Crosstalk between TEMs and endothelial cells modulates angiogenesis and metastasis via IGF1-IGF1R signalling in epithelial ovarian cancer

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Background: Epithelial ovarian cancer (EOC) is the leading cause of death from gynaecologic malignancies and has a poor prognosis due to metastasis. Drugs targeting the angiogenesis pathway significantly improve patient outcome. However, the key factors linking angiogenesis and metastasis have not been elucidated. In this study, we found Tie2 expressing monocytes (CD14$^+$Tie2$^+$, TEMs) as key contributors to angiogenesis and metastasis of EOC.

Methods: Tissue slides were evaluated by immunofluorescence for the presence of total tissue macrophages and TEMs. The correlation between microvascular density (MVD) values and the TEMs number or ratio was calculated in both ovarian cancer tissues and peritoneum. The rate of TEMs in monocytes was evaluated in the peripheral blood of female healthy donors, benign cysts patients, and EOC patients using flow cytometry. The TEMs rate in ascites from EOC patients was also evaluated by flow cytometry. The concentration of Ang2, as the ligand of Tie2, was examined by ELISA in serum samples of EOC patients, benign cysts patients, and ascites samples of EOC patients. The effects of Ang2 on the migration and the cytokine expression of TEMs were further examined. The pro- angiogenesis activity of TEMs via IGF1 was performed in both *in vivo* and *in vitro*. And the IGF1 blocking test was performed using neutralising antibody.

Results: TEMs were significantly higher in tumour foci, peripheral blood and ascites in EOC patients. The proportion of TEMs among total tissue macrophages was positively correlated with tumour MVD. *In vivo* animal results showed that TEMs promoted EOC angiogenesis and metastasis. Further functional and mechanisms studies revealed that concentration of angiopoietin 2 (Ang2), a ligand of Tie2, was elevated in EOC ascites which further recruit TEMs in a dose-dependent manner as a powerful chemokine to TEMs. Recruited TEMs promoted endothelial cell function through IGF1-activated downstream signalling. Blocking secreted IGF1 using inhibiting antibody reduced TEMs mediated angiogenesis and metastasis.

Conclusions: TEMs significantly increased in EOC patients and were recruited to tumour loci by the increased Ang2. The increased TEMs have diagnostic value in ovarian cancer and were positively correlated with the MVD in ovarian cancer tissue. Furthermore, TEMs promote angiogenesis via IGF1 in both *in vivo* and *in vitro* experimental systems after stimulation by Ang2. Altogether, this study paves the way to develop novel therapy targets as the axis of Ang2-TEMs-IGF1 in EOC.

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Epithelial ovarian cancer (EOC) is the most deadly cancer of the female genital tract and ranks as the seventh leading cause of all cancer-related deaths among women worldwide (Jemal *et al*, 2011). Nearly 70% of EOC patients are diagnosed at an advanced stage, presenting with widely distributed metastases on the surface of peritoneum, and displaying a poor prognosis caused by the progression of the disease (Seidman *et al*, 2004).

The peritoneum is organised to protect the integrity of intra-abdominal organs, which facilitates infiltration of inflammatory cells to sites of tumour implants and might also serve to facilitate the promotion of tumour growth and spread. Our previous study has suggested that more than 75% of immune cells infiltrated in the peritoneum of EOC were CD68$^+$ macrophage with M2 polarisation, and less than 25% were CD3$^+$ T cells (Wang *et al*, 2006). Examination of surgically resected EOC samples revealed frequent changes in the peritoneum harbouring tumour, including thickening or oedema, enhanced vascular patterns, and soft or firm adhesions. The peritoneum and intestinal serosa sometimes exhibited a florid appearance, similar to that found in peritonitis. Significant infiltration by macrophage was one of the feature of chronic inflammation. These tumour-associated macrophage could promote angiogenesis by interaction with EOC cells (Wang *et al*, 2013).

Recently, a novel subset of monocytes that expressed Tie2 (also known as the receptor tyrosine kinase, TEK) has been identified as Tie2 expressing monocytes (TEMs) in mouse models, human peripheral blood and several human tumour tissues (De Palma *et al*, 2003; De Palma *et al*, 2005; Venneri *et al*, 2007; Pucci *et al*, 2009). Increased numbers of TEMs in peripheral blood and liver are under consideration as novel diagnostic markers for hepatocellular carcinoma (De Palma *et al*, 2013; Matsubara *et al*, 2013). Tie2, the receptor for angiopoietins (especially angiopoietin 2, Ang2), has important functions in angiogenesis induced by the Ang2-Tie2 axis (Saharinen *et al*, 2008). Endothelial cells were previously believed to be the only cells that express Tie2 (Partanen *et al*, 1992). The specific pro-angiogenic role of TEMs has been confirmed in many studies of human tumours and non-neoplastic diseases, and this novel subset of monocytes is considered as a strong angiogenesis-promoting entity, perhaps more potent than canonical tumour-associated macrophages (Ribatti, 2009; Coffelt *et al*, 2010; Huang *et al*, 2011). Although the potency TEMs for promoting angiogenesis and their reaction to Ang2 stimulation has been reported in several human cancers, their presence, frequency and their roles in the angiogenesis promotion of EOC remain unclear.

In this study, we examined tumours from patients with EOC and benign cysts for the incidence of TEMs and their infiltration characteristics, and correlated these features with microvascular density (MVD). We evaluated Tie2 over-expressing monocytes in angiogenesis assays *in vitro* and *in vivo* to further explore the mechanism of angiogenesis-promoting activities by these cells. Elevated IGF1 secretion by TEMs was found after Ang2 stimulation. Activation of the downstream IGF1 pathway was verified in endothelial cells, as evidenced by phosphorylation of the IGF1R, together with the phosphorylation of its downstream molecules Erk1/2 and Akt.

Taken together, results of this study suggest that the axis of Ang2-TEMs-IGF1 could enhance angiogenesis by activation of the IGFR pathway in endothelial cells during progression of EOC. Targeted therapy that affects this axis in EOC might be warranted in the future.

MATERIALS AND METHODS

Patients and tissue samples. Our study was approved by the Ethics Committee of the Shanghai First Maternity and Infant Hospital, School of Medicine, Tongji University. A total of 124 patients with histologically confirmed EOC at Shanghai First Maternity and Infant Hospital, Tongji University between January 2012 and December 2014 were recruited for this study. Their diagnoses were independently reviewed by two pathologists, classified by using WHO criteria. All the patients were informed with the informed consents obtained in the present study.

Cell lines. Human monocytic cell line U937 and human ovarian cancer cell line SKOV3 were obtained from ATCC in December 2013 and the cell lines were authenticated by STR test. The last time that the cell lines were tested was December 2015.

Immunofluorescence and immunohistochemistry. CD68$^+$ Tie2$^+$ TEMs and CD68$^+$Tie2$^-$ macrophages in patient tissue slides were detected using mouse anti-human Tie2 (clone Ab33, Millipore, Billerica, MA, USA) and rabbit anti-human CD68 (MAB20401, R&D Systems, Minneapolis, MN, USA) antibodies, followed by Alexa Fluor 488-conjugated goat anti-mouse IgG (104546, Jackson, Lancaster, PA, USA) and Cy3-conjugated goat anti-rat IgG (99002, Jackson) as secondary antibodies. All cell nuclei were counterstained with DAPI (D9542, Sigma, St Louis, MO, USA).

Immunohistochemical analysis of cluster of differentiation 31 (CD31) was performed with rabbit anti-CD31 antibody (ab28364, Abcam, Cambridge, UK). IGF1 expression was evaluated by immunohistochemical staining using the anti-IGF1 antibody (ab9572, Abcam). HPR-conjugated goat anti-rabbit antibody (31461, Invitrogen, Carlsbad, CA, USA) was used as the secondary antibody. Images were captured by optical microscope, and MVD and an IGF1 immunostaining score were calculated from five random high power fields at ×20 magnifications. For IGF1 immunostaining scoring, the quantitative scoring method was used as follows: the intensity of staining was firstly divided into weak staining (score = 1), moderate staining (score = 2) and strong staining (score = 3). Then the positive cells ratio was measured as <10% (score = 0), 10–25% (score = 1), 25–50% (score = 2), 50–75% (score = 3), >75% (score = 4). We multiply the intensity of staining score by the positive cells ratio score to get the final immunostaining score of IGF1. The immunohistochemical results were valued manually by two different pathologists.

Detection of TEMs prevalence in peripheral blood and ascites. Peripheral blood mononuclear cells (PBMCs) and cells in ascites isolated from patient cohorts were double-stained with FITC-conjugated anti-CD14 (555397, BD Biosciences, Franklin, NJ, USA) and APC-conjugated anti-Tie2 (FAB3131A, R&D Systems) antibodies. Tie2$^+$CD14$^+$ TEMs were detected by flow cytometry. Numbers of TEMs were quantified by gating on CD14$^+$ cells. FITC/APC-conjugated isotype antibodies were used control antibodies.

