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Original Article

The mechanism of aspirin inhibiting ovarian cancer cell tumorigenesis

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Abstract: The mechanisms underlying the effection of Aspirin against ovarian cancer are not fully understood. Therefore, we try to investigate the mechanism in Aspirin triggering ovarian cancer. In the present study, we found the down regulation of FOXO1 in ovarian cancers samples and cancer cell lines OVCAR3 or SKOV3 compared with the normal epithelial ovarian cell line HOSEpiC. The higher FOXO1 expression level means for a better overall 5-year survival. FOXO1 over expression significantly inhibited ovarian cancer cells proliferation in vitro. Furthermore, we investigated that FOXO1 could directly inhibit the expression levels of β-catenin, cyclinD1, which were involved in the Wnt/β-catenin pathway. One of the function of Aspirin was to activate FOXO1. We also uncovered that β-catenin may act as a downstream target of FOXO1, as β-catenin blocked Aspirin-mediated proliferation and tumorigenesis inhibition. Furthermore, we disclosed the mechanism that Aspirin treatment has remarkable effect on transcriptional activity of FOXO1. Taken together, these results suggested that FOXO1 inhibited ovarian cancer cell growth by repression the Wnt/β-catenin pathway, which indicated that FOXO1 may work as a tumor suppressor for ovarian cancer prevention and therapy. In conclusion, the present study provides novel insights into the molecular circuitry of Aspirin-induced proliferation inhibition involving FOXO1-mediated tumorigenesis.

Keywords: Aspirin, ovarian cancer, tumorigenesis, FOXO1, Wnt

Introduction

Ovarian cancer is one of the deadliest form of gynecologic malignancies, the fifth cause of cancer death in women in the United States [1], occurring with 6.6 new cases per 100,000 women all over the world each year [2]. Although rapid knowledge in understanding ovarian cancer etiology [3], there were still no reliable methods to detect the early stage and no identifiable precursor for high grade serous epithelial ovarian tumors, of which more than 85% are of epithelial origin [4]. Therefore, new diagnostic biomarkers and new therapy targets are in emergency needed to reduce the morbidity and mortality in advanced stage ovarian cancer. Furthermore, the molecular mechanism of the tumor proliferation and metastasis still need exploring [5].

The mammalian forkhead transcriptional factors contain several subclasses, such as FOXO subfamily, which comprises three functionally related members FOXO1, FOXO3a and FOXO4, by distinctive forkhead DNA binding domains. The FOXO subfamily functions as downstream target of the PI3K/Akt pathway [6] to inhibit cell cycle progression, (for example, $p27^{kip}$) [7] and promote cell apoptosis in cancer cells, and FOXO1 was reported to be downregulated in several carcinomas and the deregulation of FOXO1 was associated with cancer progression [8].

Chronic inflammation was proposed as a risk factor in ovarian cancer [9]. Aspirin (acetylsalicylate), a non-steroidal anti-inflammatory drug, was one of the most commonly used agent all over the world [10]. Accumulated evidence have shown that aspirin is associated with a reduced risk of death in cancer patients, such as prostate [11], colorectal [12] Rothwell [13] endometrial cancer [14], including those with ovarian cancer [15], a previous study showed aspirin potentiated the effectiveness of histone deacetylase inhibitors by upregulating p21 in ovarian cancer cells [16].

In the present study, we further investigated the effect of aspirin by the hypothesis that aspirin might activate the expression of FOXO1 to repress the Wnt/β-catenin signaling pathway in ovarian cancer cells.

Materials and methods

Cell culture

Human ovarian cancer cell line OVCAR3 and SKOV3 were purchased from American Tissue Type Collection (Manassas, VA, USA). Normal epithelial ovarian cell line HOSEpiC were purchased from Chinese Academy of Sciences. All the above cells were cultured in DMEM medium with 10% fetal calf serum (Hyclone).

Cells were cultured at 37°C in a humidified 5% CO_2 incubator, when cells were grown to 70%, they were transfected with relative plasmids or siRNA using Lipofectamine 2000 (Invitrogen) and were routinely passaged when 90-95% confluent.

Clinical specimens

124 cases of ovarian cancer specimens and their adjacent normal tissues with complete clinicopathologic information were obtained from West China Second University Hospital from May 2009 to December 2014. The study was approved by the hospital ethical committee and all the patients were given written consent prior to the study. None of the patients were subjected to radiation therapy or chemotherapy prior to the surgery. The tissues were received after surgery immediately and stored in liquid nitrogen at -80°C until use. The survival times were calculated from the operation day to recurrence or metastasis result in death.

RNA isolation and cDNA synthesis

Total RNA was extracted using TRIZOL reagent (Invitrogen, USA), for analysis of messenger RNA (mRNA) expression, 1 μg of total RNA was reverse transcribed to cDNA using the Transcriptor First Stand cDNA synthesis kit (Roche, UK) according to manufacturer's instructions.

