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TRIM24 aggravates the progression of ovarian cancer through negatively regulating FOXM1 level

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Abstract. – OBJECTIVE: This study aims to uncover the biological functions of the feedback loop tripartite motif-containing 24 (TRIM24)/ Forkhead Box M1 (FOXM1) in the pathological progression of ovarian cancer (OC) and the underlying mechanism.

PATIENTS AND METHODS: The expression levels of TRIM24 and FOXM1 in OC tissues and cells were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The potential correlation between TRIM24 level and clinical indexes of OC patients was analyzed. The Kaplan-Meier curves were depicted for evaluating the prognostic potentials of TRIM24 and FOXM1 in OC patients. The regulatory effects of TRIM24 and FOXM1 on proliferative, migratory, and invasive capacities of SKOV3 and OVCAR3 cells were assessed through functional experiments. The rescue experiments were performed to clarify the feedback loop TRIM24/FOXM1 in influencing the progression of OC.

RESULTS: TRIM24 was upregulated in OC tissues and cells. The high level of TRIM24 was linked to higher rates of lymphatic and distant metastasis and worse survival in OC patients. The silence of TRIM24 attenuated proliferative, migratory, and invasive capacities of SKOV3 and OVCAR3 cells. FOXM1 level was negatively regulated by TRIM24, which was downregulated in OC. The low level of FOXM1 predicted worse survival in OC patients. Besides, the rescue experiments demonstrated that the feedback loop TRIM24/FOXM1 aggravated the malignant progression of OC.

CONCLUSIONS: TRIM24 is upregulated in OC tissues, and closely linked to the occurrence of lymphatic and distant metastasis. Through negatively regulating FOXM1 level, TRIM24 aggravates the progression of OC.

Key Words:
TRIM24, FOXM1, OC, Malignant progression.

Introduction

The mortality of ovarian cancer (OC) ranks the first in gynecological malignant tumors due to its high rate of metastasis[1-3]. The detective rate of the early-stage OC is extremely low because of the physiological structure of ovaries, atypical symptoms, and ineffective screening methods[4-6]. About 70% of OC patients are accompanied with metastases at the initial diagnosis[4-6]. The two-tier system for grading OC classifies it into high grade and low grade[7,8]. High-grade ovarian epithelial cancer accounts for 75% of OC cases, and 90% of OC-induced deaths. It is manifested as rapid onset and strong invasiveness, often accompanied by extensive pelvic and abdominal metastasis and implantation, resulting in an extremely poor prognosis[9,10]. In contrast, the low-grade ovarian epithelial cancer has a slow onset and often experiences the phenotype progression from benign, borderline, to low-grade malignancy. Patients with low-grade ovarian epithelial cancer often have a relatively good prognosis[11,12]. So far, the potential mechanisms underlying the invasion and metastasis of the high-grade ovarian epithelial cancer remain unclear[12-14].

TRIM24 (tripartite motif-containing 24), also known as TIF1α (transcription intermediary factor 1α), is a founding member of the transcriptional mediator family[15,16]. TRIM24 has a TRIM motif (RING-B box-coiled-coil, RBCC) at the amino terminus PHD and Bromo domains at the carboxy terminus[17]. RBCC exerts the activity of ubiquitin-protein ligase E3, which could mediate ubiquitination degradation of p53, while PHD and Bromo domains recognize specific modified histone molecules[17]. In addition, the LxxLL se-

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quence in the TRIM24 promoter region is capable of ligand-dependently binding to several nuclear receptors, while the PxVxL sequence contributes to chromatin structural change by binding to HP1[18,19]. Due to the diversity of its structure, the biological roles of TRIM24 in tumor diseases have been revealed[20]. For liver cancer, TRIM24 acts as a tumor-suppressor gene. Conversely, TRIM24 functions as an oncogene in granulocyte leukemia and breast cancer[21-23].

In this paper, we determined TRIM24 levels in tumor tissues and adjacent normal tissues of OC patients. The correlation between TRIM24 level and clinical indexes of OC patients was analyzed as well. Subsequently, the potential influences of TRIM24 on cellular behaviors of OC cells were assessed, as well as the underlying mechanism. Our results may provide references for clinical diagnosis and treatment of OC.

Patients and Methods

Patients and OC Samples

OC tissues and paracancerous tissues were surgically resected from 39 OC patients and pathologically diagnosed by hematoxylin-eosin staining (HE) staining. The samples were preserved at -80°C. None of OC patients were treated with preoperative anti-tumor therapy. The clinical indexes and follow-up data of the enrolled patients were collected. The patients and their families in this work have been fully informed. This study was approved by the Ethics Committee of the First People's Hospital of Fuyang District.

Cell Culture

Six human-derived OC cell lines (SKOV3, OVCAR3, PEO1, A2780, 3AO, CAOV3) and the human ovarian surface epithelial cell line (HOSEPiCs) were provided by American type culture collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA) and maintained at 37°C in a 5% CO_2 incubator.

Transfection

TRIM24 shRNAs and sh-NC were constructed by GenePharma (Shanghai, China). The cells inoculated in a 6-well plate were transfected at 40% confluence. At 48 h, the cells were harvested for subsequent experiments.

