

Upregulation of IGF1 by tumor-associated macrophages promotes the proliferation and migration of epithelial ovarian cancer cells

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Abstract. Ovarian cancer (OC), of which epithelial ovarian cancer (EOC) is the most common, is the deadliest gynecological tumor because of the difficulties in detection at early stages, and metastasis and chemoresistance at advanced stages. Tumor-associated macrophages (TAMs) differentiate through alternative pathways and play important roles in tumor growth and metastasis. However, the underlying mechanism remains unclear. Here, we established a mouse TAM model using bone marrow monocytes and conditioned medium (CM) of TAMs to culture ID8 mouse EOC cells. The results showed that TAM CM accelerated the proliferation and migration of ID8 cells. In a previous study, gene chip analysis showed that human TAMs expressed significantly higher levels of insulin-like growth factor-1 (IGF1) than undifferentiated M0 myeloid cells. In the present study, we observed that the IGF1 level was higher in human EOC specimens than that in benign ovarian tumor specimens, and further analysis showed that a higher level of IGF1 was related to more advanced clinical stage and liver metastasis. Therefore, we hypothesized that TAMs may accelerate the proliferation and migration of EOC cells by upregulating IGF1. As expected, increased IGF1 expression at both the mRNA and protein levels was observed in ID8 cells cultured with TAM CM, whereas blockade of the IGF1 pathway in ID8 cells with an IGF1 neutralizing antibody effectively reversed the promotion of proliferation and migration. Finally, we inhibited the phosphorylation of insulin-like

growth factor-1 receptor (IGF1R) and its downstream molecules Akt and Erk with the IGF1R inhibitor linsitinib, and observed that the treatment effectively suppressed the proliferation and migration of ID8 cells exposed to TAM CM. Thus, we demonstrated that TAMs may promote the growth and metastasis of EOC via the activation of the IGF1 pathway; thus, targeting the IGF1 pathway may be promising for EOC therapy.

Introduction

Ovarian cancer (OC) is the most fatal gynecological malignant tumor, causing 151,900 deaths worldwide in 2012 (1). According to recent statistics, OC is still the fifth leading cause of cancer-related female deaths in the United States (2). Because there are no effective methods to screen OC, 75% of cases are diagnosed at an advanced stage, with tumor cells spreading widely throughout the abdominal cavity (3). Although many therapeutic methods, including surgery and cisplatin-based chemotherapy, have been used, severe metastasis and chemoresistance lead to a 5-year overall survival rate in no more than 25-35% of cases (3,4). EOC accounts for 90% of OC (5); therefore, it is important to investigate how this cancer progresses.

Although cancer cells unlimitedly proliferate and survive, a tumor microenvironment (TME) is required for the formation and growth of clinically relevant tumors (6). The TME, comprising mesenchymal stem cells, cancer-associated fibroblasts (CAFs), myeloid cells, mesothelial cells and factors released by these cells, contributes to tumor growth, immune escape, distant metastasis and chemoresistance of cancer (6-8). For example, CAF-derived exosomes (tiny vesicles formed during endocytosis and 30-150 nm in size) can promote the survival and proliferation of pancreatic cancer cells, thus affecting responses to the standard chemotherapeutic agent gemcitabine (9). Mesothelial cells stimulated with TGF- β can promote OC cell attachment and proliferation by activating the promoters of matrix metalloprotein-2 and matrix metalloprotein-9 (10).

TAMs, the most common immune cells in the TME (11), primarily refer to macrophages infiltrating into tumor tissues (12). These cells are derived from circulating monocytes and induced to differentiate through alternative pathways through various factors in the TME, mainly due

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Abbreviations: OC, ovarian cancer; EOC, epithelial ovarian cancer; IGF1, insulin-like growth factor-1; IGF1R, insulin-like growth factor-1 receptor; TAMs, tumor-associated macrophages; CM, conditioned medium; TME, tumor microenvironment; CAFs, cancer-associated fibroblasts

Key words: tumor-associated macrophages, epithelial ovarian cancer, IGF1, proliferation, migration

to the activation of the Notch pathway (11,12). In addition to the microenvironment within tumor tissues, TAMs are also distributed in some special organs and lymph nodes, associated with metastasis to these regions (13,14). TAMs infiltrate into OC tissues in large numbers (15-17), contributing to the progression of OC (18,19), thus negatively affecting the progression-free survival rates and overall survival rates of these patients (16). However, the precise mechanism of how TAMs contribute to the progression of OC remains unclear.

The IGF1 pathway comprises three receptor tyrosine kinases [IGF-1R, insulin-like growth factor-2 receptor (IGF-2R), and insulin receptor (IR)], three ligands (insulin, IGF-1, and IGF-2), and six serum insulin-like growth factor binding proteins (IGFBPs), which are important regulators of this pathway (20). This pathway can enhance the proliferation and development of cells by initiating the anti-apoptotic PI3K/Akt/mTOR and the mitogenic Ras/Raf/Mek/Erk pathways (21,22). The IGF1 pathway is associated with the growth, metastasis and clinical outcome of various cancers, including prostate cancer, gastric cancer, lung cancer and breast cancer (23-27). For example, CAFs can increase the invasion ability of pancreatic cancer cells via paracrine IGF1/IGF1R signaling, particularly under hypoxia (27), and IGF1R is highly expressed in chronic lymphocytic leukemia cells, whereas the inhibition of IGF1R can enhance the death of CLL cells (28).