Quantitative real-time PCR

Quantitative real time PCR was performed with the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, USA) using Syber green Master Mix (Roche, UK) with 1.5 μg of cDNA. The PCR amplification was first performed at 95°C for 1 min, followed by 95°C 15 s, 60°C 15 s, 72°C 45 s for 30 cycles. Data were analyzed using SDS2.4 Software (Applied Biosystems, USA). GAPDH was used as an internal control. The specific primers were as follows:

Antibodies and reagents

The antibodies and reagents used are as follows: anti-β-catenin antibody (Santa Cruz; Santa Cruz, CA, USA); anti-FOXO1 antibody (Cell Signaling Technology, Danvers, MA, USA); and anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA). Aspirin (ASA) were purchased from Sigma (St. Louis, MO). Non-targeting siRNA (NC), small interfering RNAs (siRNAs) targeting the sequences human β-catenin were synthesized (Genechem, shanghai, China). After allowing cellular attachment to the plates, cells were treated with ASA, plasmids or siRNAs, the combination and details are described in Results. RNA or Protein was collected 72 hours following treatment.

Western blot

Whole cell lysates were collected and solubilized using EDTA lysis buffer, fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Millipore). 5% nonfat dry milk in Tris-buffered saline Tween-20 (TBST) was used to block the nonspecific proteins for 2 h at room temperature. After incubated in primary antibodies at 4° overnight, the membranes were washed 5 times with TBST for 5 min, the primary antibodies were anti-β-catenin (1:1000) and anti-GAPDH (1:2000). Followed by incubation for 1 hour with the secondary antibody (Santa Cruz), the bands were visualized using the enhanced chemiluminescence detection kit (GE Healthcare, Piscataway, NJ).

Chromatin immunoprecipitation assay

The Flag-FOXO1 plasmid was transfected into OVCAR3 and SKOV3 cells for 48 hours, chromatin immunoprecipitation (ChIP) assay was performed using a ChIP assay kit (Millipore, USA) according to manufacturer's instructions. Anti-Flag antibody-enriched β-catenin promoter fragments were qPCR amplified. IsotypeIgG (Abcam) was used as a negative control. PCR

Products were visualized on an ABI 7500 system.

Luciferase assays

The pcDNA3.1-Flag-β-catenin plasmid was obtained by amplified β-catenin from human genomic DNA using PCR and the PCR products were then inserted into the pcDNA3.1 vector (Promega, Madison, WI, USA). Ovarian cancer cells were seeded to approximately 50% confluence in six-well plates (20000 cells per well), then transiently transfected with β-catenin promoter subcloned into pGL2-basic luciferase reporter vector, or pGL3-Basic empty vector as well as siFOXO1 or Flag-FOXO1 and co-transfected with Renilla expression construct. Transfected cells were incubated for 24 hours, and lysed with Passive Lysis Buffer (Promega), the luciferase activities were assessed using a dual-luciferase reporter assay kit (Promega), Renilla was normalized to measure the Reporter luciferase activity, according to manufacturer's structions.

MTT assay

The cell proliferation assays were performed using the MTT assay, cells were seeded into the 24-well plate at a density of 10000 cells per well, at 24, 48, 72 or 96 h, each well was washed twice with phenol red-free media and added 10 μL of 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; 5 mg/mL in PBS) (Sigma-Aldrich), the plate was incubated for 4 h at 37°C. The MTT solution was removed and 500 μl/well dimethyl sulfoxide (Sigma-Aldrich) was replaced to terminate the reaction. The plates were placed on a shaker to thoroughly dissolve the MTT color product for 10 min. The optical absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA), according to the optical density values, the cell quantification viability was determined.

Statistical analysis

Each experiment was repeated at least 3 times. All statistical analyses were carried out using SPSS 18.0 software. Data were analyzed by comparing mean values (± SD) using a Student's t-test for independent groups. $P<0.05$ was considered as statistically significant. One-way analysis of variance (ANOVA) with SNK-q test for multiple comparisons was used to analyze the data from the transwell migration assay and Western blot. The Kaplan-Meier plot was used to establish survival curves, significance of differences was compared using the log-rank test. Cox regression model was used to perform multivariate analysis. *$P<0.05$, **$P<0.01$.

Results

FOXO1 is downregulated in ovarian cancer cell lines and tissues

In order to explore the role of FOXO1 during the progress of ovarian cancer, RT-qPCR analysis (**Figure 1A**) and Western blot analysis (**Figure 1B**) were performed in ovarian cancer cell lines OVCAR3, SKOV3 cells and normal epithelial ovarian cell line HOSEpiC to detect the FOXO1 expression. As compared with the normal cell line HOSEpiC, the expression of FOXO1 was shown to be significantly downregulated inOVCAR3, SKOV3 cells in both RNA and protein level.