Cell Proliferation Assay

2.0×10^3 cells per well were inoculated in a 96-well plate. The viability was determined at the appointed time points using the Cell Counting Kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan). Absorbance at 490 nm was recorded for plotting the viability curve.

Colony Formation Assay

The cells were seeded in a 6-well plate with 200 cells per well and incubated for 14 days. The medium was replaced once in the first week and twice in the second week. Subsequently, the cells were fixed in 100% methanol and dyed with 0.5% crystal violet for 20 min. The colonies were captured and calculated in a good light environment.

Transwell Cell Migration and Invasion Assay

The transfected cells for 48 h were digested and adjusted to 5.0×10^5/mL. 200 μL/well suspension was applied in the upper side of the Matrigel-coated transwell chamber (Millipore, Billerica, MA, USA). In the bottom side, 500 μL of medium containing 10% FBS was applied. After 48 h of incubation, the invasive cells were fixed in methanol for 15 min, dyed with 0.2% crystal violet for 20 min, and counted using a microscope. The penetrating cells were counted in 10 randomly selected fields per sample. The transwell migration assay was conducted in the same procedures except for Matrigel pre-coating.

Wound Healing Assay

The cells were seeded in a 6-well plate with 5.0×10^5/well. Until 90% confluence, an artificial wound was created in the confluent cell monolayer using a 1 mL pipette tip. Wound closure images were taken at 0 and 24 h using an inverted microscope, respectively. The percentage of wound closure was calculated.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

We extracted the total RNA from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and purified them by DNase I treatment. The extracted RNA was reversely transcribed into complementary deoxyribonucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The cDNA was amplified by Real Time quantitative-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). β-actin was used as an internal reference. The primer sequences

were as follows: TRIM24: forward: 5'-ACTCGGGACATACCTGCTCT-3', reverse: 5'-GCCCTAGGGGAGTGACTACA-3'; FOXM1: forward: 5'-AAGGACAGCAAGAAGAAGG-3', reverse: 5'-GAGGAACAGGTGGAGAGT-3'; β-actin: forward: 5›-CCTGGCACCCAGCACAAT-3›, reverse: 5›-GCTGATCCACATCTGCTGGAA-3›.

Western Blot

The cells were lysed for extracting the total protein using radioimmunoprecipitation assay (RIPA), quantified by bicinchoninic acid (BCA) method and loaded for electrophoresis (Beyotime, Shanghai, China). After transferring on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. The bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (Media Cybernetics, Silver Springs, MD, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The Student's *t*-test was applied for analyzing differences between the two groups. Kaplan-Meier was introduced for survival analysis, followed by Log-rank test for comparing two curves. $p<0.05$ was considered statistically significant.

Results

TRIM24 Was Highly Expressed in OC

Totally, 39 matched OC tissues and adjacent normal tissues were collected. Compared with normal ones, TRIM24 was upregulated in OC tissues (Figures 1A, 1B). Similarly, *in vitro* abundance of TRIM24 was higher in OC cells relative to that of the human ovarian surface epithelial cell HOSEPiCs (Figure 1C).

