Neoplasma 2025; 72(1–2): 106–117

doi:10.4149/neo_2025_240820N357

CCL18 derived from M2-polarized tumor-associated macrophages promotes endometrial cancer progression by activating the TWIST1/HMGA1 axis

Hongxia LI*, Jiansheng LI

Department of Biomedical Engineering, Third Hospital of Shanxi Medical University, Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Tongji Shanxi Hospital, Taiyuan, Shanxi, China

**Correspondence: hongxiali@sina.com*

Received August 20, 2024 / Accepted April 9, 2025

CCL18, originating from M2-polarized tumor-associated macrophages (M2-TAMs) is recognized for its vital role in endometrial cancer (EC) development. Nonetheless, its precise mechanisms remain largely undefined. The primary objective of this research was to elucidate the underlying mechanism of M2-TAM-isolated CCL18 in EC progression. TWIST1 and HMGA1 expressions were assessed in EC tissues and cells by qRT-PCR or western blotting. M2 macrophages were differentiated from human monocyte THP-1 cells, and characterized via flow cytometry and western blotting. CCL18 levels were evaluated using western blotting and ELISA assay. CCK8, Transwell, and wound healing assays were employed to assess EC cell vitality, invasion, and migration, respectively, while western blotting was utilized to measure related protein markers. The binding relationship between TWIST1 and HMGA1 was validated via ChIP and dual-luciferase reporter assays. TWIST1 and HMGA1 were increased in EC tissues and cells. After being treated with M2-TAM-isolated CCL18, EC cell vitality, migration, and invasion were enhanced. Additionally, CCL18 derived from M2-TAM upregulated TWIST1 levels in EC cells. Further mechanistic analyses unveiled that TWIST could positively regulate HMGA1 in EC cells. Notably, HMGA1 knockdown restrained the malignancy of EC cells, which was reversed by TWIST1 overexpression. M2-TAM-isolated CCL18 facilitated EC progression by activating the TWIST1/HMGA1 axis. These observations might offer new directions for developing targeted curative interventions for EC.

Key words: endometrial cancer; M2-polarized tumor-associated macrophage; CCL18; TWIST1; HMGA1

Endometrial cancer (EC) is an epithelial malignancy that develops in the endometrium and ranks among the top three most prevalent cancers of the female reproductive system [1]. Despite recent advances in early diagnosis and treatment, individuals with advanced EC experience recurrence or metastasis [2]. The statistics on the 5-year survival rate of patients with advanced EC are indeed concerning, with only 17% of patients surviving for 5 years after diagnosis [3]. Therefore, it is crucial to enhance our comprehension of the pathological processes of EC to develop more effective therapeutical approaches.

Recently, there has been a growing recognition that the tumor microenvironment (TME) plays a pivotal role in the onset and development of EC [4, 5]. Within the TME, tumor-associated macrophages (TAMs) are the most predominant immune cells and serve as crucial effector cells [6, 7]. Generally, TAMs are split into two primary subtypes: the M1 phenotype, known for its anti-tumor properties, and the M2 phenotype, with its pro-tumor characteristics [8]. Notably, TAMs often obtain a polarized M2 phenotype in various tumors, which correlates with poor prognosis [9]. A negative association has been defined between M2-polarized TAM (M2-TAM) infiltration and EC prognosis [10]. Moreover, current research findings have proven that the deterioration of EC could be linked to the polarization of M2 macrophages [11, 12]. Accordingly, M2-TAMs are likely pivotal mediators in the intricate interplay between EC cells and their microenvironment, warranting further investigation into the underlying mechanisms.

To our knowledge, cytokines and chemokines released by TAMs are crucial participators in cancer progression [13]. Among these, the C-C motif chemokine ligand (CCL) 18 is a vital chemokine stemming from M2-TAMs [14]. CCL18 has been determined to exert pro-tumor effects on diverse human cancers, such as gastric cancer [15], oral cancer [16], hepatoblastoma [17], and breast cancer [13]. In EC, a positive

relation was documented between CCL18 expression and malignancy degree [18]. Intriguingly, a prior investigation demonstrated that M2-TAM-derived CCL18 could foster epithelial-mesenchymal transition (EMT) in EC by triggering the activation of the PI3K/AKT/mTOR axis [19]. Additionally, the study noted a positive regulatory effect of CCL18 on twist family bHLH transcription factor (TWIST)1, a pivotal transcriptional factor of EMT [19]. Indeed, TWIST1 has been recognized for its capacity to induce EMT and propel tumor progression in various cancers, including EC [20–24]. Nonetheless, the downstream mechanisms of TWIST1 modulated by M2-TAM-derived CCL18 in EC pathogenesis remain largely unexplored.

Herein, we delved into the possible role of the M2-TAM-derived CCL18/TWIST1 axis in EC. Furthermore, utilizing bioinformatics analysis, we pinpointed high mobility group AT-hook (HMGA) 1 as the downstream target of TWIST1, which has been reported to act as an oncogene in EC [25–27]. Our research unveiled a novel pathway regulated by CCL18 in EC, specifically highlighting the promotive effect on TWIST1/HMGA1 axis. To sum up, our observations offer fresh insights into the molecular mechanisms of EC and furnish innovative targeted approaches for this malignancy treatment.

Patients and methods

Clinical specimen collection. Five sets of EC tumors and neighboring normal tissues were acquired from EC patients who received resection without preoperative chemotherapy or radiotherapy at Shanxi Bethune Hospital. All participants provided informed consent, and this research received approval from the Ethical Committee of Third Hospital of Shanxi Medical University, Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Tongji Shanxi Hospital (Approval No. SBQLL-2023-078).

The tissue samples were stored in liquid nitrogen at a temperature of –80 °C for subsequent investigations.

Cell culture. Human EC cell lines RL952, AN3 CA, HEC-1-A, and KLE, along with monocyte THP-1 cells, were procured from American Type Cell Culture (Rockville, MD, USA), while human EC cell line Ishikawa and human normal endometrial epithelial cells (hEECs) were acquired from PriCells (Wuhan, China). Ishikawa and RL952 cells, as well as hEECs, were cultivated in DMEM/F12 (Gibco, Carlsbad, CA, USA). HEC-1-A cells were maintained with high-glucose DMEM (Gibco). RPMI-1640 medium (Procell, Wuhan, China) was used for cultivating AN3 CA, KLE, and THP-1 cells. Each medium was supplemented with 10% fetal bovine serum (Procell) and 1% penicillin-streptomycin (Procell), and maintained at a temperature of 37 °C in an environment enriched with 5% CO_2.

Macrophage polarization induction. As per the previous method [28], human monocyte THP-1 cells were stimulated to differentiate into M0 and M2 macrophages. To induce M0 macrophage, THP-1 cells underwent stimulation with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Subsequently, the transformation into M2 macrophages was accomplished by exposing M0 macrophages to 20 ng/ml IL-4 (PeproTech, Rocky Hill, NJ, USA) and 20 ng/ml IL-13 (PeproTech) for another 24 h. Following induction, the levels of M2 macrophage markers (CD206 and CD163) were evaluated using flow cytometry and western blot analysis.

Cell transfection. All overexpressed plasmids (oe-CCL18: NM_002988.4, oe-TWIST1: NM_000474.4), short hairpin RNAs (sh-CCL18, sh-HMGA1), as well as the control plasmids for comparison (sh-NC and oe-NC), were obtained from GeneChem in Shanghai, China. Stable transfection was performed via Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA). Following a period of 48 h, the efficiency of the transfection process was determined through quantitative real-time polymerase chain reaction (qRT-PCR) or western blotting. The sequences of sh-CCL18 and sh-HMGA1 are: CL18: AATTGCAATAAGAAGTGGGTCCAGAATCA-AGAGttctggacccacttcttattgTTTTTT; sh-HMGA1: AATTG-CAACTCCAGGAAGGAAACCAATCAAGAGttg-gtttccttcctggagttgTTTTTT;

Flow cytometry. M2 macrophage markers were evaluated through flow cytometry. Briefly, M2 macrophages (1×10^6) were suspended in phosphate buffer saline (100 μl; Beyotime, Shanghai, China), then incubated with anti-CD206 (5 μl/test; #12-2069-42, eBioscience, San Diego, CA, USA) and anti-CD163 (5 μl/test; #17-1639-42, eBioscience) antibodies at 4 °C for 30 min. The levels of CD206 and CD163 were determined through flow cytometry analysis utilizing a cytometer from BD Biosciences in Franklin Lakes, NJ, USA.