Further, FOXO1 expression was examined, using qRT-PCR in the 124 ovarian cancer samples collected, compared with the corresponding noncancerous tissues, in ovarian cancer samples FOXO1 expression was decreased (**Figure 1C**). Patients were divided into two groups based on FOXO1 expression levels, Kaplan-Meier curves were drawn to detect the overall survival times. Those with more than median of FOXO1 expression levels means for a better overall 5-year survival (the Hazard Ratio: 0.5208, $P=0.0003<0.05$) (**Figure 1D**).

Aspirin activates FOXO1 to inhibit ovarian cancer cell proliferation in vitro

To further understand the impact of FOXO1 in ovarian cancer progression, we established an FOXO1 expression construct and transfected it in the ovarian cancer cell lines OVCAR3 and SKOV3 to detect whether FOXO1 could participate in cell proliferation. RT-qPCR and western blot analysis was used to measure the transfection efficiency. As shown in **Figure 2A**, in OVCAR3 and SKOV3 cells, FOXO1 mRNA expression was significantly increased compared with those transfected with the vector ($P<0.05$). Consistently with the RT-qPCR, FOXO1 protein expression was markedly increased (**Figure**

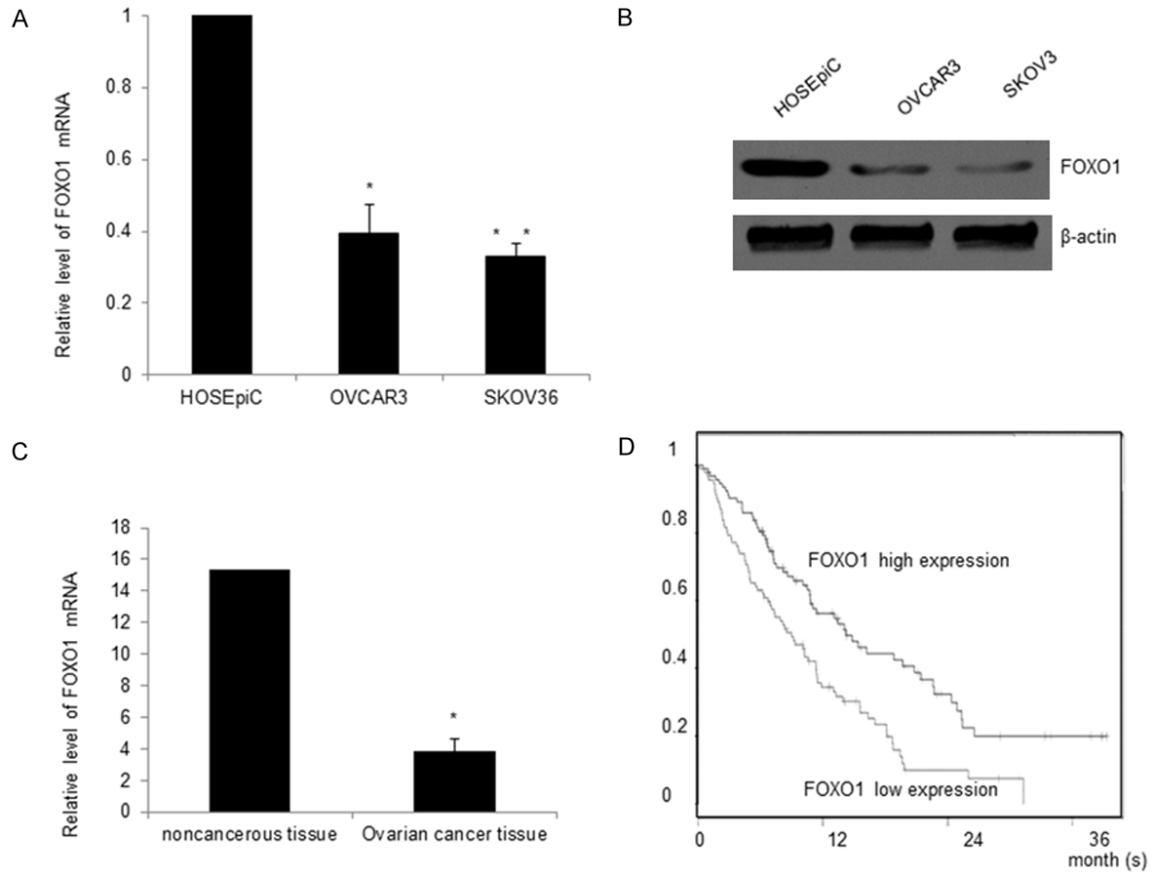

Figure 1. FOXO1 is downregulated in ovarian cancer cell lines and tissues. A: RT-qPCR was used to measure the mRNA expression of FOXO1 in the ovarian cancer cell lines, compared with the normal cell lines HOSEpiC. Data were represented as mean ± standard, for triplicate measurements. *P<0.05, **P<0.01. B: Representative protein expression of FOXO1 in the relative ovarian cancer cell lines, measured by Western blot. C: Representative results of FOXO1 mRNA expression in ovarian cancer tissues, compared with the corresponding noncancerous tissues. D: Patients were collected to measure the FOXO1 expression in the impact of overall 5-year survival rate, Kaplan-Meier curves were drawn. In univariate and multivariate models, Cox proportional hazards regression was used to test the prognostic significance. P<0.05 was considered significant.