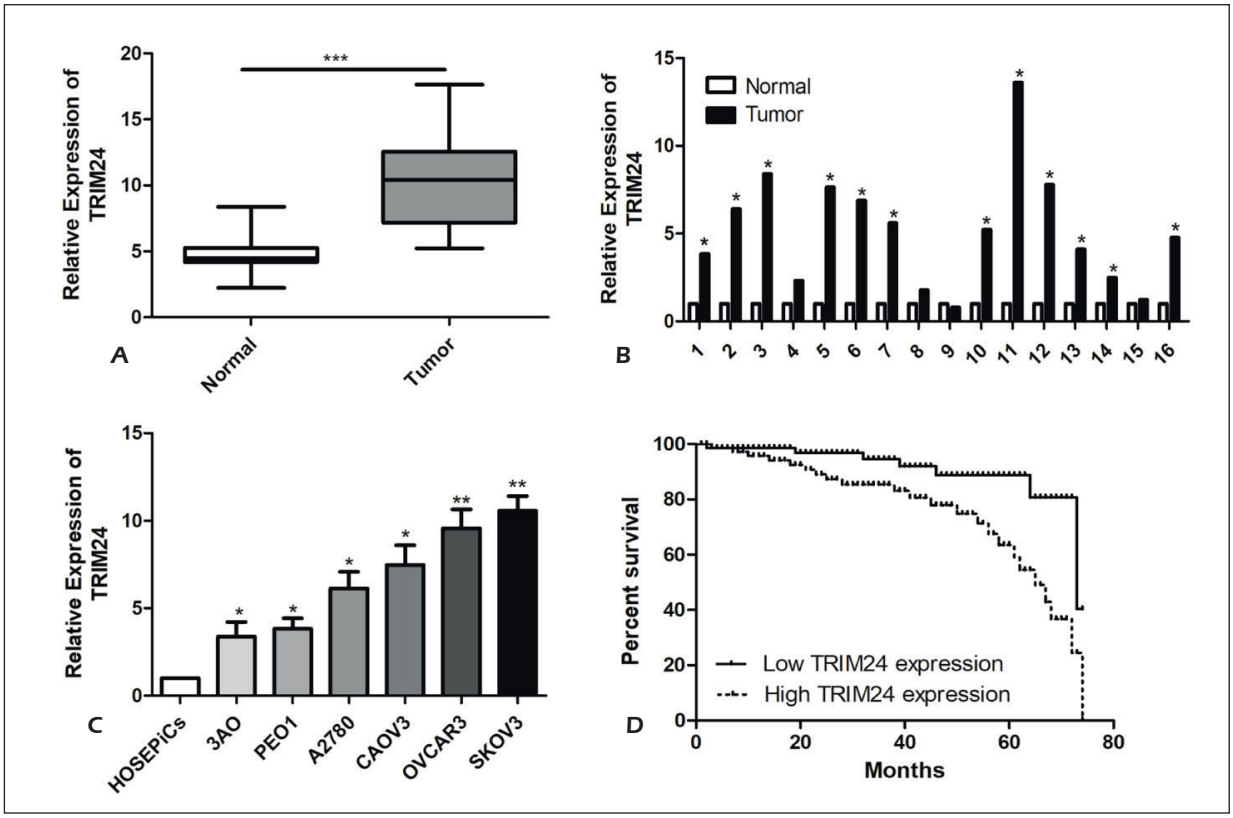

Figure 1. TRIM24 was highly expressed in OC. **A**-**B**, TRIM24 levels in OC tissues and adjacent normal ones. **C**, TRIM24 levels in the human ovarian surface epithelial cells (HOSEPiCs) and OC cells (3AO, PEO1, A2780, CAOV3, OVCAR3 and SKOV3). **D**, Survival in OC patients expressing high and low level of TRIM24.

Table I. Association of TRIM24 expression with clinicopathologic characteristics of ovarian cancer.

Parameters	Number of cases	TRIM24 expression		*p*-value*
		Low (%)	High (%)	
Age (years)				0.819
<60	13	7	6	
≥60	26	15	11	
T stage				0.896
T1-T2	24	14	10	
T3-T4	15	9	7	
Lymphatic metastasis				0.022
No	26	18	8	
Yes	13	4	9	
Distant metastasis				0.007
No	29	20	9	
Yes	10	2	8	

TRIM24 Expression Was Correlated to Metastasis in OC Patients

The clinical indexes of the enrolled OC patients were recorded. Based on the median level of TRIM24, they were classified into high-level and low-level groups, respectively. Our analysis uncovered that TRIM24 level was positively correlated to lymphatic and distant metastasis of OC patients, rather than other indexes (Table I). The Kaplan-Meier curves illustrated worse survival in OC patients of the high-level group (Figure 1D). Therefore, we considered that TRIM24 may be a prognostic marker for predicting the malignant progression of OC.

Silence of TRIM24 S uppressed OC to Proliferate and Metastasize

Before conducting functional experiments, three TRIM24 sh-RNAs were constructed. All of them were able to remarkably downregulate TRIM24 in SKOV3 and OVCAR3 cells, and the former one showed the most pronounced efficacy (Figure 2A). As CCK-8 results depicted, the transfection of sh-TRIM24#1 markedly decreased the viability in OC cells (Figure 2B). Meanwhile, the relative colony number declined after the silence of TRIM24, showing attenuated clonality (Figure 2C).

In addition, the transwell assay showed a decline in the migratory and invasive cell numbers after transfection of sh-TRIM24#1 in OC cells (Figure 3A). Wound healing assay further revealed a reduced percentage of wound closure due to the downregulated TRIM24 in SKOV3 and OVCAR3 cells (Figure 3B). Collectively, the silence of TRIM24 greatly decreased the proliferative, migratory, and invasive capacities of OC cells.

TRIM24 Negatively Regulated FOXM1 Level

The protein level of FOXM1 was markedly upregulated in SKOV3 and OVCAR3 cells transfected with sh-TRIM24#1 (Figure 4A). Identically, TRIM24 was upregulated after the silence of FOXM1 in OC cells (Figure 4B). A negative correlation was identified between the expression levels of TRIM24 and FOXM1 in OC tissues (Figure 4F). It is shown that FOXM1 was lowly expressed in OC tissues and cells (Figures 4C, 4D). OC patients expressing a low level of FOXM1 suffered worse survival than those with a high level (Figure 4E).

TRIM24 Influenced OC Progression Through Negatively Regulating FOXM1

We speculated that FOXM1 was involved in TRIM24-regulated progression of OC. Compared with OC cells transfected with si-FOXM1, the TRIM24 level was markedly lower in those co-transfected with si-FOXM1 and sh-TRIM24#1 (Figure 5A). Importantly, the silence of FOXM1 elevated the migratory cell numbers in SKOV3 and OVCAR3 cells, which was partially abolished by the co-transfection of si-FOXM1 and sh-TRIM24#1 (Figure 5B). Therefore, FOXM1 was necessary during the progression of OC regulated by TRIM24.

