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Platelets promote invasion and induce epithelial to mesenchymal transition in ovarian cancer cells by TGF-β signaling pathway

Yi Guo, Wei Cui *, Yuqing Pei, Danfei Xu

State Key Laboratory of Molecular Oncology, Department of Clinical Laboratory, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100021, China

HIGHLIGHTS

- Ovarian cancer patients with elevated platelets showed higher incidence of metastasis.
- Platelets increase invasive ability and induce EMT in ovarian cancer cells.
- Serum TGF-β level was higher in ovarian cancer patients with elevated platelet.
- TGF-β/Smad pathway was activated in platelet-treated ovarian cancer cells.
- TGF-β type I receptor inhibitor A83-01 abolished platelets-induced invasion and EMT *in vitro* and *in vivo*.

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ABSTRACT

Objective. To test whether platelets could increase invasion potential and initiate EMT in ovarian cancer cells *via* a TGF-β signaling pathway.

Methods. Blood samples were collected in 69 patients with ovarian cancer, 16 patients with benign ovarian tumor and 64 healthy donors. SK-OV-3 and OVCAR-3 ovarian cancer cells were treated with platelets. Transwell assays were used to analyze the invasive capacity, and EMT was assessed by microarray analysis, quantitative real-time PCR (qPCR) and Western blotting. Activation of TGF-β pathway was examined by ELISA and Western blotting. TGF-β type I receptor (TβR I) inhibitor A83-01 was used to confirm the role of TGF-β pathway *in vitro* and *in vivo*.

Results. Clinical data showed ovarian cancer patients with elevated platelet counts had a higher incidence of advanced stages. Treatment with platelets increased the invasive properties of both cell lines. Mesenchymal markers (snail family transcriptional repressor-1, vimentin, neural cadherin, fibronectin-1 and matrix metalloproteinase-2) were up-regulated in platelet-treated cells, while the epithelial marker (epithelial cadherin) was down-regulated. Higher TGF-β level was observed in patients with elevated platelet counts when compared to the subjects. Higher levels of TGF-β were also found in culture medium treated with platelets, and cells treated with platelets also showed increased phosphorylation of Smad2. TβR I inhibitor A83–01 reversed the EMT-like alterations and inhibited platelet-induced invasion *in vitro* and *in vivo*.

Conclusion. Platelet increased invasion potential and induced EMT in ovarian cancer cells in a TGF-β dependent pathway. Platelet-derived TGF-β may be useful as a new target treatment for ovarian cancer.

1. Introduction

Ovarian cancer is the seventh most frequently occurring cancer among women in the world [1]. Regardless of its relatively low incidence, ovarian cancer is the leading cause of death among all gynecological malignancies [2,3]. The annual mortality rate of ovarian cancer continues to rise, especially in developing countries [4,5]. Its poor prognosis is partly attributed to its metastasis, which often presents as intraperitoneal dissemination in ovarian cancer [6,7].

Platelets may be involved in the progression of metastasis in ovarian cancer. Clinical data show that ovarian cancer patients with thrombocytosis are more likely to progress to advanced stages, with shorter survival time and higher incidence of metastasis [8,9]. In resected tumor specimens, platelets are always aggregated in the

* Corresponding author at: State Key Laboratory of Molecular Oncology, Department of Clinical Laboratory, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, No.17 Panjiayuan Nanli, Chaoyang District, Beijing 100021, China.
E-mail addresses: wendycuiwei@sina.cn, 962077959@qq.com (W. Cui).

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0090-8258/

Table 1
Sequences of primer pairs for quantitative real-time PCR.

Gene	Sequence (5′-3′)	Reference
SNAI1	F: CCCCAATCGGAAGCCTAACT R: GCTGGA AGGTAAACTCTGGATTAGA	[21]
VIM	F: TGAAGGAGGAAATGGCTCGTC R: GTTTGGAAGAGGCAGAGAAATCC	[22]
E-cadherin	F: TGGAGGAATTCTTGCTTTGC R: CGCTCTCCTCCGAAGAAAC	[23]
N-cadherin	F: ACCAGGTTTGGAATGGGACAG R: ATGTTGGGTGAAGGGGTGCTTG	[22]
FN1	F: TGAAAGACCAGCAGAGGCATAAG R: CTCATCTCCAACGGCATAATGG	[22]
MMP2	F: CCAACTACAACTTCTTCCCTCGC R: GCAAAGGCATCATCCACTGTCTC	[22]
GAPDH	F: TGACAACTTTGGTATCGTGGAAGG R: AGGGATGATGTTCTGGAGAGCC	[22]

Table 2
Demographics and clinical characteristics of ovarian cancer patients with normal platelets and increased platelets.

Characteristics		PLT ≤ 350 (n = 36)	PLT > 350 (n = 33)	*p*-value
Age (years)		51.58 ± 11.36	53.64 ± 7.85	0.390
Preoperative	PLT ($\times 10^9$/L)	255.14 ± 56.83	469.42 ± 84.04	< 0.001
	WBC ($\times 10^{12}$/L)	6.07 ± 1.63	6.42 ± 1.54	0.360
	Hb (g/L)	125.11 ± 14.78	122.00 ± 14.35	0.379
	TGF-β (pg/mL)	12,146.91 ± 6147.90	17,590.13 ± 4606.21	<0.001
Tumor size (cm)		4.4 ± 2.6	5.2 ± 2.6	0.207
Histology	Serous	28 (77.8%)	29 (87.9%)	0.565
	Mucinous	1 (2.8%)	0 (0%)	
	Endometrioid	3 (8.3%)	1 (3.0%)	
	Clear cell	4 (11.1%)	3 (9.1%)	
Grade (serous)	Low	2 (7.1%)	1 (3.4%)	0.975
	High	26 (92.9%)	28 (96.6%)	
Clinical stage	I + II	11 (30.6%)	3 (9.1%)	0.027
	III + IV	25 (69.4%)	30 (90.9%)	
Involvement	Lymph nodes	12 (33.3%)	10 (30.3%)	0.787
	Omentum	18 (50.0%)	27 (81.8%)	0.006
	Mesentery	11 (30.6%)	19 (57.6%)	0.024
	Other sites	20 (55.6%)	29 (87.9%)	0.003

Other sites: Peritoneum, Diaphragm and Liver.

invasive front, which suggests their pro-metastatic potential [10]. It was also demonstrated that platelets may promote invasive ability of ovarian cancer cells [11]. However, the role of platelets in increasing invasive ability of ovarian cancer remains unclear.

Epithelial to mesenchymal transition (EMT) is mainly responsible for the changes of invasive ability in malignant cancer cells [12,13]. During EMT, polarized epithelial cells transform into motile mesenchymal cells which results in the loss of the apical-basal polarity, disruption of cell-cell junction, and acquisition of an invasive behavior[14]. The EMT process can be initiated by many extracellular factors, such as transforming growth factor-beta (TGF-β) [15,16]. Studies have shown that TGF-β could trigger EMT in carcinoma cells and increase their metastatic seeding in distant organs *via* Smad-dependent pathway [17–19]. As platelets harbor high levels of TGF-β [20], we proposed that platelet might induced EMT in human ovarian cancer cells by activating TGF-β signaling pathway to increase their invasion.

In the current study, we investigated whether platelets promoted invasion in ovarian cancer cells by inducing EMT, and whether TGF-β signaling pathway was activated. TGF-β type I receptor (TβR I) inhibitor was used to test whether platelet-induced invasion and EMT could be reversed by blocking the pathway.

2. Methods

2.1. Ethics statement

Oral and written informed consent was provided by all enrolled patients and healthy individuals. This study was approved by the Institution Ethics Review Board for Human Studies at the National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College.

2.2. Patients and clinical data

Eighty-five patients with ovarian tumor admitted to the National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences between August 2017 and April 2018 and 64 age-matched healthy donors were enrolled for this study. The patient cohort comprised of 16 patients with benign tumor and 69 patients with malignant tumor. None of patients suffered from anemia, infections or other inflammatory diseases. All patients were histologically diagnosed by laparoscopic or surgical specimen examination. Information regarding clinical stage, tumor size and the affected organs in the peritoneal cavity were collected from electronic medical files.