2B). Cell proliferation assay (MTT assay) indicated that ectopic expression of FOXO1 significantly inhibited the OVCAR3 cells (**Figure 2C** left panel) and SKOV3 cells proliferation (**Figure 2C** right panel). As mentioned early, Aspirin treatment could inhibit ovarian cancer cell growth, and FOXO1 could also inhibit proliferation. So we focus our interest on whether Aspirin has an effect on the FOXO1.

We measured Aspirin treatment on the expression level of FOXO1 in OVCAR3 cells, levels of relative mRNA and protein increased markedly upon 12 and 24 h Aspirin treatment in OVCAR3 cells (**Figure 2D** left panel). The Aspirin-mediated activation of FOXO1 was also observed in the SKOV3 cell line (**Figure 2D** right panel).

FOXO1 negative regulates the Wnt/β-catenin signaling

Since in the ovarian cancer pathogenesis, the aberrant activation of Wnt/β-catenin [17] signaling pathway was considered as to acquire chemoresistant and EMT phenotype.

To investigate whether FOXO1 has a role in the regulation of the potential signaling pathway, RT-qPCR and western blot analysis was used to detect the mRNA and protein levels of β-catenin and cyclinD1. As measured, compared with the vector group, in FOXO1 transfected OVCAR3 cells, β-catenin and cyclinD1 were significantly decreased (**Figure 3A**), the similar results were also observed in SKOV3 cells (**Figure 3B**), (P<0.05).