ELISA. Following the manufacturer's directions, a CCL18 ELISA kit (#ml058388, mlbio, Shanghai, China) was utilized to evaluate CCL18 levels in cell supernatant. The optical density (OD) readings were measured at a wavelength of 450 nm using a microplate reader (model DR-3518G, manufactured by Hiwell Diatek in Wuxi, China).

Cell counting kit-8 (CCK-8) assay. The viability of EC cells was evaluated using a CCK-8 assay. HEC-1-A and Ishikawa cells were inoculated into 96-well plates at a density of 8×10^3cell/well and incubated at 37 °C with 5% CO_2 for a duration of 24 h. 10 μl CCK-8 solution (#C0037, Beyotime, Shanghai, China) was introduced into every well, followed by a 2 h incubation period for the cells. Subsequently, the optical density values were measured at a wavelength of 450 nm using the microplate reader with the model number DR-3518G from Hiwell Diatek.

Wound healing assay. HEC-1-A and Ishikawa cells were placed in 6-well plates with a density of 3×10^5 cells/well and cultivated at 37 °C overnight. Following this, the cell monolayer was scratched using a 200 μl pipette tip, and cells were subjected to 24 h incubation in DMEM without serum at 37 °C with 5% CO_2. Ultimately, the cells were observed under a microscope at 0 and 24 h (CKX53, Olympus, Tokyo, Japan).

Transwell assay. The upper Transwell chamber was primed with Matrigel (200 μg/ml) and serum-free medium (200 μl), and HEC-1-A and Ishikawa cells (2×10^5) were seeded into the upper Transwell chamber. The lower chamber was filled with a complete medium (800 μl) as a chemoattractant. Following 48 h incubation, cells in the upper side of the membrane were eliminated with a cotton swab. In the meantime, cells located at the lower side were treated with 4% formaldehyde for 10 min followed by a 30 min staining session using 0.01% crystal violet at a temperature of 37 °C. Subsequently, the cells were captured under the microscope (CKX53, Olympus).

qRT-PCR. Cells were processed to isolate total RNA with the assistance of TRIzol reagents (#15596026CN, Invitrogen). Subsequently, reverse transcription was carried out using FastKing-RT SuperMix (#KR118-01, Tiangen, Beijing, China). Then, qRT-PCR (Bio-Rad, CA, USA) was executed on the CFX Connect RT-PCR detection system with SYBR Green PCR Master Mix (#D7507, Beyotime). The expression levels were standardized to GAPDH and quantified using the $2^{-\Delta\Delta CT}$ method. Detailed information regarding the primers utilized can be found in Table 1.

Western blotting. Protein extraction from the tissues or cells was conducted using the cutting-edge Total Protein Extraction Kits (#PP3001, Aidlab Biotech, Beijing, China), The protein concentration was then meticulously assessed with the aid of a cutting-edge bicinchoninic acid kit (Solarbio, Beijing, China). Subsequently, proteins were isolated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beyotime) and migrated to polyvinylidene difluoride membranes (Beyotime). After 1 h blocking with 5% nonfat milk (Beyotime), membranes were incubated with primary antibodies at 4 °C overnight and goat anti-rabbit IgG H&L (HRP) (1:2,000; #ab6702, Abcam, Cambridge, UK) or anti-mouse IgG H&L (HRP) (1:2,000; #ab205719, Abcam) secondary antibody. Enhanced chemiluminescence kits (APPLYGEN, Beijing, China) were employed for membrane visualization, and ImageJ software (National Institutes of Health) was for band density quantification. The utilized primary antibodies included anti-TWIST1 (1:1,000; #ab175430, Abcam), anti-HMGA1 (1:2,000; #ab129153, Abcam), anti-CD206 (1:5,000; #60143-1, Proteintech, Chicago, IL, USA), anti-CD163 (1:5,000; #68218-1, Proteintech), anti-CCL18 (1:1,000; #ab300057, Abcam), anti-MMP-2 (1:2,000; #ab92536, Abcam), anti-Ki-67 (1:5,000; #ab92742, Abcam), anti-MMP-9 (1:1,000; #ab283575, Abcam), and anti-GAPDH (1:5,000; #ab8245, Abcam).

Chromatin immunoprecipitation (ChIP) assay. As per the manufacturer's guidelines, a ChIP assay kit (#P2078, Beyotime) was utilized to identify the interplay between TWIST1 and HMGA1 in EC cells. Chromatin fragments (200–500 bp) were immunoprecipitated using specific antibodies or negative control antibodies. The DNA released from the protein-DNA complex purified by magnetic beads was analyzed by RT-qPCR. In short, cells were treated with Tan 2A (20 μM) at 37 °C for 16 h. In addition, 270 μl of 37% formaldehyde (Sangon Biotech Co., Ltd.) was added to 10 ml of growth medium, and the protein was crosslinked with chromatin at room temperature for 10 min. Then, it was immediately quenched with a final concentration of 125 mM glycine at room temperature for 5 min. The cells were harvested in cold phosphate-buffered saline. The nuclear particles were resuspended in the ultrasound processing buffer. The cell suspension was obtained by ultrasonic treatment (MiSonix Sonictor 3000) on ice with an on/off time of 30/30 s for 10 min. 1 μg IgG (#30000-0-AP, Proteintech) or anti-TWIST1 (#25465-1-AP, Proteintech) antibody bound to magnetic protein beads (CST, #14179, Shanghai, China) at a 4 °C. Subsequently, the protein/DNA complex was cross-linked with separated DNA, followed by analysis of the ChIP-enriched DNA. The primer sequences of ChIP are listed below: Forward: 5'-CTCACCAATCAGCCACACGC-3'; Reverse: 5'-CGCTCGTTAGAGAATTCCGCG-3'.

Table 1. Primer sequences.

Gene	Primer sequences (5' – 3')
TWIST1	Forward: AGCTGAGCAAGATTCAGACCCTCA Reverse: CTGCAGCTTGCCATCTTGGAGT
HMGA1	Forward: CATCCGCATTTGCTACCAGC Reverse: GGGCTCCTTCTGACTCCCTA
GAPDH	Forward: TGCACCACCAACTGCTTAGC Reverse: GGCATGGACTGTGGTCATGAG

Dual-luciferase reporter assays. The designated binding locations of TWIST1 on HMGA1 (HMGA1-WT) and its mutated counterpart (HMGA1-MUT) were strategically incorporated into the firefly luciferase gene housed within the psiCHECK2 vector (Promega, Madison, WI, USA) (Supplementary Table S1). EC cells were grown for 12 h and then co-transfected with either the HMGA1-WT or HMGA1-MUT reporter gene plasmid along with either TWIST1 or a control. Following a 48 h period of incubation, the Dual-Luciferase Reporter Assay System from Promega was employed to evaluate the activities of firefly and Renilla luciferase.

Statistical analysis. The experiments were repeated three times for accuracy. Data are presented as means ± standard deviation, with GraphPad Prism 7 (GraphPad, La Jolla, CA, USA) employed for analysis. Student's t-test was employed to define differences between the two groups, and for evaluating differences among multiple groups, a one-way analysis of variance was conducted, followed by Tukey's post-hoc test. A p-value <0.05 signified a significant discrepancy.

Results

TWIST1 and HMGA1 were elevated in EC tissues and cells. Tumor tissues and adjacent tissues were obtained from patients with EC, we have compared the expressions of TWIST1 and HMGA1 in EC tumor tissues and adjacent

tissues. In comparison with adjacent tissues, it was noted that the levels of TWIST1 and HMGA1 were significantly increased in EC tumor tissues (Figure 1A). Moreover, we also evaluated TWIST1 and HMGA1 levels in hEECs and EC cell lines (Ishikawa, RL952, AN3CA, HEC-1-A, and KLE). Our data demonstrated that TWIST1 and HMGA1 were significantly increased in EC cell lines (Figures 1B, 1C). Collectively, the dysregulation of TWIST1 and HMGA1 might be related to EC progression.