Blood samples were obtained before patients received any treatments. Three milliliters of peripheral blood was drawn from each patient into collection tubes coated with EDTA for measuring platelets counts, white blood cell counts (WBC), and hemoglobin (Hb) with an automatic blood cell analyzer (SYSMEX XN Series). Five milliliters blood was collected into serum-separating tubes for detecting TGF-β. Serum TGF-β was measured using human TGF-beta ELISA kit according

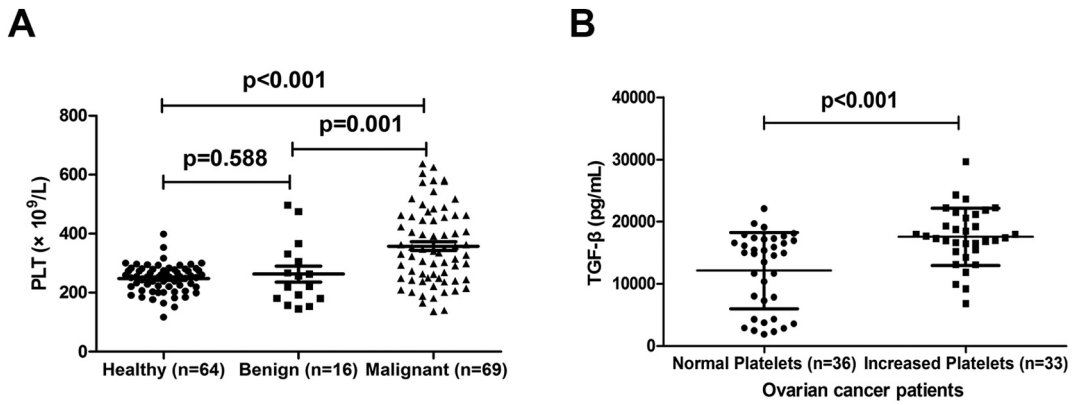


Fig. 1. Comparison of patients in the study according to pathology or platelet counts. (A) Comparison of preoperative platelet count between healthy donors, benign ovarian tumor and ovarian cancer. (B) Comparison of preoperative TGF-β levels between ovarian cancer patients with normal platelet counts and increased platelet counts. Normal platelet counts: PLT ≤ 350 × 10^9/L, increased platelet counts: PLT > 350 × 10^9/L.

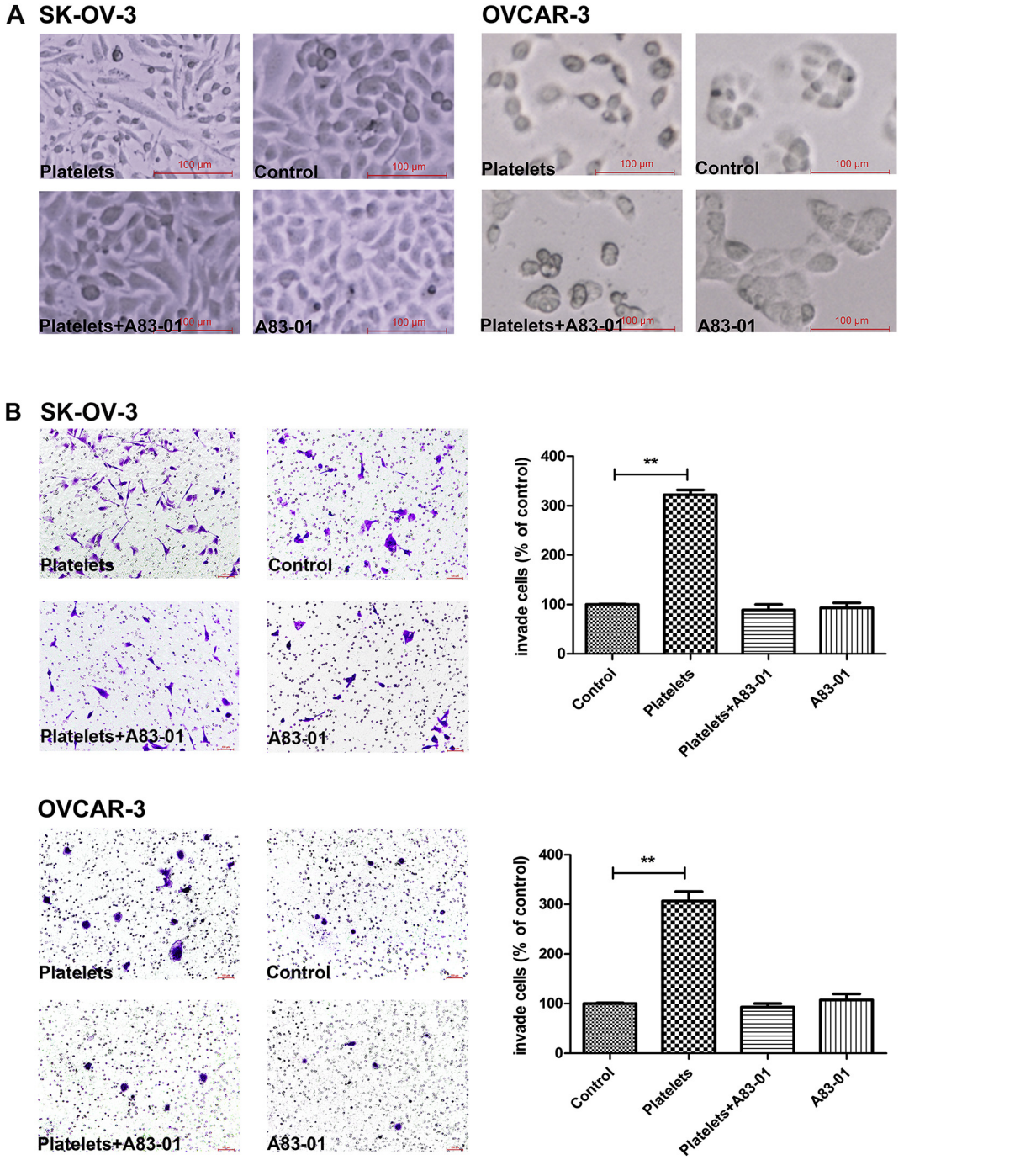


Fig. 2. Platelets promote invasion in ovarian cancer cells, and the effect could be abolished by TβR I inhibitor A83-01. (A) Photomicrographs of SK-OV-3 and OVCAR-3 cells treated with platelets, A83-01 or their combination. (B) Images of Tranwell assays for SK-OV-3 and OVCAR-3 treated with platelets, A83-01 or their combination. Quantitative results are expressed as the percentage of cells passing through invasion chambers relative to control ones (n = 3). **: $p < 0.01$. TβR I: TGF-β type I receptor, EMT: epithelial to mesenchymal transition.

to the manufacturer's instructions (ProteinTech Group Co. Ltd., Chicago, Illinois, USA).

2.3. Cell lines

Human ovarian cancer cell lines SK-OV-3 and OVCAR-3 were obtained from the National Infrastructure of Cell Line Resource (NSTI, Beijing, China). SKOV-3 cells were grown in RPMI Medium 1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), and OVCAR-3 cells were maintained in RPMI Medium 1640 with 20% FBS. SK-OV-3 cells expressing luciferase (SK-OV-3-Luc) were purchased from GENECHEM (Shanghai, China), and were grown in RPMI Medium 1640 with 10% FBS.

2.4. Isolation of platelets

EDTA-anticoagulated whole blood from healthy donors was centrifuged at 100*g* for 10 min, and the top 2/3 of the supernatant plasma was aspirated (platelet-rich plasma, PRP). The PRP was then centrifuged at 1000*g* for 10 min, and pelleted platelets were re-suspended in RPMI Medium 1640.

