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FoxO1 promotes ovarian cancer by increasing transcription and METTL14-mediated m^{6}A modification of SMC4

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Abstract

The transcription factor forkhead box protein O1 (FoxO1) is closely related to the occurrence and development of ovarian cancer (OC), however its role and molecular mechanisms remain unclear. Herein, we found that FoxO1 was highly expressed in clinical samples of OC patients and was significantly correlated with poor prognosis. FoxO1 knockdown inhibited the proliferation of OC cells in vitro and in vivo. ChIP-seq combined with GEPIA2 and Kaplan–Meier database analysis showed that structural maintenance of chromosome 4 (SMC4) is a downstream target of FoxO1, and FoxO1 promotes *SMC4* transcription by binding to its −1400/−1390 bp promoter. The high expression of SMC4 significantly blocked the tumor inhibition effect of FoxO1 knockdown. Furtherly, FoxO1 increased *SMC4* mRNA abundance by transcriptionally activating methyltransferase-like 14 (METTL14) and increasing *SMC4* m^{6}A methylation on its coding sequence region. The Cancer Genome Atlas dataset analysis confirmed a significant positive correlation between FoxO1, SMC4, and METTL14 expression in OC. In summary, this study revealed the molecular mechanisms of FoxO1 regulating SMC4 and established a clinical link between the expression of FoxO1/METTL14/SMC4 in the occurrence of OC, thus providing a potential diagnostic target and therapeutic strategy.

KEYWORDS
FoxO1, m^{6}A modification, METTL14, ovarian cancer progression, SMC4

1 | INTRODUCTION

Ovarian cancer (OC) is one of the three common malignant tumors affecting the female reproductive system. According to available statistics, about 314,000 women worldwide were diagnosed with OC in 2020, and 207,000 died of this disease.[1] OC ranks first for the mortality rates among gynecological malignant tumors. Due to the non-specificity of early clinical symptoms and the low efficiency of current OC screening, >75% of patients were in the late stage of clinical diagnosis, with a 5-year survival rate of about 20%.[2,3] The most commonly used method for clinical screening of OC is to detect serum carbohydrate antigen 125 (CA125) level, combined with or without transvaginal ultrasound (TVUS). Nonetheless, the sensitivity, specificity, and survival benefit of

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CA125 are insufficient for routine screening.[4] The limitation of the traditional TVUS resolution level and the lack of malignant change targets hinder its application.[5] An in-depth understanding of the pathogenesis and molecular mechanisms could help to identify new biomarkers for OC, which in turn could promote OC diagnosis.

Forkhead box protein O1 (FoxO1), which is also known as forkhead rhabdomyosarcoma transcription factor, belongs to the forkhead box O (FoxO) transcription factor family and is in the center of the tumor molecular regulatory network.[6,7] Numerous studies have shown that the imbalance of FoxO1 is closely related to the pathological process of tumor development, invasion, and metastasis.[7–11] However, there is no unified conclusion about the effect of FoxO1 in OC. Some studies have shown that FoxO1 has a tumor suppressor role in OC. As a downstream molecule, FoxO1 promotes the progression of OC after targeted inhibition by miR-27a.[12] It can also inhibit the viability, proliferation, and migration of OC cells and promote apoptosis after eliminating the EZH2 targeted inhibition effect.[13] On the contrary, as a direct research object, FoxO1 can promote the occurrence and development of OC. In their study, Han et al. found that FoxO1 was significantly upregulated in OC tissues compared with borderline tumors, benign tumors, and non-adjacent normal epithelium and was significantly associated with poor prognosis.[14] Liu et al. also found that FoxO1 was highly expressed in OC compared with adjacent tissues.[15] These findings suggest that FoxO1 has an important role in the occurrence and development of OC; however, its cancer-promoting reports are mainly limited to the correlation analysis of clinical indicators, and its molecular mechanism remains unclear. In the present study, we further clarified the role of FoxO1 in OC and explored the internal mechanism, which is of great significance for improving the pathogenesis of OC and finding potential diagnostic markers.

Structural maintenance of chromosome 4 (SMC4) is a member of the chromosome adenosine triphosphatase family of enzymes (ATPase), which has a key role in the cell cycle and mitosis.[16] The expression of SMC4 can accelerate the cell cycle from the G1 phase to S phase, ultimately promoting cell proliferation.[17] SMC4 has been found to be highly expressed in various tumors. It has been reported that SMC4 can promote tumor cell proliferation, migration and invasion in the liver,[18] lung,[19] breast,[20] and colorectal[21] cancer. SMC4 overexpression can activate the downstream NF-κB pathway,[22] TGF-β/Smad signaling[23] or JAK2/STAT3 pathway[24] to promote cancer, but there was still a lack of research in OC. In the upstream mechanism study, transcriptional regulation has a major role in the expression of SMC4. In addition to transcription factors,[16,25] miRNAs[24,26,27] and lncRNAs[28] can directly target *SMC4* mRNA for transcriptional regulation. However, the transcriptional regulation of FoxO1 on SMC4 has not been found. In OC, only bioinformatics analysis showed that the expression of SMC4 may be related to cell cycle, spliceosome, ubiquitin-mediated proteolysis, and adhesion connection,[29] while animal or cell-level verification and specific mechanisms to explore are lacking.

Our study discussed the role and molecular mechanism of FoxO1 in OC, and demonstrated that FoxO1-driven SMC4 expression was essential for OC genesis, which may provide important insights for early diagnosis and targeted therapy of OC patients.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Murine OC cell line ID8 and human OC cell line A2780 were provided by Dr. Zhao from the Nanjing First Hospital (Nanjing, China). The cell lines ID8 or A2780 were cultured in DMEM or RPMI-1640 (Gibco, California, USA) supplemented with 10% fetal bovine serum (Gibco, California, USA) and 1% penicillin (100 U/mL)/streptomycin (100 ng/mL). All cells were cultured at 37°C in an incubator with 5% CO_2. The FoxO1 inhibitor AS1842856 (MCE, Grand Island, NY, USA) was co-incubated with ID8 in dose of 100 nM for 48 h.

2.2 | Clinical samples preparation and immunohistochemistry staining

In total, 34 pairs of matched OC and adjacent normal tissues were obtained from Nanjing First Hospital. Clinical samples were collected, embedded in paraffin and then sliced into serial sections at 4-μm thickness. The ethical approval was granted by the Ethics Committee of Nanjing First Hospital, and informed consent was obtained from each investigator. The OC tumor samples were staged according to the 2018 Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) classification. These subjects did not receive immunotherapy or neoadjuvant chemotherapy when the tumor tissues were obtained.

Immunohistochemistry (IHC) sections were dewaxed and rehydrated using xylene and graded ethanol, respectively. Then, 3% hydrogen peroxide was used to deactivate endogenous peroxidase. After the sections were blocked with normal goat serum, the slides were incubated with primary antibodies against FoxO1 overnight at 4°C and then incubated with species-specific secondary antibodies (Table S2). Protein expression levels were calculated using the following equation: IHC score (IS) = staining intensity (0, no staining; 1, weak, light yellow; 2, moderate, yellow-brown; 3, strong, brown) × percentage of positive cells (1, <10%; 2, 10%–35%; 3, 35%–70%; 4, >70%).[30] The classification of immunostaining intensity was carried out by three independent observers.

2.3 | Tumor murine model

Female C57BL/6 specific pathogen-free (SPF) mice (6–8 weeks old) were obtained from Cavens Biotechnology Co., ltd. (Nanjing, China) and housed in a SPF facility with a relative humidity 55 ± 5%, room temperature 22 ± 2°C and 12 h/12 h light/dark cycle. These

animals were free to eat and drink during the whole experiment. All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing First Hospital, Nanjing Medical University (DWLL20220719-4). All animal studies followed the ARRIVE guidelines.

To establish the OC mouse model, different treatment of ID8 cells were resuspended at a density of 5×10^6 cells/100 μL mixed with Matrigel (ABWBIO, China) and injected into the dorsal flank of the mice. The tumor volume of mice was measured every week and calculated using the following formula: $Volume\ (mm^3) = (d^2 \times D)/2$, where d is the shortest and D is the longest diameter of the tumor. For the intratumoral injection experiment of lentivirus, the mice received SMC4 high expression or control virus ordered from Nanjing Zebrafish Biotech Co., Ltd. at a dose of 1×10^7 TU per mouse once a week for 6 weeks. About 11 weeks after the cell injections, the animals were sacrificed to analyze the size and weight of tumors.

2.4 | Statistical analysis

GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) was used for data and statistical analyses. Data were presented as the mean ± standard error of the mean. Student's *t*-test was used to compare two variables. The χ^2 test was used to compare the categorical variables between the two groups. Spearman's correlation analysis was used to describe the correlation between quantitative variables without a normal distribution. A p-value < 0.05 represented statistical significance. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

More detailed information on materials and methods is shown in the Appendix S1.