CCL18 derived from M2-TAMs facilitated the malignant phenotypes of EC cells. CCL18 is a well-recognized chemokine secreted by M2-TAMs and plays a critical role in EC development [19], which prompted us to further investigate its specific impact on EC cell proliferation, migration, and invasion. Initially, human monocyte THP-1 cells were utilized to induce M0 and M2 macrophages as per the prior approach [28]. As revealed in Figures 2A and 2B, the number of $CD206^{+}F4/80^{+}$ and $CD163^{+}F4/80^{+}$ cells were markedly higher in the M2 macrophages compared to the M0 macrophages, denoting successful M2 macrophage induction. Next, we examined CCL18 levels in M0 and M2 macrophages and found that CCL18 was elevated in M2 macrophages compared to M0 macrophages (Figure 2B). Consistently, the level of CCL18 was upregulated in the supernatant of M2 macrophages (Figure 2C). Subsequently, according to the results in Figure 1, HEC-1-A and Ishikawa cells with relatively less increases in TWIST1 and HMGA1 levels compared to other EC cell lines (RL952, AN3CA, and KLE) were selected as the research subjects to explore the implications of M2-TAMs-secreted CCL18 for EC development. The cell viability (Figure 2D), migration (Figure 2E), and invasion (Figure 2F) of EC cells were observably enhanced after being treated with M2-TAM culture medium (CM). Moreover, the expressions of cell proliferation-related protein (Ki-67), along with proteins linked to migration and invasion (MMP-2 and MMP-9) were markedly upregulated in EC cells after culturing with M2-TAMs CM (Figure 2G). Altogether, the above results suggested a promotive role of M2-TAMs in EC progression, which was related to CCL18 secretion.

M2-TAM-derived CCL18 promoted EC cells' malignant behaviors by activating TWIST1. M2-TAMs were transfected with sh-CCL18, oe-CCL18, and their respective control plasmids (sh-NC and oe-NC) plasmids, and collected corresponding CM for subsequent experiments. The transfection efficiencies were verified by western blotting, the CCL18 expression was inhibited by sh-CCL18, while

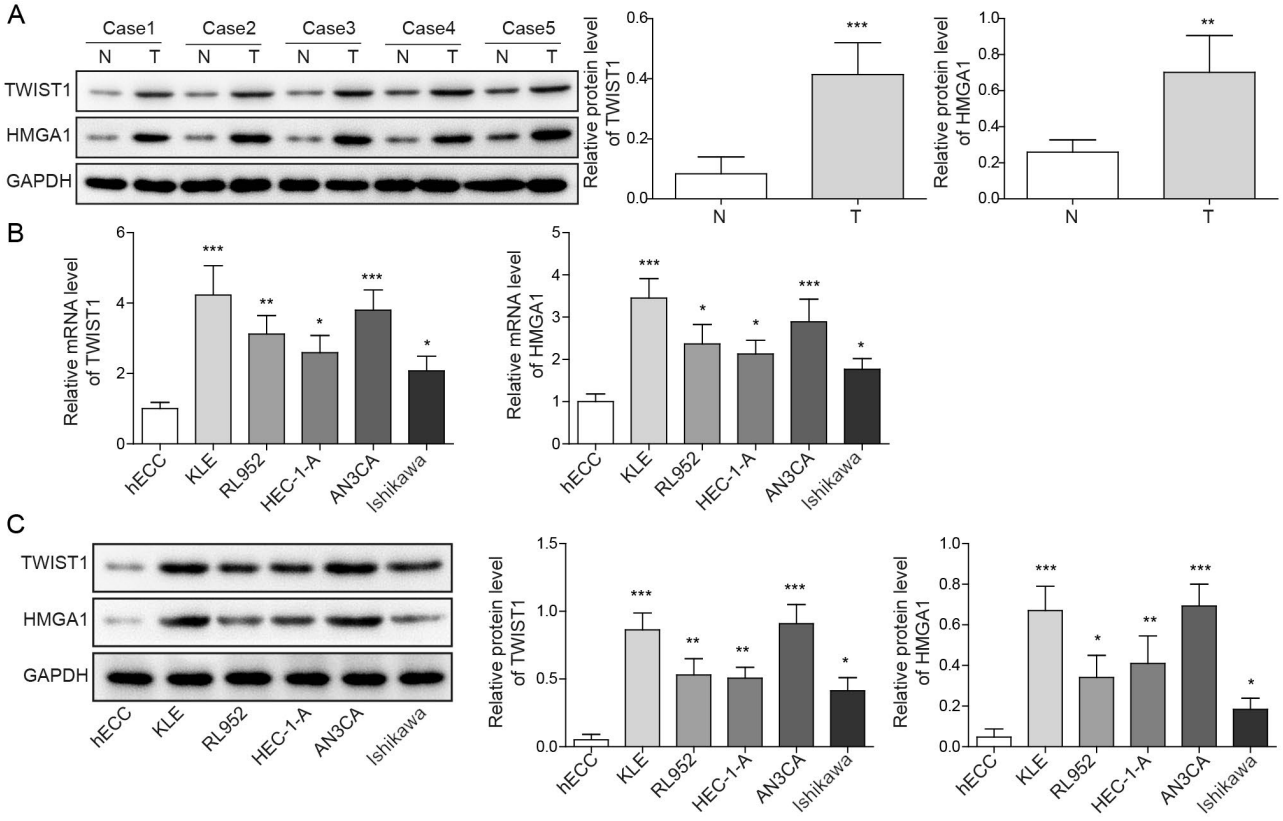

Figure 1. TWIST1 and HMGA1 were elevated in EC tissues and cells. A) Western blotting was conducted to assess the levels of TWIST1 and HMGA1 in 5 pairs of EC tissues and adjacent tissues. B, C) The expressions of TWIST1 and HMGA1 in hEECs and human EC cell lines (Ishikawa, RL952, AN3 CA, HEC-1-A, and KLE) were measured by qRT-PCR and western blotting. Each trial was conducted three times, and the results were presented as means ± SD. *p<0.05; **p<0.01; ***p<0.001

elevated by CCL18 overexpression plasmid (Figure 3A). In addition, the level of CCL18 in M2-TAM also decreased with the knockdown of CCL18 and increased with the overexpression of CCL18 (Figure 3B). These data suggested the successful establishment of CCL18 deficient and overexpressing M2-TAMs. Then, the malignant behaviors of EC cells were evaluated after the treatment with CCL18-knockdown or -overexpressing M2-TAM CM. In comparison

