ARTICLE IN PRESS

Cancer Letters xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Up-regulation of Foxp3 inhibits cell proliferation, migration and invasion in epithelial ovarian cancer

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ARTICLE INFO

Article history:
Received 12 February 2009
Received in revised form 12 May 2009
Accepted 2 June 2009
Available online xxxx

Keywords:
Epithelial ovarian cancer
Foxp3
Proliferation
Invasion

ABSTRACT

The transcription factor Forkhead Box P3 (*Foxp3*) has been shown to play important roles in the occurring of regulatory T cells (Tregs). Limited evidence indicated that it was also expressed in tissues other than thymus and spleen, while, very recently, it was identified as a suppressor gene in breast cancer. However, the precise role and molecular mechanism of the action of *Foxp3* in ovarian cancer remained unclear. To elucidate the function of *Foxp3*, we examined the expression of *Foxp3* in ovarian cancerous cells and the consequences of up-regulation of *Foxp3* in epithelial ovarian cancer cell lines, respectively. By multiple cellular and molecular approaches such as gene transfection, CCK-8 assay, flow cytometry, RT-PCR, in-cell western, wound healing assay, and invasion assay, we found that *Foxp3* was weakly/no expressed in ovarian cancerous cells. Up-regulation of *Foxp3* inhibited cell proliferation, decreased cell migration, and reduced cell invasion. Compared with control, *Foxp3* up-regulated cells showed decreased expression of *Ki-67* and cyclin-dependent kinases (*CDKs*). Moreover, up-regulation of *Foxp3* reduced the expression of matrix metalloproteinase-*2* (*MMP-2*) and urokinase-type plasminogen activator (*uPA*), resulting in the inhibition of cell migration and invasion. In addition, *Foxp3* up-regulation inhibited the activation of mammalian target of rapamycin (mTOR) and NF-κB signaling. These findings suggested that up-regulation of *Foxp3* could be a novel approach for inhibiting ovarian cancer progression.

1. Introduction

Epithelial ovarian cancer (EOC) is the leading killer among all gynecological malignancies and the 5-year survival rate for all ovarian cancer patients remains at less than 40% for the past 30 years [1]. This could be due to the fact that no effective methods of early diagnosis are currently available, as well as due to the lack of effective systemic therapies resulting in the high mortality of patients diagnosed with EOC. This disappointing outcome strongly suggests that there is a dire need for innovative research that will lead to a dramatic improvement in the survival of patients diagnosed with this deadly disease.

Foxp3 is a new member of the Forkhead/winged helix family of the transcription factors. It was identified during position cloning of *Scurfin*, a gene responsible for X-linked autoimmune diseases in mice and human (immune deregulation, polyendopathy, enterophathy, X-linked, IPEX) [2,3]. Mutation of *Foxp3* gene resulted in ablation of the suppressor function of regulatory T cells, although it was still debatable whether such mutation ablated the regulatory T lineages, as was originally proposed [4,5].

Limited evidence indicated that it was also expressed in tissues other than thymus and spleen, while, very recently, *Foxp3* mRNA as well as protein was detected in some tumor cell lines, albeit in variable levels, not related to the tissue of origin. That expression correlated with the expression levels of IL-10 and TGF-β1 [6]. In support of the above data, a very recent publication described the expression of *Foxp3* in pancreatic carcinoma cells providing

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doi:10.1016/j.canlet.2009.06.001

Please cite this article in press as: H.-Y. Zhang, H. Sun, Up-regulation of Foxp3 inhibits cell proliferation, migration and invasion in epithelial ovarian cancer, Cancer Lett. (2009), doi:10.1016/j.canlet.2009.06.001

evidence that this could be an important tumor escape mechanism [7]. Moreover, *Foxp3* was noted as a transcriptional repressor of the *HER2/ERBB2* oncogene, and interacted with *SKP2* gene directly in breast cancer [8], while widespread deletion and somatic mutations of *Foxp3* were observed in human breast cancer. In addition, germline mutation of *Foxp3* resulted in a high rate of spontaneous breast cancer and increased susceptibility to carcinogens in a mouse model [9]. According to the above findings, *Foxp3* was identified as a suppressor gene in breast cancer. However, it also suppressed cell growth and induced cell death in tumor cells without *HER2/ERBB2* over-expression [9], which suggested that *Foxp3* was involved in more other signaling pathways. Since the role and molecular mechanism of *Foxp3* in ovarian cancer remained unknown, this study was scheduled to investigate whether expressions of *Foxp3* transcripts and mature protein could occur in ovarian cancerous cells as well as whether it might play a suppressive role in ovarian cancer.

2. Materials and methods

2.1. Samples and immunohistochemistry

A total of seven normal ovarian epithelium and 27 malignant epithelial ovarian tumors were obtained from patients (age: 40.35 ± 9.02 years and 55 ± 14.03 years) admitted at The Hospital of Obstetrics and Gynecology of Fudan University after the approval of the local ethics committee and informed consent were obtained according to the Declaration of Helsinki from 2006 to 2007. Immunohistochemical staining of deparaffinized tumoral and normal ovarian tissues was done according to standard protocols using *Foxp3* antibody (236A/E7, eBioscience, USA). The staining intensities were graded as strong, moderate, and weak, respectively, by two pathologists specialized in ovarian cancer.