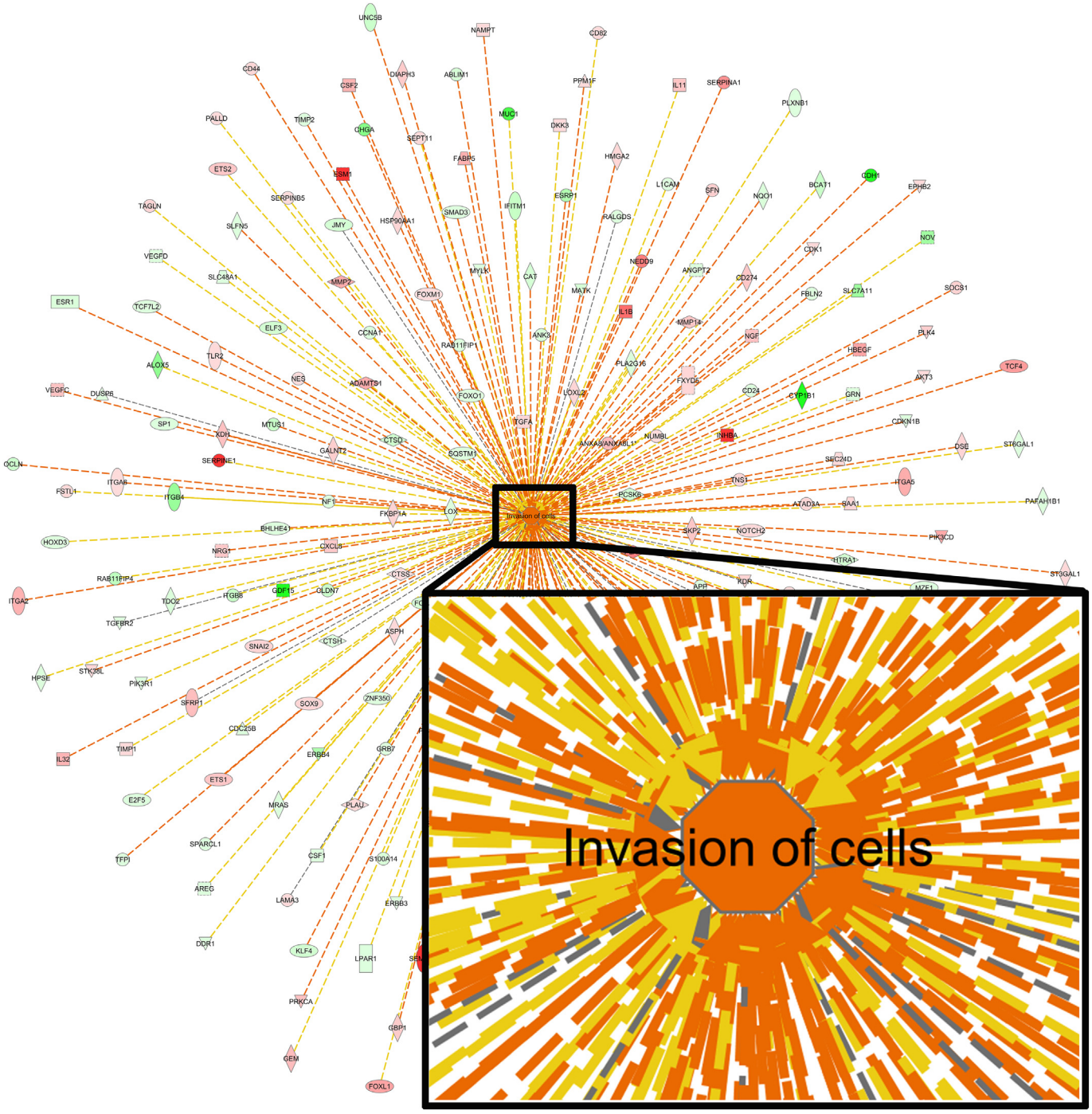


Fig. 3. Disease and Function Network in the platelet-treated SK-OV-3 ovarian cancer cells. The network shows activation of invasive phenotype in the SK-OV-3 cells treated with platelets according to the significantly differentially expressed genes related to invasion. The orange color indicates the activating expression status, the green color indicates the inhibitory expression status and the grey color indicates that no information related to the expression status is found in the database.

2.5. Treatment of ovarian cancer cells

SK-OV-3 and OVCAR-3 cells were seeded in 6-well culture plates at the densities of 2.4×10^5/well and 2×10^6/well, respectively. Cells were treated with either the platelets (prepared from 1 mL PRP), or with 25 μM of TβR I inhibitor A83-01 (MCE, Monmouth Junction, NJ, USA) or both for 24 h. Untreated cells were used as controls. After the first 24 h culture, platelets (prepared from 1 mL PRP) and/or A83-01 (25 μM) were added to the respective wells, and then cells were incubated for another 24 h. Following incubation, cell morphology was observed and photographed.

2.6. Transwell assays

Transwell inserts with polycarbonate membranes of 8.0 mm pore size (Costar, Cambridge, MA, USA) were placed in 24 well cell culture plates (Costar). The upper chamber was coated with 80 μL Matrigel (Corning, Wiesbaden, Germany) and medium mixture (1:8). SKOV-3 and OVCAR-3 cells were incubated with platelets and (or) A83-01 for 48 h, following which 1×10^5 cells were seeded into the upper chamber with serum-free medium. The lower chambers were flooded with 600 μL complete medium (10% FBS for SK-OV-3 and 20% FBS for OVCAR-3). After incubation for 20 h at 37 °C, the cells on the upper surface of the filters were wiped