TEMs isolation and culture. Monocytes were enriched from PBMCs by positive immunomagnetic selection using anti-CD14 MicroBeads (130-050-201, Miltenyi, Cologne, Germany). Tie2$^+$ CD14$^+$ TEMs were sorted using FITC-conjugated anti-CD14 and APC-conjugated anti-Tie2 antibodies. Sorted TEMs were cultured using RPMI 1640 medium containing 10% FBS and 1% penicillin-streptomycin in a humidified 37 °C incubator with 5% CO_2.

Tie2 over-expression in U937 cells. To over-express TEK gene, the gene was amplified by PCR, with the following primers: TEK-F:5′-GAGGATCCCCGGGTACCGGTCGCCACCATGGACTCTT TAGCCAGCTTAGTTCTCTG-3′, TEK-R:5′-TCCTTGTAGTC-CATACCGGCCGCTTCTTCAGCAGAAC AGTCAATTC-3′.

Then the products of PCR were cloned into the pUbi-MCS-3FLAG-SV40-EGFP Expression Vector System (Genechem, Shanghai, China). The recombinant vector pUbi-TEK was confirmed by sequencing (Shenggong, Shanghai, China). HEK-293T cells were cultured in DMEM supplemented with 10% FBS.
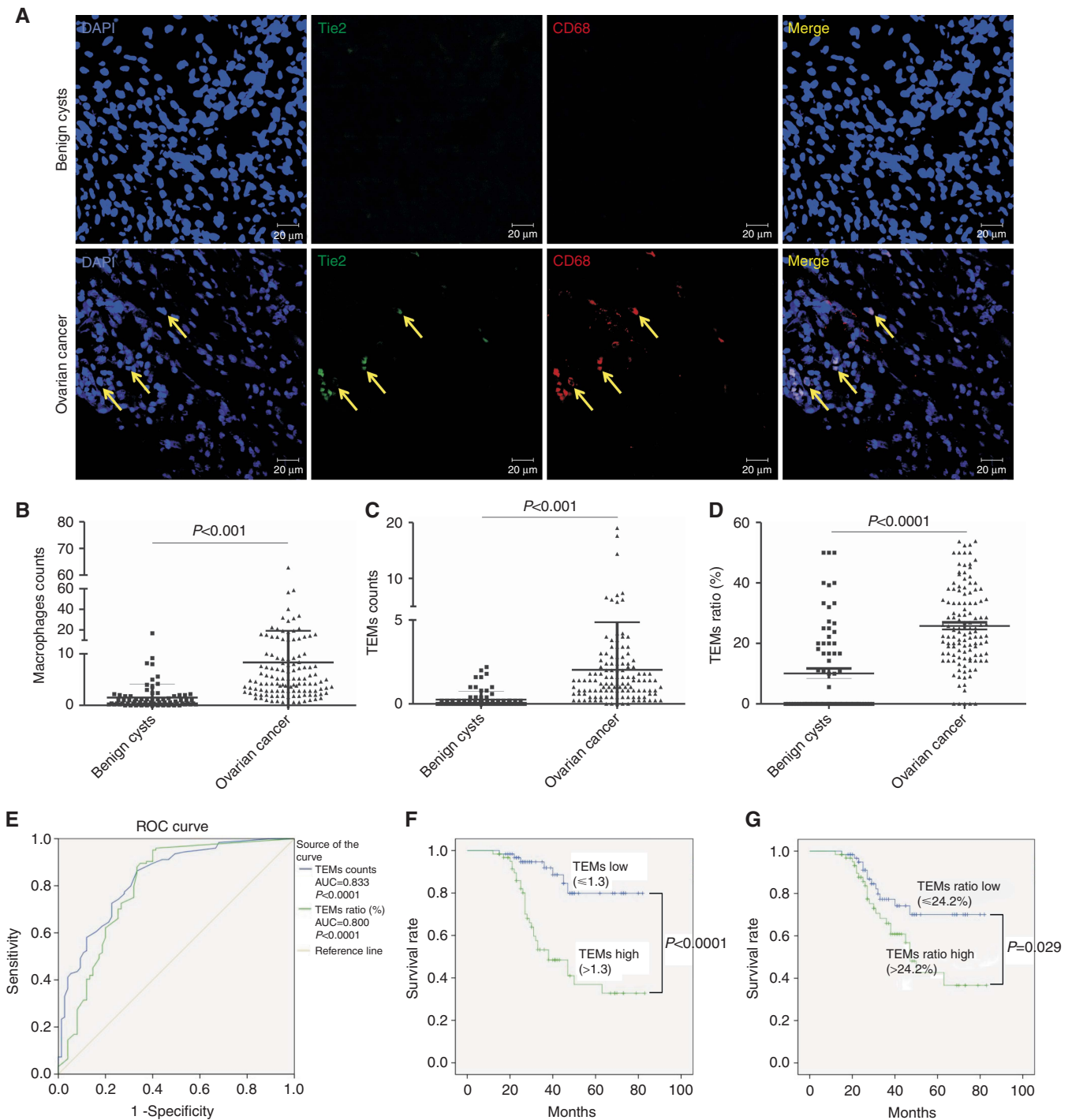
Figure 1. Increased TEMs in tissue as a biomarker for EOC and inform the patients' outcome. (A) TEMs were stained with DAPI (blue, nuclei), Tie2 (green), and CD68 (red) in benign ovarian tumour tissue ($n=75$) and in EOC tissue ($n=124$). (B–D) The macrophages, TEMs and the TEMs ratio were all significantly increased in EOC tissues compared with benign ovarian cysts (student's t-test). (E) ROC analyses were performed in order to assess the diagnostic value of TEM number and ratio in ovarian tissue for differentiating EOC ($n=124$) from benign ovarian cysts ($n=75$). (F) In EOC patients, the overall survival rate was compared between patients with TEMs high (TEMs >1.3, $n=62$) and TEM low (TEMs $\leqslant 1.3$, $n=62$) using the Kaplan–Meier method, with the log-rank test for comparison. (G) In EOC patients, the overall survival rate was compared between patients with TEMs ratio high (TEMs ratio $>24.2\%$, $n=62$) and TEMs ratio low (TEMs ratio $\leqslant 24.2\%$, $n=62$) using the Kaplan–Meier method, with the log-rank test for comparison.

The lentivirus-mediated TEK packaging system (TEK-L.V.) was co-transfected utilising lipofectamine 2000 into HEK-293T cells in a 24-well culture plate according to the manufacturer's protocol. The supernatant was collected 48 h post infection, filtered with a 0.45 micrometer filter, and stored at $-80\,^{\circ}C$. U937 cells was transduced with TEK-L.V. in the presence of $5\,\mu g\,ml^{-1}$ polybrene

(Sigma) and Eni.S (Genechem, Shanghai, China) for 12 h, after which the medium was replaced by RPMI1640 supplemented with 10% FBS. The efficiency of infection of TEK-L.V. was measured using fluorescent microscope 72 h post infection. Moreover, the efficiency of infection is confirmed by immunofluorescence detection or FACS. For further study, GFP$^+$ U937 was isolated

by FACS. Over-expression of TEK was determined by western blot analysis, respectively. The lentivirus-mediated non-specific control packaging system (NC-L.V.) was used as control.

TEMs and TEK-U937 migration assays. Migration assays were performed in a 24-well transwell apparatus containing 8-μm-pore inserts (BD Biosciences). Ang2 (623-AN, R&D Systems) was diluted as the concentration of $10\,\mathrm{ng\,ml}^{-1}$, $100\,\mathrm{ng\,ml}^{-1}$, or $200\,\mathrm{ng\,ml}^{-1}$ in serum-free RPMI1640 medium (800 μl) at the bottom compartment of the chamber with various concentrations, and 10^5 TEMs or TEK-U937 pre-treated by M-CSF (216-MC, R&D Systems) were resuspended in 300 μl RPMI1640 then added to the top compartment. After 3 h incubation for TEMs or 6 h incubation for TEK-U937, migrating cells were labelled with $5\,\mu\mathrm{g\,ml}^{-1}$ calcein-AM (C3099, Molecular Probes, Carlsbad, CA, USA, Life Technologies, Carlsbad, CA, USA) and counted under a fluorescence microscope. For blocking analysis, TEMs were pre-incubated with neutralising anti-Tie2 antibodies for 20 min, and the Ang2 was inactivated by heat at 95 °C for 30 min.