The IGF1 pathway has also been associated with the progression of OC. Women carrying haplotype 2C of the IGF1 gene have a decreased risk of OC, whereas those carrying haplotype 1D or 2D have an increased risk (29). Serous ovarian carcinoma cells are strongly positive for IGF1, and IGF1 could downregulate the expression of E-cadherin and upregulate Snail and Slug expression, thereby promoting the epithelial to mesenchymal transition of human OC cells (30,31). Low IGFBP-3 expression is clinically correlated with high tumor grade, advanced stage and poor survival of ovarian endometrioid cancer patients (32). The IGF1/PI3K/NF κ B/Erk pathway was found to be upregulated in ovarian specimens of patients demonstrating relative resistance compared with those demonstrating sensitivity (33). Furthermore, high circulating IGF-1 levels have been correlated with decreased OC risk, and overall and progression-free survival were significantly prolonged in patients with higher serum IGF1 levels (34-36). However, the underlying reason for the abnormality of IGF1 in OC patients remains unknown.

In the present study, we showed that TAMs enhanced the proliferation and migration of mouse OC ID8 cells by upregulating IGF1, and inhibition of the IGF1 pathway using an IGF1 inhibitor effectively suppressed the proliferation and migration of ID8 cells exposed to TAM-conditioned medium (CM). These results indicate that targeting the IGF1 pathway is a promising EOC therapy.

Materials and methods

TAM model establishment. A TAM model was established according to Lin *et al* (37), and the protocols for the treatment of animals were approved by the Ethics Committee of Tongji University prior to the study. The C57 mice were euthanized, and their hind legs were removed and placed in 75% alcohol

for 5 min. Soft tissues were removed, a 26-G needle attached to a 1-cc syringe was inserted into the bone marrow cavity to wash out cells with RPMI-1640 (Gibco, Foster city, CA, USA) containing 10% fetal bovine serum (FBS; Gibco) until the bone became white. The medium with bone marrow cells was passed through a cell strainer with 70- μ m pores (Merck Millipore, Billerica, MA, USA) into a 50-ml centrifuge tube. The cells were centrifuged for 10 min at 1,350 rpm, and the supernatant was subsequently discarded. Next, the cells were resuspended in 5 ml RPMI-1640 supplemented with 10% FBS and centrifuged for 10 min at 1,350 rpm. The cells were resuspended in RPMI-1640 containing 10% FBS and 1% penicillin-streptomycin (Gibco) to a final concentration of 5×10^6 cells/ml. Then, 1×10^7 cells/well were plated in 6-well plates. M-CSF (10 ng/ml; R&D Systems, Minneapolis, MN, USA) was added to the medium for M0 cell formation, or M-CSF, IL-4, IL-13, and IL-10 (10 ng/ml; R&D Systems) were added to the medium for TAM formation. The cells were incubated at 37°C in a humidified, 5% CO₂ incubator, and medium containing cytokines was changed daily for 4 days.

Cell line. ID8 mouse EOC cells were purchased from Fuheng Biotechnology Co., Ltd. (Shanghai, China) and maintained in DMEM (Gibco) medium supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were cultured at 37°C in a humidified, 5% CO₂ incubator.

TAM CM preparation, transfer and coculture. The TAM CM transfer was performed according to Richards *et al* (9). TAM medium was centrifuged at 2,500 rpm for 30 min to remove cell debris and then mixed with complete DMEM at the ratio of 1:1. Complete RPMI-1640, and DMEM was mixed at the ratio of 1:1 as normal medium. TAM CM or normal medium was transferred to plates with ID8 cells for coculture daily. IGF1 neutralizing antibody (monoclonal, rabbit to mouse; Abcam, Cambridge, MA, USA) was dissolved in PBS and linsitinib (Selleck, Houston, TX, USA) was dissolved in DMSO before use.

RNA collection and quantitative real-time PCR (qRT-PCR). Total RNA was extracted from macrophages and ID8 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed using a Prime Script™ II 1st Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. qRT-PCR was performed using Talent qPCR PreMix (SYBR-Green) (Tiangen Biotech Co., Ltd., Beijing, China) and the StepOnePlus™ Real-time PCR system (Invitrogen) according to the manufacturer's instructions. The following primer sequences were used: CD204 forward, 5'-TGGAGGAGAGAATCGAAAGCA-3' and reverse, 5'-CTGGACTGACGAAATCAAGGAA-3'; IGF1 forward, 5'-CACATCATGTCTCTTCACACC-3' and reverse, 5'-GGAAGCAACACTCATCCACAATG-3'; and GAPDH forward, 5'-AGGTCGGTGTGAACGGATTG-3' and reverse, 5'-GGGTCGTTGATGGCAACA-3'. The final concentration of all reagents was 2X Talent qPCR PreMix (with SYBR-Green I), 1X; 50X ROX Reference Dye, 5X; and forward and reverse primers: 0.3 μ M. The PCR reactions cycling conditions included an initial cycle at 95°C for 15 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 15 sec.

The data were calculated using the $2^{-\Delta\Delta C_q}$ method, with GAPDH as an internal normalization control.