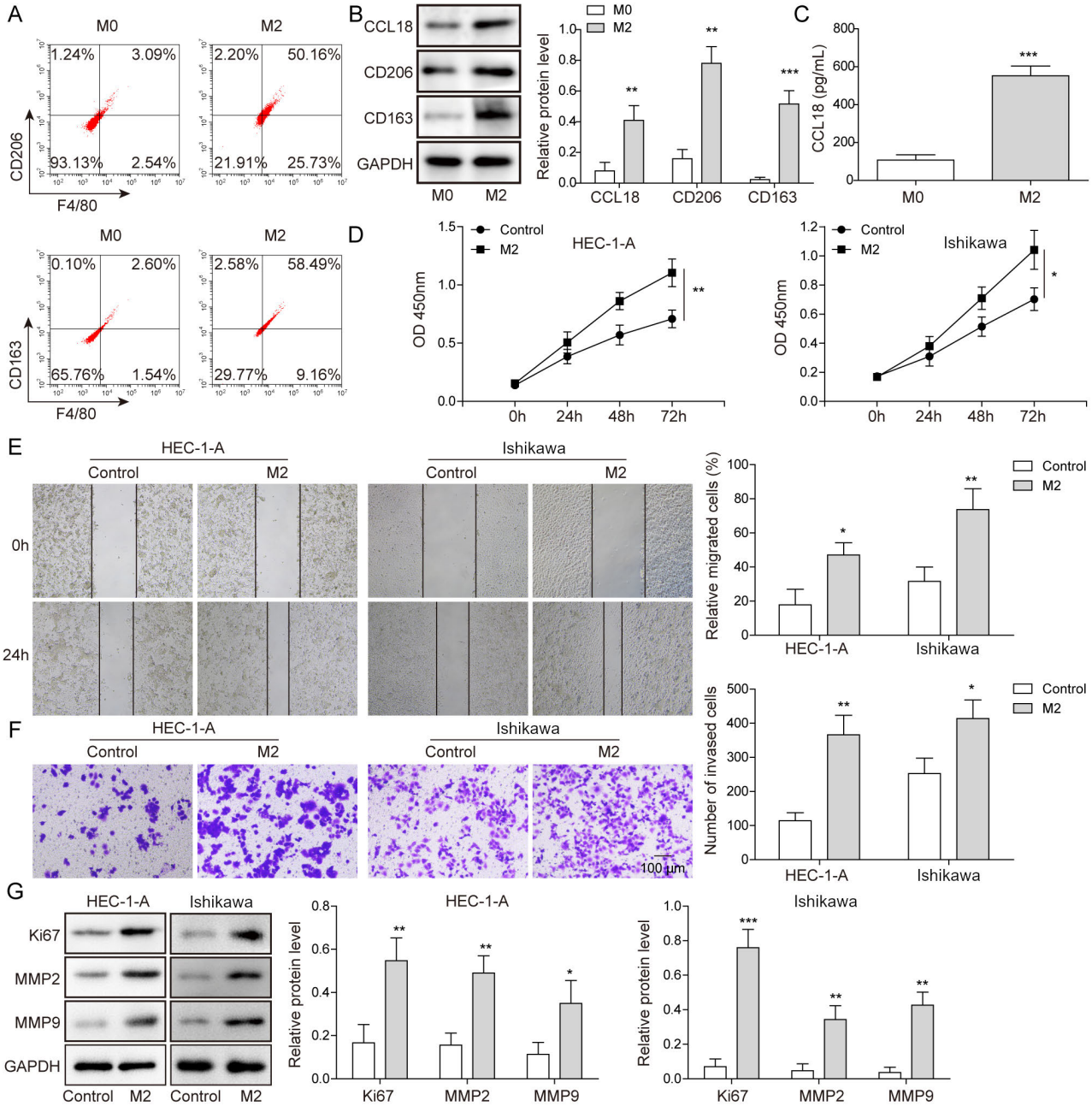

Figure 2. CCL18 derived from M2-TAMs facilitated the malignant progression of EC cells. Human monocyte THP-1 cells were differentiated into M0 macrophages by PMA stimulation and then polarized into M2 macrophages with IL-13 and IL-4. A) The levels of $CD206^{+}F4/80^{+}$ and $CD163^{+}F4/80^{+}$ M2 macrophage numbers were examined by flow cytometry. B) Western blotting was carried out to assess the CD206, CD163, and CCL18 levels. C) ELISA was conducted to measure CCL18 levels. EC cells were incubated with M2-TAMs CM. D) CCK-8 assay was performed to assess the cell viability of HEC-1-A and Ishikawa cells. E) Wound healing assay was conducted to evaluate the migration of EC cells. F) The cell invasion of HEC-1-A and Ishikawa cells was detected by Transwell assays (magnification ×100). G) Western blotting was carried out to assess the levels of Ki-67, MMP-2, and MMP-9 in HEC-1-A and Ishikawa cells. Each trial was conducted three times, and the results were presented as means ± SD. *p<0.05, **p<0.01, ***p<0.001

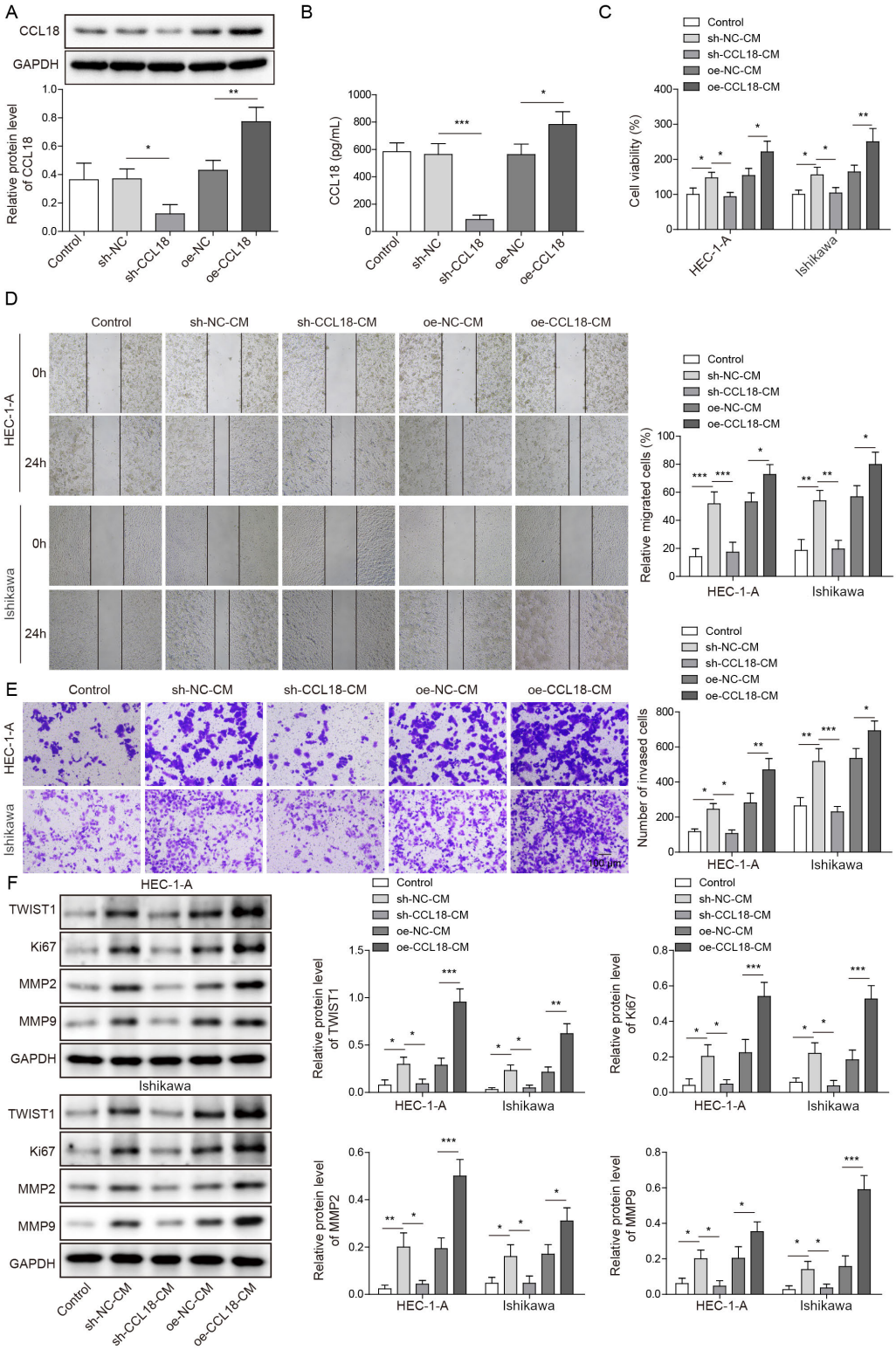

Figure 3. M2-TAM-derived CCL18 promoted EC cells' malignant behaviors by activating TWIST1. M2 macrophages transfected with sh-CCL18 and oe-CCL18 plasmids. A, B) The CCL18 expression was checked by western blotting and ELISA. C) The viability of HEC-1-A and Ishikawa cells was assessed by using CCK-8 assay. D) Migration of EC cells was evaluated by wound healing assay. E) Transwell assays were performed to detect the invasion of HEC-1-A and Ishikawa cells (magnification ×100). F) The levels of TWIST1, Ki-67, MMP-2, and MMP-9 in EC cells were determined by western blotting. Each trial was conducted three times, and the results were presented as means ± SD. *p<0.05, **p<0.01, ***p<0.001

with the sh-NC-CM or oe-NC-CM groups, the cell viability (Figure 3C), migration (Figure 3D), and invasion (Figure 3E) of EC cells were significantly enhanced by CCL18 upregulation, but the CCL18-knockdown M2-TAMs CM had no effects on EC cells progression. Additionally, CCL18 overexpression further promoted the expressions of TWIST1, Ki-67, MMP-2, and MMP-9 in EC cells than M2 TAMs CM, after knocking down CCL18 in M2 TAMs, the promoting effect of M2 TAMs on TWIST1, Ki-67, MMP-2, and MMP-9 expressions were eliminated (Figure 3F). Taken together, CCL18 derived from M2 TAMs could activate TWIST1 and promote EC cell proliferation, migration, and invasion.