Quantitative real-time PCR. The RNA of TEK-U937/NC-U937 was collected using TRIzol (15596-018, Life Technologies) before Ang2 stimulation or after Ang2 treatment for 6 h. For q-PCR analysis, 500 ng of total RNA was reversed transcribed to cDNA, and amplified by PCR cycling conditions: 5 s at 95 °C and 30 s at 60 °C for 40 cycles. Differences in gene expression were determined by the $2^{\Delta\Delta}$CT method (β-actin was used for calibration) using as: 5′-CCTGGCACCCAGCACAAT-3′ and 5′-GGGCCGGACTCG TCATACT-3′ (β-actin), 5′-CAGGATACGAACCATGAAGAT GC-3′ and 5′-GGGG CACTGAATGGATGAAG-3′ (Tie2), 5′-CAG GATACGAACCATGAAGATGC-3′ and 5′-GGGGCACTGAAT GGATGAAG-3′ (IL-8), 5′-CAGGATACGAACCATGAA GATG C-3′ and 5′-GGGGCACTGAATGGATGAAG-3′(IL-10), 5′-CTGG AGTGTG TGCCCACTGA-3′ and 5′-TCCTATGTGCTGGCC TTGGT-3′(VEGFA), 5′-TCCCACGAAATCCAGGATGC-3′ and 5′-GGATGTTCAGGTTGACCATCAC-3′ (IL-17A), 5′-GCTCTT CAGTTCGTGTGTGGA-3′ and 5′-GCCTCCTTAGATCACAG CTCC-3′(IGF1).

Soluble protein analysis. Serum and ascites were collected from patient cohorts. The Ang2 and IGF1 levels were detected by ELISA using human Angiopoietin2 (DANG20, R&D Systems) or human IGF1 Quantikine ELISA kit (DG100, R&D Systems).

EC activation assays *in vitro*. Conditioned medium was collected from TEMs/TEK-U937 or NC-Monocytes/NC-U937 after treatment with Ang2 ($100\,\mathrm{ng\,ml}^{-1}$). After 6 h stimulation, cells were washed and the conditioned mediums were collected 24 h later. In the tubule formation assays, serum-starved HUVECs (5×10^4 per 96-well plate) were re-suspended in conditioned medium and seeded into growth factor-reduced Matrigel (356234, BD Biosciences). Tubules were counted after incubation for 6 h. Recombinant human IGF-I protein (291-G1, R&D Systems) was used as a positive control. For IGF1 block analysis, the anti-IGF1 antibody (ab9572, Abcam) was used. To determine the activation of the IGF1-IGF1R pathway in HUVECs, phosphorylation IGF1R, Akt, and Erk1/2 were detected by western blot after treatment with conditioned medium.

Mice model of ovarian cancer. Female athymic nude mice (4–6 weeks old) weighing 14–16 g were purchased from Shanghai JiaoTong University School of Medicine and bred under specific-pathogen-free conditions. Prior to the study, the protocols for the treatment of animals were approved by Medical Animal Care. 5×10^5 human ovarian cancer cell line SKOV3 transfected with luciferase (SKOV3-Luc) cells together with 1×10^5 TEK-U937 or NC-U937 cells in 30 μl DMEM were injected into the left ovarian parenchyma of the nude mice. TEK-U937 and NC-U937 cells were stimulated by Ang2 ($100\,\mathrm{ng\,ml}^{-1}$) for 24 h before injection. Then all the nude mice were separated into three groups according to the injected cell types as follows: TEK-U937 group (SKOV3-Luc cells were injected with TEK-U937), NC-U937 group (SKOV3-Luc cells were injected with NC-U937), and control group (SKOV3-Luc were injected alone). There were six mice in each group.

For long-term experiments to assess tumour growth, 1×10^5 TEK-U937 or NC-U937 cells were injected intravenously through the tail vein every 3 days after the third day of the orthotopic injection. For the control group, 200 μl RPMI1640 media was injected each time. For IGF1 block analysis, the anti-IGF1 antibody (ab9572, Abcam) was used every 3 days after the third day of the orthotopic injection through intraperitoneal injection.

After 4 weeks, following euthanasia, the left ovary and enterocelia of the regions showing clear luciferase signal were dissected. Tumour total flux, tumour weight, and the distribution of the tumour were recorded. Tumour tissues were fixed in 4% neutral buffered formalin for frozen slide preparation.

Statistics analysis. The one-way analysis of variance (ANOVA, for all groups), t-test, Pearson Chi-Square test and Mann–Whitney test were used to compare groups statistically using the SPSS19.0 statistical software package. Each test for each experimental group was shown in results. The correlation between groups was assessed by Pearson's analysis. Overall survival was calculated using the Kaplan–Meier method. All tests were two-tailed, and a P-value of less than 0.05 was considered statistically significant.

RESULTS

TEMs amplified in EOC patients as EOC biomarker and inform the overall survival. Ovarian tissue slides from 124 EOC patients and 75 patients with benign ovarian cysts were evaluated by immunofluorescence for the presence of total tissue macrophages and TEMs (Figure 1A). The number of total tissue macrophages identified as CD68$^+$ cells is significantly higher ($P<0.001$, student's t-test, Figure 1B) in EOC patients' ovarian tissue (8.12 ± 10.89) than in benign cysts (1.53 ± 2.60). The number of CD68$^+$Tie2$^+$ TEMs in EOC patients (1.89 ± 2.85) is also significantly higher ($P<0.001$, student's t-test, Figure 1C) as compared with the benign cysts patients (0.26 ± 0.50). Moreover, the ratio of TEMs/total tissue macrophages in EOC patients'

Table 1. Univariate and multivariate analysis of overall survival in 124 EOC patients

| Variables | Overall survival | | | |
| | Univariate analysis | | Multivariate analysis | |
	Mean ± s.e.	P	95% CI	P
TEMs number				
Low (⩽1.3)	72.94 ± 3.16			
High (>1.3)	49.37 ± 3.89	0.001	1.59–8.64	0.002
Age (years)				
Low (⩽50)	61.96 ± 4.01			
High (>50)	54.26 ± 3.39	0.802	0.45–1.67	0.668
Grade				
LGSC	78.49 ± 3.03			
HGSC	49.65 ± 2.80	<0.0001	1.26–23.54	0.023
MVD				
Low (⩽4.6)	65.37 ± 3.87			
High (>4.6)	54.47 ± 3.92	0.052	0.93–3.53	0.083

Abbreviations: CI = confidence interval; EOC = epithelial ovarian cancer; HGSC = high grade serous carcinoma; LGSC = low grade serous carcinoma; MVD = microvascular density; s.e. = standard error; TEM = Tie2 expressing monocytes.
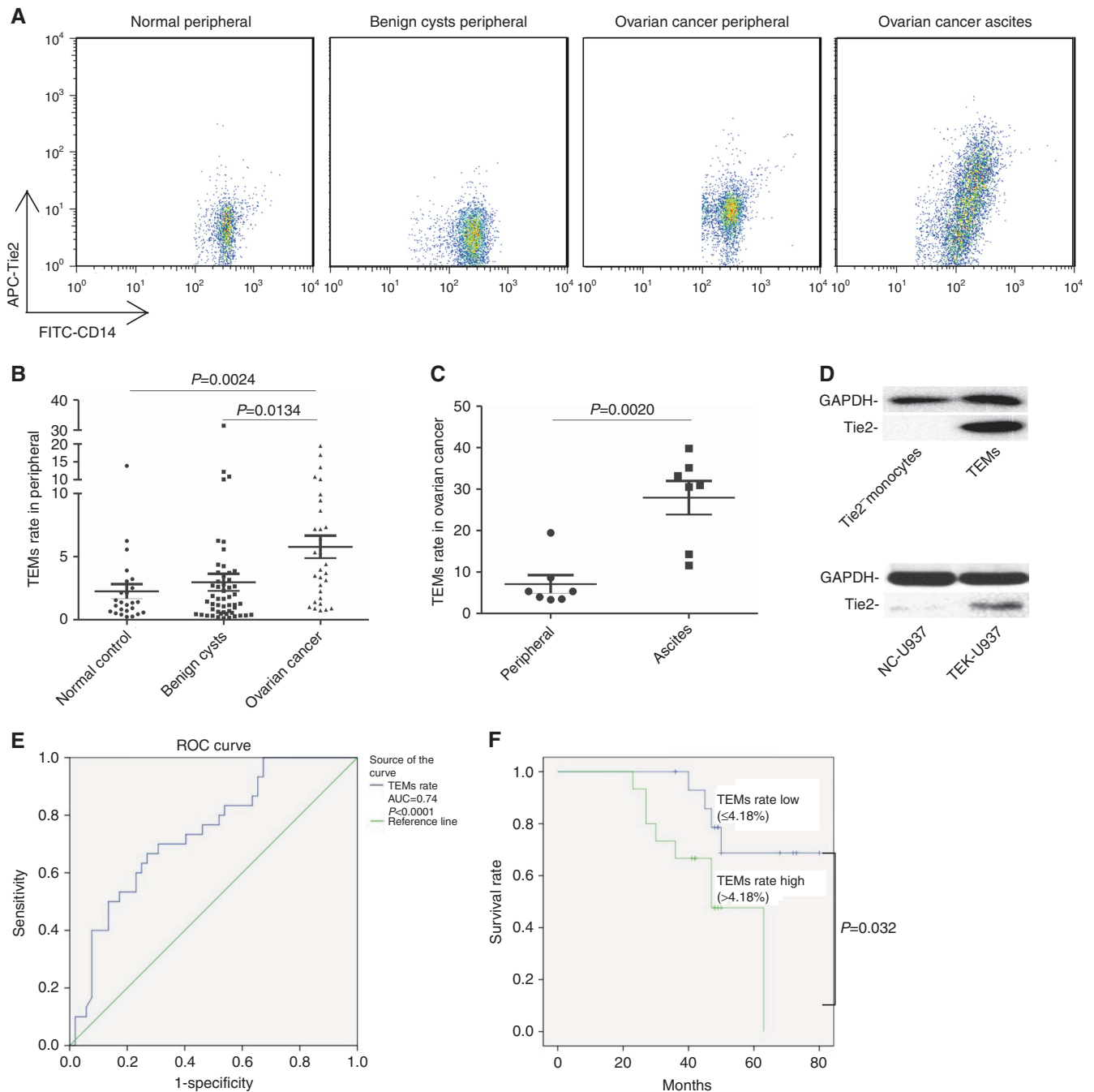
Figure 2. Increased TEMs in peripheral as a biomarker for EOC and inform the patients' outcome. (A) The CD14$^+$Tie2$^+$ TEMs rate in total CD14$^+$ monocytes was detected by flow cytometry in peripheral of healthy female donors ($n = 25$), benign ovarian cysts patients ($n = 52$), EOC patients ($n = 30$) and ascites of EOC patients ($n = 7$). **(B)** TEMs rate in peripheral of EOC patients was significantly higher compared with benign ovarian cysts patients and healthy control (student's t-test). **(C)** TEMs rate in ascites compared with matched peripheral blood of the same EOC patients (student's t-test). **(D)** Tie2 expression was detected in primary TEMs and TEK-overexpressing monocyte TEK-U937 by WB. **(E)** ROC analysis was performed in order to assess the diagnostic value of TEM rate in peripheral for differentiating EOC ($n = 30$) from benign ovarian cysts ($n = 52$). **(F)** In EOC patients, the overall survival rate was compared between patients with TEMs rate high (TEMs rate in peripheral $> 4.18\%$, $n = 15$) and TEMs ratio low (TEMs ratio $\leqslant 4.18\%$, $n = 15$) using the Kaplan–Meier method, with the log-rank test for comparison.

