have no conflict of interest.

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