2.2. Cells and cell culture

Human epithelial ovarian cancer cell lines SKOV3 and ES-2 were obtained from American Type Culture Collection. OMC685 were generously provided by Dr. Xu (M.D., Ph.D., Fudan University, Shanghai, China). All cells were cultured in RPMI1640 medium with 10% fetal bovine serum and 1% penicillin and streptomycin in 5% CO_2-humidified atmosphere at 37 °C.

2.3. Selecting a population of cells that stably express Foxp3

The pEF-1α-*Foxp3* plasmid containing *Foxp3* full-length cDNA was generously provided by Dr. Yang Liu (University of Michigan, USA). SKOV3 cells were seeded in 6-well plates, and transfected with pEF-1α-*Foxp3* and pEF-1α-vector, respectively, using Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) for 24 h without antibiotic selection. Then cells were cultured in medium containing 1600 μg/ml G418 (Sigma, St. Louis, MO) until all of the cells in the non-transfected control culture were killed. The antibiotic-resistant cells were pooled and passaged in medium containing 800 μg/ml G418. Cells were named as SKOV3/pEF-1α-*Foxp3* and SKOV3/pEF-1α-vector, respectively, for further experiments.

2.4. Cell growth assay

Cell growth was analyzed using a WST-8 Cell Counting Kit-8 (Beyotime, Jiangsu, China). Cells (7.5×10^3) suspended in RPMI1640 medium (100 μl) containing 10% fetal bovine serum were seeded in 96-well plates and incubated for 2d, 4d, and 6d, respectively. CCK-8 solution (10 μl) was added to each well and the cultures were incubated at 37 °C for 90 min. Absorbance at 450 nm was measured using an immunoreader. The results were plotted as means ± SD of three separate experiments having four determinations per experiment for each experimental condition.

2.5. Clone formation assay

Cells were seeded at 1.0×10^4 cells/well in 6-well plates and left to form clones over a period. Cultures were stained with 0.1% crystal violet and the number of clones in a 2×2 cm grid (on the culture plates) was scored to determine the clone-forming ability of the cells. Clones containing over 50 cells were counted.

2.6. Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry. After indicated treatments, cells were trypsinized, rinsed with PBS, fixed with 70% ethanol at 4 °C overnight, and treated with RNaseA (0.02 mg/ml) in the dark at room temperature for 30 min. Cells were resuspended in 0.05 mg/ml propidium iodide and analyzed with flow cytometry (Becton Dickinson). DNA histograms were analyzed by the ModFit LT V2.0 software. For each sample, at least 10^4 events were recorded.

2.7. Western blot analysis

Two types of western blot, regular western blot and in-cell western, were conducted. Regular western blot was according to previous documents. In-cell western was a promoted method [10]. Briefly, cells were seeded in 96-well plates at 5×10^4 cells/well and cultured for 12 h. After being washed twice by PBS, cells were fixed with 4% paraforma1dehyde for 20 min, incubated with 0.1% Triton-100 for 20 min, and then washed twice by PBS. Blocking was carried out with 5% BSA for 1 h at room temperature. Each well was incubated overnight at 4 °C with target primary antibody [anti-*MMP-2* 1:50; anti-*MMP-9* 1:50, anti-*uPA* 1:50; anti-*Ki-67* 1:50, anti-*PCNA* 1:50, anti-*CDKs* 1:50, anti-NF-κB 1:50 (RnD, Minneapolis, USA), anti-AKT 1:50; anti-phospho-AKT 1:50; anti-mTOR 1:50; anti-phospho-mTOR 1:50; anti-ERK 1:50; anti-phospho-ERK 1:50 (Cell Signaling Technology, Beverly, MA)] and β-actin 1:100 as control, followed by IRDye38 conjugated anti-mouse (800 nm) or Alexa Fluor®680 conjugated anti-rabbit (700 nm) secondary antibody 1:5000 (BD PharMingen, California, US) incubation at room temperature for 1 h in the

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dark. Fluorescence was visualized, scanned and analyzed on Odyssey (LI-COR, US).

2.8. Real-time RT-PCR analysis

Total RNA was isolated with TRI-zol (Tarkara, Japan) according to the manufacturer's protocols. RNA concentration was determined by Ultrospec3000 spectrophotometer (Pharmcia, Sweden). First strand reverse transcription kit was obtained by MBI (Fermentas, Lithuania, USA). Each reaction mixture (final volume 20 μl) contained RT template or negative control (2 μl), ddH_2O, primers (0.3 μM), and SYBR Green taq-enzyme mix buffer (2×, 10 μl) (Takara, Japan). GAPDH was used as the control reaction. GAPDH: sense primer 5′-GAA GGT GAA GGT CGG AGTC-3′ and anti-sense primer 5′-GAA GAT GGT GAT GGG ATT TC-3′. *Foxp3*: sense primer 5′-TCA CCT ACG CCA CGC TCA T-3′ and anti-sense primer 5′-ACT CAG GTT GTG CGG ATGG-3′. Data were analyzed with ABI7000 software. Reaction products were also analyzed on 2% agarose gels, and visualized by ethidium bromide staining.

2.9. Wound healing assay

Cells were cultured and grown to 100% confluence. A clear area was then scraped in the monolayer with a 200 μl pipette tip. After being washed with serum-free RPMI1640 medium, cells were incubated at 37 °C in RPMI1640 medium containing 5% fetal bovine serum. Migration of cells into wounded areas was evaluated at the indicated times with an inverted microscope and photographed. The healing rate was quantified with measurements of the gap size during the culture. Three different areas in each assay were chosen to measure the distance of migrating cells to the origin of the wound.