ovarian tissue ($25.78 \pm 13.05\%$) is also significantly higher ($P < 0.001$, student's t-test, Figure 1D) than in benign cysts patients ($25.78 \pm 13.05\%$). Similar results were found in 15 peritoneum slides obtained from ovarian cancer patients and 10 peritoneum slides of benign ovarian cysts patients. Both TAMs (10.57 ± 4.50 vs 1.60 ± 2.09, $P < 0.0001$, t-test) and TEMs (1.93 ± 1.14 vs 0.26 ± 0.31, $P = 0.0002$, student's t-test) were increased in peritoneum of ovarian cancer patients (Supplementary Figure 1A–C). Immunofluorescence staining of

Tie2 together with CD31 in blood vessels of ovarian cancer tissue showed Tie2 is also expressed in the vessels (Supplementary Figure 4A).

The ovarian tissue infiltrated TEMs number and ratio at different levels to determine the most optimal cutoff value to predict the ovarian tumour as benign cyst or EOC was evaluated. The ROC curve analyses showed that the TEMs number of 1.9 yielded the most optimal predictive value with the area under the curve (AUC) as 0.833 (95% CI, 0.776 to 0.891, $P < 0.0001$). And the
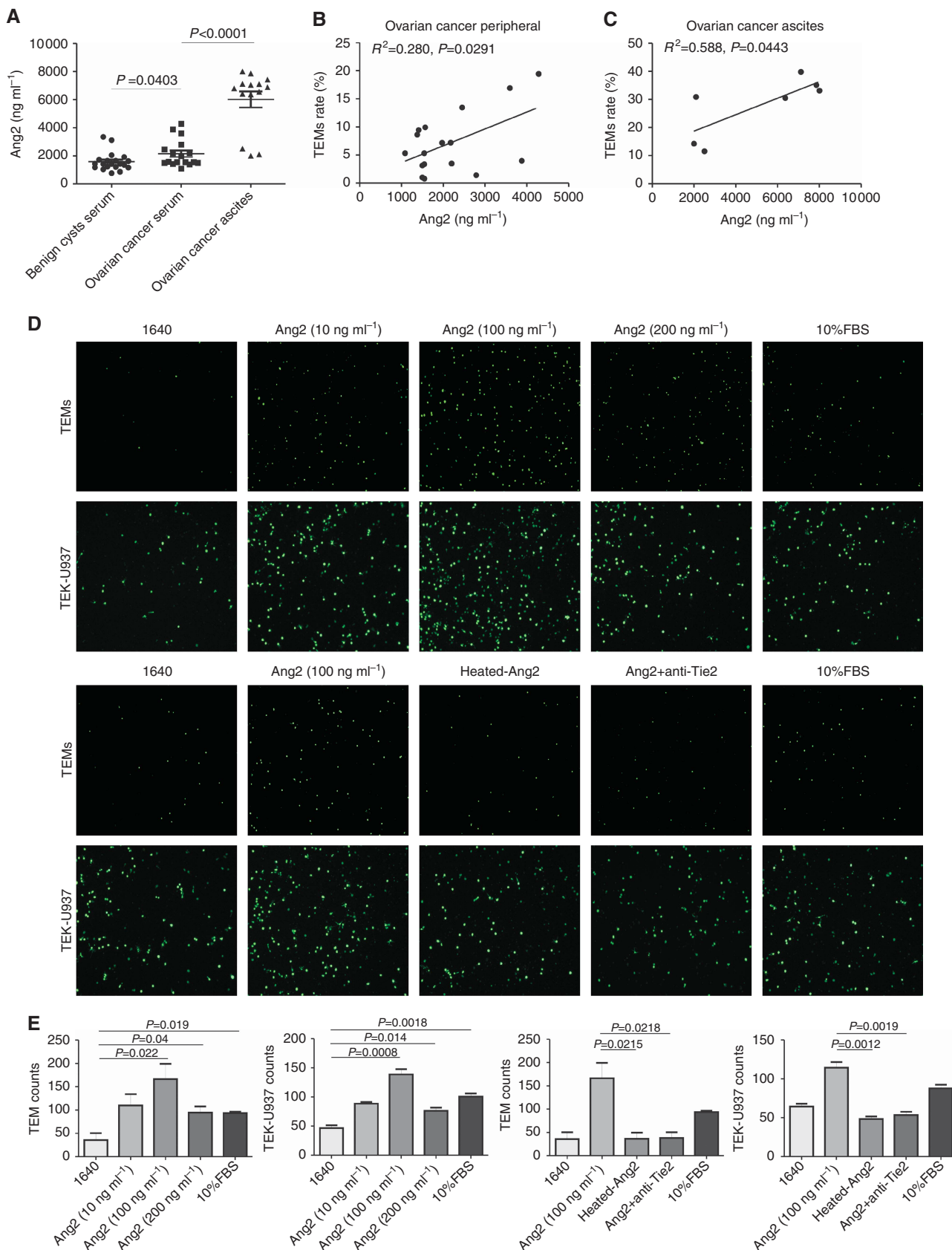
Figure 3. Increased Ang2 recruited TEMs and TEK-U937. (**A**) The Ang2 level was significantly higher in the ascites ($n = 14$) and serum ($n = 17$) of EOC patients compared with the serum of benign ovarian cysts patients ($n = 20$, student's t-test) according to the ELISA assay. (**B** and **C**) Increased Ang2 was positively correlated to the TEMs rate in peripheral and ascites of EOC patients. (**D**) The migration ability of TEMs or TEK-U937 to medium alone (1640) or medium containing various doses of Ang2 from 10 to 200 ng ml^{-1} or 10% FBS and the Ang2-Tie2 blocking assays were conducted. (**E**) The migration was significantly enhanced by Ang2 at the dose of 100 ng ml^{-1} compared with 1640 as a negative control and heat-inactivation of Ang2 or treatment with neutralising anti-Tie2 antibodies inhibited cell migration in response to Ang2.
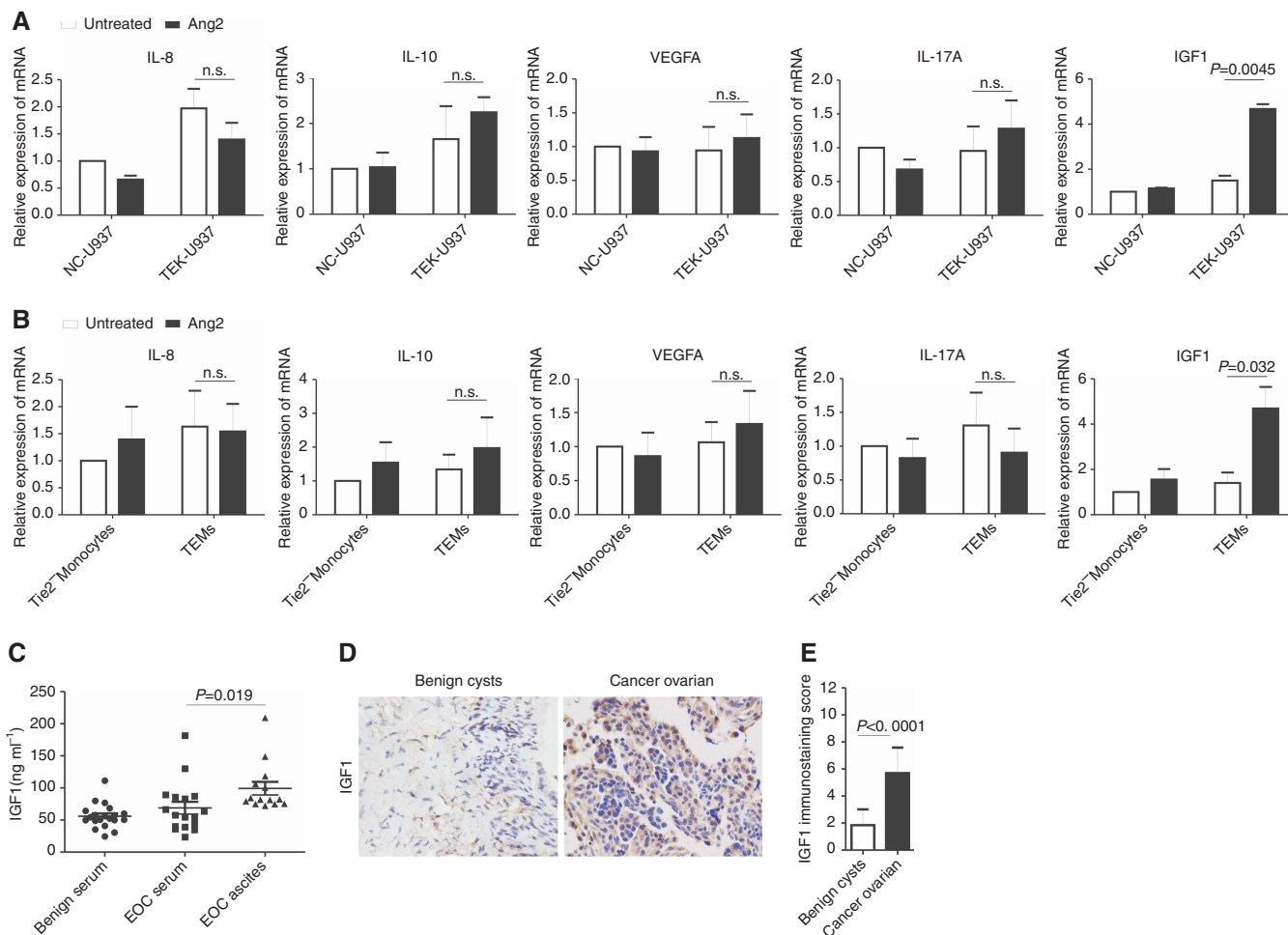
Figure 4. Increased Ang2 enhanced the IGF1 secretion of TEMs and TEK-U937. (**A** and **B**) Ang2 stimulation increased the IGF1 expression in TEK-U937 ($n = 3$) and primary TEMs ($n = 3$, student's t-test). (**C**) IGF1 level was significantly increased in ascites of EOC patients ($n = 14$) compared with the serum of EOC patients ($n = 17$) and benign ovarian cysts patients ($n = 20$, student's t-test) according to the ELISA assay. (**D**) IGF1 level in ovarian tissues of EOC patients ($n = 124$) and benign ovarian cysts patients ($n = 75$) was detected by immunohistochemistry. (**E**) The IGF1 expression is significantly higher in EOC ovarian tissues compared with benign ovarian cysts patients (student's t-test).