3 | RESULTS

3.1 | Intratumoral FoxO1 is overexpressed and correlated with poor prognosis in OC

Although research reports have suggested that FoxO1 is involved in the progression of OC, its impact on tumor outcomes remains controversial.[12–15] We first clarified the expression of FoxO1 in OC and explored its correlation with clinical indicators. We used the microarray data deposited in the public Gene Expression Omnibus (GEO) database to evaluate whether FoxO1 was present between cancer and normal tissues. As shown in Figure 1A, FoxO1 expression was significantly higher than that in normal ovarian tissues ($p<0.001$). Next, we verified the protein level of FoxO1 in 34 pairs of paired primary OC samples and adjacent non-cancerous epithelial tissues by IHC, and then divided them into negative, weak, moderate, or strong groups according to the staining intensity (Figure 1B). Consistent with the GEO data, the protein level of FoxO1 in OC tissues was significantly upregulated compared with that in adjacent tissues ($p<0.0001$; Figure 1C,D). In addition, 34 patients were divided into either a FoxO1 high expression group ($n=17$) or low expression group ($n=17$) according to the median of IHC score (IS = 6). The results showed that the protein level of FoxO1 was not significantly correlated with OC patient age and FIGO stage, which indicated that the expression of FoxO1 in OC was universal in all ages and stages (Table 1). Importantly, Kaplan–Meier survival analysis of TCGA database and the GSE30161, GSE26712, and GSE9891 datasets of the GEO database showed that overall survival (OS) and progression-free survival (PFS) of OC patients with high *FoxO1* mRNA levels were shorter than those of OC patients with low levels (Figure 1E). The above results indicate that FoxO1 is highly expressed in OC patients and is significantly associated with poor prognosis.

3.2 | FoxO1 promotes growth, migration and invasion of OC cells in vitro

Based on the database and our clinical sample analysis, high intratumoral expression of FoxO1 may be associated with poor OC prognosis. In order to explore the relationship between endogenous FoxO1 and tumor growth or invasion, we used a plasmid to overexpress FoxO1 in ID8 and A2780 cells (Figure 2A,B). CCK-8 assay, colony formation assay and 5-ethynyl-2′-deoxyuridine (EdU) detection revealed that upregulation of FoxO1 promotes the viability, colony formation and cell proliferation of OC cells (Figure 2C–E). Accordingly, PI staining and G1–S phase markers detection showed that the proportion of cells decreased in the G0/G1 phase and increased the S phase (Figure 2F,G). In addition, overexpression of FoxO1 also inhibited cell apoptosis (Figure 2H). The results of wound healing showed that, when compared with the vector group, the incidence of wound closure in OC cells with FoxO1 overexpression was significantly increased after 48 h, indicating that the migration ability was enhanced after FoxO1 overexpression (Figure 2I). Subsequently, transwell assay revealed that, compared with the contrast, the migration ability of OC cells was significantly increased after FoxO1 overexpression (Figure 2J). In addition, FoxO1 overexpression also significantly increased the number of cells invading the Matrigel (Figure 2K).

Alternatively, we silenced the expression of FoxO1 in ID8 and A2780 cells using small interfering RNA (siRNA; Figure 3A,B). CCK8, colony formation and EdU experiments showed that FoxO1 knockdown could inhibit the viability, colony formation and cell proliferation of OC cells (Figure 3C–E). In addition, downregulation of FoxO1 induced cell cycle arrest in OC cells (Figure 3F,G). In contrast with overexpression of FoxO1, decreased FoxO1 expression promoted cell apoptosis (Figure 3H) and inhibited cell migration and invasion in vitro (Figure 3I–K). The above data indicated that FoxO1 can promote the proliferation, migration, and invasion of OC cells, thus suggesting that high expression of FoxO1 in OC cells could be a sign of malignant degeneration.

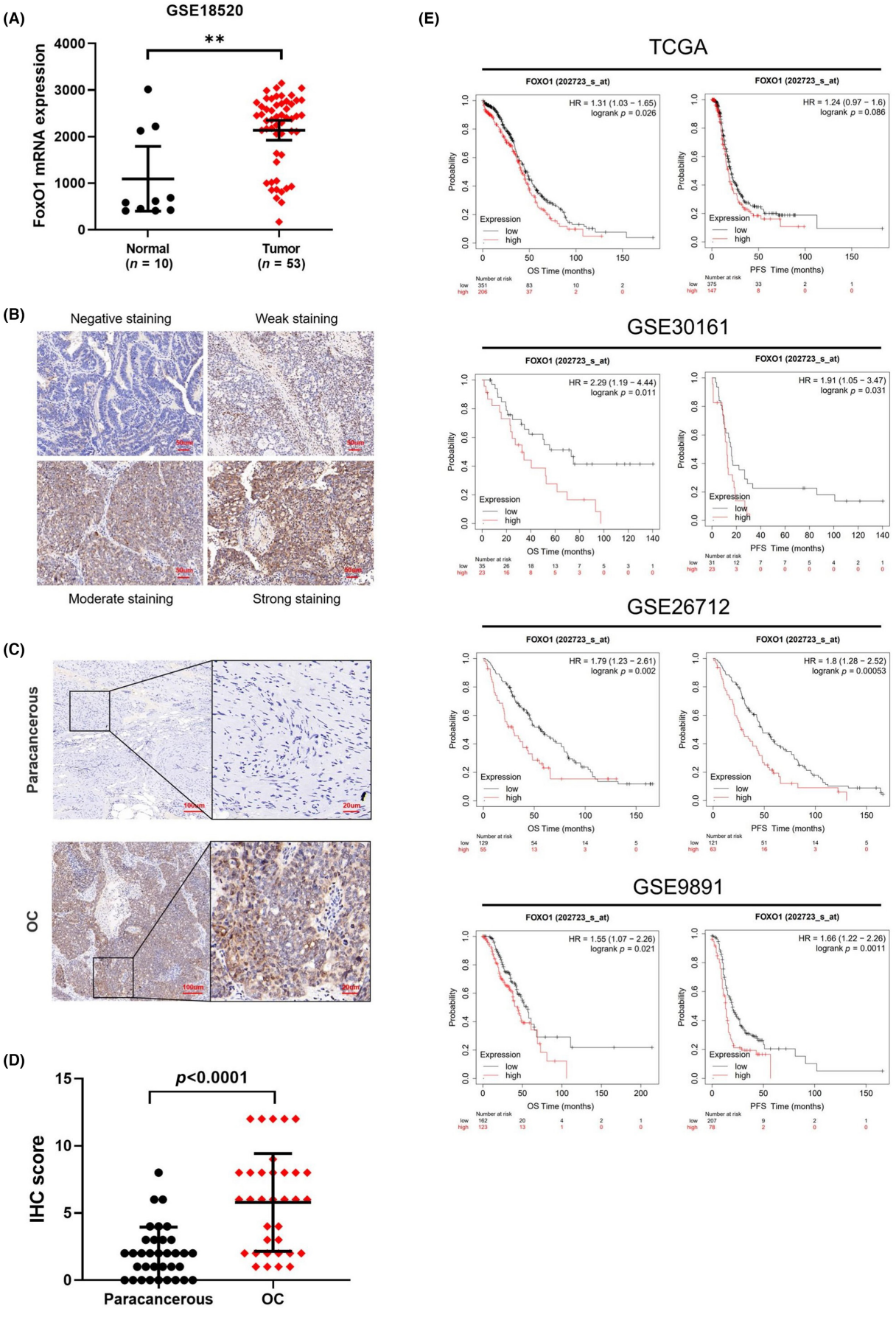
(A)
GSE18520
**
FoxO1 mRNA expression
4000
3000
2000
1000
0
Normal
(n = 10)
Tumor
(n = 53)
(B)
Negative staining
Weak staining
Moderate staining
Strong staining
(C)
Paracancerous
OC
100um
20um
(D)
p<0.0001
IHC score
15
10
5
0
Paracancerous
OC
(E)
TCGA
GSE30161
GSE26712
GSE9891
FOXO1 (202723_s_at)
HR = 1.31 (1.03 − 1.65)
logrank p = 0.026
HR = 1.24 (0.97 − 1.6)
logrank p = 0.086
HR = 2.29 (1.19 − 4.44)
logrank p = 0.011
HR = 1.91 (1.05 − 3.47)
logrank p = 0.031
HR = 1.79 (1.23 − 2.61)
logrank p = 0.002
HR = 1.8 (1.28 − 2.52)
logrank p = 0.00053
HR = 1.55 (1.07 − 2.26)
logrank p = 0.021
HR = 1.66 (1.22 − 2.26)
logrank p = 0.0011
Probability
Expression
low
high
OS Time (months)
PFS Time (months)
Number at risk

FIGURE 1 FoxO1 is upregulated in OC tissues, and high expression of FoxO1 is associated with a poor prognosis. (A) The expression level of the *FoxO1* gene in OC and normal ovarian tissues in GSE18520. (B) The representative images of FoxO1 expression in 34 cases of OC tissues and adjacent normal control tissues detected by IHC. (C, D) Representative images of FoxO1 staining intensity (C) and quantitative analysis (D). (E) The OS and PFS of OC patients in TCGA and GEO datasets were analyzed using the Kaplan–Meier database.

TABLE 1 The relationship between FOXO1 protein level and clinicopathological features in OC patients.

Variables	FOXO1 expression ($n=34$)		Total	p-value
	Low/%	High/%		
Age				
<60 years	10 (58.8)	7 (41.2)	17	0.3035
≥60 years	7 (41.2)	10 (58.8)	17	
FIGO stage				
I–II	3 (50.0)	3 (50.0)	6	>0.9999
III–IV	14 (50.0)	14 (50.0)	28	

3.3 | Knockdown of FoxO1 inhibits OC tumorigenesis in vivo

In order to further clarify the comprehensive function of FoxO1 in OC mice, we constructed a stable knockdown of FoxO1 in ID8 cells by lentivirus (Figure 4A,B). Subsequently, the ID8 mouse subcutaneous injection and xenotransplantation model were established to verify the roles of FoxO1 in OC progression. FoxO1 knockdown group had a significant reduction in tumor volume and weight compared with shNC group (Figure 4C,E). In vivo bioluminescence imaging system analysis showed that tumor growth was significantly reduced in FoxO1 knockdown cells (Figure 4F). Immunohistochemistry further revealed that the expression level of Ki-67 in FoxO1 knockdown tumor cells was significantly reduced compared with shNC group (Figure 4G). These data show that silencing FoxO1 inhibits OC tumorigenesis in vivo.