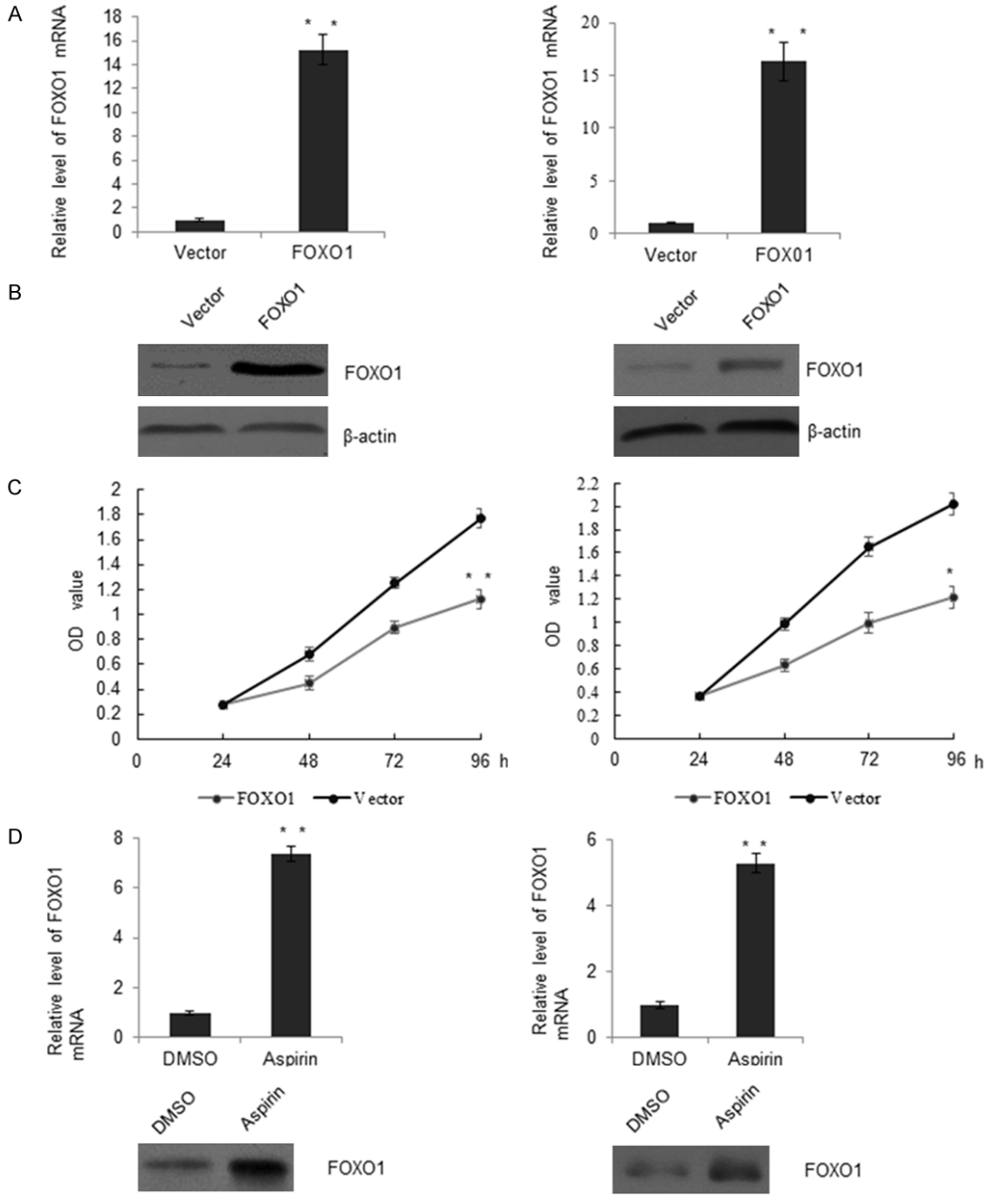

Figure 2. Aspirin activates FOXO1 to inhibit ovarian cancer cell proliferation in vitro. A: RT-qPCR was used to detect FOXO1 mRNA in transfected ovarian cancer cell linesOVCAR3 and SKOV3 cells, when compared with the vector group. Data were represented as mean ± SD, of three independent experiments. **P<0.01. B: Western blotting analysis was used to measure the FOXO1 protein expression in the above cell lines. C: MTT assay was used to measure the OVCAR3 and SKOV3 cells proliferation, cells were transfected with FOXO1 or relative vector group (vector). D: qRT-PCR and western blot analysis for FOXO1 mRNA levels and protein levels in OVCAR3 and SKOV3cells treated with DMSO (control) or Aspirin for 16-24 h. Results shown are mean ± SD. *P<0.05.

To confirm whether β-catenin was involved in FOXO1 triggering ovarian cancer proliferation, in the FOXO1-transfected OVCAR3 cells, β-catenin was overexpressed, the inhibition effect of FOXO1 on ovarian cancer cell proliferation was identified.