Western blot analyses. Total protein was collected from M0 cells, TAMs, ID8 cells treated with or without TAM CM or linsitinib using RIPA lysis buffer (Beyotime, Shanghai, China). A BCA kit (Thermo Fisher, Rockford, IL, USA) was used for protein quantification according to the manufacturer's instructions. Protein was separated on 10% SDS-PAGE gels (EpiZyme, Shanghai, China) at a consistent voltage of 80 V in the stacking gel and 120 V in the separating gel and subsequently transferred onto polyvinylidene difluoride membranes at consistent current of 300 mA for 80 min. The membranes were blocked in TBST buffer with 5% bovine serum albumin (BSA) for 2 h at room temperature. Then, the membranes were incubated with the following primary antibodies at 4°C overnight: IGF1 (cat. no. ab9572; 1:1,000, monoclonal, rabbit to mouse; Abcam), CD204 (cat. no. ab15707; 1:1,000, polyclonal, rabbit to mouse; Abcam), IGF1R (cat. no. 9750; 1:1,000, monoclonal, rabbit to mouse; Cell Signaling Technology, Inc., Danvers, MA, USA), phospho-IGF1R (cat. no. 3918; 1:1,000, monoclonal, rabbit to mouse; Cell Signaling Technology, Inc.), Akt (cat. no. 4685; 1:1,000, monoclonal, rabbit to mouse; Cell Signaling Technology, Inc.), phospho-Akt (cat. no. ab81283; 1:1,000, monoclonal, rabbit to mouse; Abcam), Erk (cat. no. 4695; 1:1,000, monoclonal, rabbit to mouse; Cell Signaling Technology, Inc.), phospho-Erk (cat. no. 4370; 1:1,000, monoclonal, rabbit to mouse; Cell Signaling Technology, Inc.), GAPDH (cat. no. AB0036; 1:5,000; AB2000, monoclonal, rabbit to mouse; Abways, Shanghai, China), and the HRP-conjugated rabbit secondary antibody (cat. no. 16402-1-AP; 1:5,000; Proteintech, Wuhan, China) was used to incubate the membranes at room temperature for 1 h. The protein signals were detected using enhanced chemiluminescent HRP substrate (Merck Millipore).

Immunohistochemistry (IHC). Ovarian benign tumor specimens (patient ages ranged from 21 to 70 years) and EOC specimens (patient ages ranged from 33 to 74 years) used for tissue microarrays were obtained from the specimen repository of Shanghai First Maternity and Infant Hospital, after obtaining consent from each patient and the Ethics Committee of Tongji University. IHC was performed using an IGF1 primary antibody (1:125, monoclonal, rabbit to mouse; Abcam). Specimens (4- μ m thick) were subjected to heat-induced epitope retrieval and then dewaxed in xylene and hydrated through a graded series of alcohol. The specimens were incubated in 0.3% H₂O₂ for 30 min to inactivate the endogenous peroxidase. Next, the specimens were incubated with 1% goat serum (Invitrogen) for 20 min at room temperature, followed by incubation with IGF1 antibody overnight at 4°C and then rabbit secondary antibody for 1 h at 37°C. A Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) was used according to the manufacturer's instructions for color development. The specimens were counterstained with hematoxylin and then dehydrated through a graded series of alcohol, cleared in xylene and mounted. Tumor cells with immunohistochemical expression in the cytoplasm were regarded as IGF1-positive, and the expression level was determined using the IRS system by multiplication of the staining intensity (0, no; 1, weak;

2, moderate; and 3, strong staining) and the percentage of positively stained cells (0, no staining; 1, <10% of cells; 2, 11-50% of cells; 3, 51-80% of cells; and 4, >81% of cells stained), according to Remmele and Stegner (38). The total score per sample therefore ranged from 0 to 12; a score <6 indicates low expression, whereas a score of 6-12 indicates high expression. The slides were examined in a blinded manner by two experienced investigators.

MTS proliferation assay. ID8 cells (1.5×10^3) were seeded onto 96-well plates and cultured with 100 μ l of medium for 72 h. Then, 20 μ l of MTS Solution Reagent (PR Omega Biosciences, USA) was added to each well followed by incubation at 37°C in 5% CO₂ for 2 h. The absorbance was measured at 490 nm using a 96-well plate reader. Each group had six duplicates.

Cell migration assays. Transwell chambers (Corning, Glendale, AZ, USA) containing 8- μ m inserts were used to measure the migration of tumor cells. ID8 cells (5×10^4 cells) in 200 μ l DMEM containing 2% FBS was plated in the top chambers. The bottom of the wells was filled with 800 μ l complete DMEM containing 10% FBS. Calcein AM (Invitrogen) was used to stain the cells in the bottom of the filter membrane. The images of cells were captured using a fluorescence microscope at x100. ImageJ software was used to count the cells that had migrated to the bottom of the filter membrane. Each group had two duplicates, and five images of different fields for each duplicate were captured.

Statistical analysis. All experiments were independently performed three or more times. Statistical analysis was performed using SPSS 19.0 (SPSS, Inc., Chicago, IL, USA). The data are expressed as the means \pm SEMs or means \pm SDs and analyzed using Student's t-test or the Mann-Whitney test. The correlation of IGF1 expression and clinical and pathological factors was analyzed using the Chi-square test or the Fisher's exact test. P-value <0.05 was considered statistically significant.

Results

The TAM model is established using mouse bone marrow monocytes. TAMs have been characterized as a popularized M2 macrophage phenotype different from the M1 type, which differentiates through a classic pathway. In the present study, a TAM model was established using isolated primary mouse bone marrow cells. Monocytes were induced to become undifferentiated M0 cells using MCSF. To induce M0 cells to differentiate into TAMs, IL-10, IL-13, and IL-4 were continuously used.