3.4 | SMC4 is transcriptionally regulated by FoxO1

Previous studies have shown that the function of FoxO1, also known as a nuclear transcription factor, depends on the regulation of downstream targets.[31] In order to explore the specific molecular mechanism underlying FoxO1 promotion on OC progression, GEPIA2 database analysis and ChIP-seq were integrated to identify potential transcription targets for FoxO1, which were closely related to the OC poor prognosis. As shown in Figure 5A, we first screened out 4917 differentially expressed genes (DEGs) between OC (TCGA, $N=426$) and normal (GTEx, $N=88$) ovarian tissue samples. The DEGs contained 1742 upregulated and 3175 downregulated genes, shown in the volcano plot (Figure 5B). Importantly, Gene Ontology (GO) enrichment analysis indicated that OC tissues had typical features such as abnormal proliferation, migration, and cell cycle, which contained 1251 DEGs (Figure 5C). Furthermore, we performed ChIP-seq analysis on ID8 cells (Figure 5A), identifying 7530 peaks corresponding to 7160 RefSeq genes, of which 17.04% were located at the promoter-transcription start site (Figure 5D). Next, we performed intersection analysis of 1251 DEGs on proliferation, migration and cell cycle and 1220 potential downstream targets of FoxO1 on ChIP-seq data. In total, 77 candidate functional transcription targets that were directly regulated by FoxO1 were screened in OC cells (Figure 5E). Subsequently, prognosis analysis of these 77 candidate targets in OC was carried out using the Kaplan–Meier database (Figure S1), where 36 genes showed a similar prognosis to that of FoxO1, verified by RT-qPCR in FoxO1stable knockdown ID8 cells (Figure 5A). As shown in Figure 5F, SMC4 was significantly downregulated by FoxO1 knockdown. Accordingly, we hypothesized that *SMC4* may be a potential target gene of FoxO1.

RT-qPCR and western blot analysis confirmed that SMC4 increased after FoxO1 overexpression and decreased after FoxO1 knockdown in OC cells (Figure 5G,H). Moreover, ChIP-seq data showed that FoxO1 was significantly enriched at sites (chr: 68,936, 142–68,936, 295) in the promoter region upstream of *SMC4* (Figure 5I). Subsequently, we predicted the sequence to obtain the potential binding motif of FoxO1 on the *SMC4* promoter using the JASPAR database, finding that the significant FoxO1-binding site (AGTTGTTTCTA) was located at the nucleotides −1400 bp to −1390 bp upstream of the *SMC4* transcription initiation site (Figure 5J). ChIP-qPCR analysis further confirmed that this site in the promoter region of *SMC4* was enriched in FoxO1 signals (Figure 5K). We further conducted luciferase assays using reporter constructs, such as SMC4-WT (wild-type), SMC4-MUT (mutation of the binding site), and SMC4-TRU (truncation of the binding site) (Figure 5L). Our results revealed that FoxO1 increased the transcriptional activity of SMC4-WT, while the stimulatory effects disappeared when the promoter region was further truncated or mutated (Figure 5M). In addition, when FoxO1 was complemented in stable shFoxO1 ID8 cells, the luciferase activity of the WT binding site was reversed (Figure 5N). In order to further confirm that SMC4 expression depends on the transcriptional activity of FoxO1, OC cells were treated with FoxO1 inhibitor AS1842856, and *SMC4* mRNA and protein levels were validated to decrease significantly (Figure S2A,B). In addition, AS1842856 significantly damaged the proliferation, migration

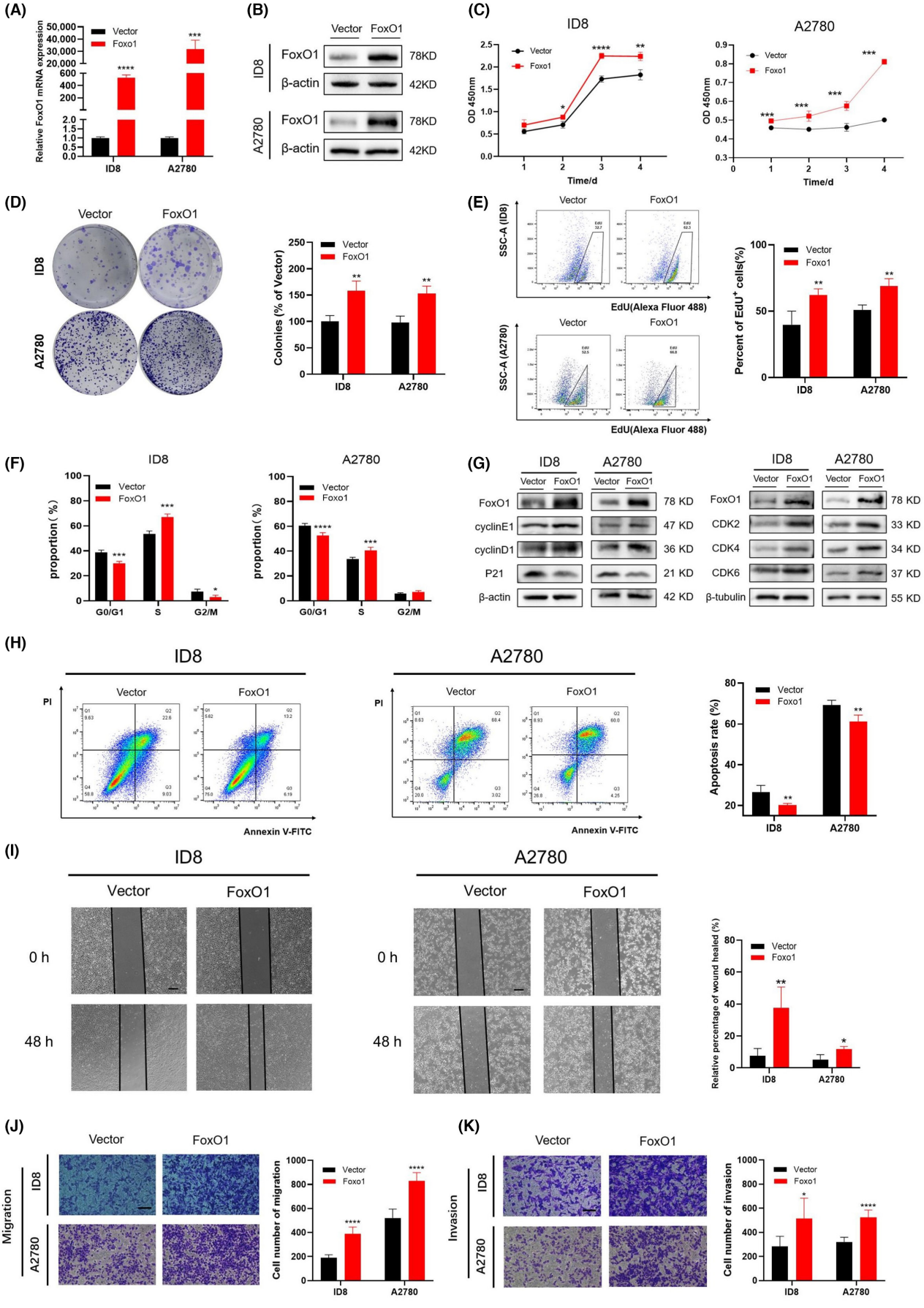
(A)
Relative FoxO1 mRNA expression
Vector
Foxo1
ID8
A2780
(B)
Vector FoxO1
ID8
A2780
FoxO1
β-actin
78KD
42KD
(C)
ID8
A2780
OD 450nm
Time/d
(D)
Vector
FoxO1
ID8
A2780
Colonies (% of Vector)
(E)
SSC-A (ID8)
SSC-A (A2780)
EdU(Alexa Fluor 488)
Percent of EdU+ cells(%)
(F)
ID8
A2780
proportion (%)
G0/G1
S
G2/M
(G)
FoxO1
cyclinE1
cyclinD1
P21
β-actin
CDK2
CDK4
CDK6
β-tubulin
78 KD
47 KD
36 KD
21 KD
42 KD
33 KD
34 KD
37 KD
55 KD
(H)
PI
Annexin V-FITC
Apoptosis rate (%)
(I)
0 h
48 h
Relative percentage of wound healed (%)
(J)
Migration
Cell number of migration
(K)
Invasion
Cell number of invasion

FIGURE 2 Overexpression of FoxO1 promotes proliferation, migration, and invasion and inhibits apoptosis of OC cells in vitro. (A, B) Comparison of *FoxO1* mRNA and protein expression in OC cell lines transfected with FoxO1 overexpression plasmid or empty vector. (C) Cell viability was analyzed using the CCK-8 method. (D) Representative images of colonies determined by colony formation experiment. (E–H) The cell proliferation detected by EdU (E), cell cycle (F), and apoptosis (H) were determined by flow cytometry; (G) G1/S phase marker proteins were detected by western blot. (I) Scratch healing assay was used to detect cell migration. Scale bar = 400 μm. (J, K) Cell migration (J) and invasion (K) ability were detected by transwell. Scale bar = 200 μm.

and invasion of OC cells and promoted apoptosis (Figure S2C–J). These data together suggest that FoxO1 can transcriptionally activate *SMC4* expression by binding to its promoter region in OC cells.