2.10. Cell invasion assay

Invasion assay was performed using 24-well Transwell units with 8 μm pore size polycarbonate inserts. The polycarbonate membranes were coated with Matrigel (Becton Dickinson) and cultured at 37 °C for 1 h. Cells (1.0×10^4) suspended in 200 μl of RPMI1640 medium containing 5% fetal bovine serum were seeded in the upper compartment of the Transwell unit. 800 μl of RPMI1640 medium containing 10% fetal bovine serum was added into the lower compartment as a chemoattractant. After 24 ~ 48 h incubation, cells on the upper side of the membrane were then removed, whereas the cells that migrated through the membrane to the underside were fixed and stained with 0.1% crystal violet. Cell numbers were counted in five separate fields using light microscopy at 200× magnification. The data were expressed as the mean value of cells in five fields based on three independent experiments.

2.11. Statistical analysis

Experiments were independently done three or more times. Comparisons were made between SKOV3/pEF1α-vector and SKOV3/pEF1α-*Foxp3* cells. Two-tailed Student's *t* test or one-way ANOVA (SPSS software version 11.5) was used to analyze. $P < 0.05$ was considered to indicate statistical significance.

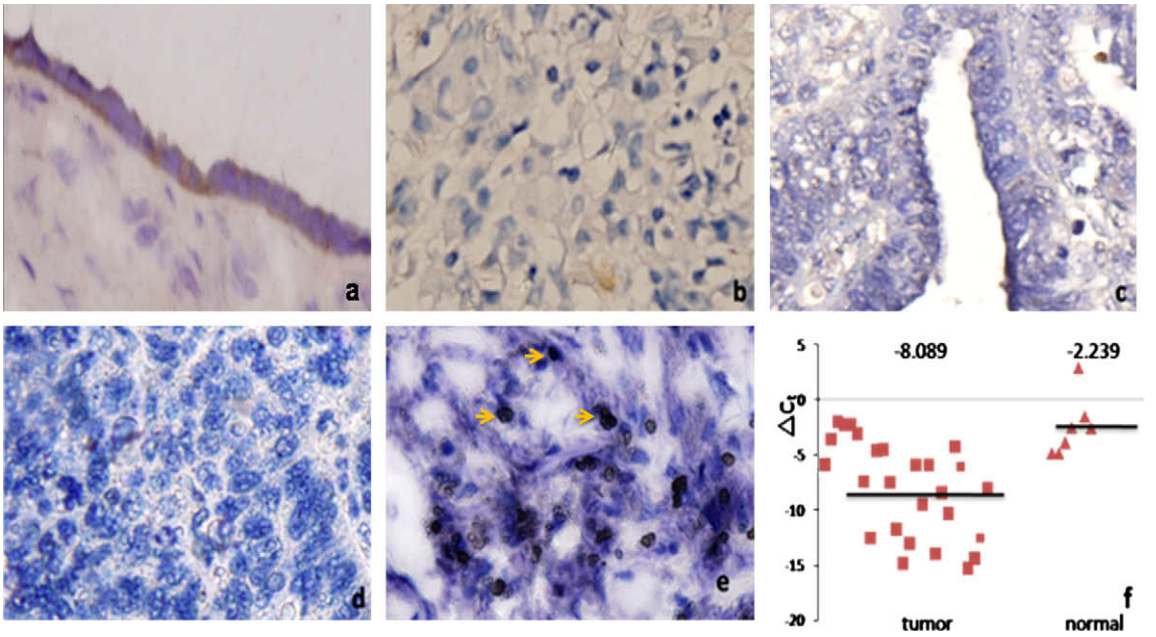

Fig. 1. Immunohistochemical staining revealed *Foxp3* expression in paraffin-embedded normal and cancerous ovarian tissues (200× magnification). (a) Cytoplasmic *Foxp3* staining was observed in normal ovarian epithelium. In 26 patients with ovarian cancer, nuclear and cytoplasmic *Foxp3* staining patterns were not detected as showed in b (serous type), c (clear cell type), and d (mucin type). Arrows in e showed nuclear *Foxp3* staining in tumor-infiltrating Tregs. (f) Real-time RT-PCR analysis detected *Foxp3* transcript level in ovarian cancer tissues ($n = 26$, exclusion the existence of *Foxp3*-positive Tregs) and normal ovarian epithelium ($n = 7$). ΔC_t was calculated as C_t (GAPDH)-C_t (*Foxp3*). Statistical significance was shown using unpaired *t* test, two-tailed, $P < 0.01$.

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3. Results

3.1. *Foxp3* expression in normal and cancerous ovarian tissues

We assessed *Foxp3* expression in human epithelial ovarian cancer specimens ($n = 27$) and normal ovarian epithelium ($n = 7$) by immunohistochemistry. *Foxp3* protein was detected in normal ovarian epithelium, whereas no or weak expression was determined in tumor cells (three histological subtypes, Fig. 1a–d). Moreover, the existence of *Foxp3*-positive Tregs was observed in 1/27 cancerous ovarian tissue (Fig. 1e). Real-time RT-PCR analysis confirmed the results obtained by immunohistochemistry. Analysis of transcript level showed that the expression of *Foxp3* gene in 26 epithelial ovarian cancer specimens (exclusion the existence of *Foxp3*-positive Tregs) was lower than in normal ovarian epithelium (Tumor $\Delta C_t = -8.089$ *vs*. normal $\Delta C_t = -2.239$, $P < 0.01$.) (Fig. 1f).