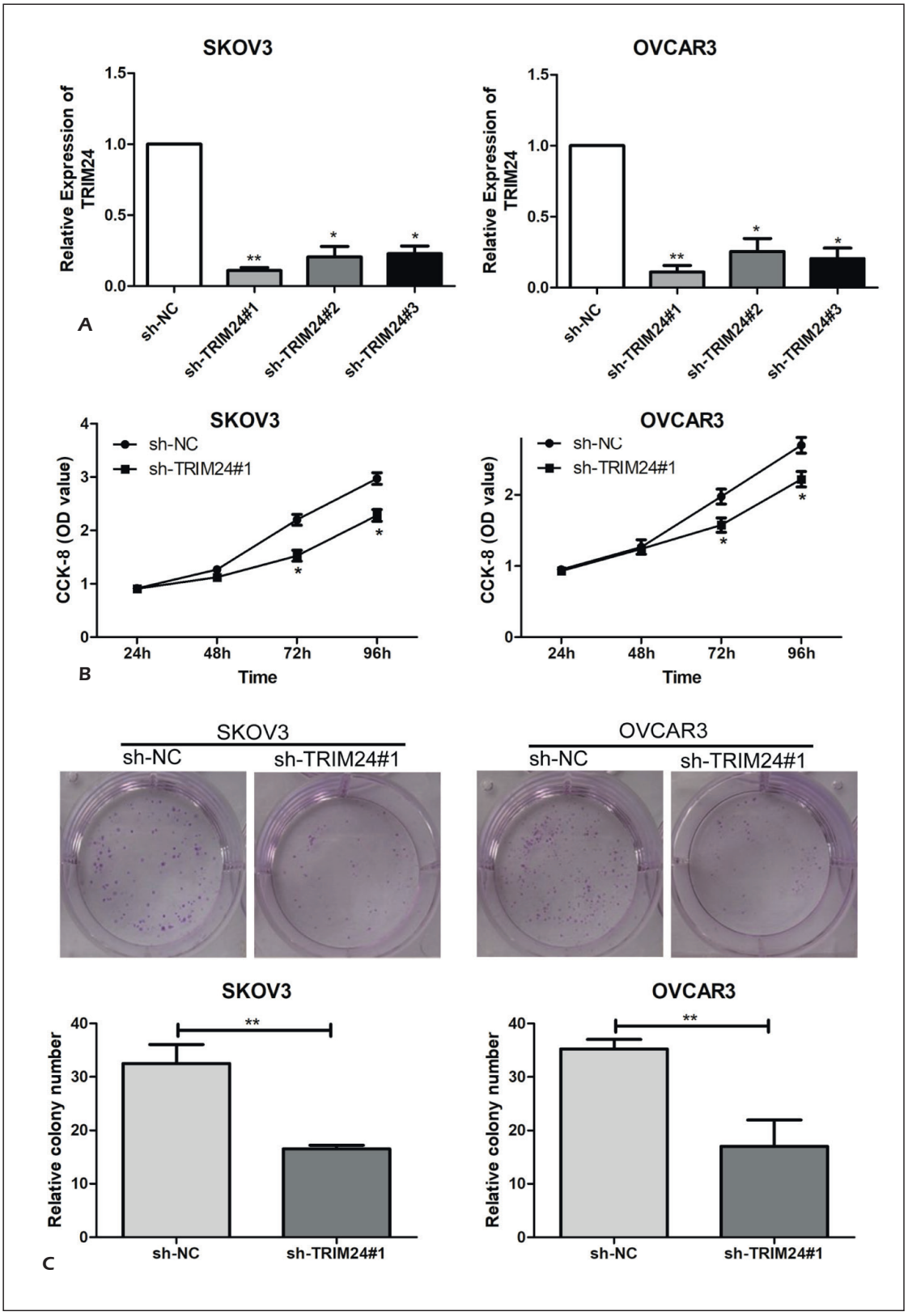

Figure 2. Silence of TRIM24 inhibited proliferation of OC. **A**, Transfection efficacy of sh-TRIM24#1, sh-TRIM24#2 and sh-TRIM24#3 in SKOV3 and OVCAR3 cells. **B**, CCK-8 assay showed viability in SKOV3 and OVCAR3 cells transfected with sh-NC or sh-TRIM24#1. **C**, Clonality in SKOV3 and OVCAR3 cells transfected with sh-NC or sh-TRIM24#1 (magnification 20×).