To determine whether the TAM model was successful, analyses of cell morphology and the expression level of the TAM-specific marker CD204 was used. As shown, TAMs were larger and more stacked in morphology (Fig. 1A). The expression of CD204 was higher in TAMs at both the mRNA (Fig. 1B) and protein levels (Fig. 1C).

TAM CM enhances the proliferation and migration of ID8 cells. As TAMs are associated with the enhanced progression of various cancers, including OC, and the number of TAMs in

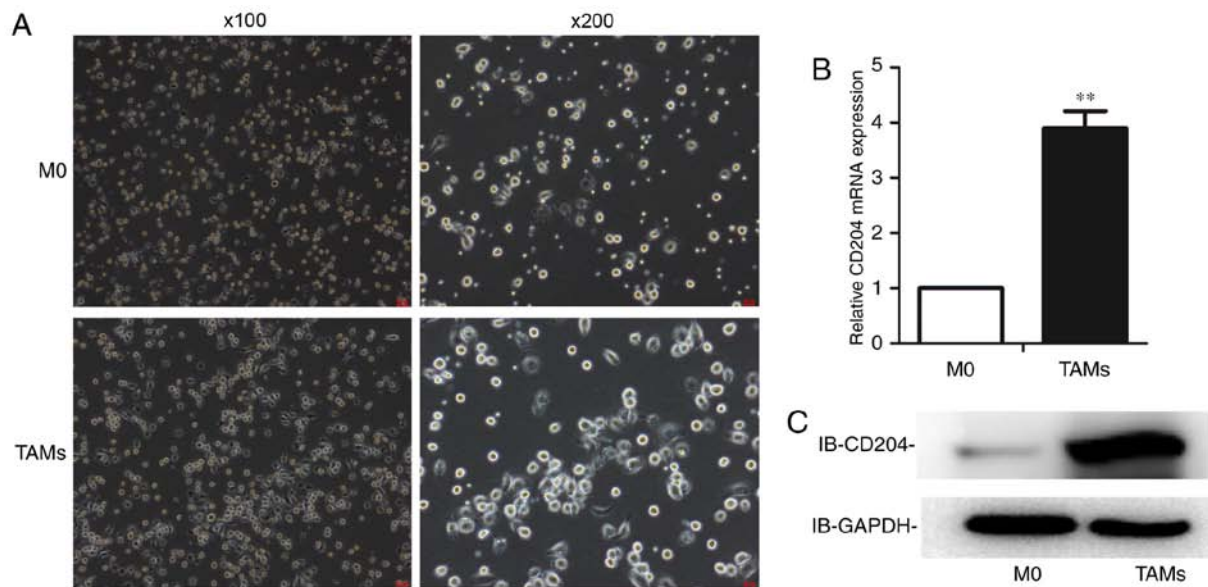


Figure 1. Mouse bone marrow monocytes are induced to become TAMs. (A) Morphology parameters, including shape, length and size, were compared between M0 cells and TAMs using a microscope. (B and C) Expression level of the TAM-specific marker CD204 was compared between M0 cells and TAMs using qRT-PCR and western blotting. The bar graphs represent the means \pm SDs of three independent experiments; ** $P < 0.001$. TAMs, tumor-associated macrophages. qRT-PCR, quantitative real-time PCR. Scale bar, 10 μ m.

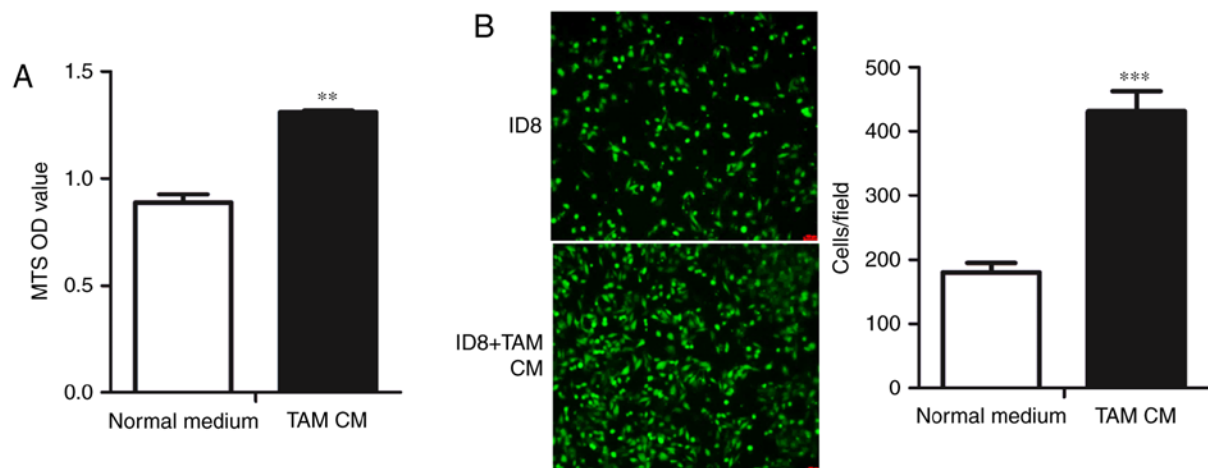


Figure 2. TAM CM promotes the proliferation and migration of EOC cells. (A) ID8 cells were cultured with normal medium or TAM CM for 72 h. A cell proliferation assay (MTS assay) was performed to observe the effect of TAMs on the proliferation of ID8 cells. (B) After growing in normal medium or TAM CM for 48 h, a cell migration assay was performed to assess the effect of TAMs on the migration of ID8 cells. The cells were counted 16 h after plating in Transwell chambers. The bar graphs represent the means \pm SDs of three independent experiments; ** $P < 0.01$, *** $P < 0.001$. TAMs, tumor-associated macrophages. CM, conditioned medium. EOC, epithelial ovarian cancer. Scale bar, 100 μ m.