TEMs/total tissue macrophage ratio 5.76% the most optimal predictive value with the AUC as 0.800 (95% CI, 0.730 to 0.869, $P < 0.0001$, Figure 1E). The median TEMs number in EOC ovarian tissue (TEMs number = 1.3) was determined as the cutoff value of TEMs low group and TEMs high group. The EOC patients with high TEMs infiltrated number in ovarian tissue exhibited a significantly shorter overall survival time (49.37 ± 3.89) compared with those with low TEMs infiltrated number (72.94 ± 3.16, $P < 0.0001$, Log-Rank test, Figure 1F, Table 1). Also, the median TEMs/total tissue macrophage ratio in EOC ovarian tissue (TEMs ratio = 24.2%) was determined as the cutoff value of TEMs ratio low group and TEMs ratio high group. The EOC patients with high TEMs ratio exhibited a significantly shorter overall survival time (53.54 ± 3.65) compared with those with low TEMs ratio (66.72 ± 3.65, $P < 0.029$, Log-Rank test, Figure 1G).

Moreover, the rate of TEMs ($Tie2^+CD14^+$) in monocytes ($CD14^+$) was evaluated in the peripheral blood of 25 female healthy donors, 52 benign cysts patients, and 30 EOC patients using flow cytometry (Figure 1A). The TEMs rate ($5.77\% \pm 4.86\%$) in the peripheral blood in EOC patients was significantly higher ($P = 0.0024$ vs healthy controls, $P = 0.0124$ vs benign ovarian cysts, Student's t-test, Figure 2B) than in healthy controls ($2.25\% \pm 2.89\%$) and patients with benign ovarian cysts ($2.96\% \pm 4.85\%$). The TEMs rate in ascites ($27.90\% \pm 10.72\%$) from seven EOC patients was also evaluated (Figure 2A) and the

frequency in ascites was significantly higher ($P = 0.002$, paired t-test, Figure 2C) than that of matched peripheral blood samples ($7.07\% \pm 5.75\%$) in each patient. The Tie2 expression in primary TEMs isolated by FACS and in the TEK over-expression monocytes (TEK-U937) were confirmed by WB (Figure 2D). The gating strategy and the isotype used in the flow cytometry were shown in Supplementary Figure 2 and the TEK over-expression in TEK-U937 was also measured by fluorescent microscope and FACS detection of GFP (Supplementary Figure 3).

The peripheral TEMs rate at different levels to determine the most optimal cutoff value to predict the ovarian tumour as benign cyst or EOC was evaluated. The ROC curve analysis showed that the TEMs rate in peripheral of 3.08% yielded the most optimal predictive value with the AUC as 0.74 (95% CI, 0.631 to 0.849, $P < 0.0001$, Figure 2E). The median peripheral TEMs rate in EOC patients (TEMs rate = 4.18%) was determined as the cutoff value of TEMs rate low group and TEMs rate high group. The EOC patients with high peripheral TEMs rate exhibited a significantly shorter overall survival time (48.49 ± 4.40) compared with those with low peripheral TEMs rate (69.34 ± 4.50, $P = 0.032$, Log-Rank test, Figure 2F).