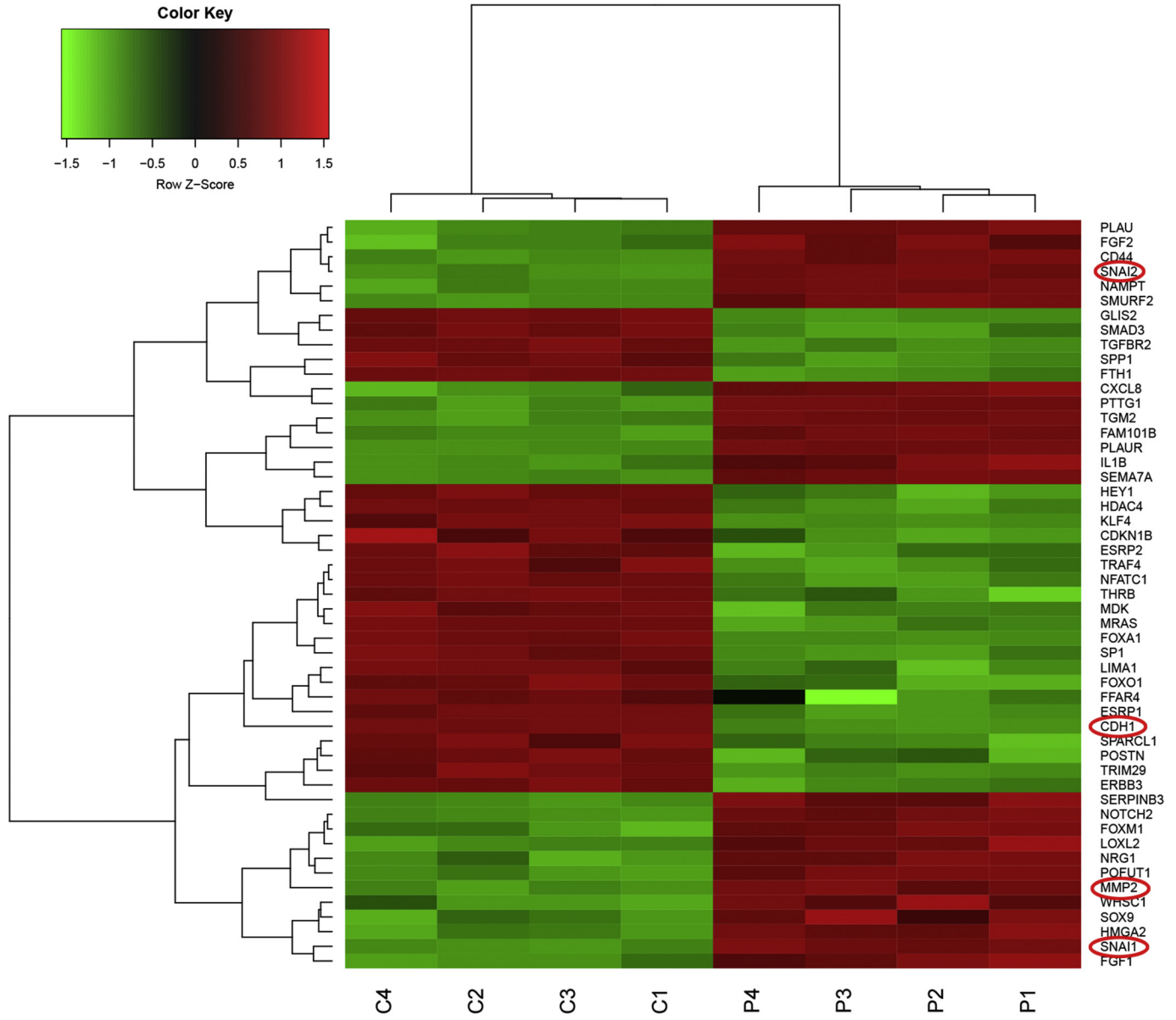


Fig. 4. Heatmap depicting EMT-related genes that were significantly differentially expressed between SK-OV-3 cells treated with platelets (P1–P4) and control ones (C1–C4). The heatmap shows that epidermal-related genes (*e.g. CDH1*) are downregulated, while mesenchymal-related ones are upregulated (*e.g. SNAI1*, *SNAI2* and *MMP2*) in the platelet-treated SK-OV-3 cells. Rows and columns represent genes and samples, respectively. Up-regulated gene expression is indicated by red color, and down-regulated one is shown as green color. EMT: epithelial to mesenchymal transition.

out using cotton swabs, and the invading cells on the lower surface were fixed with methanol for 20 min and stained with 0.1% crystal violet for 5 min. Cell invasion was determined by counting the number of cells, and three visual fields were randomly selected for each membrane. The average number of cells was calculated as the number of invading cells. The data was expressed as percentage invasion relative to the control.

2.7. RNA extraction, reverse transcription and quantitative real-time PCR (qPCR)

Total RNA was extracted using TRIzol Reagent (Invitrogen and Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. cDNA was synthesized from 1 μg RNA by a reverse transcriptase kit (Takara Bio Inc., Otsu, Shiga, Japan). The mRNA expression of EMT related genes [snail family transcriptional repressor 1 (*SNAI1*), vimentin (*VIM*), epithelial cadherin (*E-cadherin*), neural cadherin (*N-cadherin*), fibronectin-1 (*FN1*) and matrix metalloproteinase-2 (*MMP2*)] was analyzed by qPCR with LightCycler480 (Roche Diagnostics Ltd., Shanghai, China), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping control. The real-time PCR mixture consisted of SYBR green premix (Takara Bio Inc.), specific forward and reverse primers (Sangon Biotech, Shanghai, China) and ddH2O. The primer pair sequences were obtained from other studies [21–23] and are listed in Table 1. The PCR reaction parameters were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 20 s and 60 °C for 30 s. Relative fold-change expression of mRNAs was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.8. Microarray analysis

Total RNA was isolated from control or platelet-treated SK-OV-3 cells (4 replicates each, n = 8). cDNA samples were then synthesized and hybridized onto GeneChip PrimeView™ Human Gene Expression Array (Affymetrix). Functional pathway analysis was performed using the commercially available software Ingenuity Pathways Analysis (IPA) with the assistance of GENECHEM. We used |fold change| ≥ 2.0

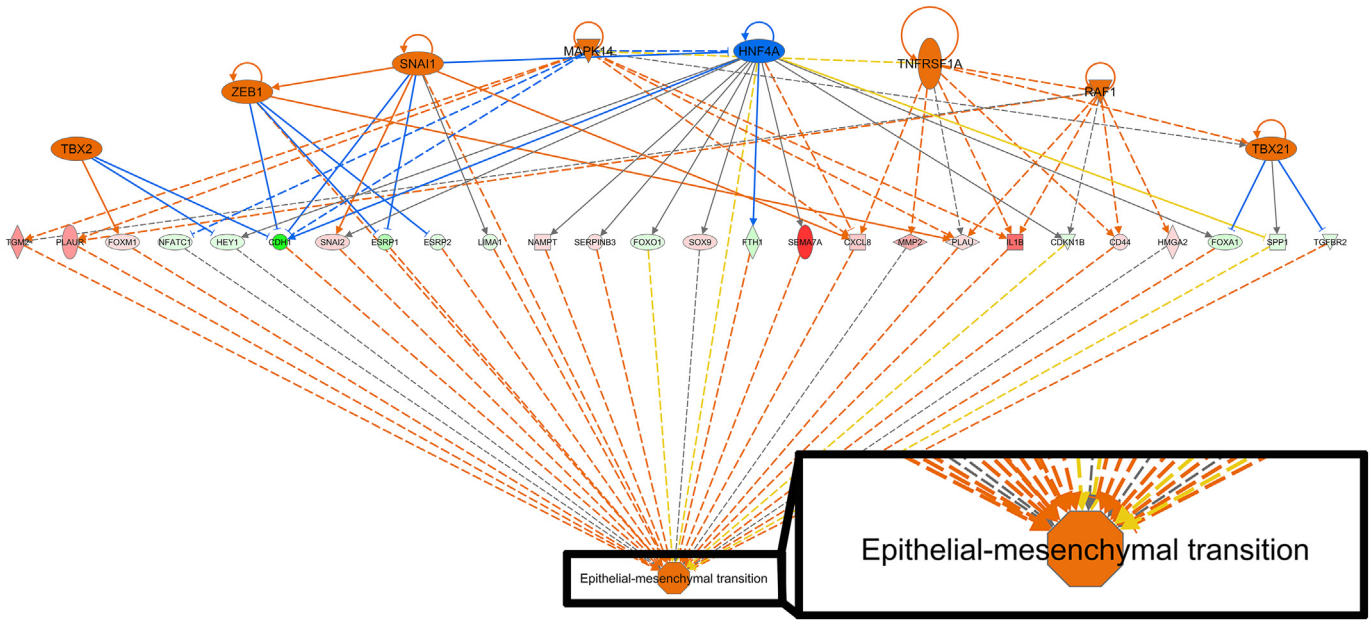


Fig. 5. Regulator Effects Network in the platelet-treated SK-OV-3 ovarian cells. The network shows the activation of EMT regulated by significantly differentially expressed factors. The orange color indicates the activating expression status, the blue color indicates the inhibitory expression status, and the grey color indicates that no information related to the expression status is found in the database. EMT: epithelial to mesenchymal transition.

and a false discovery rate (FDR) <0.05 as a threshold for screening significantly differentially expressed genes. Moreover, we mixed platelets isolated from PRP with SK-OV-3 cells, and immediately extracted RNA from the mixture. Compared to the untreated SK-OV-3 cells, we observed that significantly differentially expressed genes, as revealed by IPA, were mainly derived from platelets. Next, these genes were excluded in the analysis of differentially expressed genes between platelet-treated SK-OV-3 cells and untreated controls to eliminate the mRNAs derived from platelets.

2.9. Western blotting

Cultured cells were washed with cold PBS and lysed with RIPA lysing buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) supplemented with protease and phosphatase inhibitors (Solarbio). Protein concentration of the lysate was determined using the BCA Protein Assay Kit (Applygen Technologies Inc., Beijing, China). Equal amounts (20 μg) of protein samples were separated by sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis on a 5% stacking gel for 15 min at 100 V and 10% separating gel for 90 min at 130 V. Protein bands were transferred to polyvinylidene difluoride (PVDF) membranes, and blocked with 5% skim milk/Tris-buffered saline with 0.1% (*v*/v) Tween 20 (TBST) for 1 h at room temperature (RT). Blots were then incubated at RT with primary antibodies to E-cadherin (1:1000), N-cadherin (1:1000), MMP-2 (1:1000) (Proteintech Group) and GAPDH (1:1000) (ZHGB-BIO, Beijing, China) for 2 h. Incubation with the primary antibodies against pSmad2 and Smad2 (1:1000) (Proteintech Group) was performed at 4 °C with gentle shaking overnight. Blots were washed three times in TBST and incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibodies (1:2500) (Proteintech Group) for 1 h at RT. Blots were washed four times and signal was detected using a chemiluminescence kit (Thermo Fisher Co. Ltd., Shanghai, China).