TWIST1 transcriptionally activated HMGA1 in EC cells. Using the JASPAR database, there were potential interaction sites between TWIST1 and HMGA1. As depicted in Figure 4A, TWIST1 could directly combine with the HMGA1 promoter region. ChIP indicated an enhanced enrichment of HMGA1 promoter by TWIST1 antibody relative to IgG in Ishikawa and HEC-1-A cells (Figure 4B). Furthermore, dual-luciferase reporter assay evidenced that TWIST1 overexpression sharply enhanced the luciferase activity in the HMGA1-WT group of Ishikawa and HEC-1-A cells, without affecting the MUT group (Figure 4C). These observations illustrated that TWIST1 directly targeted and upregulated HMGA1 in EC cells.

M2-TAM-secreted CCL18 promoted EC cell malignancy by activating the TWIST1/HMGA1 axis. To explore whether M2-TAM-derived CCL18 promoted EC progression through the TWIST1/HMGA1 axis, Ishikawa and HEC-1-A cells were introduced with oe-TWIST1, and/or sh-HMGA1 plasmids, and then treated with M2-TAM-derived CCL18. M2-TAMs-secreted CCL18 increased the levels of TWIST1 and HMGA1 in EC cells and were further enhanced by TWIST1 overexpression. sh-HMGA1 did not affect the TWIST1 expression but inhibited the HMGA1 level in M2-TAMs-secreted CCL18 and/or oe-TWIST1 cultured EC cells (Figures 5A, 5B). Functional assays revealed that M2-TAM-derived CCL18 facilitated EC cell viability, migration, and invasion and was further enhanced by TWIST1 upregulation, while HMGA1 knockdown reversed this result. Moreover, the knockdown of HMGA1 relieved the promoting effect of M2-TAM-derived CCL18 on the malignant behavior of EC cells (Figures 5C–5E). Additionally, TWIST1 overexpression further upregulated the levels of Ki-67, MMP-2, and MMP-9 in EC cells which cultured with M2-TAM-secreted CCL18, HMGA1 knockdown markedly reduced the expression of Ki-67, MMP-2, and MMP-9 expressions in M2-TAM-derived CCL18 and/or oe-TWIST1 incubated EC cells (Figure 5F). Collectively, these results indicated that M2-TAM-derived CCL18 accelerated EC cell proliferation, migration, and invasion by activating the TWIST1/HMGA1 axis.

Discussion

EC is a prevalent gynecological cancer with an unfavorable prognosis and limited therapeutic approaches [29, 30], rendering it urgent to further grasp its pathological

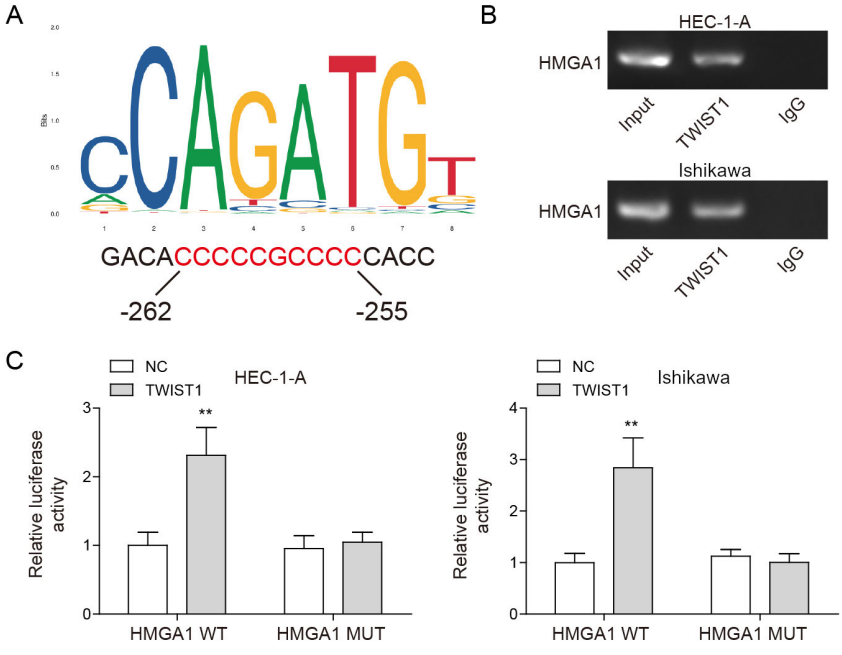

Figure 4. TWIST1 transcriptionally activated HMGA1 in EC cells. A) JASPAR database was used to predict the binding motif of TWIST1 and the binding sites on the HMGA1 promoter. B, C) ChIP and dual-luciferase reporter assays were conducted to investigate the binding site of TWIST1 on the HMGA1 promoter. Each trial was conducted three times, and the results were presented as means ± SD. **p<0.01