Increased Ang2 recruited primary TEMs and TEK-U937. The concentration of Ang2, as the ligand of Tie2, was examined by ELISA in serum samples of 17 EOC patients, 20 benign cysts
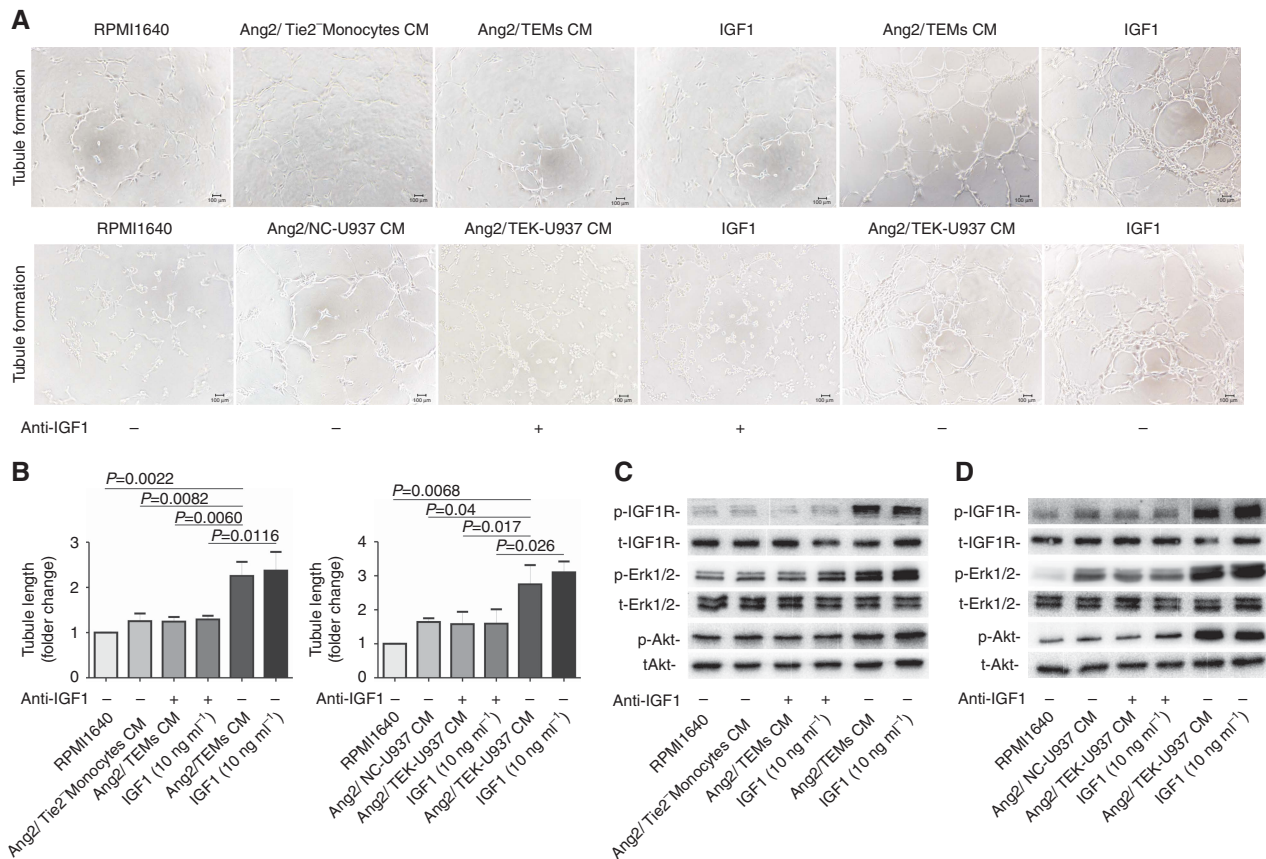
Figure 5. Ang2 enhanced the TEMs and TEK-U937 promoting tubule formation via IGF1 pathway *in vitro*. (**A**) HUVEC tubule formation assay *in vitro* was conducted. (**B**) The Ang2 stimulated primary TEMs or TEK-U937 conditioned medium (CM) induced significantly more tubule formation by calculating the tubule length ($n=3$ in each group, student's *t*-test). (**C** and **D**) The Ang2 stimulated primary TEMs or TEK-U937 conditioned medium (CM) induced the phosphorylation of IGF1R, Akt and Erk1/2 detected in HUVECs by WB.

patients, and ascites samples of 14 EOC patients (Figure 3A). Ang2 levels in the serum of EOC patients ($2142 \pm 958.6 \, \mathrm{ng \, ml^{-1}}$) were significantly higher ($P=0.0403$, student's *t*-test) than benign cysts patients ($1575 \pm 651.5 \, \mathrm{ng \, ml^{-1}}$). Moreover, Ang2 level in the ascites of EOC patients ($6013 \pm 2123 \, \mathrm{ng \, ml^{-1}}$) was significantly higher than that in the patient-matched serum samples ($P<0.001$, student's *t*-test) in each patient. The increased Ang2 was positively correlated to the TEMs rate in peripheral ($R^2=0.280$, $P=0.0291$) and ascites of EOC patients ($R^2=0.588$, $P=0.0443$, Figure 3B and C).

The effects of Ang2 on the migration of human TEMs and TEK-U937 were further examined (Figure 3D). We found that Ang2 induced significant migration of both TEMs and TEK-U937, with the highest response corresponding to an Ang2 concentration of $100 \, \mathrm{ng \, ml^{-1}}$ (Figure 3E, $P=0.022$ or 0.0008 *vs* RPMI1640 control). To confirm that the observed chemotactic response was induced by specific interactions between Ang2 and Tie2, TEMs or TEK-U937 were pretreated with neutralising anti-Tie2 antibodies. Anti-Tie2 antibodies or heat inactivation of Ang2 significantly blocked Ang2-induced cell migration (Figure 3E).

Ang2 induced IGF1 increasing in primary TEMs and TEK-U937. The effects of Ang2 on cytokine expression in TEMs and TEK-U937 were evaluated by qPCR before and after treatment with Ang2 (Figure 4A and B). The qPCR results showed that IGF1 was significantly increased in both TEMs (($P=0.032$) and TEK-U937 cells after treatment ($P=0.0045$, student's *t*-test), whereas the mRNA levels of interleukin-8 (IL-8), interleukin-10 (IL-10), vascular endothelial growth factor-A (VEGFA), and interleukin-17A (IL-17A) were not changed. Anti-Tie2 antibodies significantly reserved the IGF1 increased in TEMs and TEK-U937 induced by

Ang2 (Supplementary Figure 5). Moreover, ELISA assays showed that the IGF1 levels in ascites of the EOC patients was significantly higher than in serum ($P=0.019$, student's *t*-test, Figure 4C). Also, IGF1 expression level was significantly increased in 124 EOC patients compared with 75 benign cysts patients as evidenced by a significantly higher immunostaining score ($P<0.0001$, student's *t*-test, Figure 4D and E). Moreover co-immunostaining of IGF1 and TEMs in ovarian tissues of EOC was performed by immunofluorescence and the TEMs expressed high IGF1 level was observed (Supplementary Figure 4B).

TEMs promote angiogenesis via IGF1. Primary TEMs or TEK-U937 cells were firstly stimulated by Ang2 ($100 \, \mathrm{ng \, ml^{-1}}$) for 24 h and the conditioned medium (CM) was collected for assays of endothelial tubule formation (Figure 5A). IGF1 with the concentration of $10 \, \mathrm{ng \, ml^{-1}}$ served as the positive control. The results showed that Ang2-stimulated primary TEMs or TEK-U937 cells CM significantly increased capillary tubule formation compared with the Ang2-stimulated-NC CM (Figure 5B, $P=0.04$, student's *t*-test). Addition of an IGF1 inhibiting antibody significantly reversed tubule formation induced by Ang2-stimulated TEMs or TEK-U937 cells CM (Figure 5B, $P=0.017$, student's *t*-test). The phosphorylation status of IGF1R, Erk1/2 and Akt in HUVECs were significantly increased after stimulation with Ang2-stimulated TEMs or TEK-U937 CM and addition of anti-IGF1 blocked the phosphorylation of these proteins in HUVECs (Figure 5C and D). After TEMs or TEK-U937 pre-treated with anti-Tie2 blocking antibody, the Ang2-stimulated TEMs or TEK-U937 CM induced phosphorylation of IGF1-IGF1R pathway proteins in HUVECs was reserved (Supplementary Figure 5).
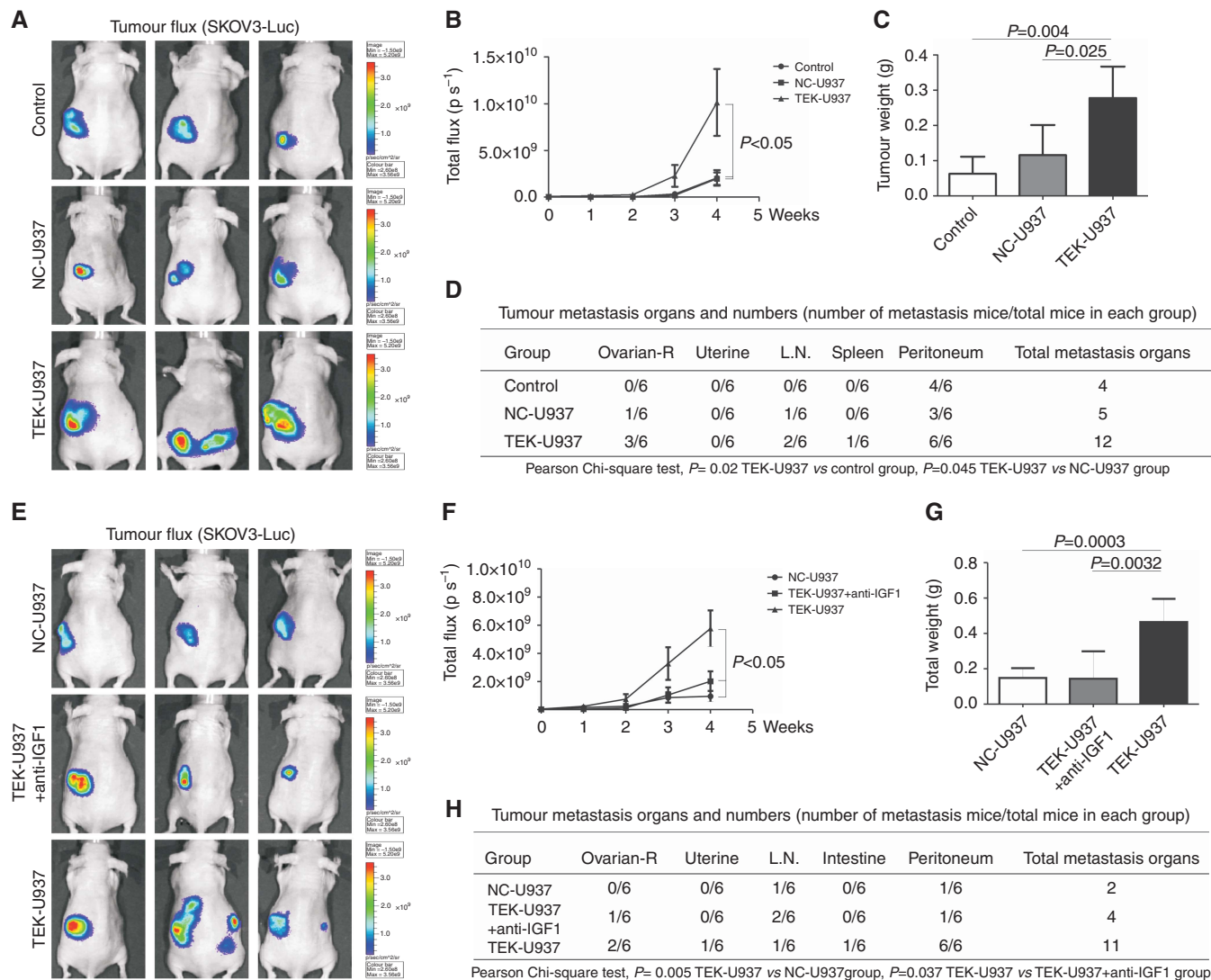
Figure 6. TEK-U937-promoted tumour development and metastasis *in vivo*. (A) SKOV3-Luc expression was detected by BLI in the tumour burden mouse model. (**B** and **C**) The TEK-U937 group showed significantly higher flux and tumour weight compared with the NC-U937 and control groups ($n = 6$ in each group, Mann–Whitney test). (**D**) TEK-U937 significantly promoted tumour metastasis compared to the NC-U937 and control groups (Pearson Chi-square test). (**E–H**) IGF1 blocking antibody reversed the increased tumour flux, tumour weight and tumour metastasis promoted by TEK-U937 ($n = 6$).