3.5 | SMC4 mediates FoxO1 to aggravate OC malignant phenotype in vivo

In order to clarify the prognostic role of SMC4 in OC based on the GEO database, GSE26712 and GSE18520 datasets were collected. SMC4 was found to be significantly overexpressed in tumors compared with normal epithelial tissues based on overall differences (Figure 6A,B). Taking the median expression value of SMC4 as the cut-off value, the OS performance of OC patients in the high-expression group was even worse than that in the low-expression group (Figure 6C). Kaplan–Meier survival analysis for OC PFS also generated similar results (Figure 6D). Based on the above clinical studies, we assessed the biological function of SMC4 in OC cells. *SMC4* was overexpressed or knocked down in ID8 cells (Figure 6E,F). We found that SMC4 significantly promoted the proliferation (Figure 6G–I), reduced the proportion of G0/G1 phase cells, and increased the proportion of S phase cells (Figure 6J,K). After knocking down SMC4, cell viability decreased, and cell cycle was arrested (Figure 6G–K). In addition, SMC4 also significantly inhibited apoptosis and improved cell migration and invasion, while low expression of SMC4 had the opposite effect (Figure 6L–N). These results suggest that SMC4 has a functional phenotype similar to FoxO1, proposing that SMC4 is a key downstream target of FoxO1.

Above, we only proved that SMC4 promotes proliferation, migration, and invasion of OC cells in vitro, however it remained unclear whether FoxO1 promotes tumorigenesis through the participation of SMC4. It has been reported that SMC4 participates in tumor progression by activating TGF-β/Smad and JAK2/STAT3 signal pathways.[23,24] Gain- and loss-of-function of SMC4 could significantly regulate TGF-β/Smad and JAK2/STAT3 signaling pathways, while the increased proliferation and invasion of OC cells were induced by SMC4 (Figure 6O). Accordingly, we constructed a mouse model of subcutaneous xenotransplantation by stably knocking down FoxO1 ID8 cells and intratumoral injection of high-expression SMC4 lentivirus or control virus. After we confirmed the high-expression effect of SMC4 lentivirus (Figure 6P), in vivo experiments showed that SMC4 overexpression significantly reversed the inhibitory effect of FoxO1 knockdown on OC progression. More precisely, compared with the control virus group, the tumor volume and weight were significantly increased, and the growth rate was faster in the high-expression SMC4 lentivirus injection group (Figure 6Q–S). Therefore, these results confirmed that SMC4 is required for FoxO1-induced OC malignant phenotype.

3.6 | FoxO1 promotes *SMC4* mRNA stability via METTL14-mediated m^{6}A modification

Recent studies have found that variations in N^6-methyladenosine (m^{6}A) levels and disorders of m^{6}A modulators are associated with the occurrence, development, or prognosis of OC[32]; however, the role of m^{6}A regulatory genes in OC has not been fully elucidated. As m^{6}A affects almost every stage of mRNA metabolism,[33] we found that the low expression of FoxO1 in ID8 cells significantly accelerated the degradation of *SMC4* mRNA (Figure 7A), suggesting the post-transcriptional regulation of SMC4 by FoxO1. Furthermore, several m^{6}A modulators were detected to explore the regulatory effect of FoxO1 on m^{6}A modification. The results showed that overexpression of FoxO1 induced a significant increase in the mRNA and protein expression of methyltransferase-like 14 (METTL14) in OC cells (Figure 7B,C). In addition, compared with shNC group, the mRNA and protein expression of METTL14 were significantly downregulated in stable shFoxO1 ID8 cells (Figure S3A,B). METTL14 is a key component of the methylase complex, which promotes the recognition of m^{6}A.[34] In the Kaplan–Meier database, a higher *METTL14* mRNA level was associated with poorer OS and PFS in OC patients (Figure 7D). By transfecting *METLL14* overexpression plasmids or siRNA fragments in ID8 cells, we confirmed the positive regulatory effect of METTL14 on SMC4 (Figure 7E,F). We further demonstrated that FoxO1 could increase SMC4 via METTL14 by supplementing METTL14 in the context of FoxO1 knockdown (Figure 7G). Therefore, we postulated that METTL14-induced dysfunction of SMC4 probably accounted for the FoxO1-mediated OC promotion signature.

Next, we examined whether FoxO1-mediated METTL14 overexpression increases SMC4 levels through m^{6}A-mediated post-transcriptional modification. RIP-qPCR experiments showed that the knockdown of FoxO1 significantly reduced the binding level of METTL14 to *SMC4* mRNA (Figure 7H). Subsequently, to identify the specific m^{6}A site affected by METTL14, the mRNA sequence of *SMC4* was predicted using the SRAMP database. The prediction results revealed four potential m^{6}A modification sites in the coding sequence (CDS) region with very high confidence (Figure 7I). As sites 1, 2 or 3, 4 were close to each other, we divided the two adjacent m^{6}A modification sites into one segment and designed primers for each segment separately (Table S1). MeRIP-qPCR indicated that knockdown of METTL14 significantly decreased the level of m^{6}A at the

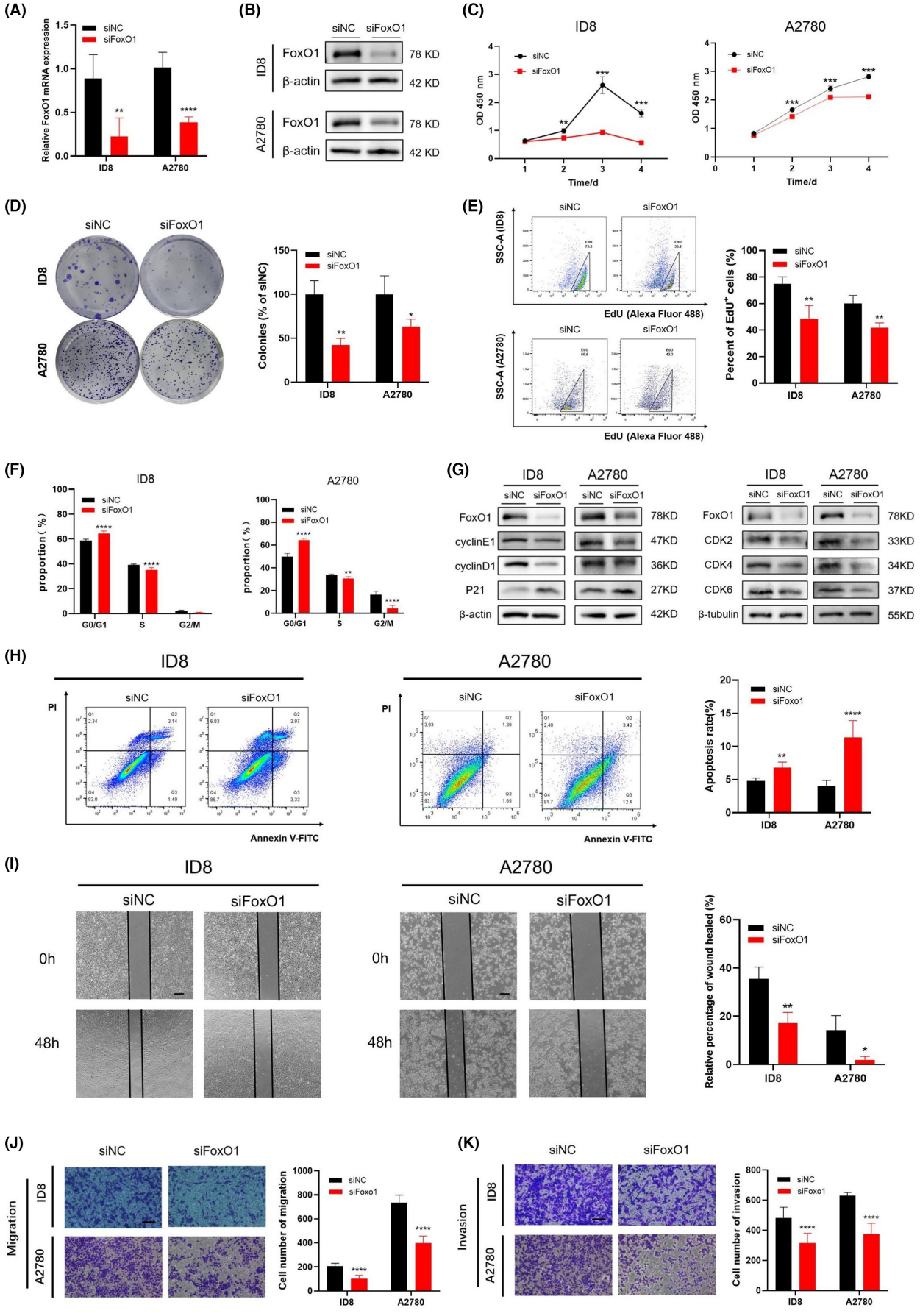
(A)
Relative FoxO1 mRNA expression
siNC
siFoxO1
ID8
A2780
(B)
siNC
siFoxO1
ID8
FoxO1
β-actin
78 KD
42 KD
A2780
(C)
ID8
A2780
OD 450 nm
Time/d
(D)
siNC
siFoxO1
ID8
A2780
Colonies (% of siNC)
(E)
SSC-A (ID8)
SSC-A (A2780)
EdU (Alexa Fluor 488)
Percent of EdU+ cells (%)
(F)
ID8
A2780
proportion (%)
G0/G1
S
G2/M
(G)
FoxO1
cyclinE1
cyclinD1
P21
β-actin
CDK2
CDK4
CDK6
β-tubulin
78KD
47KD
36KD
27KD
42KD
33KD
34KD
37KD
55KD
(H)
PI
Annexin V-FITC
Apoptosis rate(%)
siFoxo1
(I)
0h
48h
Relative percentage of wound healed (%)
(J)
Migration
Cell number of migration
(K)
Invasion
Cell number of invasion