OC specimens is correlated with poor prognosis, we assessed whether TAMs similarly affect the physiological activity of mouse EOC cells. We first studied the effect of TAMs on the proliferation of ID8 cells, observing that TAM CM promoted the proliferation of ID8 cells (Fig. 2A). We next determined whether TAMs could change the migration of ID8 cells, and the results showed that the migration of ID8 cells treated with TAM CM was significantly increased compared with the control group (Fig. 2B). Taken together, these data showed that TAMs could accelerate the proliferation and migration of ID8 cells.

TAMs express higher levels of IGF1 than M0 cells. In a previous study, we performed a gene chip analysis to compare

the expression profile of human TAMs and M0 cells, and observed that TAMs expressed significantly higher levels of IGF1 than M0 cells. We identified this result at both the mRNA and protein levels in mouse TAMs. Consistent with the gene chip analysis, the expression level of IGF1 was higher in mouse TAMs at both the mRNA (Fig. 3A) and protein levels (Fig. 3B).

Human EOC specimens express higher levels of IGF1. The IGF1 level is higher in prostate cancer and in gastric cancer compared with corresponding benign tumors (24,25); thus, we examined whether EOC would have a similar phenomenon. First, we used western blot analysis to assess the level of IGF1 in fresh ovarian specimens, and observed that EOC specimens

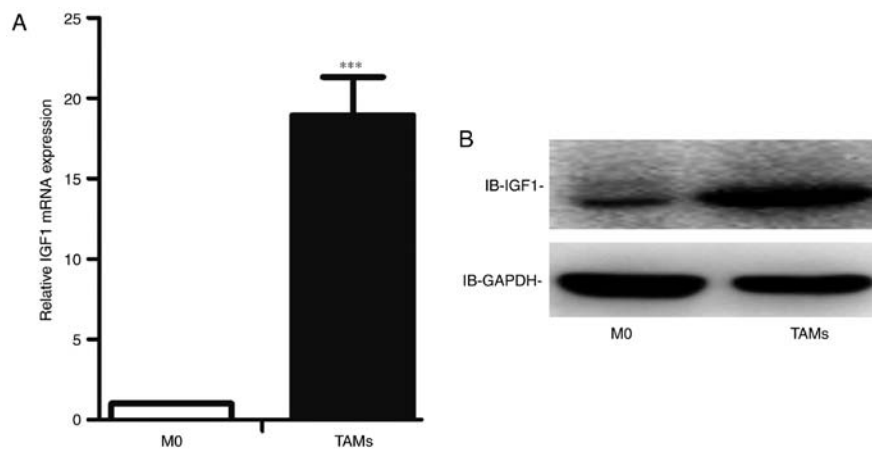


Figure 3. TAMs express higher levels of IGF1. (A and B) To verify the result of the gene chip analysis, qRT-PCR was used to compare expression levels of IGF1 between M0 cells and TAMs at the mRNA level, and western blotting was used to compare IGF1 expression at the protein level. The bar graphs represents the means \pm SDs of three independent experiments; *** P <0.001. TAMs, tumor-associated macrophages. qRT-PCR, quantitative real-time PCR. IGF1, insulin-like growth factor-1.