TEK-U937 promote angiogenesis and progress of EOC *in vivo*. TEK-U937 cells behaved highly similar to primary TEMs *in vitro*, and was used for further study *in vivo*. Tumour development occurred in 100% of orthotopically injected ovaries in mice and longitudinal bioluminescence imaging for fluorescent tumour burden was assessed (Figure 6A). The test of BLI signal intensity confirmed that in the TEK-U937 group ($2.27 \times 10^9 \pm 2.85 \times 10^9$ by week 3, $1.01 \times 10^{10} \pm 8.77 \times 10^9$ by week 4), tumour growth (total flux, ps^{-1}) was enhanced compared with the NC-U937 group ($1.98 \times 10^8 \pm 1.92 \times 10^8$ by week 3, $1.97 \times 10^9 \pm 1.63 \times 10^9$ by week 4) and the control group ($3.26 \times 10^8 \pm 3.93 \times 10^8$ by week 3, $2.05 \times 10^9 \pm 2.05 \times 10^9$ by week 4, $n = 6$ in each group, Figure 6B).The tumour weight (g) of the TEK-U937 group (0.28 ± 0.09 g) was also much higher at the time of killing than that of the NC-U937 (0.12 ± 0.09 g) and control groups (0.06 ± 0.05 g, Figure 6C).

Within the TEK-U937 group, tumour metastases were found in the contra lateral ovary, lymph nodes, spleen and peritoneum. Moreover, the peritoneum metastases were found in all the six mice of TEK-U937 group. There are significantly more tumour metastases in TEK-U937 group compared with the NC-U937

group ($P = 0.045$, Pearson Chi-square test) and the control group ($P = 0.004$, Pearson Chi-square test, Figure 6D).

To evaluate tumour angiogenesis, MVD was calculated by anti-CD31 staining in murine tumour tissues and the mice peritoneum (Figure 7A). MVD was significantly higher in ovarian tumour tissue of TEK-U937 group (23.70 ± 4.78) compared with in NC-U937 group (14.63 ± 2.94, $P = 0.003$, student's *t*-test) and in control group (13.83 ± 2.85, $P = 0.001$, student's *t*-test, Figure 7B). Furthermore, MVD was significantly higher in peritoneum of TEK-U937 group (27.27 ± 6.48) compared with in NC-U937 group (20.37 ± 1.52 $P = 0.029$, student's *t*-test) and in control group (15.13 ± 1.68, $P = 0.001$, student's *t*-test, Figure 7C).

Further study of IGF1 blocking was performed and anti-IGF1 blocking antibody significantly reversed the tumour growth, tumour metastases and the angiogenesis promoted by TEK-937 *in vivo* (Figures 6E–H, 7D–F).

Immunofluorescence detection of GFP was performed and TEK-U937-GFP cells and NC-U937-GFP cells were homogeneously distributed in the mice tumour tissue but not in the control group (Supplementary Figure 6).
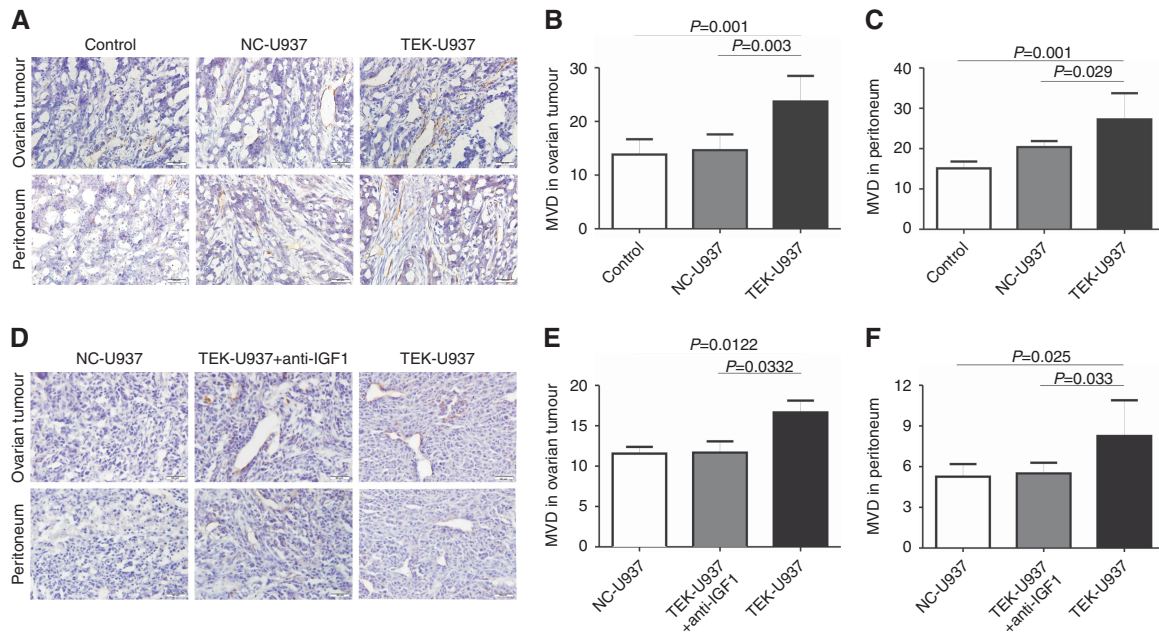
Figure 7. TEK-U937-promoted tumour angiogenesis *in vivo*. (**A**) MVD value was calculated in mice tumour tissues and the corresponding peritoneum by immunohistochemistry staining of CD31 ($n = 6$). (**B** and **C**) The MVD value of TEK-U937 group is significantly increased in tumour local and corresponding peritoneum (Mann–Whitney test). (**D–F**) IGF1 blocking antibody reversed the angiogenesis promoted by TEK-U937 ($n = 6$).
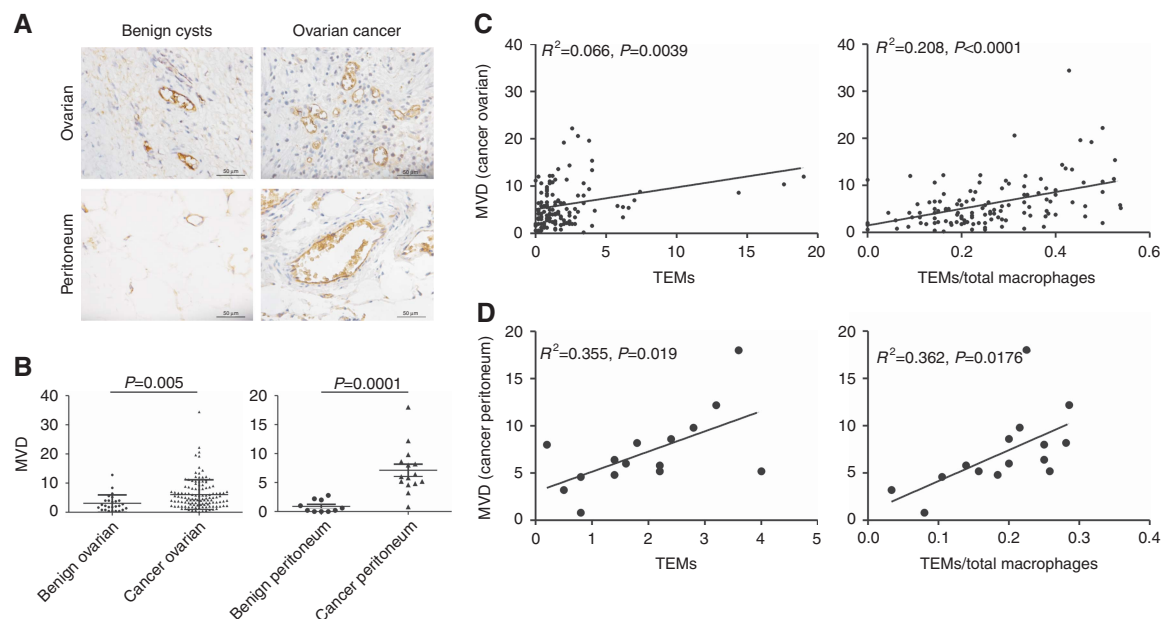