3.2. Foxp3 decreased cell growth and inhibited cell cycle associated proteins

Several epithelial ovarian cancer cell lines included SKOV3, OMC685, and ES-2 were taken to detect *Foxp3* expression. However, the results showed *Foxp3* expression only at mRNA level but devoid on protein level compared with activated T cells as positive control (Fig. 2a and b). To explore whether *Foxp3* gene suppressed the growth of epithelial ovarian cancer cells, we transfected the empty vector or the vectors carrying *Foxp3* cDNA into SKOV3 cells. The untranfected cells were removed by a selection with G418, and stable clones survived, named as SKOV3/pEF1α-vector and SKOV3/pEF1α-*Foxp3* cells. Western blot analysis confirmed that *Foxp3* protein expression was significantly increased in SKOV3/pEF1α-*Foxp3* cells (Fig. 2b). While the empty-vector-transfected cells grew into large clones, the *Foxp3*-transfected cells grew slowly (Fig 3a). CCK-8 assay demonstrated that pEF1α-*Foxp3* inhibited cell proliferation compared with SKOV3/pEF1α-vector cells (Δ cell viability rate >10%, $P < 0.05$) (Fig. 3b). Cell cycle analysis showed that *Foxp3* over-expression caused a significant accumulation of cells in the G0–G1 phase, with a concomitant decrease of cells in the G2–S phase compared with empty-vector-transfected cells (G0–G1 93.7 ± 2.5% *vs*. 78.4 ± 1.8%, $P < 0.05$) (Fig. 3c). To study cell proliferation in further detail, some proteins involved in cell cycle control were examined. In-cell western analysis detected that the levels of *Ki-67* and *CDKs* were inhibited by *Foxp3* up-regulation (Fig. 3d).

3.3. Foxp3 decreased cell migration and invasion

Wound healing assay was carried out to test cell migration. SKOV3/pEF1α-*Foxp3* cells had a much slower wound-healing rate compared with SKOV3/pEF1α-vector cells (0.25 ± 0.053:1, $P < 0.05$) (Fig. 4a). Matrigel-Transwell assay was used to determine effects of *Foxp3* on cell invasion. SKOV3/pEF1α-vector cells showed about 5.89 ± 0.97-fold penetration rate through the Matrigel-coated membrane compared with SKOV3/pEF1α-*Foxp3* cells (Fig. 4b), which indicated that *Foxp3* reduced the invasion ability of tumor cells. To further characterize above effects, several regulatory factors involved in cell migration and invasion were examined. The expressions of *MMP-2* and *uPA* at protein level were decreased in SKOV3/pEF1α-*Foxp3* cells (Fig. 4c).

3.4. Foxp3 inhibited mTOR and NF-κB signaling

To further explore the mechanism of *Foxp3*, several signaling pathways were tested. In-cell western analysis showed that *Foxp3* inhibited the activation of mTOR and NF-κB, but almost no effects on PI3K/AKT and ERK1/2 signaling (Fig. 5).

4. Discussion

Foxp3 was considered as a master control gene of the function of thymically derived naturally occurring regulatory T cells [3,4]. Due to the regulatory T lineage specification by *Foxp3*, its tissue expression was expected in thymus, spleen and lymph nodes which had been well documented. Recent data showed that quantification of *Foxp3* mRNA in tissues was represented an independent prognostic variable in terms of overall survival and progression-free survival [11]. However, those data were mostly focused on the expression of *Foxp3* in Tregs. The large number of Tregs infiltrated in cancerous tissues was associated with a dismal prognosis in ovarian cancer [12]. Quantification of *Foxp3* mRNA serve as a surrogate marker for the ovarian cancer tissue infiltration grade by Tregs, and immunohistochemical staining of *Foxp3* on a tissue microarray corroborated *Foxp3* mRNA data in terms of the identification of tumors with *Foxp3*-positive Tregs [11,12].

Albeit to a far lesser extent, *Foxp3* expression had also been observed in other normal and cancerous tissues [6,7]. The present study extended those findings by investigating *Foxp3* expression in ovarian cancerous cells. First, immunohistochemistry staining was used to detect *Foxp3*-positive Tregs in cancerous tissues. As a result, the

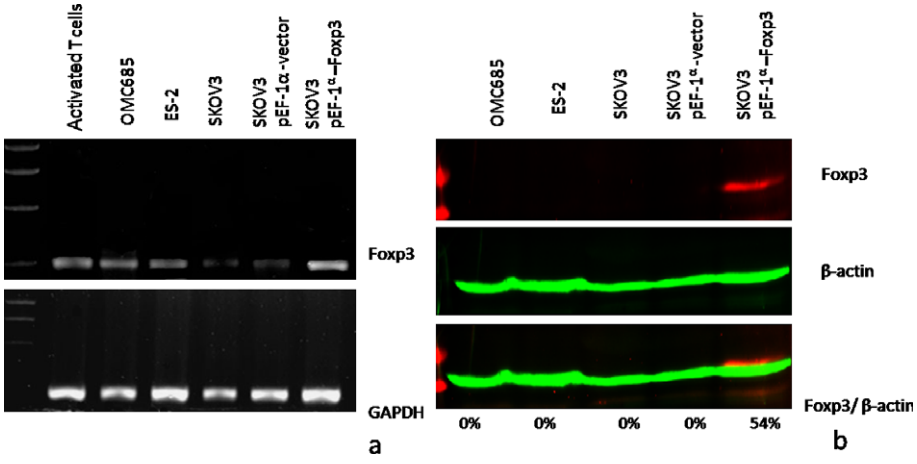

Fig. 2. (a) RT-PCR analysis detected *Foxp3* mRNA in different ovarian cancer cell lines. Activated T cells were used as positive control. *Foxp3* mRNA was observed in all cell lines, especially over-expressed in SKOV3/pEF1α-*Foxp3* cells. (b) Western blot analysis showed *Foxp3* protein in different ovarian cancer cell lines. Relative *Foxp3* expression determined by densitometry and normalized to β-actin was depicted in percent. *Foxp3* was only detected in SKOV3/pEF1α-*Foxp3* cells, not detectable in OMC685, ES-2, and SKOV3 cells.