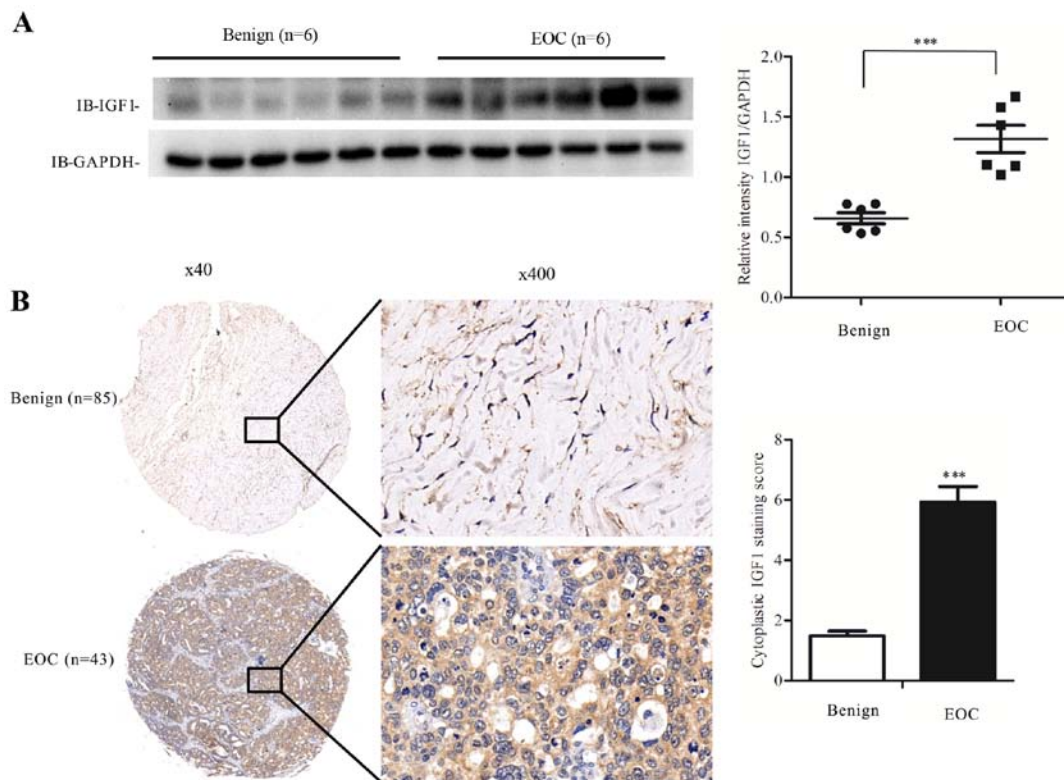


Figure 4. Human EOC specimens express higher levels of IGF1 than ovarian benign tumor tissues. (A) Western blotting was performed to compare the IGF1 level in benign ovarian tumor and EOC specimens. (B) Immunohistochemistry was used to determine the expression of IGF1 in benign ovarian tumor and EOC tissue microarrays. The bar graphs represents the means \pm SDs (A) or means \pm SEMs (B) of three independent experiments; *** P <0.001. EOC, epithelial ovarian cancer. IGF1, insulin-like growth factor-1.

expressed higher levels of IGF1 compared with levels in the benign ovarian tumors (Fig. 4A).

Next, we used IHC staining to assess the expression level of IGF1 in tissue microarrays of benign ovarian tumors and EOC. The results showed that the mean IRS score of EOC specimens was significantly higher than that of the benign ovarian tumors (Fig. 4B). Then, we classified the EOC specimens into high and low expression groups, and used the Chi-square test or Fisher's exact test to identify the correlation

between clinical and pathological factors and IGF1 expression levels. The median age of the patients was 56 years. Patients with a high IGF1 expression were in a more advanced stage and tended to have undergone liver metastasis more frequently (Table I). These results indicated that high IGF1 expression may enhance the progression of EOC.

TAM CM upregulates the expression of IGF1 in ID8 cells. Based on the preceding results, we determined whether TAMs

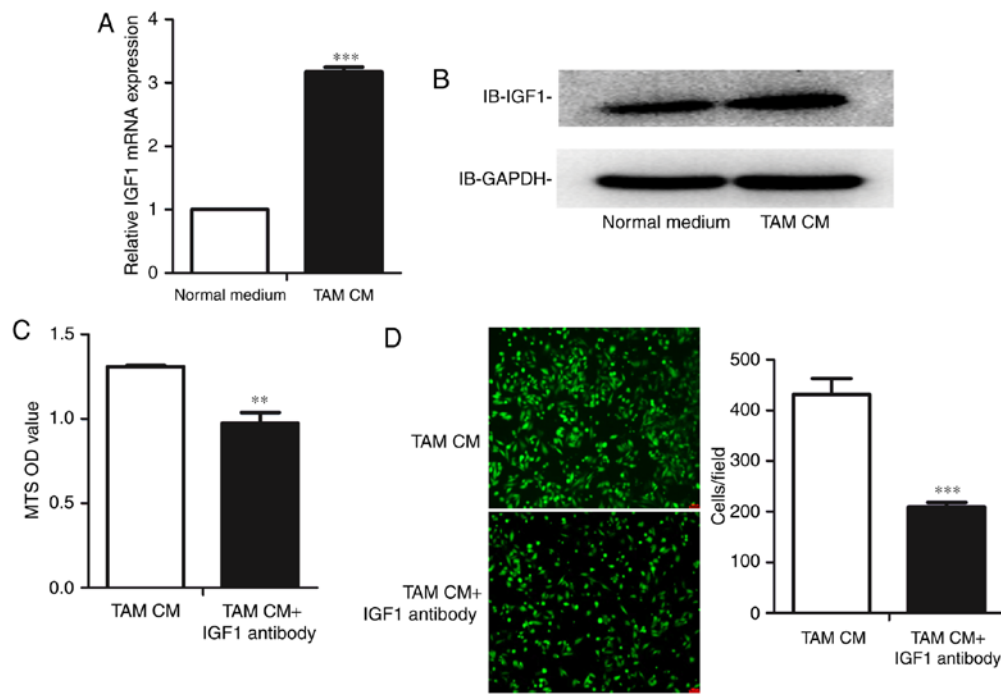


Figure 5. TAM CM upregulates IGF1 in ID8 cells, and blockade of IGF1 reverses the increase in proliferation and migration. (A and B) ID8 cells were cultured with normal medium or TAM CM for 72 h. qRT-PCR was used to determine the effect of TAMs on expression of IGF1 at the mRNA level, whereas western blotting was used to determine IGF1 expression at the protein level. (C) ID8 cells were cultured in TAM CM or TAM CM containing IGF1 neutralizing antibody (2 μ g/ml) for 72 h. A cell proliferation assay (MTS assay) was used to measure the proliferation. (D) After culturing in TAM CM or TAM CM containing IGF1 neutralizing antibody for 48 h, a cell migration assay was performed to assess the effect of TAMs on the migration of ID8 cells. The cells were counted at 16 h after plating in the Transwell chambers. The bar graphs represent the means \pm SDs of three independent experiments; * P <0.01, *** P <0.001. TAMs, tumor-associated macrophages. CM, conditioned medium. IGF1, insulin-like growth factor-1. Scale bar, 100 μ m.