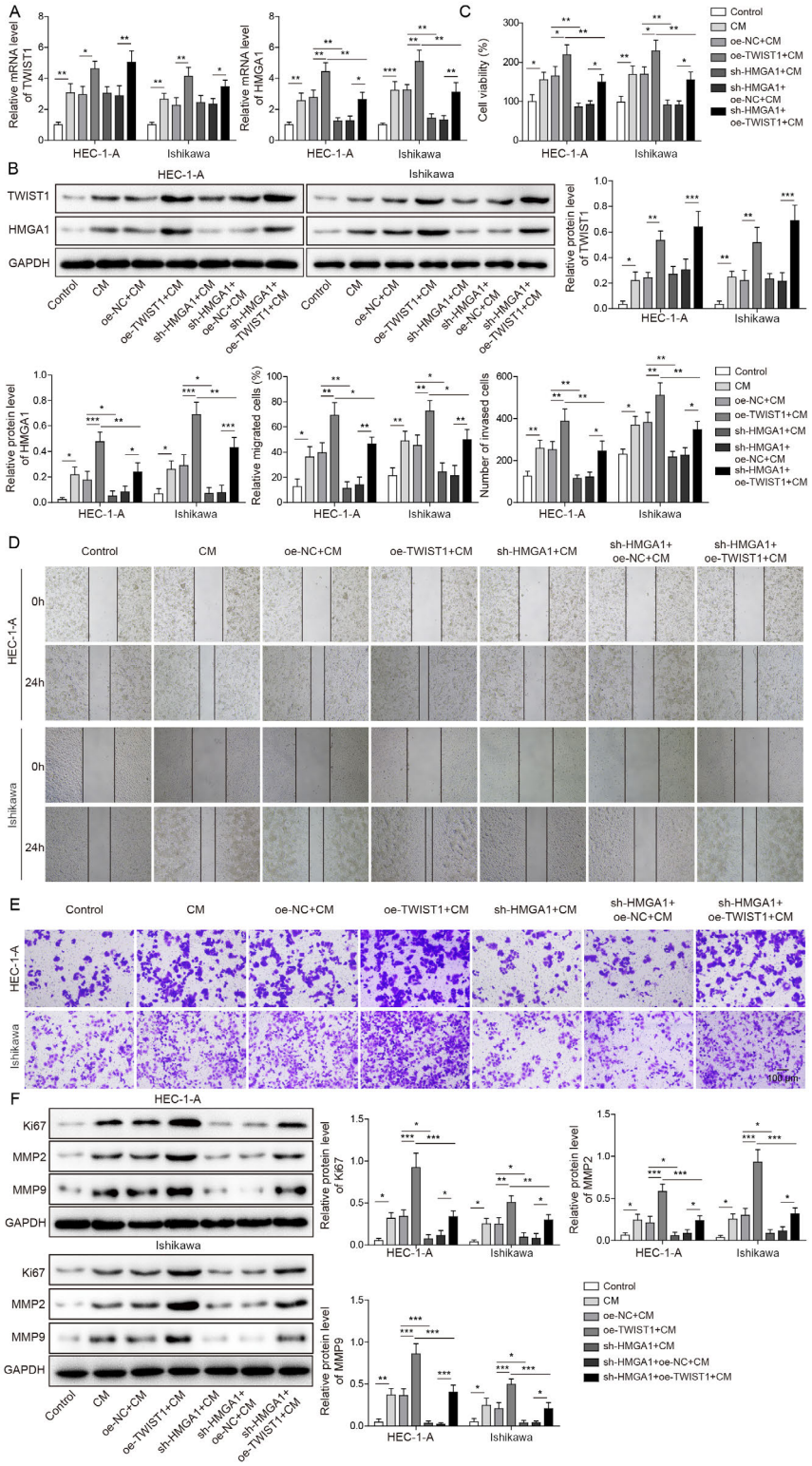

Figure 5. M2-TAM-secreted CCL18 promoted EC cell malignancy by activating the TWIST1/HMGA1 axis. EC cells were treated with oe-NC, oe-TWIST1, and/or sh-HMGA1 plasmids, and then treated with M2-TAM CM. A, B) The expressions of TWIST1 and HMGA1 were measured by qRT-PCR and western blotting. C) The viability of EC cells was assessed by using CCK-8 assay. D) To evaluate the migration of EC cells, wound healing assay was conducted. E) Transwell assay was performed to detect the invasion of EC cells (magnification ×100). F) The Ki-67, MMP-2, and MMP-9 expressions were determined by western blotting. Each trial was conducted three times, and the results were presented as means ± SD. Two-way ANOVA was used to analyze the statistical significance. *p<0.05, **p<0.01, ***p<0.001

mechanisms to develop more effective therapies. As recently reported, anti-tumor strategies aimed at reshaping the TME have attracted a lot of attention, and TAMs were the main components of immune cells in the TME, regulating TAMs M1 or M2 polarization determined tumor development [31]. Existing research has revealed the promotive role of M2-TAMs in EC progression, miR-192-5p-modified TAM-derived exosomes inhibit the progression of endometrial cancer by targeting the IRAK1/NF-κB signaling pathway [32]. Nonetheless, the exact molecular processes responsible for the significant impact of M2-TAMs on EC progression are still not fully understood. Our study revealed that M2-TAMs-secreted CCL18 could promote the proliferation, migration, and invasion of EC cells through modulation of the TWIST1/HMGA1 axis.

TAMs represent a major tumor-infiltrating immune cell subgroup and are typically classified into two distinct types: classically activated M1 macrophages and alternatively activated M2 macrophages [33]. In the context of tumors, TAMs principally display an M2 phenotype that drives tumor growth and progression [9, 34, 35]. Consequently, the idea of targeting TAMs for cancer treatment focuses on suppressing the recruitment and accumulation of M2 macrophages [36]. A previous study demonstrated that the poor prognosis of EC was attributed to high M2 macrophage infiltration, M2 polarized macrophages promoted phagocytosis of tumor cells in EC [37]. Different co-culture models reveal the pivotal role of TBBPA-promoted M2 macrophage polarization in the deterioration of endometrial cancer. In line with previous findings [32], our observations indicated that the proliferation, migration, and invasion of EC cells were enhanced after coculturing with M2-TAMs. These findings indicated that M2-TAMs may play a supportive function in the advancement of EC malignancy.

In fact, M2-TAMs produce a variety of cytokines and chemokines that have important implications for cancer progression, extracellular vesicles originating from M2-TAMs have been found to facilitate immune evasion in ovarian cancer through the NEAT1/miR-101-3p/ZEB1/PD-L1 pathway [38]. Among these, CCL18 stands out as a pivotal chemokine known for its significant role in promoting the growth, proliferation, migration, EMT, and angiogenesis of cancer cells [39]. CCL18 has been identified as a key driver in the progression of various cancers, including gastric cancer [40] and oral squamous cell carcinoma, promoting tumor proliferation and spread [41]. Furthermore, it is documented that CCL18 expressed by M2-TAMs can promote renal cell carcinoma progression *in vivo* [42]. Notably, a recent study showed that M2-TAMs accelerated EC progression through CCL18. The CCL18 secreted from M2-TAMs upregulated KIF5B expression and activated the PI3K/AKT/mTOR signaling pathway in EC to promote EMT [19]. Following incubation with M2-TAMs-secreted CCL18, we noticed a significant boost in proliferation, migration, and invasion of EC cells, while CCL18 downregulation reversed this result. Collectively, these results demonstrated that M2-TAM-derived CCL18 could promote EC cell malignancy.

Further, we delved into the downstream mechanism of M2-TAM-derived CCL18 fostering EC progression. We uncovered a novel axis, TWIST1/HMGA1, implicated in the pro-tumor mechanisms of M2-TAM-derived CCL18 in EC. As a crucial transcription factor to induce EMT, TWIST1 is overexpressed in multiple human cancers and is closely linked to tumor progression and metastasis [43]. In EC, heightened TWIST1 expression could exacerbate the malignant traits of tumor cells [24]. Consistent with this, our research identified increased TWIST1 expression in EC tissues and cells in comparison with normal counterparts. Significantly, we found that TWIST1 expression was positively modulated by M2-TAM-derived CCL18 in EC progression. However, we noticed that this is inconsistent with the proteomic data on the Human Protein Atlas (HPA) website (https://www.proteinatlas.org), where there is a significant decrease in TWIST1 in EC (https://www.proteinatlas.org/ENSG00000122691-TWIST1/cancer). However, this does not affect the conclusion of this study. In line with many previously published results, the upregulation of TWIST1 in EC tissues and cells can be used as a potential therapeutic target for EC [44, 45]. For example, miR-214-3p inhibits epithelial-mesenchymal transition and metastasis of EC cells by targeting TWIST1 [24], and NOL6 and TWIST1 synergistically promote the development of EC [46]. In addition, miR-106b can inhibit EMT and cell invasion by regulating TWIST1 in EC cells [47]. The above research fully demonstrates that TWIST1 upregulation is involved in the malignant progression of EC.

On the other hand, HMGA1 has been identified as a vital contributing factor and prognostic biomarker in EC [48, 49]. Our study demonstrated HMGA1 overexpressed in EC tumor tissues and EC cells than in normal counterparts. In terms of the mechanisms related to HMGA1 in EC, previous studies focused on its interaction with noncoding RNAs [25–27]. Here, we presented groundbreaking evidence indicating that HMGA1 could be targeted and positively modulated by TWIST1 in EC progression. Subsequent functional assays revealed that HMGA1 knockdown could restrain the malignancy of M2-TAM-derived CCL18 cultured EC cells. Furthermore, TWIST1 overexpression eliminated the restraint effects of HMGA1 knockdown on EC progression under CCL18 overexpression M2-TAMs CM. Taken together, these observations proved that the oncogenicity of M2-TAM-derived CCL18 in EC could be attributed to the activated TWIST1/HMGA1 axis.

Nevertheless, this work has several limitations. This study predominantly counted on *in vitro* assays without *in vivo* animal experiments to substantiate the observations. Additionally, while the relationship between TWIST1 and CCL18 or HMGA1 expression was defined in EC cells, their correlation was not interpreted at the clinical level. Hence, our future research will incorporate *in vivo* animal experi-

ments and clinical data to further verify the role and mechanism of M2-TAM-derived CCL18 in EC.

In conclusion, this research unveiled that CCL18 derived by M2-TAMs contributed to the proliferation, migration, and invasion of EC cells. The biological functions of M2-TAM-derived CCL18 in EC were achieved by targeting the TWIST1/HMGA1 axis. The results of our study provided new perspectives on the cellular and molecular processes involved in EC progression, potentially paving the way for innovative and precise treatment approaches for this cancer.

Supplementary information is available in the online version of the paper.

Acknowledgments: This work was supported by the Youth project of Science and Technology Department of Shanxi Province (No. 202203021212105) and the Shanxi Bethune Hospital talent project (2022RC05).