2.10. Detection of TGF-β in conditioned media by ELISA

Culture media from control and platelet-treated ovarian cancer cells were collected after 48 h incubation. TGF-β levels were measured using Human TGF-beta1 ELISA kit according to the manufacturer's instructions (ProteinTech Group).

2.11. Animal experiments

4 to 6-week-old female Balb/c nude mice (BEIJING HUAFUKANG BIOSCIENCE CO.INC, Beijing, China) were housed and maintained under specific pathogen-free conditions. The experimental protocols were approved by the Animal Care and Use Committee of the Cancer Hospital of the Chinese Academy of Medical Sciences.

In the preliminary assay, platelets were isolated from 2 mL PRP and re-suspended in 500 μL 1640 culture media. Mice were divided into 4 groups based on the interventions: 1) platelets, 2) vehicle (1640 culture media), 3) platelets + A83-01 (150 μg/body) and 4) A83-01 (150 μg/body). Each group was administered intraperitoneally with 500 μL volume of reagents (n = 10/group).The following day, mice were injected intraperitoneally with 2×10^6 SKOV3-Luc cells. Mice in the four groups were subsequently injected with respective reagents (platelets, vehicle, platelets+A83-01, or A83-01 alone)) every three days. Tumor-producing luciferase signals were monitored seven days after cell inoculation. Four weeks later, mice were euthanized and exploratory laparotomy was performed. Organs in the abdominal cavity were observed and photographed for intraperitoneal dissemination. Subsequently, tumors were harvested and embedded in paraffin for hematoxylin and eosin (H&E) staining and immunohistochemistry. Three tumor specimens were harvested from three animals in each group.

2.12. Immunohistochemical staining

Immunohistochemical staining of E-cadherin, N-cadherin was carried out using SP-POD kit based on the streptavidin-biotin-peroxidase method (Solarbio). Briefly, formalin-fixed paraffin-embedded tissue sections were deparaffinized by xylene and rehydrated by ethanol. Antigen retrieval was performed by boiling the samples in 0.01 M sodium citrate buffer (pH 6.0). Endogen peroxidase was quenched with 3% hydrogen peroxidase for 10 min at RT. The slides were incubated with primary anti-E-cadherin and anti-N-cadherin antibodies (1:50) (Proteintech Group) overnight at 4 °C followed by incubation with biotinylated goat anti-rabbit secondary antibodies (Solarbio) at RT for 30 min. After incubation with Peroxidase (Solarbio) at RT for 30 min, the antibody staining was visualised with Diaminobenzidine (DAB)

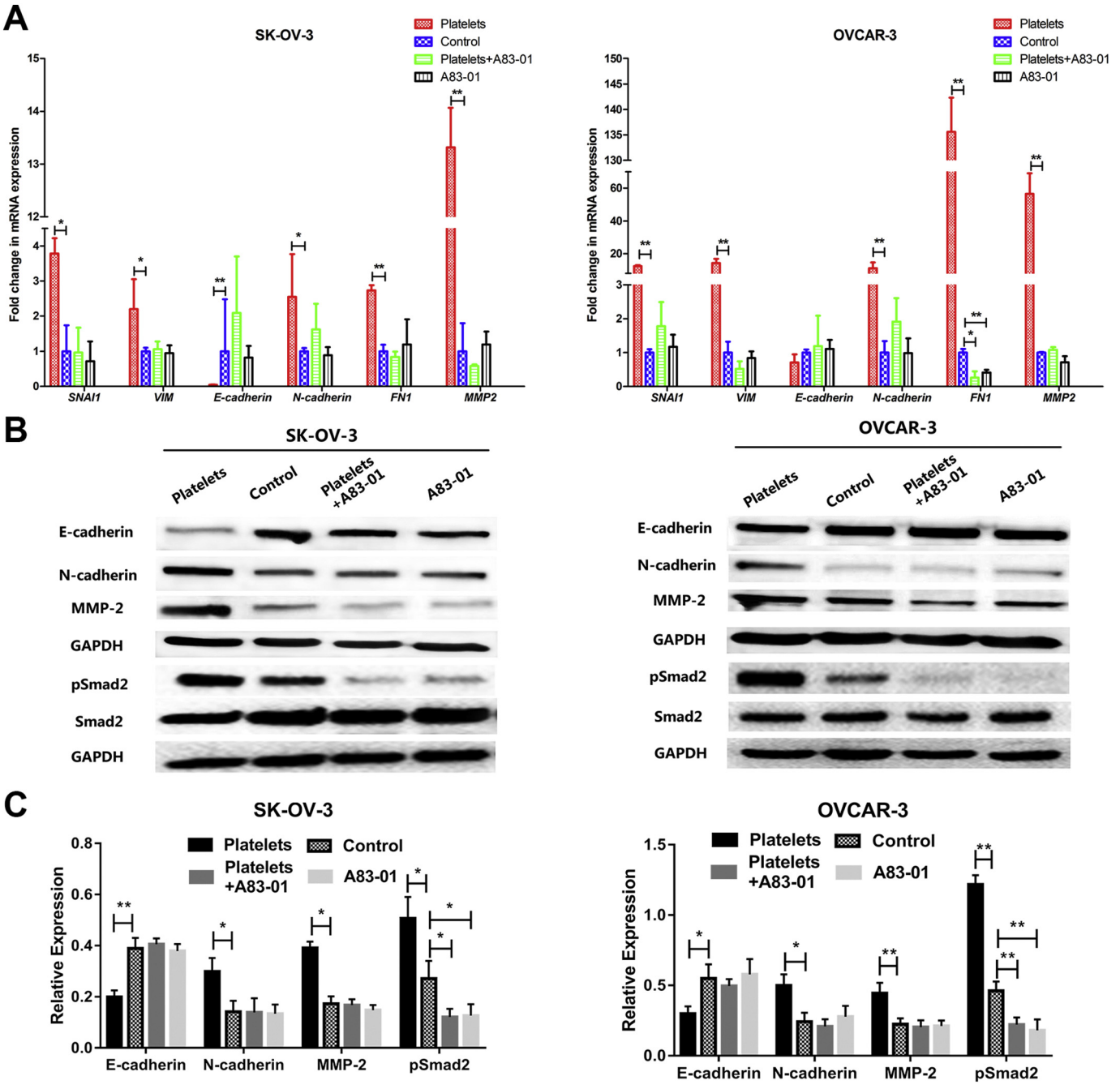


Fig. 6. Platelets induce EMT in ovarian cancer cells, and the effect could be abolished by TβR I inhibitor A83-01. (A) Analysis of EMT-related genes in SK-OV-3 and OVCAR-3 cells treated with platelets, A83-01 or their combination at mRNA level. Results are expressed as fold changes relative to control ones (n = 3). Values are normalized to GAPDH. Data are shown as mean ± SD. (B, C) Analysis of E-cadherin, N-cadherin, MMP-2 and pSmad2 in SK-OV-3 and OVCAR-3 cells treated with platelets, A83–01 or their combination at protein levels (n = 3). GAPDH is used as loading control. *: $p < 0.05$, **: $p < 0.01$. TβR I: TGF-β type I receptor, EMT: epithelial to mesenchymal transition.

(Solarbio) at RT for 15 min. Finally, the section slides were counter-stained with Mayer's hematoxylin, dehydrated and mounted. Quantification is presented as the intensity of image using Image J software. The mean intensity value of three tumor specimens represented the protein levels in an animal (n = 3/group).