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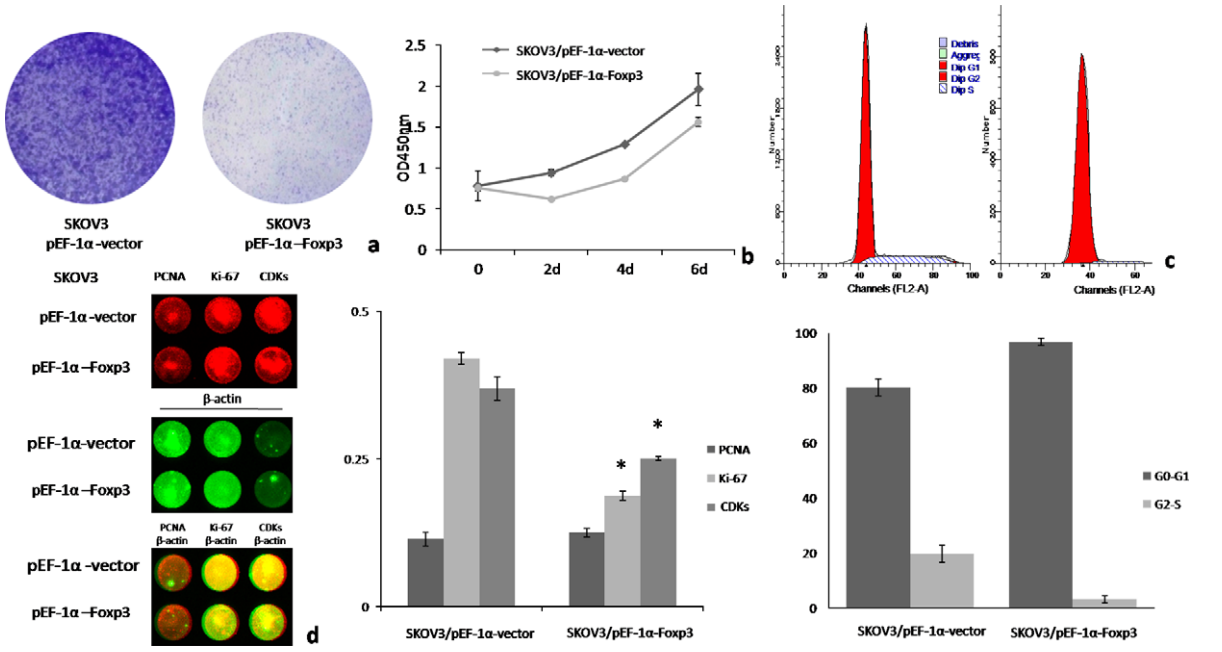

Fig. 3. (a) Less and smaller clones were formed in SKOV3/pEF1α-*Foxp3* cells by crystal violet staining compared with SKOV3/pEF1α-vector cells. (b) CCK-8 assay detected the growth condition of SKOV3/pEF1α-*Foxp3* and SKOV3/pEF1α-vector cells. Points, means of three separate experiments. Bars, SD (Δ cell viability rate >10%, $P < 0.05$). (c) *Foxp3* over-expression caused a significant accumulation of cells in the G0–G1 phase and a marked decrease in the G2–S phase compared with empty-vector-transfected cells (G0–G1 93.7 ± 2.5% *vs.* 78.4 ± 1.8%, $P < 0.05$) by flow cytometry. (d) In-cell western analysis showed decreased expression of *Ki-67* and *CDKs* in SKOV3/pEF1α-*Foxp3* cells. Relative protein expression normalized to β-actin was depicted. ∗, $P < 0.05$, relative to empty-vector-transfected cells.