FIGURE 3 Silencing FoxO1 inhibits the proliferation, migration, and invasion and promotes apoptosis of OC cells in vitro. (A, B) Comparison of *FoxO1* mRNA and protein expression in OC cell lines transfected with siFoxO1 or siNC. (C) Cell viability was analyzed using the CCK-8 assay. (D) Representative images of colonies determined by colony formation experiment. (E–H) The cell proliferation detected by EdU (E), cell cycle (F) and apoptosis (H) were determined by flow cytometry; (G) G1/S phase marker proteins were detected by western blot. (I) Scratch healing assay was used to detect cell migration. Scale bar = 400 μm. (J, K) Cell migration (J) and invasion (K) ability were detected by transwell. Scale bar = 200 μm.

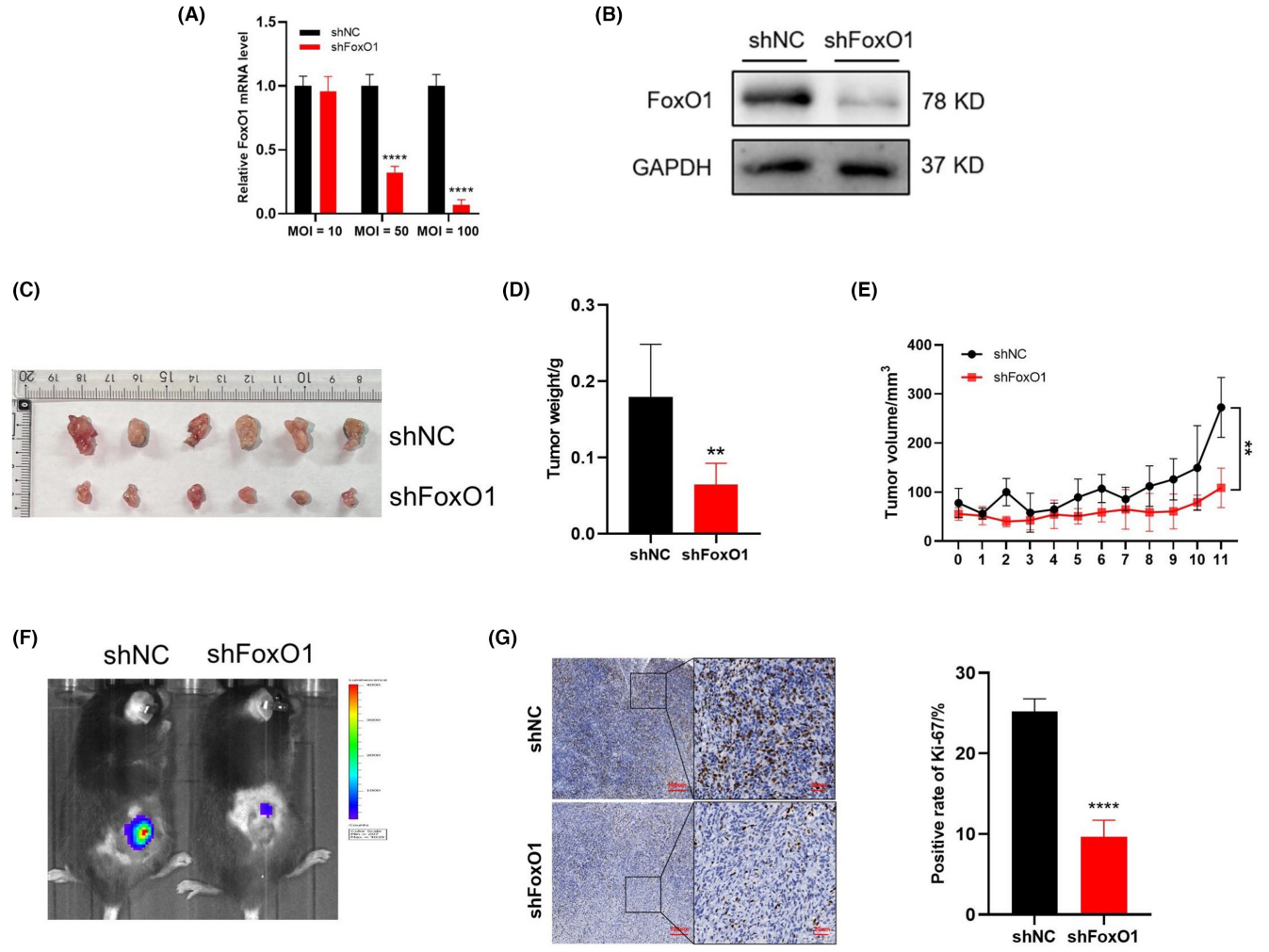

FIGURE 4 FoxO1 knockdown inhibited the growth of ID8-derived orthotopic xenografts in C57BL/6 mice. ID8 cells stably transfected with shNC or shRNA targeting FoxO1 were injected subcutaneously into the dorsal flank of C57BL/6 mice ($n = 6$). (A, B) Lentivirus-generated stable FoxO1 knockdown cell lines verified by RT-qPCR and western blot. (C) Tumor nodules, (D) tumor weight, and (E) the growth curve of tumor volume of mouse xenografts. (F) In vivo imaging of small animals to detect the fluorescence intensity of xenograft tumors. (G) The expression of Ki-67 in shNC and shFoxO1 tumor tissues was detected by IHC.

two segments (Figure 7J). Furthermore, we confirmed the positive regulation of METTL14 on *SMC4* mRNA stability by transfection of METTL14 overexpression plasmids or siRNA fragments (Figure 7K). Then, the dCas13b-FTO fusion protein was engineered to induce demethylation at four predicted m^{6}A modification sites in the CDS region on *SMC4* mRNA, and the results showed that the removal of m^{6}A modification significantly damaged the stability of *SMC4* mRNA in ID8 cells (Figure 7L), and reduced the mRNA and protein levels of SMC4 (Figure 7M). At the same time, the upregulation of SMC4 induced by METTL14 disappeared (Figure S3E). Furthermore, stably knocked-down FoxO1 significantly reduced m^{6}A modification at specific sites on *SMC4* mRNA (Figure 7N).

In addition, we further speculated that METTL14 could be transcriptionally regulated by FoxO1. The JASPAR database predicted that FoxO1 bound to the *METTL14* promoter at site 1 (CTTTGGTTTCA) and site 2 (GTTTGGTTTTATT), located at −1801 bp to −1791 bp and −578 bp to −568 bp, respectively, upstream of the *METTL14* transcription initiation site (Figure S3C). ChIP-qPCR analysis confirmed that site 1 in METTL14 was enriched in FoxO1 signals (Figure 7O). Luciferase assays also validated this result