References

[1] SUNG H, FERLAY J, SIEGEL RL, LAVERSANNE M, SOERJOMATARAM I et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 2021; 71: 209–249. https://doi.org/10.3322/caac.21660

[2] NIENHAUS A, RAJAKULENDRAN R, BERNAD E. A 10-Year Retrospective Cohort Study of Endometrial Cancer Outcomes and Associations with Lymphovascular Invasion: A Single-Center Study from Germany. Diagnostics (Basel) 2024; 14: 1686. https://doi.org/10.3390/diagnostics14151686

[3] TRONCONI F, NERO C, GIUDICE E, SALUTARI V, MUSACCHIO L et al. Advanced and recurrent endometrial cancer: State of the art and future perspectives. Crit Rev Oncol Hematol 2022; 180: 103851. https://doi.org/10.1016/j.critrevonc.2022.103851

[4] XIAO Y, YU D. Tumor microenvironment as a therapeutic target in cancer. Pharmacol Ther 2021; 221: 107753. https://doi.org/10.1016/j.pharmthera.2020.107753

[5] LEE M, JUNG W, KANG J, LEE KH, LEE SJ et al. Prognostic Significance of the Immune Microenvironment in Endometrial Cancer. Lab Invest 2024; 104: 102126. https://doi.org/10.1016/j.labinv.2024.102126

[6] LI M, HE L, ZHU J, ZHANG P, LIANG S. Targeting tumor-associated macrophages for cancer treatment. Cell Biosci 2022; 12: 85. https://doi.org/10.1186/s13578-022-00823-5

[7] YUAN D, HU J, JU X, PUTZ EM, ZHENG S et al. NMDAR antagonists suppress tumor progression by regulating tumor-associated macrophages. Proc Natl Acad Sci U S A 2023; 120: e2302126120. https://doi.org/10.1073/pnas.2302126120

[8] WU K, LIN K, LI X, YUAN X, XU P et al. Redefining Tumor-Associated Macrophage Subpopulations and Functions in the Tumor Microenvironment. Front Immunol 2020; 11: 1731. https://doi.org/10.3389/fimmu.2020.01731

[9] ZHANG, Q, SIOUD M. Tumor-Associated Macrophage Subsets: Shaping Polarization and Targeting. Int J Mol Sci 2023; 24: 7493. https://doi.org/10.3390/ijms24087493

[10] GU S, NI T, WANG J, LIU Y, FAN Q. CD47 Blockade Inhibits Tumor Progression through Promoting Phagocytosis of Tumor Cells by M2 Polarized Macrophages in Endometrial Cancer. J Immunol Res 2018; 2018: 6156757. https://doi.org/10.1155/2018/6156757

[11] JIN X, SU H, XU L, WANG Y, SU R et al. Different co-culture models reveal the pivotal role of TBBPA-promoted M2 macrophage polarization in the deterioration of endometrial cancer. J Hazard Mater 2021; 413: 125337. https://doi.org/10.1016/j.jhazmat.2021.125337

[12] GU X, SHI Y, DONG M, JIANG L, YANG J et al. Exosomal transfer of tumor-associated macrophage-derived hsa_circ_0001610 reduces radiosensitivity in endometrial cancer. Cell Death Dis 2021; 12: 818. https://doi.org/10.1038/s41419-021-04087-8

[13] HUANG X, LAI S, QU F, LI Z, FU X et al. CCL18 promotes breast cancer progression by exosomal miR-760 activation of ARF6/Src/PI3K/Akt pathway. Mol Ther Oncolytics 2022; 25: 1–15. https://doi.org/10.1016/j.omto.2022.03.004

[14] WANG J, QIN Y, ZHU G, HUANG D, WEI M et al. High serum CCL18 predicts a poor prognosis in patients with laryngeal squamous cell carcinoma. J Cancer 2019; 10: 6910–6914. https://doi.org/10.7150/jca.37515

[15] LIU J, LI SM. MiR-484 suppressed proliferation, migration, invasion and induced apoptosis of gastric cancer via targeting CCL-18. Int J Exp Pathol 2020; 101: 203–214. https://doi.org/10.1111/iep.12366

[16] Jiang X, Huang Z, Sun X, Zheng X, Liu J et al. CCL18-NIR1 promotes oral cancer cell growth and metastasis by activating the JAK2/STAT3 signaling pathway. BMC Cancer 2020; 20: 632. https://doi.org/10.1186/s12885-020-07073-z

[17] ZHANG H, LIN P, FU L, LI Z, DING Y. Propofol Suppresses Cell Progression by Inhibiting CCL18 Expression in Hepatoblastoma. J Oncol 2021; 2021: 6880473. https://doi.org/10.1155/2021/6880473

[18] SAKANE R, TSUBAMOTO H, SAKATA K, INOUE K, OGINO M et al. Expression of chemokine ligand 18 in stage IA low-grade endometrial cancer. Anticancer Res 2014; 34: 5331–5336.

[19] JING X, PENG J, DOU Y, SUN J, MA C et al. Macrophage ERα promoted invasion of endometrial cancer cell by mTOR/KIF5B-mediated epithelial to mesenchymal transition. Immunol Cell Biol 2019; 97: 563–576. https://doi.org/10.1111/imcb.12245

[20] Zhou Z, Jin L, Shen J, Shi W, Xu Y et al. COM33 suppresses carboplatin-induced epithelial-mesenchymal transition via inhibition of Twist1 in ovarian cancer. Acta Biochim Biophys Sin (Shanghai) 2022; 55: 34–42. https://doi.org/10.3724/abbs.2022195

[21] PAN J, FANG S, TIAN H, ZHOU C, ZHAO X et al. lncRNA JPX/miR-33a-5p/Twist1 axis regulates tumorigenesis and metastasis of lung cancer by activating Wnt/β-catenin signaling. Mol Cancer 2020; 19: 9. https://doi.org/10.1186/s12943-020-1133-9

[22] HUANG X, LIU N, XIONG X. ZNF24 is upregulated in prostate cancer and facilitates the epithelial-to-mesenchymal transition through the regulation of Twist1. Oncol Lett 2020; 19: 3593–3601. https://doi.org/10.3892/ol.2020.11456

[23] KHALES SA, MOZAFFARI-JOVIN S, GEERTS D, ABBASZADEGAN MR. TWIST1 activates cancer stem cell marker genes to promote epithelial-mesenchymal transition and tumorigenesis in esophageal squamous cell carcinoma. BMC Cancer 2022; 22: 1272. https://doi.org/10.1186/s12885-022-10252-9

[24] Fang YY, Tan MR, Zhou J, Liang L, Liu XY et al. miR-214-3p inhibits epithelial-to-mesenchymal transition and metastasis of endometrial cancer cells by targeting TWIST1. Onco Targets Ther 2019; 12: 9449–9458. https://doi.org/10.2147/OTT.S181037

[25] LIU Y, CHANG Y., CAI Y. Circ_0067835 sponges miR-324-5p to induce HMGA1 expression in endometrial carcinoma cells. J Cell Mol Med 2020; 24: 13927–13937. https://doi.org/10.1111/jcmm.15996

[26] CAI Y, HAO M, CHANG Y, LIU Y. LINC00665 enhances tumorigenicity of endometrial carcinoma by interacting with high mobility group AT-hook 1. Cancer Cell Int 2021; 21: 8. https://doi.org/10.1186/s12935-020-01657-2

[27] ZHOU Y, PAN A, ZHANG Y, LI X. Hsa_circ_0039569 facilitates the progression of endometrial carcinoma by targeting the miR-197/high mobility group protein A1 axis. Bioengineered 2022; 13: 4212–4225. https://doi.org/10.1080/21655979.2022.2027060

[28] LIU R, SUN X, HU Z, PENG C, WU T. Knockdown of long non-coding RNA MIR155HG suppresses melanoma cell proliferation, and deregulated MIR155HG in melanoma is associated with M1/M2 balance and macrophage infiltration. Cells Dev 2022; 170: 203768. https://doi.org/10.1016/j.cdev.2022.203768

[29] LIU H, BEI Q, LUO X. MLN4924 inhibits cell proliferation by targeting the activated neddylation pathway in endometrial carcinoma. J Int Med Res 2021; 49: 3000605211018592. https://doi.org/10.1177/03000605211018592