2.13. Statistical analysis

Statistical analysis was performed using SPSS (Statistical Package for the Social Sciences, Chicago, IL, USA) 13.0 software. Student's-test was used to compare age, platelet counts, TGF-β levels, WBC, Hb and tumor sizes in the patient groups, and results were presented as mean ± SD. Association between platelet counts and other clinico-pathological variables was determined by Chi-Square Tests. Student's *t*-test and One-Way ANOVA were used to compare means of two or more groups in the *in vitro* and *in vivo* studies. Data were shown as mean ± SD. All tests were two-tailed, and *p* value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Platelets increase invasiveness and induce EMT in ovarian cancer cells

In the study, we observed a significantly higher number of platelets in patients with ovarian cancer malignant tumors ($357.62 \pm 128.89 \times 10^9$) when compared to the patients with benign tumors (262.75 ± 108.22

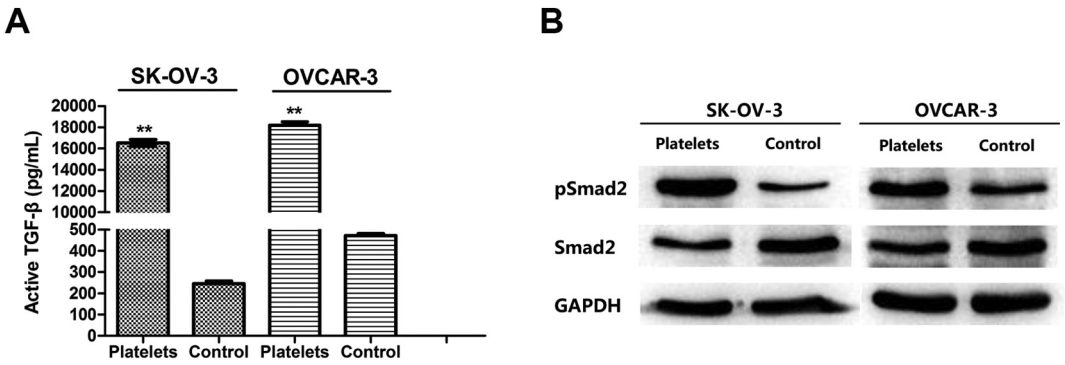


Fig. 7. Platelets induce EMT in ovarian cancer cells by activating TGF-β/Smad pathway. (A) Concentration of active TGF-β in conditioned medium from SK-OV-3 and OVCAR-3 cells treated with or without platelet by ELISA (n = 3). Data are shown as mean ± SD. (B) Detection of the levels of pSmad2 in platelet-treated cells or control ones by western blot. GAPDH is used as loading control. **: $p < 0.01$. EMT: epithelial to mesenchymal transition. ELISA: enzyme-linked immunosorbent assay.

$\times 10^9$) and healthy individuals ($247.64 \pm 47.30 \times 10^9$) (Fig. 1A). With the platelet count threshold set at 350×10^9/L, 33 patients in the ovarian cancer group had increased platelet counts at the initial diagnosis (Table 2). With respect to the clinical stage, more patients showed advanced stages (FIGO III and IV) in the increased platelet counts group ($p < 0.01$, Table 2). Similarly, a significantly higher incidence of metastasis to omentum ($p < 0.01$), mesentery ($p < 0.05$) and other sites ($p < 0.01$) was observed in ovarian cancer patients with increased platelet counts than those with normal platelet counts (Table 2).

To try explaining the reason why platelets promote metastasis in ovarian cancer, the effects of platelets on morphology and invasiveness were firstly tested in SK-OV-3 and OVCAR-3 ovarian cancer cells. After co-incubated with purified platelets for 48 h, both cell lines exhibited a more invasive phenotype, such as elongated shape and loss of cell-cell contact (Fig. 2A). In addition, transwell assay showed that SK-OV-3 and OVCAR-3 cells incubated with platelets had a 3.2-fold ($p < 0.001$) and 3.1-fold ($p < 0.001$) higher invasion capacity, respectively when compared to the untreated cells (Fig. 2B). From the results of high-throughput microarrays, IPA predicted that invasive phenotype was activated when SK-OV-3 cells were treated with platelets (Fig. 3).

To investigate the underlying mechanisms of invasion changes, we further tested the molecular alterations of the platelet-treated ovarian cancer cells. Heat maps from microarrays revealed that epidermal-related genes were down-regulated, while mesenchymal-related genes were up-regulated in the platelet-treated SK-OV-3 cells when compared to the control ones (Fig. 4). Moreover, IPA analysis showed that many biological processes, including EMT (Fig. 5), were significantly enriched in SK-OV-3 cells treated with platelets. To confirm the results of the microarray, the expression of six EMT-associated genes was analyzed by real-time PCR. The mesenchymal markers *SNAI1*, *VIM*, *N-cadherin*, *FN1* and *MMP2* were significantly up-regulated in both cell lines treated with platelets in comparison to control cells (Fig. 6A). In contrast, the epithelial marker *E-cadherin* was significantly down-regulated in platelet-treated SK-OV-3 cells (Fig. 6A). The mRNA levels of *E-cadherin* were reduced in platelet-treated OVCAR-3 cells, but this difference was not statistically significant from control cells (Fig. 6A). Thus, our qPCR analysis validated our findings of microchip array. Next, we analyzed the protein expression of these markers by western blot. We found that E-cadherin expression was significantly reduced and N-cadherin protein levels were significantly increased in platelet-treated SK-OV-3 and OVCAR-3 cells when compared to untreated cells (Fig. 6B, C). Similarity, the protein levels of MMP-2 were significantly increased in comparison to the untreated controls (Fig. 6B, C), suggesting that platelet treated cells have a higher capacity to degrade the extracellular matrix (ECM) and to invade the surrounding environment.

Taken together, these results suggest that platelets promote a more invasive and mesenchymal phenotype in ovarian cancer cells.

3.2. Platelets activate TGF-β/Smad pathway in ovarian cancer cells

Clinical data showed that ovarian cancer patients with platelet counts $>350 \times 10^9$/L also had significantly higher levels of TGF-βin the serum in comparison to subjects with normal platelet counts ($p < 0.001$). The distribution of TGF-β levels in the different cohort is shown in Fig. 1B. The tumor sizes did not differ significantly between the two patient groups stratified by platelet counts (Table 2), so they did not affect TGF-β levels.

Based on observations, we hypothesized that platelet-induced invasion and EMT in ovarian cancer cells may be mediated by platelet-derived TGF-β. Results showed that the levels of TGF-β in the culture media of ovarian cancer cells treated with platelets were markedly higher than those in untreated cells (Fig. 7A). IPA analysis also predicted that TGF-β was the activated as an upstream regulator in the SK-OV-3 treated with platelets (Fig. 8).

Moreover, SK-OV-3 and OVCAR-3 cells treated with platelets showed increased phosphorylation of Smad2, a TGF-β signaling effector, when compared to the untreated controls (Fig. 7B), indicating that platelets activate the TGF-β/Smad pathway in ovarian cancer cells.

3.3. TβR I inhibitor A83-01 abolished platelet-induced invasiveness and EMT in ovarian cancer cells

To further validate the role of TGF-β/Smad pathway in platelet-mediated invasiveness and EMT, we cultured the platelet-treated cells with or without TβR I inhibitor A83-01 (25 μM). We found that in the presence of A83-01, platelets failed to induce mesenchymal phenotype in SK-OV-3 and OVCAR-3 cells (Fig. 2A). Concordantly, exposure of the platelet-treated SK-OV-3 and OVCAR-3 cells to A83–01 resulted in 2.3-fold ($p < 0.001$) and 2.1-fold ($p < 0.001$) reduction in their invasion activity compared to platelet-treated cells alone, respectively (Fig. 2B). The EMT-gene expression pattern was also reversed by A83-01 in the ovarian cancer cells treated with the platelets (Fig. 6A). The levels of *FN1* mRNA in the OVCAR-3 cells treated with A83-01 were even lower than those in the control group (Fig. 6A). A83-01 treatment also restored the protein expression of E-cadherin and repressed N-cadherin, MMP-2 and pSmad2 expression in both cell lines (Fig. 6B, C).