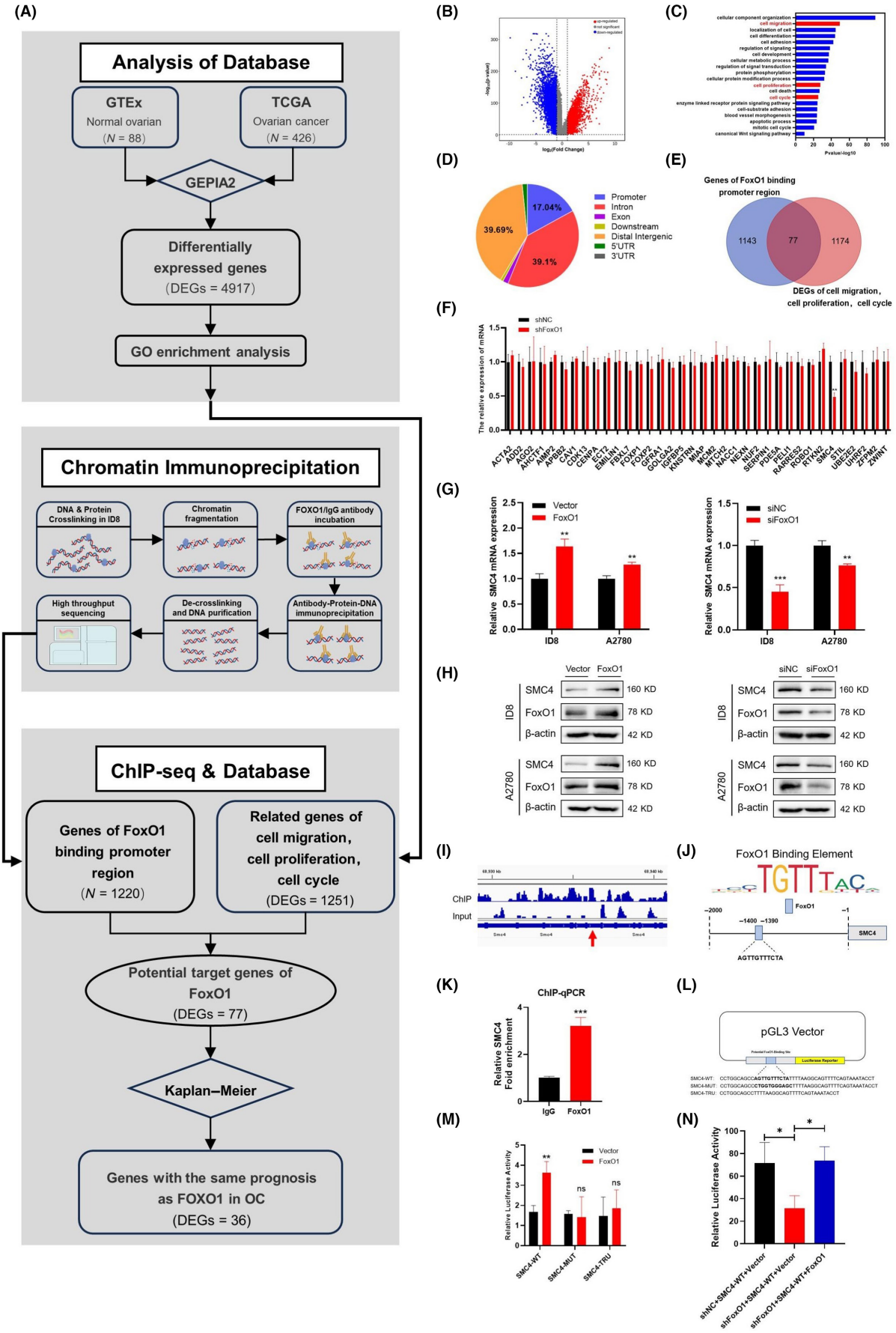
(A)
Analysis of Database
GTEx
Normal ovarian
(N = 88)
TCGA
Ovarian cancer
(N = 426)
GEPIA2
Differentially expressed genes
(DEGs = 4917)
GO enrichment analysis
Chromatin Immunoprecipitation
DNA & Protein Crosslinking in ID8
Chromatin fragmentation
FOXO1/IgG antibody incubation
Antibody-Protein-DNA immunoprecipitation
De-crosslinking and DNA purification
High throughput sequencing
ChIP-seq & Database
Genes of FoxO1 binding promoter region
(N = 1220)
Related genes of cell migration, cell proliferation, cell cycle
(DEGs = 1251)
Potential target genes of FoxO1
(DEGs = 77)
Kaplan–Meier
Genes with the same prognosis as FOXO1 in OC
(DEGs = 36)
(B)
(C)
(D)
Promoter
Intron
Exon
Downstream
Distal Intergenic
5'UTR
3'UTR
17.04%
39.69%
39.1%
(E)
Genes of FoxO1 binding promoter region
1143
77
1174
DEGs of cell migration, cell proliferation, cell cycle
(F)
shNC
shFoxO1
The relative expression of mRNA
(G)
Vector
FoxO1
Relative SMC4 mRNA expression
ID8
A2780
siNC
siFoxO1
(H)
Vector FoxO1
siNC siFoxO1
SMC4
FoxO1
β-actin
160 KD
78 KD
42 KD
(I)
ChIP
Input
(J)
FoxO1 Binding Element
FoxO1
SMC4
AGTTGTTTCTA
(K)
ChIP-qPCR
Relative SMC4 Fold enrichment
IgG
FoxO1
(L)
pGL3 Vector
(M)
Relative Luciferase Activity
SMC4-WT
SMC4-MUT
SMC4-TRU
ns
(N)
Relative Luciferase Activity
shNC+SMC4-WT+Vector
shFoxO1+SMC4-WT+Vector
shFoxO1+SMC4-WT+FoxO1

FIGURE 5 FoxO1 directly activates SMC4 transcription in OC. (A) Screening strategy diagram of FoxO1 downstream targets in OC. (B) The volcano plot of DEGs between OC and normal ovarian tissue samples was analyzed using the GEPIA2 database. (C) Gene Ontology (GO) enrichment analysis for DEGs (top 20 are listed). (D) Distribution of FoxO1 binding sequences across the length of the DNAs in ID8 cells detected by ChIP-seq. (E) Venn diagram of genes in the FoxO1 binding promoter region and DEGs of cell migration, proliferation, and cycle. (F) RT-qPCR used to detect the expression of 36 potential binding targets of FoxO1 with stable knockdown of FoxO1 and its control cells. (G, H) The mRNA and protein levels of SMC4 were detected after high expression or knockdown of FoxO1. (I) IGV diagram of FoxO1 binding to the SMC4 promoter sequence. (J) The JASPAR database predicted the potential FoxO1 binding site and motif in the promoter region of *SMC4*. (K) ChIP-qPCR used to determine the level of FoxO1 enrichment at the promoter of *SMC4*. (L) Schematic diagram of the *SMC4* promoter luciferase-reporter genes. (M) The luciferase activity with the SMC4-WT, SMC4-MUT or SMC4-TRU binding site was detected after transfection of FoxO1 overexpression or control plasmid. (N) The luciferase activity with the WT binding site was detected after transfection of FoxO1 overexpression or control plasmid with stable knockdown of FoxO1 and its control ID8 cells.

(Figure 7P; Figure S3D). After FoxO1 transcription activity was blocked by AS1842856, the mRNA and protein levels of METTL14 were significantly downregulated (Figure S2A,B). Collectively, these data implied that FoxO1 increased *SMC4* mRNA abundance by promoting METTL14 transcription to methylate *SMC4* at four m^{6}A residues in the CDS region.

3.7 | The expressions of FoxO1, SMC4 and METTL14 were positively correlated with each other in OC

In order to clarify the correlation among FoxO1, SMC4 and METTL14 in OC clinical data, RNA-seq data and corresponding clinical information of 376 OC tumors were obtained from TCGA database, and the correlation was analyzed through R software package *ggstatsplot*. The results showed that FoxO1 and SMC4 (Pearson $R=0.31$, $p<0.0001$), FoxO1 and METTL14 (Pearson $R=0.33$, $p<0.0001$), METTL14 and SMC4 (Pearson $R=0.33$, $p<0.0001$) were all positively correlated in OC (Figure 8A). In addition, the mRNA of tumor tissue was extracted from the subcutaneous xenotransplantation model of mice, and RT-qPCR results revealed that FoxO1 and SMC4 (Pearson $R=0.6105$, $p=0.0027$), FoxO1 and METTL14 (Pearson $R=0.6132$, $p=0.0026$), METTL14 and SMC4 (Pearson $R=0.9299$, $p<0.0001$) had significant positive correlation (Figure 8B). To conclude, the co-expression pattern of FoxO1, SMC4 and METTL14 might be regarded as an efficient prognostic factor of OC.

4 | DISCUSSION

OC has an insidious onset, and due to the lack of sensitivity and specificity of early diagnostic biomarkers, most OC patients are usually diagnosed in the late stage, which results in poor prognosis and extremely high mortality. Current studies are vigorously looking for additional protein biomarkers[35]; however, progress is slow due to the unclear pathogenesis of OC. Therefore, studying the cellular and molecular mechanisms behind the malignant biological behavior of OC is of utmost importance.

As a transcription factor in various cell functions, FoxO1 regulates biological processes such as cell proliferation, apoptosis, cell cycle and autophagy.[31,36] Its function in different cancer types remains controversial.[37–45] Our study found that FoxO1 serves as an oncogene in OC. Clinical data analysis showed that FoxO1 was highly expressed in samples of OC patients and was associated with a poor prognosis, which is consistent with the conclusions of Liu et al.[15] and Han et al.[14] Functional experiments further demonstrated FoxO1 in promoting the proliferation, migration, and invasion of OC in vitro and in vivo, suggesting that FoxO1 can be used as a useful independent prognostic marker in OC. As is well known, various post-translational modifications of FoxO1, such as phosphorylation, acetylation and ubiquitination, are closely related to tumorigenesis.[46–48] Further exploration of the modification level of FoxO1 protein in OC could better explain the mechanism of FoxO1.

In order to clarify the molecular mechanism of FoxO1 promoting OC progression, we used ChIP-seq, GEPIA2, and the Kaplan–Meier database combined with bioinformatics analysis, which showed that SMC4 was a potential downstream target of FoxO1. Multiple transcription starting points characterize the *SMC4* gene,[49] and its overexpression has been reported to be closely related to abnormal transcriptional regulation. HIF-1 and MYB can directly bind to the *SMC4* promoter region to regulate transcription.[16,25] Here, we first confirmed the transcriptional promotion of *SMC4* by FoxO1 through ChIP-qPCR and dual luciferase-reporter assay. Combined with FoxO1 transcriptional activity inhibitor treatment, it was confirmed that the expression of SMC4 depended on FoxO1 transcriptional activation. It has been reported that SMC4 can regulate FoxO1 phosphorylation level,[50] which needs to be explored and improved in our follow-up work. Further, database analysis confirmed that a high expression of SMC4 was significantly associated with a poor prognosis. Previous studies have shown that SMC4 is involved in the cell cycle, tumor proliferation, apoptosis, migration, and invasion,[22,23,51–55] and our data also revealed that SMC4 may play a role in promoting the proliferation, migration, and invasion phenotype of OC cells by activating TGF-β/Smad and JAK2/STAT3 signaling pathways.