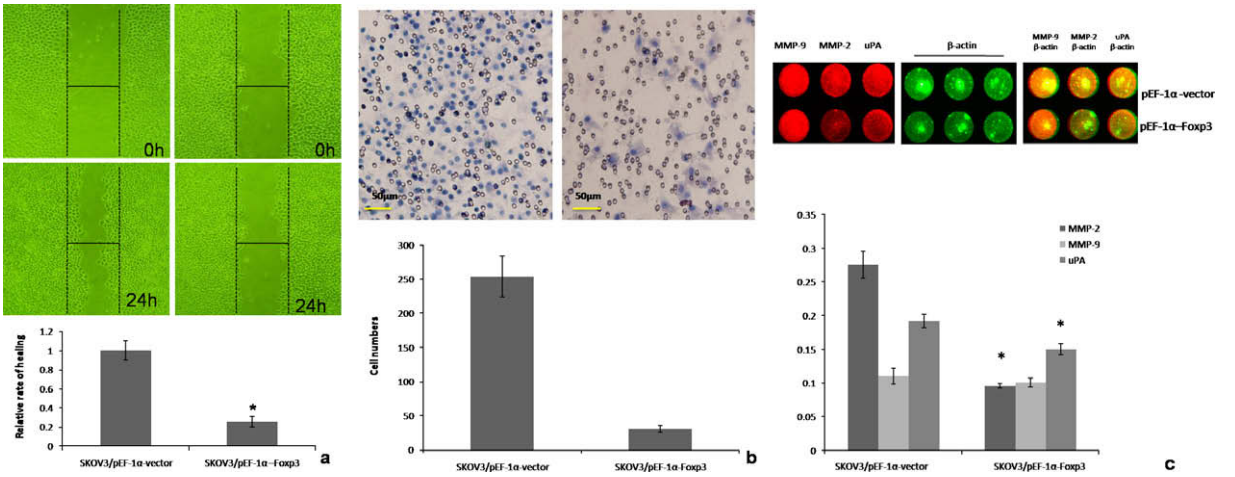

Fig. 4. (a) Would healing assay determined the migration rate of SKOV3/pEF1α-vector and SKOV3/pEF1α-*Foxp3* cells. Migration of the cells to the wound was visualized at 24 h with an inverted Olympus phase-contrast microscope (100× magnification). The healing rate of SKOV3/pEF1α-*Foxp3* cells was normalized to that of SKOV3/pEF1α-vector cells. (b) *Foxp3* expression inhibited cell invasion by Matrigel-Transwell assay. SKOV3/pEF1α-vector cells showed higher penetration rate through the Matrigel-coated membrane compared with SKOV3/pEF1α-*Foxp3* cells. (c) In-cell western analysis showed that *Foxp3* over-expression decreased the expression of *MMP-2* and *uPA* protein in SKOV3 cells. Relative protein expression normalized to β-actin was depicted. ∗, $P < 0.05$, relative to empty-vector-transfected cells.

existence of *Foxp3*-positive Tregs was observed only in 1/27 cancerous ovarian tissues by immunohistochemical staining, which might due to the small series of specimens. Of note, *Foxp3* expression was readily detectable in ovarian epithelium from healthy women, whereas no or weak expression was determined in tumor cells. Furthermore, real-time RT-PCR analysis confirmed the results obtained by immunohistochemistry. Analysis of transcript level showed that the expression of *Foxp3* gene in 26 epithelial ovarian cancer specimens (exclusion the existence of

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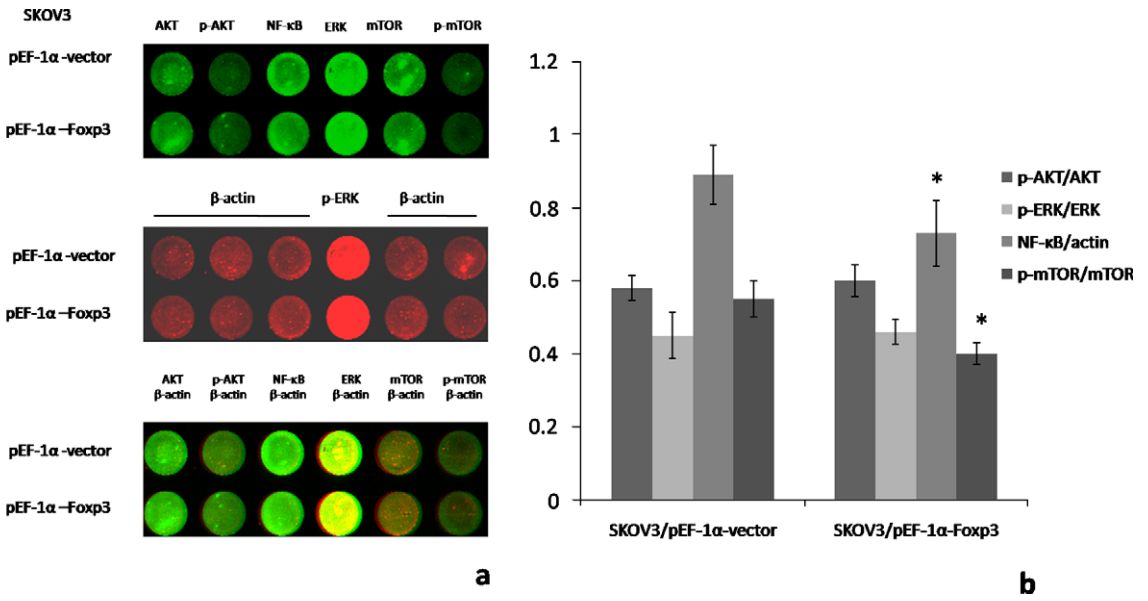

Fig. 5. In-cell western analysis detected effects of *Foxp3* over-expression on the activation of AKT, ERK, NF-κB or mTOR in SKOV3 cells. Cells were seeded in 96-well plate, low-serum cultured for 24 h, and stimulated with 10 ng/ml IGF-1 for so min. Activation of AKT, ERK, and mTOR were assessed using phospho-specific antibodies. Bar, SD.∗, $P < 0.05$, relative to empty-vector-transfected cells.

Foxp3-positive Tregs by immunohistochemical staining) was lower than in normal ovarian epithelium. Although it was a small series of specimens, a correlation between *Foxp3* expression and ovarian cancer was indicated. That *Foxp3* expression in normal ovarian epithelium but weak/no in epithelial ovarian cancerous cells and cell lines might represent an example of molecular mimicry and reveal another mechanism of etiology of ovarian cancer [13].