[30] WANG Y, YIN L, SUN X. CircRNA hsa_circ_0002577 accelerates endometrial cancer progression through activating IGF1R/PI3K/Akt pathway. J Exp Clin Cancer Res 2020; 39: 169. https://doi.org/10.1186/s13046-020-01679-8

[31] JIANG M, WANG D, SU N, LOU W, CHEN Y et al. TRIM65 knockout inhibits the development of HCC by polarization tumor-associated macrophages towards M1 phenotype via JAK1/STAT1 signaling pathway. Int Immunopharmacol 2024; 128: 111494. https://doi.org/10.1016/j.intimp.2024.111494

[32] WANG Y, MA H, LI Y, SU R. MiR-192-5p-Modified Tumor-Associated Macrophages-Derived Exosome Suppressed Endometrial Cancer Progression Through Targeting IRAK1/NF-κB Signaling. Reprod Sci 2022; 29: 436–447. https://doi.org/10.1007/s43032-021-00789-8

[33] PAN Y, YU Y, WANG X, ZHANG T. Tumor-Associated Macrophages in Tumor Immunity. Front Immunol 2020; 11: 583084. https://doi.org/10.3389/fimmu.2020.583084

[34] GENIN M, CLEMENT F, FATTACCIOLI A, RAES M, MICHIELS C. M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. BMC Cancer 2015; 15: 577. https://doi.org/10.1186/s12885-015-1546-9

[35] BOUTILIER AJ, ELSAWA SF. Macrophage Polarization States in the Tumor Microenvironment. Int J Mol Sci 2021; 22: 6955. https://doi.org/10.3390/ijms22136995

[36] ZHOU YX, ZHAO W, MAO LW, WANG YL, XIA LQ et al. Long non-coding RNA NIFK-AS1 inhibits M2 polarization of macrophages in endometrial cancer through targeting miR-146a. Int J Biochem Cell Biol 2018; 104: 25–33. https://doi.org/10.1016/j.biocel.2018.08.017

[37] ZHU X, XU Y, WANG J, XUE Z, QIU T et al. Loss of NLRP3 reduces oxidative stress and polarizes intratumor macrophages to attenuate immune attack on endometrial cancer. Front Immunol 2023; 14: 1165602. https://doi.org/10.3389/fimmu.2023.1165602

[38] YIN L, WANG Y. Extracellular vesicles derived from M2-polarized tumor-associated macrophages promote immune escape in ovarian cancer through NEAT1/miR-101-3p/ZEB1/PD-L1 axis. Cancer Immunol Immunother 2023; 72: 743–758. https://doi.org/10.1007/s00262-022-03305-2

[39] KORBECKI J, OLBROMSKI M, DZIĘGIEL P. CCL18 in the Progression of Cancer. Int J Mol Sci 2020; 21: 7955. https://doi.org/10.3390/ijms21217955

[40] HOU X, ZHANG Y, QIAO H. CCL18 promotes the invasion and migration of gastric cancer cells via ERK1/2/NF-κB signaling pathway. Tumour Biol 2016; 37: 641–651. https://doi.org/10.1007/s13277-015-3825-0

[41] JIANG X, LIU J, LI S, JIA B, HUANG Z et al. CCL18-induced LINC00319 promotes proliferation and metastasis in oral squamous cell carcinoma via the miR-199a-5p/FZD4 axis. Cell Death Dis 2020; 11: 777. https://doi.org/10.1038/s41419-020-02978-w

[42] NGUYEN TN, NGUYEN-TRAN HH, CHEN CY, HSU T. IL6 and CCL18 Mediate Cross-talk between VHL-Deficient Kidney Cells and Macrophages during Development of Renal Cell Carcinoma. Cancer Res 2022; 82: 2716–2733. https://doi.org/10.1158/0008-5472.CAN-21-3749

[43] GHAFOURI-FARD S, ABAK A, BAHROUDI Z, SHOOREI H, ABBAS RAZA SH et al. The interplay between non-coding RNAs and Twist1 signaling contribute to human disorders. Biomed Pharmacother 2021; 135: 111220. https://doi.org/10.1016/j.biopha.2021.111220

[44] SHEN J, CHEN Q, LI N, BAI X, WANG F et al. TWIST1 expression and clinical significance in type I endometrial cancer and premalignant lesions: A retrospective clinical study. Medicine (Baltimore) 2020; 99: e23397. https://doi.org/10.1097/MD.0000000000023397

[45] DONG P, KANEUCHI M, XIONG Y, CAO L, CAI M et al. Identification of KLF17 as a novel epithelial to mesenchymal transition inducer via direct activation of TWIST1 in endometrioid endometrial cancer. Carcinogenesis 2014; 35: 760–768. https://doi.org/10.1093/carcin/bgt369

[46] LIANG J, SUN W, SONG H, WANG C, LI Q et al. NOL6 promotes the proliferation and migration of endometrial cancer cells by regulating TWIST1 expression. Epigenomics 2021; 13: 1571–1585. https://doi.org/10.2217/epi-2021-0218

[47] DONG P, KANEUCHI M, WATARI H, SUDO S, SAKURAGI N. MicroRNA-106b modulates epithelial-mesenchymal transition by targeting TWIST1 in invasive endometrial cancer cell lines. Mol Carcinog 2014; 53: 349–359. https://doi.org/10.1002/mc.21983

[48] ZHANG Y, XIAO Y, ZHAO X, XU J, ZHAO H et al. IGF2BP3 is upregulated in endometrial cancer and tightly regulates the growth of drug-resistant endometrial cancer cells via HMGA1. Sci Prog 2025; 108: 368504251315008. https://doi.org/10.1177/00368504251315008

[49] PEI X, NIE H, LIU Q, XU C. Study on the expression of S100A4 and HMGA1 in endometrial carcinoma and their correlation with metastasis. Am J Transl Res 2025; 17: 221–229. https://doi.org/10.62347/QIBF6088

https://doi.org/10.4149/neo_2025_240820N357

CCL18 derived from M2-polarized tumor-associated macrophages promotes endometrial cancer progression by activating the TWIST1/HMGA1 axis

Hongxia LI*, Jiansheng LI

Supplementary Information

Supplementary Table S1. The DNA sequences used in dual-luciferase reporter assays.

Name	Sequences
HMGA1-WT	TTAGGGGGCGGCGTGTAGGCCGCGGAATTCTCTAACGAG CGCGTTTCTCTTCACCCCCTGGGCCCGCGTGGACCCCCGT CCACCCCCACACGCCCTGGGGGGGGGGCCGGGCCCACAC GCCCTGGAAGCCCCTAGAGGTGACTCTCCCTGGGACCCC TGTACCAGGGAGGAAGGATACCGCCACCCGTCACCACCC CCCGCCAGAAGCTCCTTCGTGACTCCTCTGCGCGTGCCTT CCCACACCTCCTCCGTCCGGGACTGCGAGGAGTGGGCGG TCGACTCGAGTTCGCAGCCCAGGCCTCCCACACGCCCCT CCCTGCCGTCAGCACCCACCCGCGCGGCAGCGGCGGCGG CGGCTGGCGGGCGGCCGCCCTTTTAAATCCCCGGGCTCAT TTGCATG
HMGA1-MUT	TTAGGGGGCGGCGTGTAGGCCGCGGAATTCTCTAACGAG CGCGTTTCTCTTCACCCCCTGGGCCCGCGTGGACCCCCGT CCACCCCCACACGCCCTGGGGGGGGGGCCGGGCCCACAC GCCCTGGAAGCCCCTAGAGGTGACTCTCCCTGGGACCCC TGTACCAGGGAGGAAGGATACCGCCACCCGTCACCACCC CCCGGGTCTTCGTCCTTCGTGACTCCTCTGCGCGTGCCTT CCCACACCTCCTCCGTCCGGGACTGCGAGGAGTGGGCGG TCGACTCGAGTTCGCAGCCCAGGCCTCCCACACGCCCCT CCCTGCCGTCAGCACCCACCCGCGCGGCAGCGGCGGCGG CGGCTGGCGGGCGGCCGCCCTTTTAAATCCCCGGGCTCAT TTGCATG