Table I. Correlation of IGF1 expression level with clinical and pathological factors.

Parameters	n	Low expression	High expression	P-value
Age years)				0.749
≥ 56	21	9	12	
<56	20	9	11	
Tumor size				0.169
≥ 7	26	9	17	
<7	14	8	6	
Pathologic grade				0.848
I+II	12	6	6	
III	13	6	7	
Clinical stage				0.020
I+II	14	10	4	
III+IV	27	9	18	
Ascites				0.951
Yes	23	10	13	
No	18	8	10	
Liver metastasis				0.036
Yes	11	2	9	
No	29	16	13	

P-value was generated by Chi-square test or Fisher's exact test, and P <0.05 was considered as statistically significant. As clinical information was not available for each patient, the total number for each factor was different. IGF1, insulin-like growth factor-1.

could alter the proliferation and migration of mouse OC cells by upregulating the tumor-promoting gene IGF1. Therefore, we treated ID8 cells with TAM CM. As expected, the expression level of IGF1 in ID8 cells increased following culture with TAM CM at both the mRNA (Fig. 5A) and protein levels (Fig. 5B).

Blockade of IGF1 reverses the alteration of proliferation and migration of ID8 cells. After observing that TAMs accelerate the proliferation and migration of ID8 cells and simultaneously upregulate IGF1, we finally assessed whether the blockade of IGF1 in TAM CM would reverse the changes in proliferation and migration. An IGF1 neutralizing antibody was added to TAM CM to block the IGF1 pathway. We observed that the blockade of IGF1 reduced the proliferation of ID8 cells (Fig. 5C). We then determined whether the IGF1 neutralizing antibody had the same effect on the migration of ID8 cells, observing that blockade of IGF1 reversed the increase in ID8 cell migration (Fig. 5D).

Inhibition of the activation of IGF1R suppresses the proliferation and migration of ID8 cells exposed to TAM CM. To validate the value of IGF1 pathway inhibition in EOC therapy, we used the IGF1R inhibitor linsitinib to inhibit the IGF1 pathway. As expected, the phosphorylation of IGF1R, Akt and Erk was significantly inhibited (Fig. 6A). Additionally, the proliferation and migration of ID8 cells were both significantly suppressed by linsitinib (Fig. 6B and C).

Taken together, these data suggest that IGF1 may be a key regulator by which TAMs promote the proliferation and