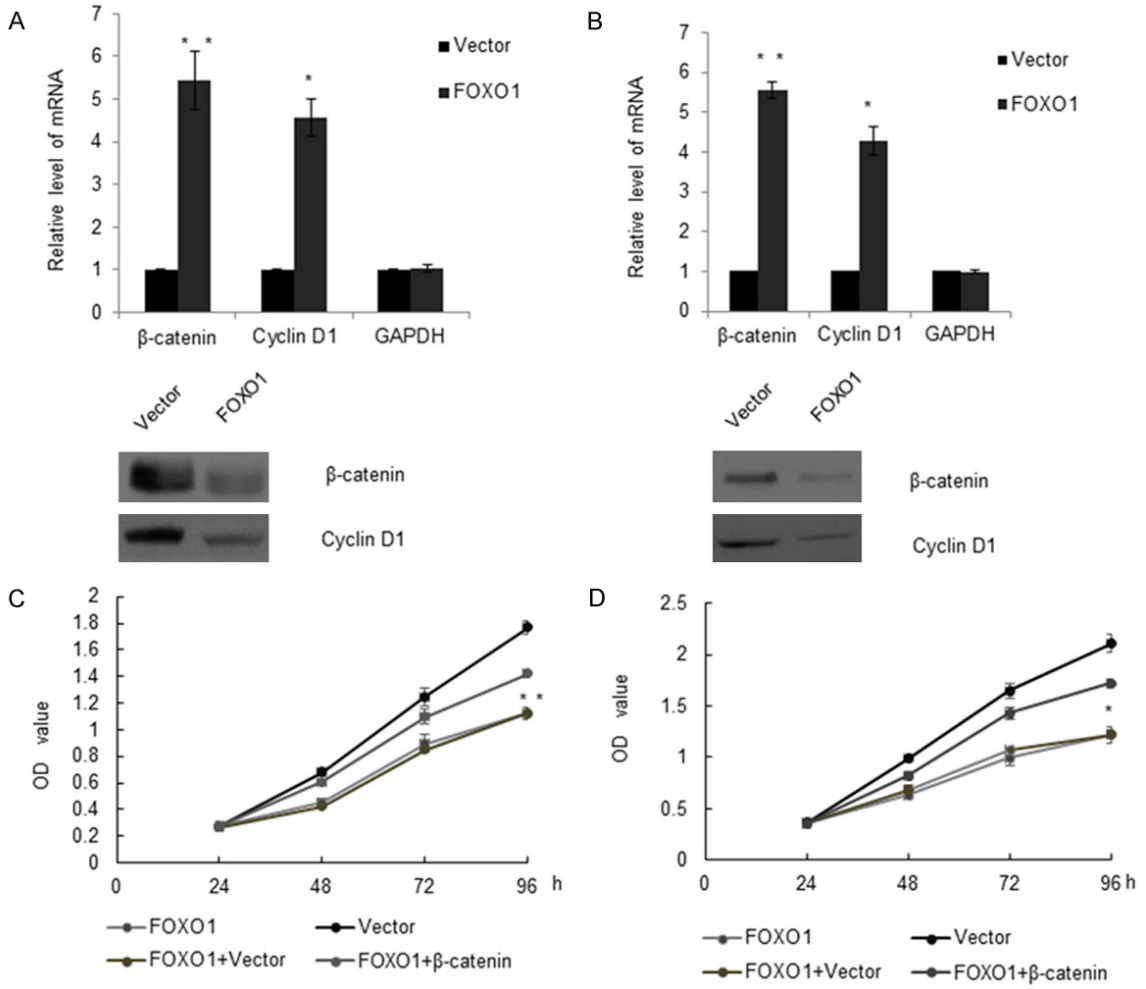

Figure 3. FOXO1 negative regulates the Wnt/β-catenin signaling. A: RT-qRCR for β-catenin and cyclinD1 mRNA and western blot for the relative protein using lysate from OVCAR3 cells transfected with a vector or FOXO1. B: The above experiment was performed in SKOV3 cells stably transfected with FOXO1 or vector as control. C: Cell proliferation assay was performed in FOXO1 transfected OVCAR3 cells, followed by transiently transfected with vector or a β-catenin, equal numbers of cells were seeded into replicate plates and counted every 12 hours. D: The similar experiment was performed in FOXO1 stable transfected SKOV3 cells, follow by transiently transfected with vector or a β-catenin, and counted every 12 hours.

The data indicated the overexpression of β-catenin could significantly reduced the effect of FOXO1 in OVCAR3 cells (**Figure 3C**). Further, we created a FOXO1 stable expression SKOV3 cell line, the similar tendency was also observed with etopic β-catenin (**Figure 3D**) ($P<0.05$). These results revealed that FOXO1 inhibited ovarian cancer cell proliferation through inhibition of the Wnt/β-catenin pathway.

β-catenin blocks Aspirin-mediated proliferation and tumorigenesis inhibition

Considering β-catenin may function as a downstream target of FOXO1, we constructed two β-catenin-targeted siRNA. After transfected OVCAR3 cells or SKOV3 cells with the relative siRNA, mRNA and protein levels of β-catenin was decreased by nearly 90% compared with the control siRNA transfection cells (**Figure 4A**). As detected by the MTT assay, promotion of cell proliferation resulting from knockdown FOXO1 was partially affected by β-catenin knockdown in either OVCAR3 cells (**Figure 4B**) or SKOV3 cells (**Figure 4C**). In other word, β-catenin had obviously meaningful impact on FOXO1's ability to inhibit cell proliferation. Furthermore, upon treatment with Aspirin in OVCAR3 cells (**Figure 4D**) or SKOV3 cells (**Figure 4E**), compared with