To determine the effects of A83-01 on platelet-induced invasiveness *in vivo*, a mouse model of peritoneal dissemination was established using SKOV3-Luc cells. As shown in Fig. 9A&B, combination of platelets and A83-01 significantly suppressed the metastasis of ovarian cancer cells in the peritoneal cavity by bioluminescence imaging when compared with only platelet injection. Consistently, the numbers of tumor nodules involved in abdominal organs were decreased in (platelets + A83-01) group and were comparable to those in the control group (Fig. 9C). Next, the expressions of E-cadherin and N-cadherin in tumor nodules were examined by immunohistochemistry. We observed that treating mice with A83-01

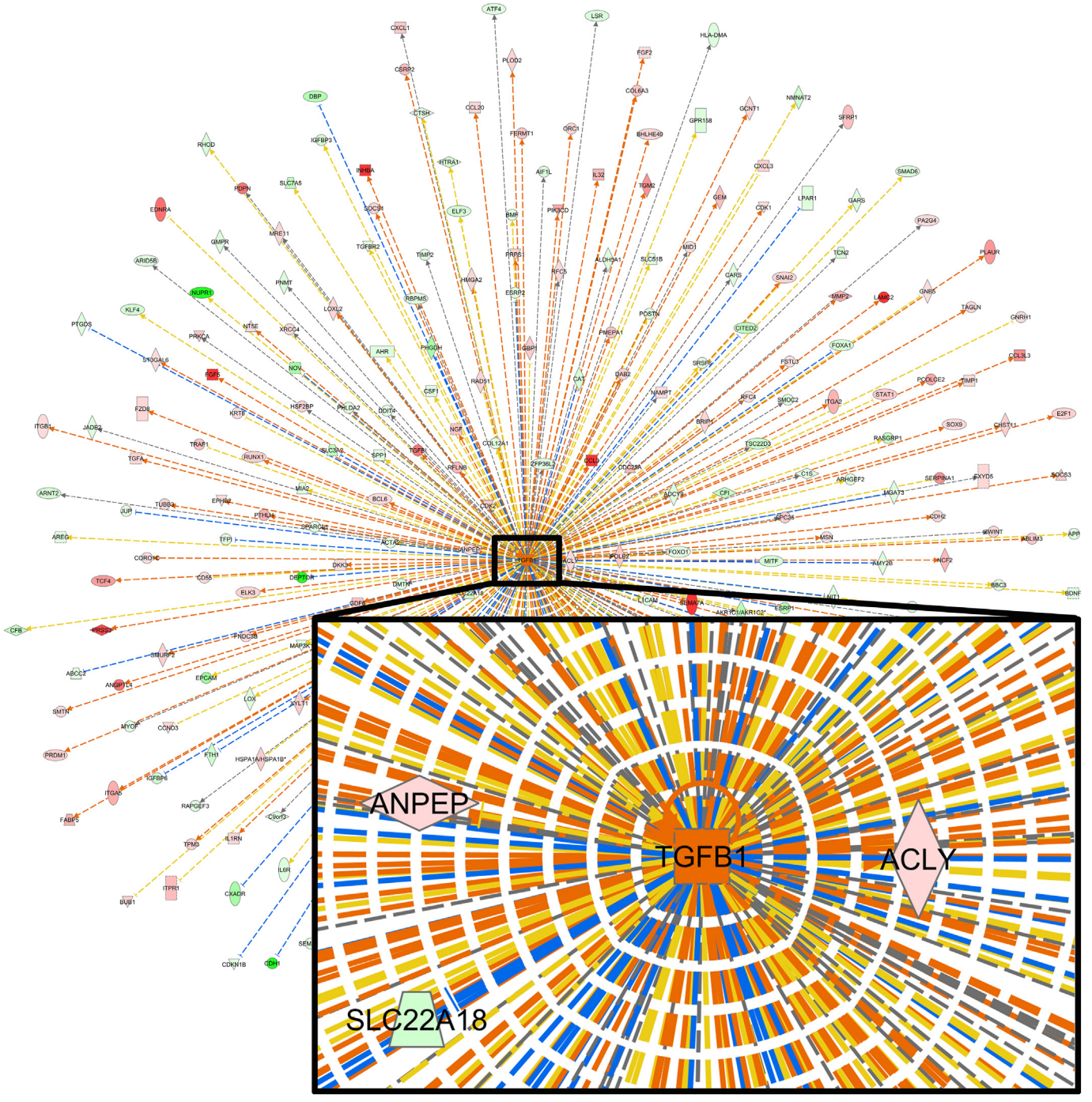


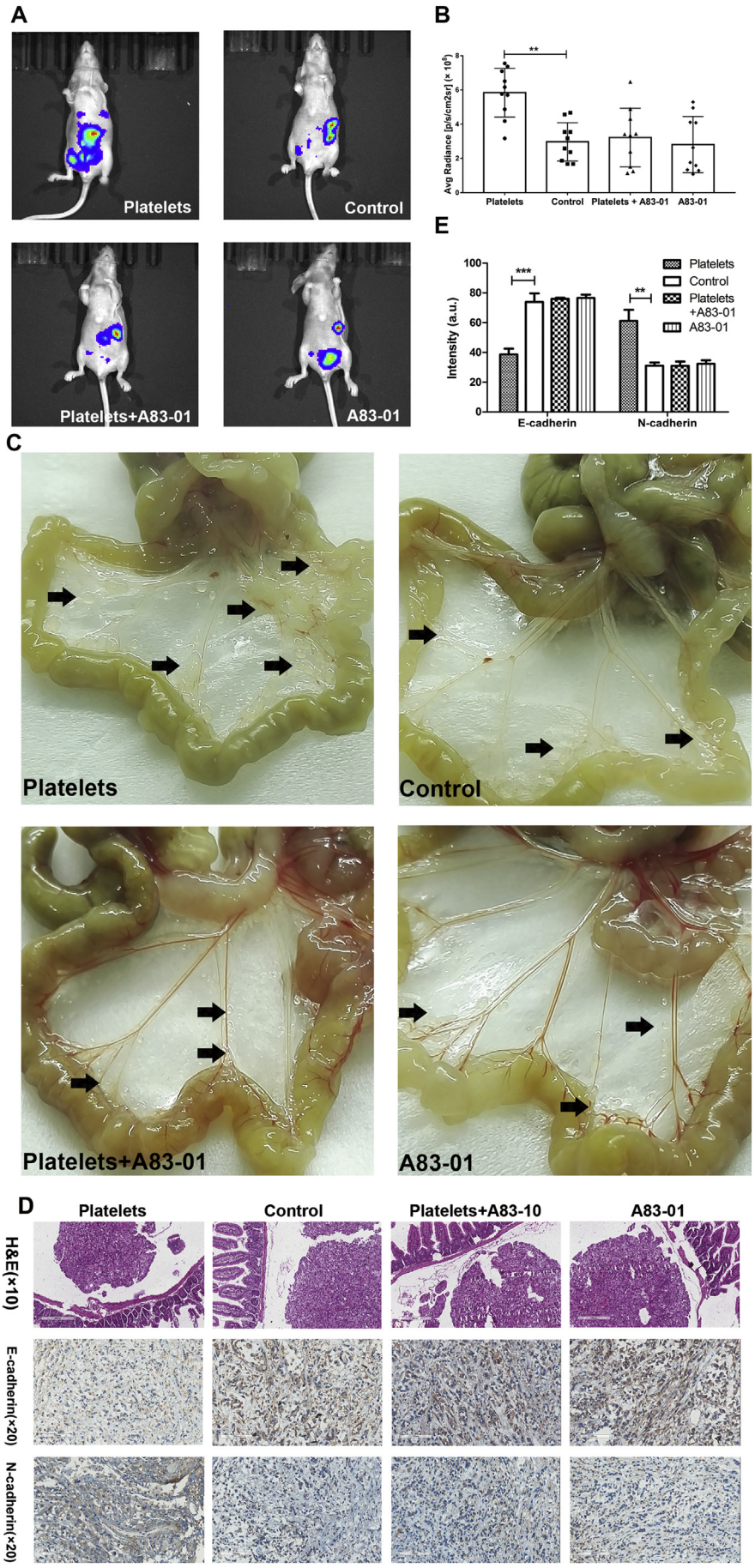
Fig. 8. Upstream prediction network in the platelet-treated SK-OV-3 ovarian cells. The network shows that TGF-β1 is predicted as the upstream regulator when SK-OV-3 cells treated with platelets. The orange color indicates the activated expression status, blue color indicates the inhibitory expression status, and the grey color indicates that no prediction information related to the expression status is found in the database.

significantly inhibited the down-regulation of E-cadherin expression and the up-regulation of N-cadherin in ovarian cancer tissues, indicating a reversal of EMT (Fig. 9D, E).