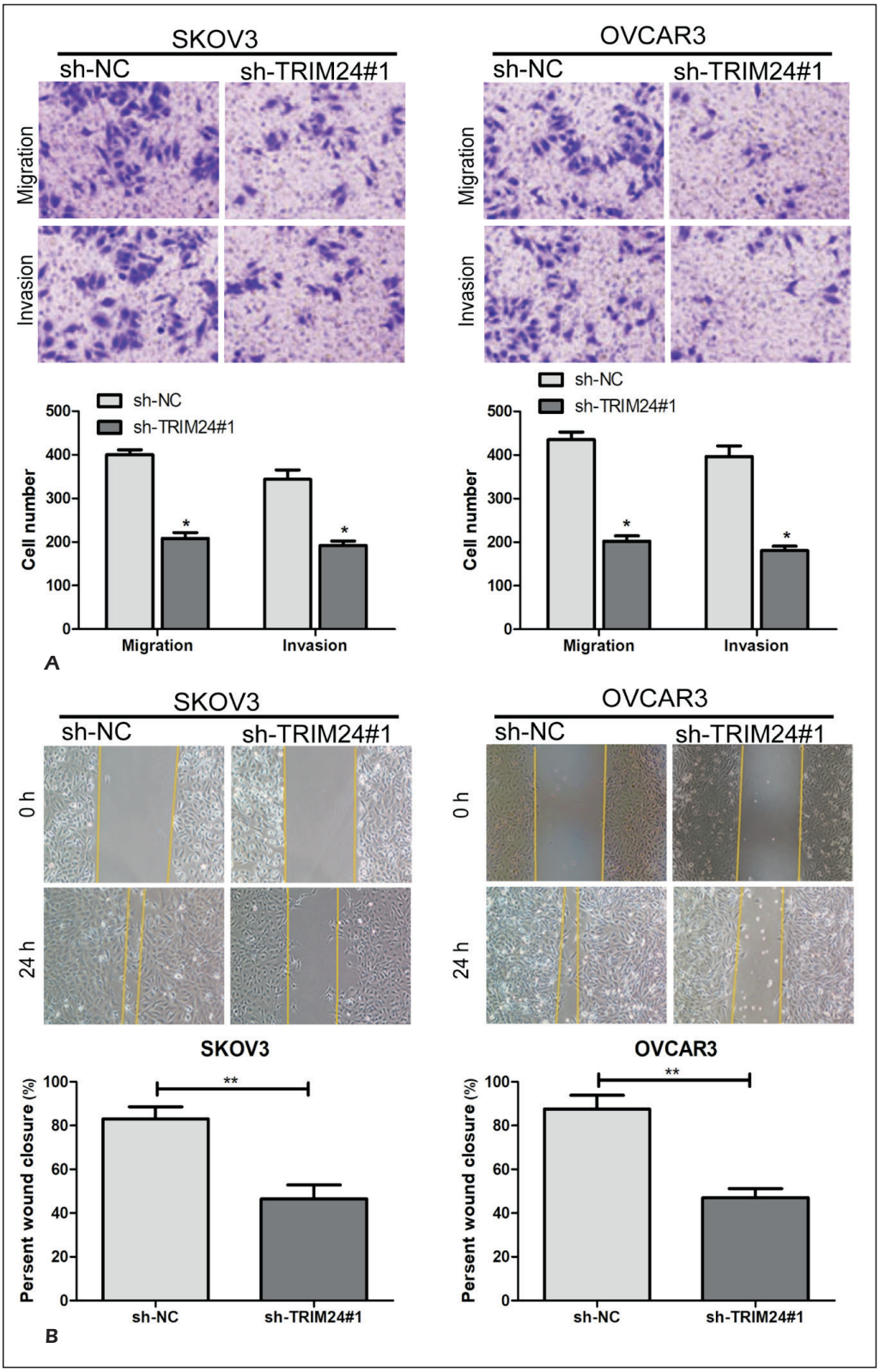

Figure 3. Silence of TRIM24 inhibited metastasis of OC. **A**, Migration and invasion in SKOV3 and OVCAR3 cells transfected with sh-NC or sh-TRIM24#1 (magnification 40×). **B**, Wound closure in SKOV3 and OVCAR3 cells transfected with sh-NC or sh-TRIM24#1 (magnification 40×).