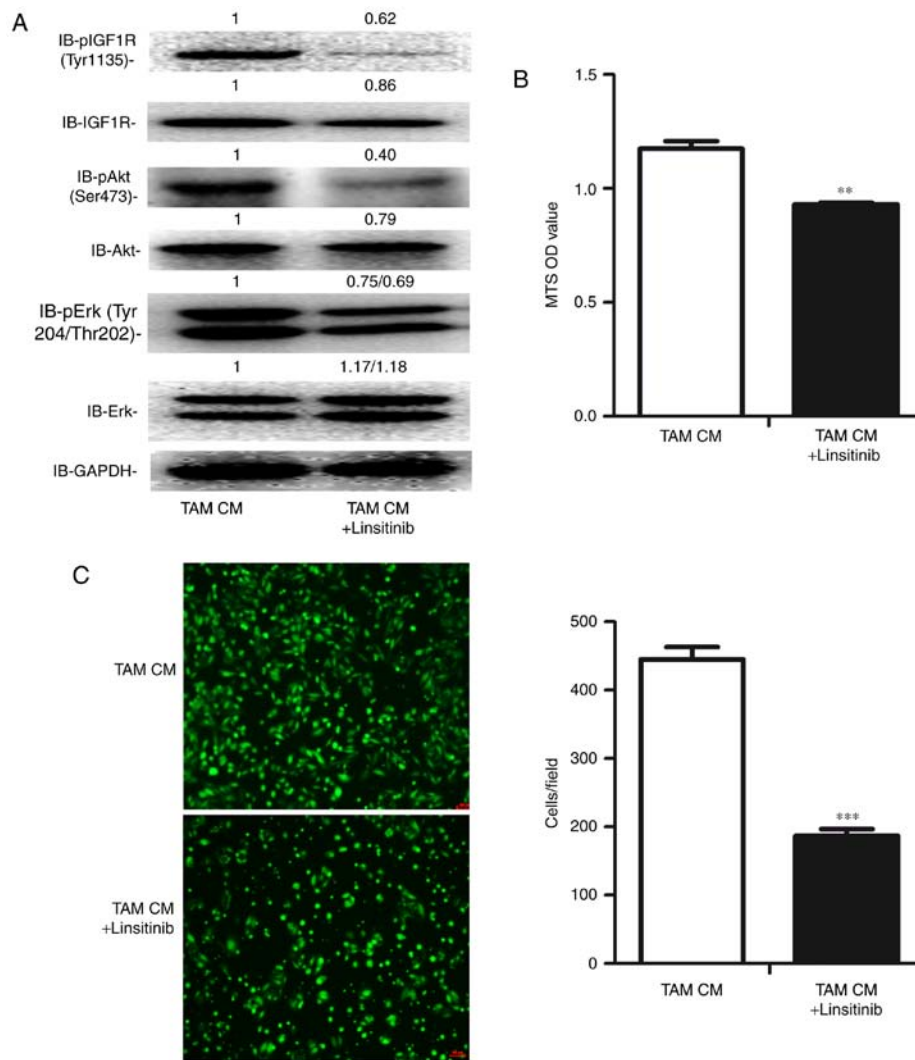


Figure 6. Inhibition of IGF1R suppresses the proliferation and migration of ID8 cells exposed to TAM CM by decreasing phosphorylated IGF1R. (A) ID8 cells were cultured in TAM CM for 48 h, and linsitinib (5 μ M) was added to the medium for 24 h. Western blotting was used to determine the protein expression of total and phosphorylated IGF1R and Akt and Erk. (B) ID8 cells were cultured in TAM CM or TAM CM containing linsitinib for 72 h (5 μ M). A cell proliferation assay (MTS assay) was used to measure the proliferation. (C) After growing in TAM CM or TAM CM containing linsitinib for 48 h, a cell migration assay was performed to assess the effect of IGF1R inhibition on the migration of ID8 cells. The cells were counted at 16 h after being plated in Transwell chambers. The bar graphs represent the means \pm SDs of three independent experiments; ** P <0.01, *** P <0.001. IGF1R, insulin-like growth factor-1 receptor. TAMs, tumor-associated macrophages. CM, conditioned medium. Scale bar, 100 μ m.

migration of OC cells, indicating the potential of IGF1 inhibition in EOC therapy.

Discussion

In the present study, we showed that IGF1 is expressed at higher levels in mouse TAMs than in M0 cells, TAMs were able to enhance the proliferation and migration of ID8 cells by upregulating IGF1, and inhibition of the IGF1R pathway effectively suppressed the proliferation and migration of ID8 cells exposed to TAM CM.

We established a mouse TAM model and observed that TAM CM accelerated the proliferation and migration of ID8 cells, supporting the role of TAMs in the progression of OC (Figs. 1 and 2). The TME is a supportive and receptive tissue microenvironment undergoing a series of molecular and cellular changes to form metastatic-designated sites, or the fertile soil in preparation for metastatic tumor cell seed

colonization, thus supporting tumor settlement in distant organs and promoting tumor metastasis (6-8). As the most common immune cells in the TME, TAMs infiltrate the TME in OC in large numbers and play an important role in the formation of the OC TME and education of OC cells to develop to become more malignant by secreting various factors, such as IL-6 and IDO (16,18,19,39,40). Indeed, therapeutic methods targeting TAMs have been demonstrated as effective for controlling OC. An inhibitor of IDO, which is associated with the formation of TAMs, can control the growth of OC *in vivo* (40). An MCSF inhibitor was found to reduce the infiltration of TAMs, and promote the survival of peritoneal blood vessels, thereby reducing the production of ascites in mice transplanted with OC cells (41).

IGF1 is a cancer progression-related gene that is associated with the growth, metastasis and chemoresistance of various cancers (23-27). In the present study, we confirmed that TAMs significantly expressed higher IGF1 levels than M0 cells as

demonstrated in a previous gene chip analysis (Fig. 3). More importantly, the level of IGF1 was high in EOC specimens and was related to more advanced clinical stage and liver metastasis (Fig. 4; Table I). Thus, we demonstrated that TAMs promoted the progression of EOC by upregulating IGF1. As expected, these results showed that TAM CM upregulated IGF1, whereas an IGF1 neutralizing antibody reversed the alteration of proliferation and migration (Fig. 5). Additionally, these results provide evidence that IGF1 may be a key factor in the promotion of EOC progression through TAMs, explaining its high expression in EOC. Finally, we propose that the mechanism of the upregulation of IGF1 in ID8 cells through TAMs may involve exosomes, which could transfer microRNAs, mRNAs, DNA fragments and proteins, particularly miRNA, from donor cells to recipient cells (42) or secrete cytokines, which also play an important role in the communication between different cells (43). However, these hypotheses need further experimental validation.

Targeting the IGF1 pathway has been demonstrated as effective in the therapy of various cancers (28,44). A typical example is that IGF1R inhibitors decreased the viability of chronic lymphocytic leukemia cells in a microenvironment context (28). Theranostic nanoparticles IGF1-IONP-Dox were found to significantly inhibit the growth of pancreatic tumors (44). Indeed, knockdown of IGF-1 using siRNA decreased the migration and invasion of ES-2 cells (45). In the present study, we used the IGF1R inhibitor linsitinib to block the phosphorylation activation of the IGF1/IGF1R/Akt and IGF1/IGF1R/Erk pathways. Remarkably, IGF1 pathway inhibition reduced the proliferation and migration of ID8 cells, even those exposed to TAM CM, suggesting that the IGF1 pathway is potentially involved in EOC therapy, even in the TME (Fig. 6). However, whether IGF1 is effective *in vivo* and whether it will be more effective combined with other targets requires additional studies.

In conclusion, these results showed that TAMs play an active role in promoting the proliferation and migration of EOC, and in upregulating the expression of IGF1, whereas the blockade of IGF1 reversed the changes in proliferation and migration. These findings may represent a new mechanism related to the growth and metastasis of EOC, and currently available IGF1 inhibitors may be potential tools for EOC intervention.

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