We selected stable cell clones which constitutively expressed *Foxp3* protein to detect the function and mechanism of *Foxp3* in epithelial ovarian cancer cell lines. Up-regulation of *Foxp3* elicited a dramatic effect on the proliferation of SKOV3 cells by CCK-8 assay and clone formation assay. To explore the mechanism of *Foxp3* in cell growth inhibition, we postulated whether it was due to cell cycle arrest. Actually, *Foxp3* up-regulation increased cell population in the G0–G1 phase and decreased cell progression into the DNA replication phase (G2–S phase). We also observed a reduction in the expressions of *CDKs* [14,15] and *Ki-67* [16,17] at protein level, which were strongly correlated with cell cycle distribution phenotype and growth suppression.

mTOR was involved in numerous regulatory functions of cell biology [18]. Inhibition of mTOR by everolimus promoted cisplatin-induced apoptosis [19], delayed tumor onset and progression in a transgenic mouse model of ovarian cancer [20], and enhanced effects of chemotherapy in advanced renal cell carcinoma by a double-blind, random, placebo-controlled phase III trial [21]. Interestingly, *Foxp3* over-expression inhibited the activation of mTOR in SKOV3 cells, which was similar to the effect of rapamycin as documented [22,23]. But almost no effects on the activation of PI3K/AKT and ERK1/2 signaling, suggesting that *Foxp3* might induce cell cycle arrest and decrease cell proliferation through down-regulating mTOR signaling.

Peritoneal metastasis was a big problem in ovarian cancer. When we observed that the up-regulation of *Foxp3* inhibited cell growth, we further examined the effects of *Foxp3* up-regulation on the migration and invasion of SKOV3 cells. We found that up-regulation of *Foxp3* inhibited the invasion rate of SKOV3 cells through the Matrigel, and reduced the migration rate in would healing assay. It was known that *MMPs* were critically involved in the processes of tumor cell invasion and metastasis [24,25], and *MMP-9* and *MMP-2* were directly associated with metastatic processes in ovarian cancer [26,27]. Here, we found that up-regulation of *Foxp3* inhibited *MMP-2* expression at protein level, suggesting that the potential anti-metastatic activities of *Foxp3* could be interpreted partly through the down-regulation of *MMP-2*. In addition, up-regulation of *Foxp3* inhibited the activity of NF-κB, which confirmed that *Foxp3* might inhibit cell migration and invasion partly through the inhibition of NF-κB and its target genes *MMPs* [28–30]. However, further in detail studies were needed to ascertain the precise molecular regulation of *Foxp3* and NF-κB and their crosstalk in elucidating the role of *Foxp3* in cell growth, invasion, and migration in animal models and human ovarian cancer.

Another important molecule involved in cell invasion and metastasis was *uPA* and its receptor. It was reported that cancer cells with high levels of *uPA* or its receptor would tend to invade surrounding tissues and subsequently migrate to blood vessels, thereby developing cancer cell metastasis [31,32]. In the present study, we found that up-regulation of *Foxp3* decreased the expression of *uPA*, which suggested *Foxp3* could potentiate the anti-metastasis activities partly through the down-regulation of *uPA*.

In summary, our study herein presented experimental evidences that supported the role of *Foxp3* up-regulation

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as anti-tumor and anti-metastatic mechanisms in ovarian cancer. From these data, we could expect that up-regulation of *Foxp3* might potentially be an effective therapeutic approach for the inhibition of cell growth, migration, and invasion of ovarian cancer.

Conflicts of interest

None declared.

Acknowledgment

We are grateful to Dr. Yang Liu (University of Michigan, USA) for providing us with *Foxp3* cDNA. Thanks Dr. Xu for generously providing OMC685 cells. Thanks Institute of Obstetrics and Gynecology of Fudan University for experiment equipments supply.