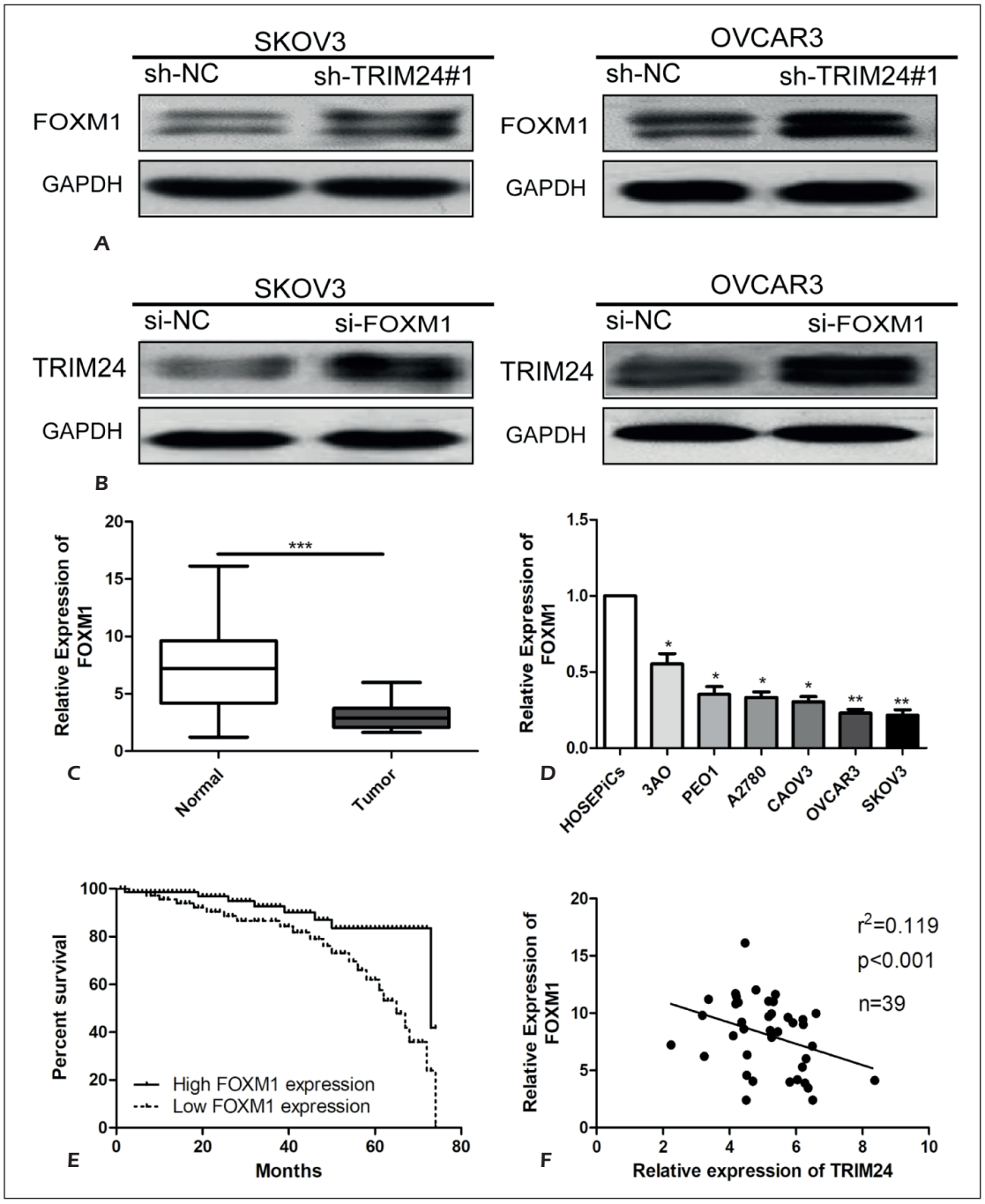

Figure 4. TRIM24 negatively regulated FOXM1 level. **A**, Protein level of FOXM1 in SKOV3 and OVCAR3 cells transfected with sh-NC or sh-TRIM24#1. **B**, Protein level of TRIM24 in SKOV3 and OVCAR3 cells transfected with si-NC or si-FOXM1. **C**, FOXM1 levels in OC tissues and adjacent normal ones. **D**, FOXM1 levels in the human ovarian surface epithelial cells (HOSEPiCs) and OC cells (3AO, PEO1, A2780, CAOV3, OVCAR3 and SKOV3). **E**, Survival in OC patients expressing high and low level of FOXM1. **F**, A negative correlation between expression levels of TRIM24 and FOXM1 in OC tissues.