Taken together, these results showed that TβR I inhibitor A83-01 prevented metastasis and EMT *in vitro* and *in vivo*, which suggests that platelet-induced invasiveness and EMT are dependent on the activation of TGF-β pathway.

4. Discussion

The role of platelets in the progression of cancer has been reported in some studied [24,25]. In ovarian cancer, an estimated 7.0% to 36.4% of the patients show thrombocytosis based on the cutoff values followed by different clinics (350×10^9/L, 400×10^9/L or 450×10^9/L) [8,9,26,27]. These patients are more likely to progress to advanced stage (FIGO III and IV) [8], and exhibit a higher frequency of organ metastasis compared to those without thrombocytosis [9]. Consistent with previous studies, a higher proportion of ovarian patients in our cohort with elevated platelet counts exhibited tumor dissemination towards omentum, mesentery and other sites in the peritoneal cavity. Insights into the underlying mechanisms of thrombocytosis in ovarian cancer patients have been forthcoming in recent years. Cooke et al. [11] found that platelets enhanced the metastatic ability of ovarian cancer cells, and their qPCR results showed the alteration of EMT-related


A
Platelets
Control
Platelets+A83-01
A83-01
B
Avg Radiance [p/s/cm2sr] (× 10^6)
**
Platelets
Control
Platelets + A83-01
A83-01
E
Intensity (a.u.)
***
**
Platelets
Control
Platelets +A83-01
A83-01
E-cadherin
N-cadherin
C
Platelets
Control
Platelets+A83-01
A83-01
D
Platelets
Control
Platelets+A83-10
A83-01
H&E(×10)
E-cadherin(×20)
N-cadherin(×20)

genes expression. In keeping with these findings, our study further confirmed that platelets promoted the invasion of ovarian cancer cells by initiating EMT at protein and microarray levels. However, their study showed that only blocking platelet adhesive surface proteins did not completely diminish platelet-induced EMT and invasion in ovarian cancer cells [11]. Therefore, we speculate that other mechanisms might be involved in the pro-metastatic effect of platelets.

In an elegant study, Labelle et al. [28] have shown that the platelet-induced EMT and invasion is partially correlated with TGF-β/Smad signaling pathway murine colon and breast cancer. Indeed, we for the first time found that serum TGF-β level was higher in ovarian patients with increased platelet counts than those with normal platelet counts. Based on the data, we hypothesized that TGF-β may drive platelet-induced pro-metastasis in ovarian cancer. To validate the underlying mechanism, we tested whether TGF-β secreted from platelets could increase ovarian cancer cell invasion *via* EMT, and activate its downstream target Smad2.

EMT refers to the trans-differentiation of epithelial cells into motile mesenchymal cells [29]. The repression of the epithelial phenotype is mainly a result of the up-regulation of Snail, [30] a transcription factors that binds to the proximal promoter region of the E-cadherin gene by zinc-finger domains and inhibits its expression [31]. Other transcriptional regulators can contribute to the malignant phenotype by activating genes like *VIM*, *N-cadherin* and *FN1* [29]. Another key event in EMT is the up-regulation of MMPs, which contribute to metastasis by degrading the extracellular matrix and other barriers [32].

In the current study, we confirmed that platelets induced EMT in ovarian cancer cells. The mRNA levels of the epithelial marker *E-cadherin* were down-regulated, and levels of mesenchymal markers, including *SNAI1*, *VIM*, *N-cadherin*, *FN1* and *MMP2*, were up-regulated in the presence of platelets. The fold changes in the expression of mesenchymal genes in platelet-treated OVCAR-3 cells (12.0 to 143.8 fold) were significantly higher compared to those in SK-OV-3 cells (2.0 to 10.8 fold), indicating that OVCAR-3 cells were more sensitive to platelet-induced EMT. Similarly, E-cadherin protein expression was reduced, and levels of N-cadherin and MMP-2 were increased in the platelet-treated cells and tissue samples harvested from platelet-treated mice. Transwell assays revealed that EMT cells showed a more invasive phenotype. By contrast, the invasive ability of SK-OV-3 cells was higher than that of OVCAR-3 cells with or without platelet-co-culture. This is likely due to the epithelial phenotype of the OVCAR-3 cells and the intermediate mesenchymal phenotype of the SK-OV-3 cells [33], which results in different invasive capabilities.

Several signaling molecules may induce EMT and influence tumor cell behavior [34]. TGF-β and its downstream effector Smad proteins play major roles in inducing EMT and promoting tumor metastasis [19,35–37]. Briefly, TGF-β binds to the TGF-β type II receptor (TβRII), which then activates type I receptor kinase by phosphorylating TβR I; Then the phosphorylated TβR I activates Smad2 and Smad3 by phosphorylation. Finally, a heterotrimeric complex of phosphorylated Smad2 (p-Smad2), phosphorylated Smad3 (p-Samd3) and a common Smad4 is formed, which translocates into the nucleus to regulate EMT-related gene expression [38].

In the current study, we found increased levels of TGF-β in platelet-treated cell culture media and increased phosphorylation of Smad2 in SK-OV-3 and OVCAR-3 cells treated with platelets which indicates that platelets activated TGF-β/Smad pathway. To further validate the role of the TGF-β pathway, we examined the effect of the TβR I inhibitor A83-01 on platelet-induced pro-metastasis *in vitro* and *in vivo*. A83-01 is a selective inhibitor of TβRI (ALK-5 kinase), and can inhibit activation of Smad2 [18]. Results showed that A83-01 blocked the pro-metastatic effects and reverse the EMT in ovarian cancer cells and tumor-bearing mice, thereby suggesting that platelet induced EMT and invasion phenotype in ovarian cancer cells is mediated *via* the TGF-β signaling pathway. In addition, we found that the mRNA expression of *FN1* in the OVCAR-3 cells treated with A83-01 were even lower than that in the control cells since TβR inhibitors may also competitively antagonize the TGF-β secreted by tumors [39]. Compared to TβR I inhibitor A83-01, neither aspirin nor $P2Y_{12}$ blocker could abolish platelet-induced alteration in the EMT-related genes completely [11]. Moreover, A83-01 had less adverse effects on platelet function than antiplatelet agents, which is helpful in maintaining physiological hemostasis. Therefore, TβR I inhibitors like A83-01 may be an effective anti-metastatic therapy in ovarian cancer.

In conclusion, our study demonstrated that higher platelet counts are significantly associated with a higher incidence of intraperitoneal dissemination and higher TGF-β levels in ovarian cancer patients. Furthermore, platelets promoted EMT and invasiveness of ovarian cancer cells by activating the TGF-β/Smad pathway. Therefore, TGF-β may be a novel target for developing treatment strategies for ovarian cancer.

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Conflict of interest statement

The authors declare that there are no conflicts of interest in this work.

Author contribution section

Yi Guo contributed to the study design, performed the experiments and wrote the main manuscript text; Wei Cui revised the manuscript and funded the study. Yuqing Pei and Danfei Xu conceived the study and contributed to statistical analysis. All the authors reviewed the manuscript.

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Fig. 9. Mouse model of intraperitoneal dissemination using SK-OV-3-Luc treated with platelets and (or) A 83-01. (A) Representative mice images injected with SK-OV-3-Luc cells pre-conditioned with platelets and (or) A 83-01 monitored by bioluminescence imaging. (B) Quantification of bioluminescence shows that tumor spread is significantly reduced in the group pre-conditioned with platelets and A 83–01 ($n = 10$). (C) The physical appearance of representative mice in each group. (D) The expression of E-cadherin and N-cadherin in tumor nodules harvested from mice treated with platelets and (or) A 83-01. (E) Quantification of immunohistochemistry shows that the expression of E-cadherin is significantly up-regulated and the expression of N-cadherin was significantly down-regulated in A83-03-treated group compared with platelet-treated group ($n = 3$). **: $p < 0.01$.

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