Surprisingly, we found that the mRNA stability of *SMC4* was significantly downregulated in FoxO1 knockdown OC cells. It is reasonable to speculate that SMC4 is also regulated by FoxO1-induced post-transcriptional regulation. m^{6}A is the most common epigenetic change in eukaryotic cells, which can regulate the alternative splicing, nuclear export, stability, and translation efficiency of mRNA. In recent years, many studies have shown that m^{6}A modification is widely involved in the proliferation, invasion, metastasis, and chemotherapy resistance

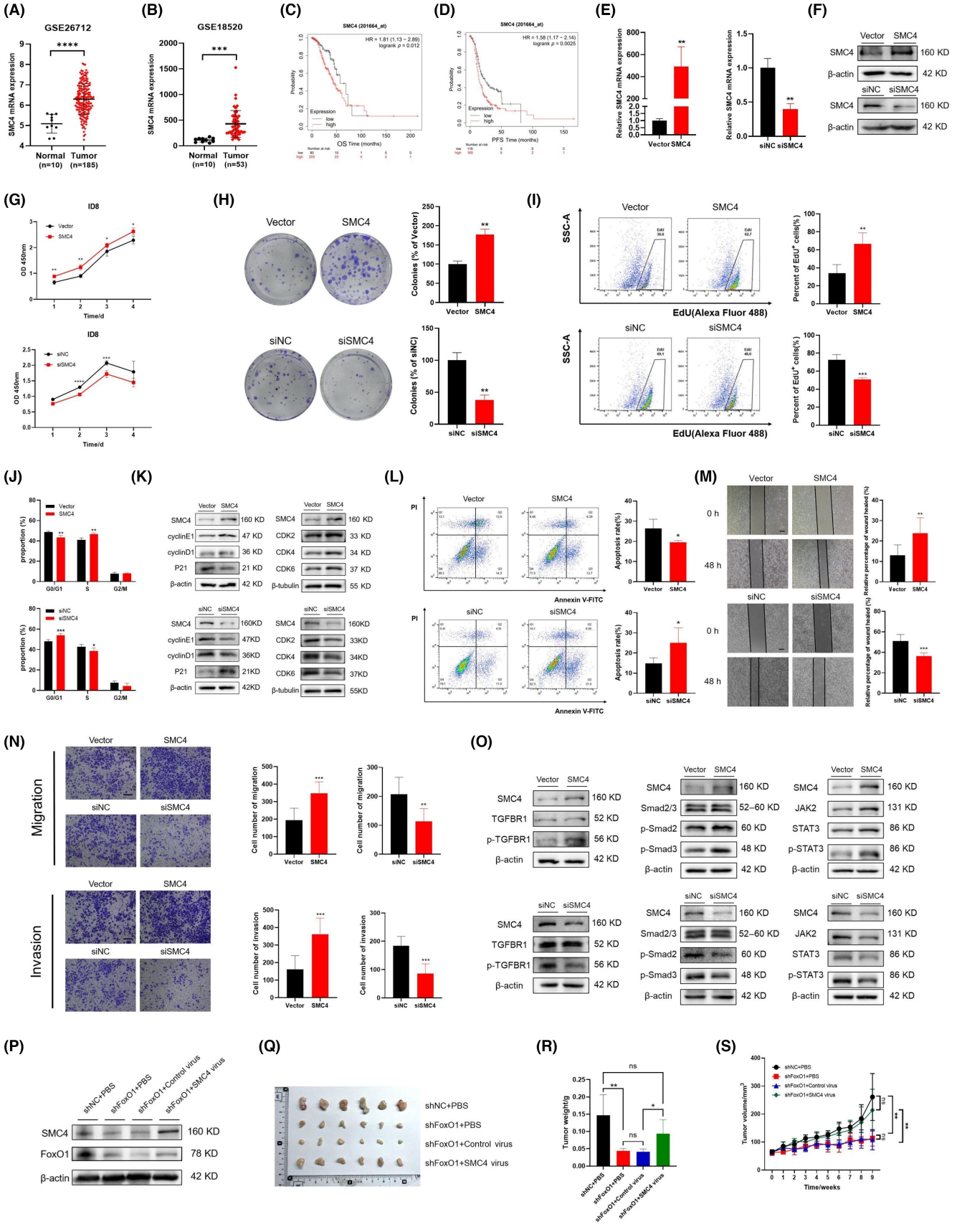

FIGURE 6 (Legend on next page)

FIGURE 6 SMC4 promotes the proliferation, migration, and invasion and inhibits apoptosis of ID8 cells, which is a key molecule in the process of FoxO1 promoting OC. (A, B) The expression level of SMC4 in OC tissues was compared with that in normal epithelial tissues in GEO datasets. (C, D) Kaplan–Meier database was used to analyze the OS and PFS of OC patients in TCGA and GSE9891 datasets. (E, F) The mRNA and protein levels of SMC4 after the transfection of SMC4 high-expression plasmid or siSMC4 were detected. (G) Cell viability was analyzed using the CCK-8 assay. (H) Representative images of colonies determined by colony formation experiment. (I–L) The cell proliferation detected by EdU (I), cell cycle (J) and apoptosis (L) were determined by flow cytometry; (K) G1/S phase marker proteins were detected by western blot. (M) Scratch healing assay used to detect cell migration. Scale bars = 400 μm. (N) Cell migration and invasion ability were detected by transwell assay. Scale bars = 200 μm. (O) The protein expression changes of TGF-β/Smad and JAK2/STAT3 pathway molecules were detected by western blot. ID8 cells stably transfected with shNC or shRNA targeting FoxO1 were injected subcutaneously into the dorsal flank of C57BL/6 mice ($n = 6$), and intratumoral injection of high-expression SMC4 lentivirus or control virus. (P) The protein expression level of SMC4 was detected in the tumor. (Q) Tumor nodules, (R) tumor weight, and (S) the growth curve of tumor volume of mouse xenografts.

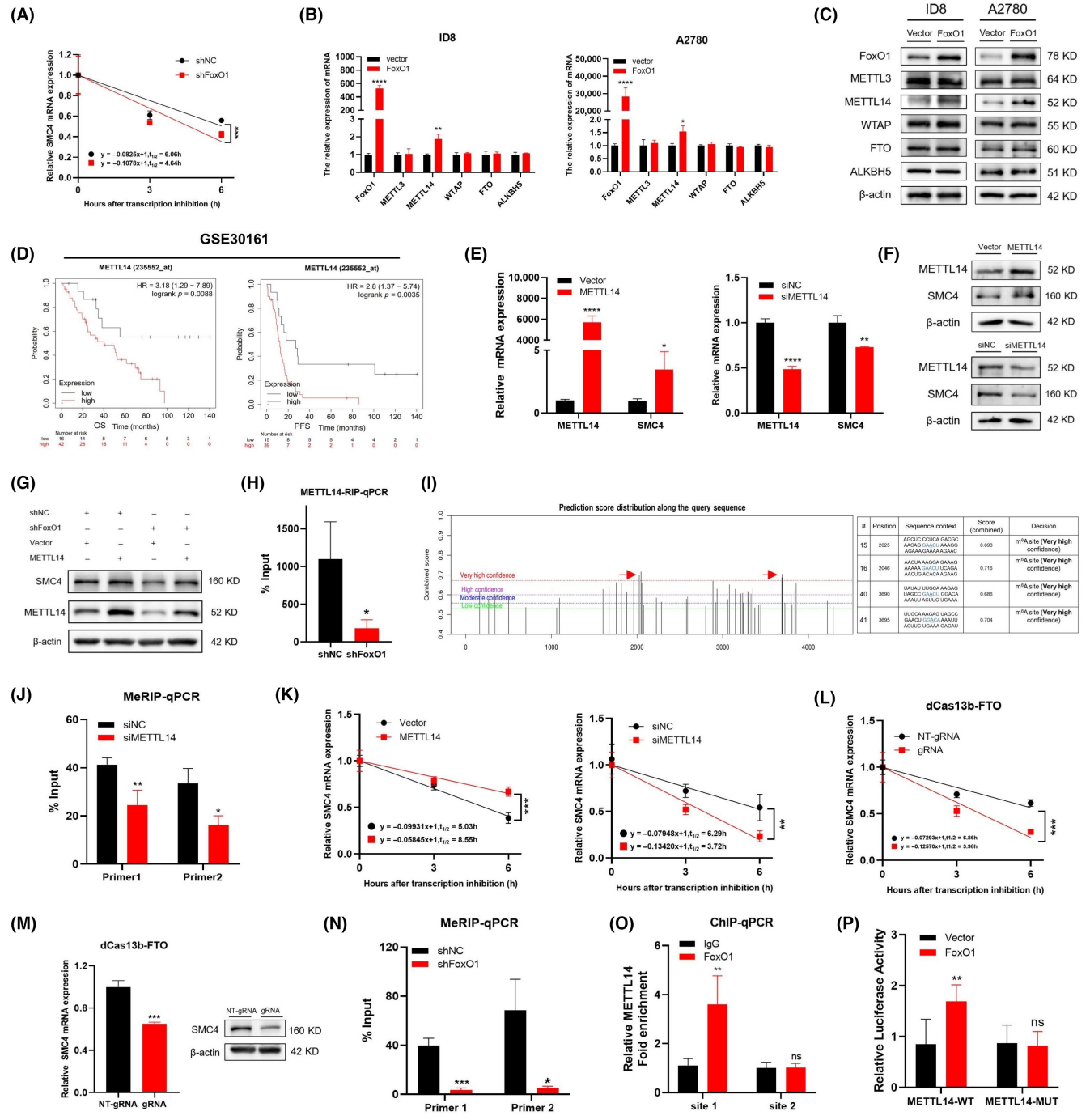

FIGURE 7 (Legend on next page)