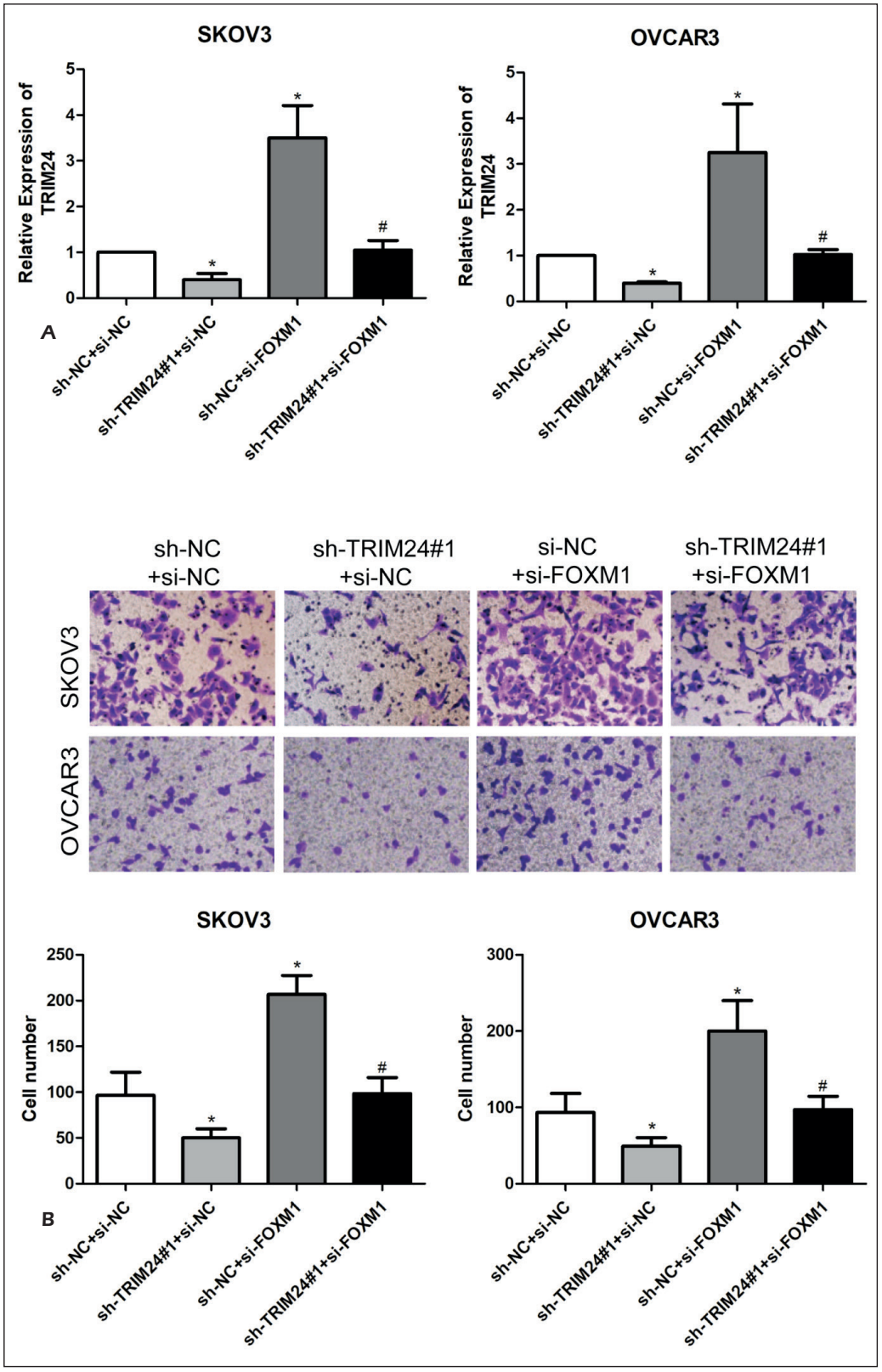

Figure 5. TRIM24 influenced OC progression through negatively regulating FOXM1 in SKOV3, and OVCAR3 cells were transfected with sh-NC+si-NC, sh-TRIM24#1+si-NC, sh-NC+si-FOXM1 or sh-TRIM24#1+si-FOXM1. **A**, TRIM24 level. **B**, Migratory cell number (magnification 40×).

Discussion

Etiology, the malignant phenotypes and progression mechanism of OC are well concerned in recent researches[8-10]. The majority of OC patients are progressed into the middle or advanced stage due to atypical signs and symptoms of OC[5,6,9]. Clinically, the OC patients in grade III or IV account for 75% of all OC cases[10,11]. Although great strides have been made in surgical procedures and cisplatin-chemotherapy, the 5-year survival of OC patients remains at 30%[12,13]. It is urgent to develop an early intervention for OC, thus improving the clinical outcomes[12-14].

The occurrence and progression of OC experience the following steps: firstly, OC cells lose the normal regulation of the cell cycle progression, and the uncontrolled proliferative tumor cells are produced. Subsequently, decreased cell adhesion and increased adhesion to the basement membrane of the extracellular matrix trigger the infiltration of tumor cells. At last, tumor cells are metastatic from blood vessels and lymphatic vessels to distant organs[9-13]. Abnormally expressed genes may be utilized for the diagnosis of OC, and they could be applied as therapeutic targets[7,13,14]. TRIM24 is reported to be upregulated in many types of tumor cells, and are closely linked to tumor progression[15-17]. In our work, TRIM24 was upregulated in OC tissues and cells. OC patients expressing a high level of TRIM24 presented higher rates of lymphatic and distant metastasis, and worse survival. It is suggested that TRIM24 may exert an oncogenic role in OC. The ability of tumor cells to migrate is of significance in tumor metastasis[24]. Extensive pelvic and abdominal cavity metastasis are the major reasons for poor prognosis of OC[13,14]. Once tumor cells adhered and further degraded the extracellular matrix, their metastatic abilities were greatly enhanced[13,14]. Our results showed that the silence of TRIM24 attenuated the proliferative, migratory, and invasive capacities of SKOV3 and OVCAR3 cells.

Appikonda et al[25] demonstrated that TRIM24 mediates tumor progression by regulating the downstream genes. It is of significance to clarify the biological functions of the downstream genes binding to TRIM24 in the progression of OC. FOXM1 is a classical transcription factor relevant to malignancies, which is upregulated in many types of tumors and affects multiple properties of tumor cell phenotypes[25-27]. In this experiment, FOXM1 level was negatively regulated by TRIM24, which was downregulated in OC. A low level of FOXM1 predicted worse survival in OC patients. Notably, the regulatory effects of TRIM24 on OC cells could be abolished by FOXM1, by verifying that FOXM1 was responsible for OC progression regulated by TRIM24.

Conclusions

The results of this study demonstrated that TRIM24 is upregulated in OC tissues, and was closely linked to the occurrence of lymphatic and distant metastasis. By negatively regulating FOXM1 level, TRIM24 aggravates the progression of OC.

Conflict of Interests

The authors declare that they have no conflict of interests.