References

[1] R.F. Ozols, M.A. Bookman, D.C. Connolly, Focus on epithelial ovarian cancer, Cancer Cell 5 (2004) 19–24.
[2] M.E. Brunkow, E.W. Jeffery, K.A. Hjerrild, et al., Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse, Nat. Genet. 27 (2001) 68–73.
[3] S. Hori, S. Sakaguchi, Foxp3: a critical regulator of the development and function of regulatory T cells, Microbes Infect. 6 (2004) 745–751.
[4] J.D. Fonteno, M.A. Gavin, A.Y. Rudensky, Foxp3 programs the development and function of CD4+CD25+ regulatory T cells, Nat. Immunol. 4 (2003) 330–336.
[5] Y.W. Yisong, A.F. Richard, Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression, Nature 445 (15) (2007) 766–770.
[6] V. Karanikas, M. Speletas, M. Zamanakou, et al., Foxp3 expression in human cancer cells, J. Transl. Med. 6 (2008) 19–26.
[7] S. Hinz, L. Pagerols-Raluy, H.H. Oberg, et al., Foxp3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer, Cancer Res. 67 (17) (2007) 8344–8350.
[8] T. Zuo, R.H. Liu, H.M. Zhang, et al., FOXP3 is a novel transcriptional repressor for the breast cancer oncogene SKP2, J. Clin. Invest. 117 (12) (2007) 3765–3773.
[9] T. Zuo, L.Z. Wang, C. Morrison, et al., FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene, Cell 129 (2007) 1275–1286.
[10] E.M. Egorina, M.A. Sovershaev, B. Osterud, In-cell western assay: a new approach to visualize tissue factor in human monocytes, J. Throm. Haemost. 4 (3) (2006) 614–620.
[11] W. Dominik, M.W. Anna, R. Holger, et al., The expression of the regulatory T cell specific Forkhead Box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer, Clin. Cancer Res. 11 (23) (2005) 8231–8236.
[12] T.J. Curiel, G. Coukos, L. Zou, et al., Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival, Nat. Med. 10 (2003) 942–949.
[13] M. Katoh, M. Katoh, Human FOX gene family, Int. J. Oncol. 25 (5) (2004) 1495–1500.
[14] H.Y. Jung, S.H. Park, Y.D. Yoo, et al., CDK2/4 regulates retinoic acid-induced G1 arrest in hepatocellular carcinoma cells, Hepatol. Res. 31 (3) (2005) 143–152.
[15] B. Davidson, B. Risberg, A. Berner, et al., Expression of cell cycle proteins in ovarian carcinoma cells in serous effusions-biological and prognostic implications, Gynecol. Oncol. 83 (2) (2001) 249–256.
[16] W. Zhong, J. Peng, H. He, et al., Ki-67 and PCNA expression in prostate cancer and benign prostatic hyperplasia, Clin. Invest. Med. 31 (1) (2008) E8–E15.
[17] M. Reitmaier, C. Rudlowski, S. Biesterfeld, et al., Comparative studies on the biological significance of the marker for proliferation Ki-67-Antigen and PCNA in primary ovarian carcinoma, ZBL Gynakol. 122 (7) (2000) 361–367.
[18] D.C. Fingar, S. Salama, C. Tsou, et al., Mammalian cell size is controlled by mTOR and its downstream targets S6K1 with 4EBP1/eIF4E, Genes Dev. 16 (2002) 1472–1487.
[19] S. Mabuchi, D.A. Altomare, M. Cheung, et al., RAD001 inhibits human ovarian cancer cell proliferation, enhances cisplatin-induced apoptosis, and prolongs survival in an ovarian cancer model, Clin. Cancer Res. 13 (14) (2007) 4261–4270.
[20] S. Mabuchi, D.A. Altomare, D.C. Connolly, et al., RAD001 (Everolimus) delays tumor onset and progression in a transgenic mouse model of ovarian cancer, Cancer Res. 67 (6) (2007) 2408–2413.
[21] R.J. Motzer, B. Escudier, S. Oudard, et al., Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial, Lancet 372 (9637) (2008) 449–456.
[22] I. Hashimoto, K. Koizumi, M. Tatematsu, et al., Blocking on the CXCR4/mTOR signalling pathway induces the anti-metastatic properties and autophagic cell death in peritoneal disseminated gastric cancer cells, Eur. J. Cancer 4 (7) (2008) 1022–1029.
[23] C. Cao, T. Subhawong, J.M. Albert, et al., Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes PTEN null prostate cancer cells, Cancer Res. 66 (20) (2006) 10040–10047.
[24] S. Sillanpää, M. Anttila, K. Voutilainen, et al., Prognostic significance of matrix metalloproteinase-9 (MMP-9) in epithelial ovarian cancer, Gynecol. Oncol. 104 (2) (2007) 296–303.
[25] S. Sillanpää, M. Anttila, K. Suhonen, et al., Prognostic significance of extracellular matrix metalloproteinase inducer and matrix metalloproteinase 2 in epithelial ovarian cancer, Tumour Biol. 28 (5) (2007) 280–289.
[26] S. Curran, G.I. Murray, Matrix metalloproteinases: molecular aspects of their roles in tumor invasion and metastasis, Eur. J. Cancer 36 (2000) 1621–1630.
[27] D. Belotti, P. Paganoni, L. Manenti, et al., Matrix metalloproteinases (MMP9 and MMP2) induce the release of vascular endothelial growth factor (VEGF) by ovarian carcinoma cells: implications for ascites formation, Cancer Res. 63 (17) (2003) 5224–5229.
[28] K. Ogawa, F. Chen, C. Kuang, et al., Suppression of matrix metalloproteinase-9 transcription by transforming growth factor-beta is mediated by a nuclear factor-kappa B site, Biochem. J. 381 (Pt 2) (2004) 413–422.
[29] M.A. Altinoz, R. Korkmaz, NF-kappa B, macrophage migration inhibitory factor and cyclooxygenase-inhibitions as likely mechanisms behind the acetaminophen- and NSAID-prevention of the ovarian cancer, Neoplasma 51 (4) (2004) 239–247.
[30] S. Fujioka, G.M. Sclabas, C. Schmidt, et al., Inhibition of constitutive NF-κB activity by IκBαM suppresses tumorigenesis, Oncogene 22 (2003) 1365–1370.
[31] H. Kobayashi, M. Suzuki, N. Kanayama, et al., A soybean Kunitz trypsin inhibitor suppresses ovarian cancer cell invasion by blocking urokinase upregulation, Clin. Exp. Metast. 21 (2) (2004) 159–166.
[32] M.J. Duffy, C. Duggan, The urokinase plasminogen activator system: a rich source of tumor markers for the individualized management of patients with cancer, Clin. Biochem. 37 (2004) 541–548.

Please cite this article in press as: H.-Y. Zhang, H. Sun, Up-regulation of Foxp3 inhibits cell proliferation, migration and invasion in epithelial ovarian cancer, Cancer Lett. (2009), doi:10.1016/j.canlet.2009.06.001