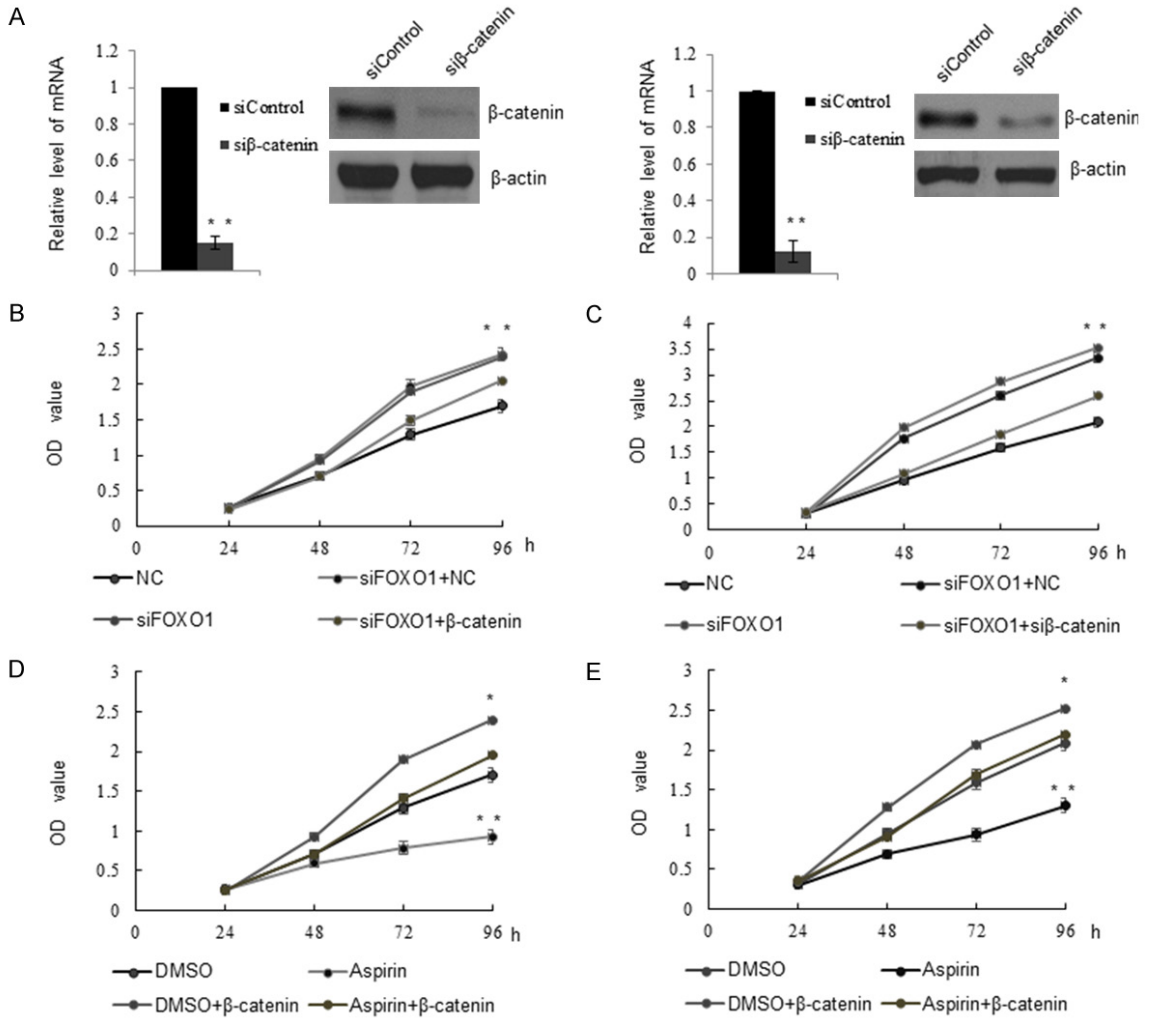

Figure 4. β-catenin blocks Aspirin-mediated proliferation and tumorigenesis inhibition. A: Knockdown efficiencies ofβ-catenin in OVCAR3 cells or SKOV3 cells wasconfirmed by Quantitative real-time PCR (left panel) and western blotting (right panel). B: Cell proliferation assay was performed in si-FOXO1 transfected OVCAR3 cells, followed by transiently transfected with a control siRNA or a β-catenin targeted siRNA and treated for 24 hours, of three independent experiments. C: The similar experiment was performed in SKOV3 cells. D: OVCAR3 cells were treated with DMSO (control) or Aspirin for 16-24 h, followed by transiently transfected with a control plasmid or a β-catenin, MTT assay was performed. E: The similar experiment was performed in SKOV3 cells.

the control plasimid-transfected cells, the proliferation was markedly augmented by β-catenin overexpression. Collectively, these results indicated that Aspirin developed its effect by activation of FOXO1 to repress β-catenin on cell proliferation.

Aspirin treatment has remarkable effect on transcriptional activity of FOXO1

As mentioned early, Aspirin treatments are shown to inhibit ovarian cancer cell proliferation, and the activation of FOXO1 was an important mechanism by Aspirin triggering growth. So we further focus our interest on whether Aspirin has an effect on the FOXO1 transcriptional activity. First, we examined the transcriptional activity of FOXO1 in OVCAR3 cells and SKOV3 cells. ChIP (qChIP) assays were performed, on the promoter of β-catenin, the bindings of FOXO1 was obviously higher than that of the normal IgG (**Figure 5A**) ($P<0.05$). Then luciferase reporter activity was carried out in the two cell lines, we declared that overexpression of FOXO1 could remarkably decrease the β-catenin luciferase reporter activity, while the knockdown of FOXO1 could increased the activity (**Figure 5B**), and the OVCAR3 cells was rela-