FIGURE 7 FoxO1 promotes SMC4 expression by regulating METTL14-mediated m^{6}A modification. (A) After treatment with actinomycin D (5 μg/mL) for 0, 3, or 6 h, RT-qPCR used to analyze the effect of low expression of FoxO1 on the half-life of *SMC4* mRNA in ID8 cells. (B) The mRNA level of m^{6}A modification key regulatory genes was detected after high expression of FoxO1. (C) The protein level of m^{6}A modification key regulatory genes was detected after high expression of FoxO1. (D) The Kaplan–Meier database was used to analyze the OS and PFS of OC patients in GSE30161 datasets. (E, F) Changes in mRNA and protein levels of METTL14 and SMC4 after transfection of high-expression METTL14 plasmid or siMETTL14. (G) Western blot used to detect the effect of high expression of METTL14 after knocking down FoxO1 on the METTL14 and SMC4 protein levels. (H) METTL14-RIP-qPCR used to detect the enrichment of *SMC4* mRNA after FoxO1 knockdown. (I) Prediction results of *SMC4* mRNA in the SRAMP database show the potential sites of m^{6}A modification. The red arrows point to sites with very high confidence. (J) MeRIP-qPCR used to detect the enrichment of *SMC4* mRNA-predicted binding sites after low expression of METTL14. (K) RT-qPCR to analyze the effect of high or low expression of METTL14 on the half-life of *SMC4* mRNA. (L) The half-life of *SMC4* mRNA in ID8 transfected with lentivirus containing RNA methylation editor and guide RNA (gRNA) or non-targeting gRNA (NT-gRNA) was detected by RT-qPCR. (M) The expression of *SMC4* mRNA and protein in ID8 transfected with lentivirus containing RNA methylation editor and gRNA or NT-gRNA were detected. (N) MeRIP-qPCR used to detect the enrichment of SMC4 mRNA-predicted binding sites after FoxO1 knockdown in ID8. (O) ChIP-qPCR used to determine the level of FoxO1 enrichment at the promoter of METTL14. (P) The luciferase activity with METTL14-WT, METTL14-MUT binding site was detected after transfection of FoxO1 overexpression or control plasmid.

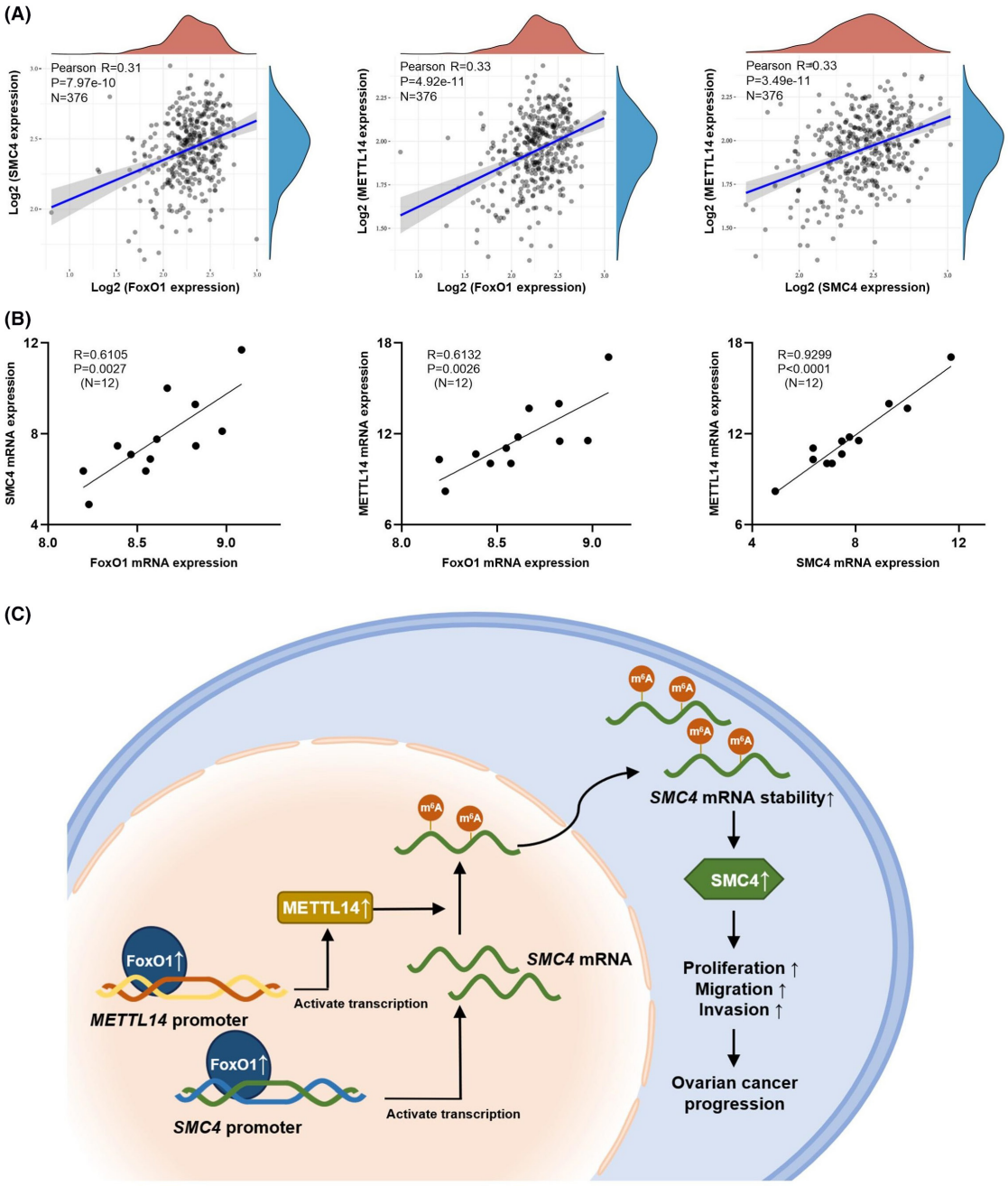

FIGURE 8 Correlation analysis of FoxO1, SMC4 and METTL14 in OC in clinical samples and mouse models. (A) The scatter plots show the correlation of *FoxO1*, *SMC4* and *METTL14* mRNA in OC tissues of 376 OC patients from TCGA. (B) Correlation among *FoxO1*, *SMC4* and *METTL14* mRNA levels in mice xenografts ($n = 12$) was measured using Spearman correlation analysis. (C) Working model of SMC4 regulation by FoxO1 through transcriptional activation and increased METTL14-mediated m^{6}A modification to promote OC. [Correction added on 9 March 2024, after first online publication: The image for Figure 8 has been updated.]

of OC.[56–61] It has been found that FoxM1, as the most homologous transcript of FoxO1, can regulate m^{6}A modification by regulating the methylated reading protein IGF6BP2[62]; however, there have been no reports of FoxO1 regulating m^{6}A modification. Our results showed that high expression of FoxO1 induced a significant increase of methyltransferase METTL14. METTL14 plays different roles in different tumors.[63–67] In OC, METTL14 is associated with poor overall survival[68] and can promote cell proliferation and migration and inhibits apoptosis.[69,70] We further confirmed that the knockdown of FoxO1 downregulated the binding level of METTL14 to *SMC4* mRNA and impaired *SMC4* mRNA stability by reducing m^{6}A modification. Existing studies have suggested that m^{6}A modification has a potential correlation with SMC4. SMC4 has been screened as an m^{6}A RNA methylation-related gene in liver cancer[71] and identified as an m^{6}A modification gene related to colorectal cancer recurrence[72]; however, its specific regulatory mechanism remains unclear. Our data reveal a new mechanism through which FoxO1 regulates *SMC4* mRNA stability through METTL14 in OC, and further confirmed that FoxO1 can transcriptionally activate the expression of METTL14. It has been reported that METTL14 can regulate FoxO1 expression.[73] Future studies are needed to address whether there is a similar mechanism in OC.

In summary, our study illustrated the oncogenic role of FoxO1 and an activated transcriptional and METTL4-mediated m^{6}A machinery of SMC4 in OC. FoxO1 could bind directly to the *SMC4* promoter to facilitate the translation of SMC4, furthermore, FoxO1 could be directly combined with the *METTL14* promoter, and also promote SMC4 stability in an m^{6}A METTL14-dependent manner, thus promoting proliferation, migration, and invasion of OC cells (Figure 8C). These findings suggest that the FoxO1/METTL14/SMC4 axis is a promising factor for diagnosing and treating OC.

AUTHOR CONTRIBUTIONS

Liping Tan: Conceptualization; data curation; methodology. **Shuangan Wang:** Data curation; formal analysis; methodology; software; writing – original draft. **Shijia Huang:** Data curation; investigation. **Yujuan Tie:** Formal analysis; methodology. **Na Sai:** Investigation; methodology. **Yichen Mao:** Methodology. **Shuli Zhao:** Conceptualization; funding acquisition; writing – review and editing. **Yayi Hou:** Conceptualization; funding acquisition. **Huan Dou:** Writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: The study was approved by the Ethics Committee of Nanjing First Hospital (approval no. KY20210628-9).

Informed Consent: Informed consent was obtained from each investigator.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing First Hospital, Nanjing Medical University (DWLL20220719-4). All animal studies have followed the ARRIVE guidelines.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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