References

1) Kossaï M, Leary A, Scoazec JY, Genestie C. Ovarian cancer: a heterogeneous disease. Pathobiology 2018; 85: 41-49.
2) Yao N, Yu L, Zhu B, Gan HY, Guo BQ. LncRNA GIHCG promotes development of ovarian cancer by regulating microRNA-429. Eur Rev Med Pharmacol Sci 2018; 22: 8127-8134.
3) Zhang Y, Zhang D, Wang H. Research on correlations of ERCC-1 with proliferation and apoptosis of ovarian cancer cells. J BUON 2018; 23: 1753-1795.
4) Akahori T, Woods DC, Tilly JL. Female fertility preservation through stem cell-based ovarian tissue reconstitution in vitro and ovarian regeneration in vivo. Clin Med Insights Reprod Health 2019; 13: 1179558119848007.
5) Zhou F, Shi LB, Zhang SY. Ovarian fibrosis: a phenomenon of concern. Chin Med J (Engl) 2017; 130: 365-371.
6) Rizzuto I, Behrens RF, Smith LA. Risk of ovarian cancer in women treated with ovarian stimulating drugs for infertility. Cochrane Database Syst Rev 2019; 6: CD008215.
7) Ledermann JA, Pujade-Lauraine E. Olaparib as maintenance treatment for patients with platinum-sensitive relapsed ovarian cancer. Ther Adv Med Oncol 2019; 11: 1758835919849753.
8) Vlad C, Dina C, Kubelac P, Vlad D, Pop B, Achimas Cadariu P. Expression of toll-like receptors in ovarian cancer. J BUON 2018; 23: 1725-1731.
9) Yu Y, Zhang X, Tian H, Zhang Z, Tian Y. Knockdown of long non-coding RNA HO-TAIR increases cisplatin sensitivity in ovarian cancer by inhibiting cisplatin-induced autophagy. J BUON 2018; 23: 1396-1401.

10) Iorio GC, Martini S, Arcadipane F, Ricardi U, Franco P. The role of radiotherapy in epi-thelial ovarian cancer: a literature overview. Med Oncol 2019; 36: 64.

11) Erol A, Niemira M, Kretowski AJ. Novel approaches in ovarian cancer research against heterogeneity, late diagnosis, drug resistance, and transcoelomic metastases. Int J Mol Sci 2019; 20: 2649.

12) Maru Y, Hippo Y. Current status of patient-derived ovarian cancer models. Cells-Basel 2019; 8: 505.

13) Yeung TL, Leung CS, Yip KP, Au YC, Wong ST, Mok SC. Cellular and molecular pro-cesses in ovarian cancer metastasis. A review in the theme: cell and molecular processes in cancer metastasis. Am J Physiol Cell Physiol 2015; 309: C444-C456.

14) Palma Flores C, García-Vázquez R, Gallardo Rincón D, Ruiz-García E, Astudillo de la Vega H, Marchat LA, Salinas Vera YM, López-Camarillo C. MicroRNAs driving invasion and metastasis in ovarian cancer: opportunities for translational medicine (Review). Int J Oncol 2017; 50: 1461-1476.

15) Appikonda S, Thakkar KN, Barton MC. Regulation of gene expression in human cancers by TRIM24. Drug Discov Today Technol 2016; 19: 57-63.

16) Stone L. Prostate cancer: TRIM24 acts as a transcriptional activator. Nat Rev Urol 2016; 13: 437.

17) Jain AK, Barton MC. Regulation of p53: TRIM24 enters the RING. Cell Cycle 2009; 8: 3668-3674.

18) Fong KW, Zhao JC, Song B, Zheng B, Yu J. TRIM28 protects TRIM24 from SPOP-mediated degradation and promotes prostate cancer progression. Nat Commun 2018; 9: 5007.

19) Wang P, Shen N, Liu D, Ning X, Wu D, Huang X. TRIM24 siRNA induced cell apop-tosis and reduced cell viability in human nasopharyngeal carcinoma cells. Mol Med Rep 2018; 18: 369-376.

20) Wang H, Xue W, Jiang X. Overexpression of TRIM24 stimulates proliferation and glu-cose metabolism of head and neck squamous cell carcinoma. Biomed Res Int 2018; 2018: 6142843.

21) Jiang S, Minter LC, Stratton SA, Yang P, Abbas HA, Akdemir ZC, Pant V, Post S, Gagea M, Lee RG, Lozano G, Barton MC. TRIM24 suppresses development of spontaneous hepatic lipid accumulation and hepatocellular carcinoma in mice. J Hepatol 2015; 62: 371-379.

22) Ma L, Yuan L, An J, Barton MC, Zhang Q, Liu Z. Histone H3 lysine 23 acetylation is associated with oncogene TRIM24 expression and a poor prognosis in breast cancer. Tumour Biol 2016; 37: 14803-14812.

23) Gechijian LN, Buckley DL, Lawlor MA, Reyes JM, Paulk J, Ott CJ, Winter GE, Erb MA, Scott TG, Xu M, Seo HS, Dhe-Paganon S, Kwiatkowski NP, Perry JA, Qi J, Gray NS, Bradner JE. Functional TRIM24 degrader via conjugation of ineffectual bromodomain and VHL ligands. Nat Chem Biol 2018; 14: 405-412.

24) Fidler IJ, Kripke ML. The challenge of targeting metastasis. Cancer Metastasis Rev 2015; 34: 635-641.

25) Appikonda S, Thakkar KN, Barton MC. Regulation of gene expression in human cancers by TRIM24. Drug Discov Today Technol 2016; 19: 57-63.

26) Gartel AL. FOXM1 in cancer: interactions and vulnerabilities. Cancer Res 2017; 77: 3135-3139.

27) Yao S, Fan LY, Lam EW. The FOXO3-FOXM1 axis: a key cancer drug target and a modulator of cancer drug resistance. Semin Cancer Biol 2018; 50: 77-89.