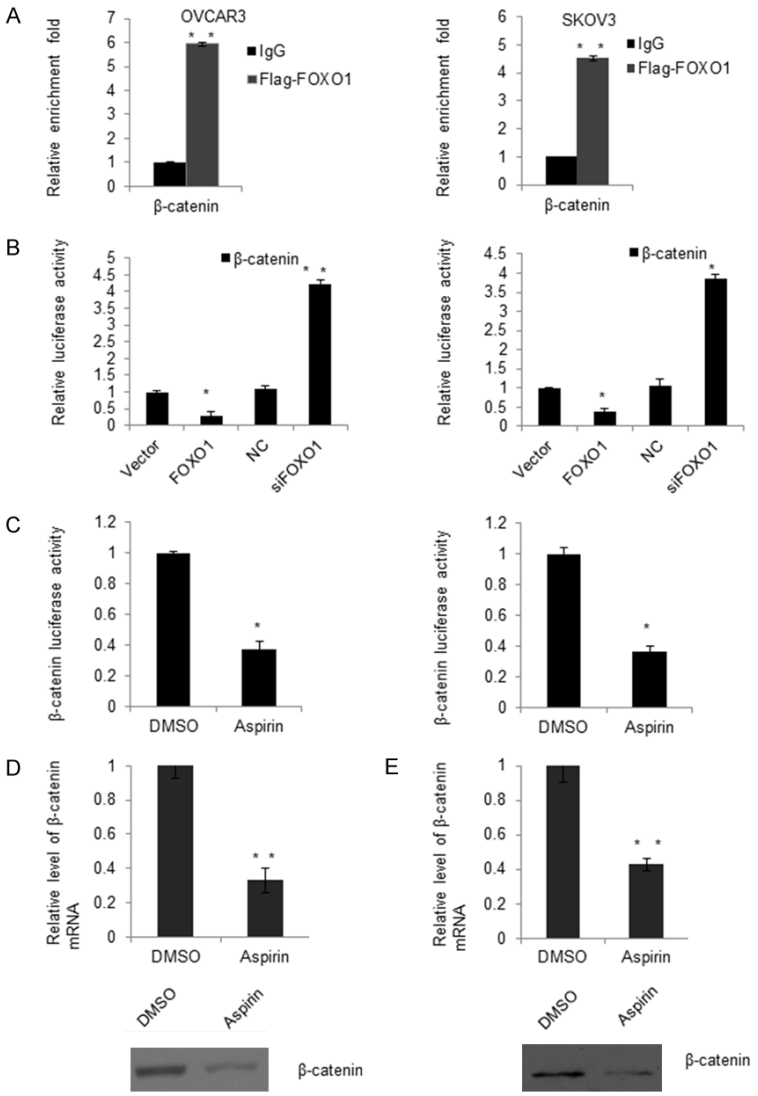

Figure 5. Aspirin treatment has remarkable effect on transcriptional activity of FOXO1. A: Recruitment of FOXO1 on β-catenin promoter. qChIP experiments were performed in OVCAR3 cells(left panel) or SKOV3 cells(right panel) with FOXO1 or normal IgG. Each bar indicates mean ± SD, of three independent experiments. B: β-catenin luciferase reporter activity was performed in OVCAR3 cells (left panel) or SKOV3 cells (right panel). Cells were transfected with promoter luciferase constructs together with FOXO1 over expression constructs or FOXO1 siRNAs. Luciferase activities were measured, experiments were performed in triplicate and all data are shown as means ± SD. C: β-catenin luciferase reporter activity was performed in OVCAR3 cells (left panel) or SKOV3 cells (right panel) after treated with DMSO or the indicated concentrations of Aspirin for 24 h. Each experiment was repeated at least three times. Results were shown as mean ± SD. (*P<0.05). D: OVCAR3 cells were treated with Aspirin (0.5 mmol/l) or DMSO, qRT-PCR and western blot analysis for β-catenin mRNA levels and protein levels in OVCAR3 cells. β-Actin was used as a loading control. E: qRT-PCR and western blot analysis for β-catenin mRNA levels and protein levels in SKOV3 cells after treated with Aspirin for 24 hours.

tively more sensitive compared with SKOV3. Considering Aspirin could activate FOXO1 expression, we further detect whether Aspirin treatment has an effect on transcriptional activity of FOXO1. After exposuredOVCAR3 and SKOV3 cells to Aspirin for 24 h, we found there was a more significant decrease in β-catenin luciferase reporter activity (**Figure 5C**). Consistently with the transcriptional activity, levels of β-catenin mRNA and protein were decreased markedly upon 12 and 24 h treatment with Aspirin inOVCAR3 (**Figure 5D**). The Aspirin mediated suppression of β-catenin was also observed in SKOV3 cells (**Figure 5E**).

Discussion

One of the primary findings in this study is that FOXO1 is downregulated in ovarian cancer cell lines and tissues, higher FOXO1 expression level means for a better overall 5-year survival. A significant decrease in MTT assay was observed upon exposure to aspirin, levels were significantly lower compared with the OVCAR3 or SKOV3 cells transferred with DMSO alone. These results suggested that aspirin had a great inhibitory effect on the ability of OVCAR3 or SKOV3 cells to proliferate. Aspirin inhibits ovarian cancer cell proliferation in aFOXO1 dependent manner. Aspirin is supposed to function primarily by activating FOXO1 activity. FOXO1 could directly inhibit the expression levels of β-catenin. Although further studies to clarify the relationship between β-catenin signaling pathways and FOXO1 in ovarian cancer are required, our results showed that β-catenin acted as a downstream target of FOXO1, leading to a significant abrogation of

Aspirin OVCAR3 cells and SKOV3 cells proliferation.

It is well-known that in the inhibition of ovarian cancer, the alternations of a large number of genes should be attributed. Thus, further exploration for the potential target of Aspirin contributes to the ovarian cancer is necessary. As reported, zinc-dependent endopeptidases MMPs are involved in the degradation of extracellular matrix. Several studies demonstrated that aspirin can inhibit the expression and activity of MMP-2 and MMP-9 [18] in liver cancer [19] or colorectal metastasis [20]. It was shown that SKOV3 cells could secrete both MMP-2 and MMP-9 [21]. Therefore, it is possible that aspirin inhibiting the release of essential MMPs might be another mechanism that contributed to the decreased invasion.

Disclosure of conflict of interest

None.

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