Figure 8. TEMs correlate with MVD in ovarian cancer tissue. (**A**) MVD value was calculated in ovarian tumour tissues of EOC patients ($n = 124$), ovarian tumour tissues of patients with benign ovarian cysts ($n = 75$), the peritoneum of EOC patients ($n = 15$) and peritoneum of patients with benign ovarian cysts ($n = 10$). (**B**) The MVD values in both ovarian tissues and peritoneum of the EOC patients are significantly increased compared with the patients with benign ovarian cysts (student's *t*-test). (**C** and **D**) The increased MVD values in EOC patients' ovarian tissues and peritoneum were positively correlated to the infiltrated TEMs number and the TEMs/total macrophages ratio.

TEMs correlate with MVD in EOC patients. To determine if infiltrated TEMs correlate with tumour angiogenesis, we measured MVD using anti-CD31 staining in ovarian tissue slides obtained from 124 ovarian cancer patients and 75 patients with benign cysts (Figure 8A). As expected, MVD in ovarian cancer patients (6.05 ± 5.08) was significantly higher (Figure 8B, $P < 0.0001$, student's *t*-test) than in patients with benign cysts

(1.91 ± 2.21). microvascular density in the perineum of 15 ovarian cancer patients (7.02 ± 4.09) was also significantly higher (Figure 8B, $P = 0.0001$, student's *t*-test) than in 10 benign cysts patients (0.90 ± 1.06). Furthermore, MVD values in both ovarian cancer tissues and peritoneum were positively correlated with the TEMs number and TEMs/total macrophages ratio (Figure 8C and D).

DISCUSSION

TEMs, a novel subset of monocytes, are considered important not only for the development of blood-vessel formation in tumours but also for other human diseases, such as ischemic limb (Patel *et al*, 2013). Previous studies have shown that monocytes are the principal population of mononuclear cells that express Tie2 in the circulation system, and one study has shown that selective elimination of TEMs impairs angiogenesis and tumour growth in a tumour-burden mouse model (De Palma *et al*, 2007). Thus, TEMs have been discussed as a potential therapeutic target to inhibit angiogenesis in tumours (Daly *et al*, 2013).

The data presented here show that both circulating TEMs and TEMs infiltrated into ovarian tissues and peritoneum are significantly increased in EOC patients compared with control groups. Moreover, the frequency of TEMs was significantly increased in ascites compared to matched samples of peripheral blood in EOC patients. ROC and survival analysis found that the ovarian tissue infiltrated TEMs number, the TEMs/total macrophages ratio in tissue, together with the TEMs rate in patients peripheral, could serve as novel biomarkers and prognosis predictors of EOC and particular EOC-derived factors may promote the recruitment of TEMs.

CCR2, the receptor for the chemokine MCP1 that plays an important role in the recruitment of monocytes, was not expressed in TEMs (Augustin *et al*, 2009), suggesting that TEMs might be attracted to tissues by other factors. Ang2, as a ligand for Tie2, is upregulated in malignant tumours as a response to hypoxia and functions as a chemoattractant for human TEMs (Huang *et al*, 2011). Interestingly, Ang2 levels were increased in the ascites fluid of EOC patients and were significantly higher than matched sets of serum. Increased Ang2 levels in EOC patients, especially in the ascites samples, may play an important role in recruiting TEMs to tissue. Pretreatment of cells with anti-Tie2 antibody reversed TEMs attraction by Ang2. These results suggest that TEMs migration into tumour tissue may be regulated by increased Ang2 levels in the tumour microenvironment and expression of Tie2 on TEMs. Similar results were observed in TEK-U937 cell line, supporting the hypothesis that the over-expressed Tie2 in monocytic cell lines interact with the ligand Ang2. These results also suggest that TEK-U937 may be used as a Tie2 expressing monocytic cell model in future studies.

As classical core molecules in tumour angiogenesis, Ang2–Tie2 interactions in endothelial cells were hypothesised to induce the abnormal angiogenesis caused by IL6 in ovarian cancer (Gopinathan *et al*, 2015). In our study, high TEMs infiltrated in ovarian cancer, another prominent cell subset that expressed Tie2 molecule, might also play an important role in angiogenesis as the target cell of Ang2. The effects of Ang2 on TEMs function have been studied (Coffelt *et al*, 2010), and the results demonstrate that Ang2 can markedly enhance the pro-angiogenic activity of TEMs, while targeting the Ang2/Tie2 pathway could inhibit the functions of TEMs (De Palma and Naldini, 2011).

In this study, we explored the gene expression changes in TEMs before and after Ang2 stimulation. The results show that the mRNA level of the cytokines IL-8 and IL-10, IL-17A, as well as VEGFA, a classical growth factor that promotes angiogenesis, were not changed in primary TEMs or TEK-U937 by Ang2 stimulation. However, the mRNA level of IGF1 is significantly increased after Ang2 stimulation. This confirmed results observed in our previous study of microarray assays of TEMs and Tie2-Monocytes isolated from peripheral blood of healthy donors, which showed that IGF1 mRNA levels were increased in TEMs (Wang *et al*, 2016). These data indicate that circulating Ang2 could upregulate the expression of IGF1 in TEMs. Further studies verified that IGF1 was also significantly increased in the ascites and the ovarian tissue of the EOC patients.

The insulin-like growth factor (IGFs) family and their receptors comprise an important growth factor system that is necessary for the function of many cells under normal physiological conditions (Rother and Accili, 2000). In pathological situations, evidence has indicated that IGFs play an important role in tumourigenesis due to powerful anti-apoptotic effects (Khandwala *et al*, 2000). In human tumours, such as prostate cancer, a high circulating level of IGF1 is correlated with a high risk of tumour development (Cohen, 1998; Wolk *et al*, 1998). With respect to the function of IGF1 in angiogenesis-promoting progression, we found that capillary tubule formation in HUVECs was significantly enhanced by TEMs/TEK-U937-CM. Evidence of activation of the IGF1-IGF1R pathway in HUVECs was provided by detection of phosphorylation of IGF1R signal transduction cascade molecules, including IGF1R, Akt and Erk1/2 (LeRoith *et al*, 1995). The results indicate that IGF1-mediated signalling may play an important role in angiogenesis induced by the Tie2 over-expressing monocytic cells.

In summary, we found that TEMs are significantly increased in peripheral blood, ascites, and tissue samples of ovarian cancer patients. And the increased TEMs have diagnostic value in ovarian cancer. Our study also showed that Ang2 was increased in ovarian cancer ascites, and could attract TEMs to tumour tissue. Furthermore, MVD in ovarian cancer tissue correlated with TEMs number and frequency. We also found that the TEMs promote angiogenesis via IGF1 in both *in vivo* and *in vitro* experimental systems after stimulation by Ang2. Thus, the Ang2-TEMs-IGF1 axis is a potential target for ovarian cancer therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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