Research Paper

Exosomal miR-15b-5p derived from gastric cancer cells enhances immune infiltrating of tumor-associated macrophages through the WIF1/WNT5A axis

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ABSTRACT

Objective: We intend to investigate the effects of gastric cancer (GC) cell-derived exosomes on tumor-associated macrophages (TAMs) and angiogenesis of vascular endothelial cells (VECs).
Methods: The exosomes from GC cells were extracted using the ultracentrifugation method. TAMs were treated with the exosomes and then co-cultured with GC cells or VECs. The viability, migration, invasion and apoptosis of GC cells were detected by the cell counting kit-8 (CCK-8) assay, transwell assay and flow cytometry, respectively. Meanwhile, the tube formation ability and viability of VECs were determined. Furthermore, gain- or loss- of function assays of miR-15b-5p were performed to probe its role in modulating the polarization of TAMs.
Results: TAMs significantly promoted GC cell proliferation, migration and invasion and repressed apoptosis. Additionally, TAMs promoted VECs' angiogenesis. Forced overexpression of miR-15b-5p in GC cell-derived exosomes enhanced the "M2" polarization of TAMs. Moreover, TAMs with overexpressed-miR-15b-5p strengthened the malignant phenotypes of GC cells and increased the angiogenesis of VECs. Further mechanism studies showed that miR-15b-5p targeted and inhibited WIF1 and up-regulated WNT5A.
Conclusions: Exosomal miR-15b-5p shuttled by GC cells facilitated the "M2" polarization of TAMs by regulating the WIF1/WNT5A pathway, thus promoting GC progression.

INTRODUCTION

The tumor microenvironment (TME) exerts a dominant function in tumor progression. Traditionally, TME contains tumor parenchymal cells, stromal cells (including immune cells, tumor-related fibroblasts, and tumor-associated macrophages), extracellular matrix, and cell-secreted soluble factors [1]. Therein, tumor-associated macrophages (TAMs) evolved from the macrophages of the peripheral blood to the solid tumor tissue, making up a large proportion of the tumor stromal cells. In recent years, emerging studies have found that TAMs, acting on the tumor-related stroma or endothelial cells by secreting special cell growth factors, promote tumor development, stimulate tumor cell growth and metastasis, induce tumor neovascularization and lymphangiogenesis, immunosuppression and contribute to other processes [2, 3]. For example, it has been confirmed that the degree of TAMs infiltration is positively correlated with tumor growth in breast cancer [4] and endometrial cancer [5]. Similarly, TAMs induce gastric cancer (GC) invasion and poor prognosis in a cyclooxygenase-2 (COX2)/matrix metalloproteinase-9 (MMP9) dependent

manner [6]. Also, Li H et al. hold that TAMs enhance GC cell invasion and metastasis by up-regulating the secreted protein Sema4D [7], which indicates that TAMs affect the malignant progression of GC. However, the interaction between TAMs and GC cells has been rarely reported.

Exosomes are vesicles around 30-200 nm in diameter with lipid bilayer structures synthesized and secreted by cells [8]. Recent studies have confirmed that cell-derived exosomes carry various omics information of parent cells for information transmission in TME and act as messengers of tumor cells to mediate the interaction between cells [9]. It has been reported that during the formation of exosomes in tumor cells, microRNAs (miRNAs), a type of non-coding small RNA, are randomly sorted into exosomes and released into the extracellular domain, and then are ingested by other cells in TME or enter the circulatory system to play a biological regulatory role [10]. For example, some studies reveal that GC cell-derived exosomal miR-23a promotes angiogenesis and provides blood supply for GC cell development by targeting PTEN [11]. In addition, Li Q et al. discovered that GC cell-derived exosomal miR-21-5p induces mesothelial mesenchymal transition (MMT) of peritoneal mesothelial cells (PMC) and accelerates the peritoneal metastasis of cancers by targeting SMAD7 to activate the TGF-β/Smad pathway [12]. However, whether GC cell-derived exosomal miR-15b-5p influences TAMs remains unknown.

Wnt inhibitory factor-1 (WIF1) is a Wnt signal inhibitor discovered in recent years. As a tumor suppressor gene, WIF1 has been found to be down-regulated in most tumors. For instance, Cai W et al. found that miR-552-5p facilitates the proliferation and metastasis of osteosarcoma cells by targeting WIF1 [13]. Similarly, miR-590-3p aggravates colon cancer development by inhibiting WIF1 and DKK1 and the Wnt/β-catenin signaling pathway [14]. WNT5A belongs to the Wnt protein family and is a prototype activator of the non-classical Wnt pathway. Its overexpression is strongly linked with the evolvement of diversified tumors by promoting cell movement, invasion, epithelial-mesenchymal transition (EMT), and metastasis [15]. For instance, studies have reported that WNT5A enhances cell invasiveness by inducing CXCL8, thereby affecting the survival rate of $HER2^+$ breast cancer [16]. Similarly, Li Y et al. found that miR-26a-5p abates GC cell proliferation and invasion by inhibiting WNT5A [17]. Nevertheless, the role of WIF1/WNT5A in TAMs remains to be studied.

Here, we obtained TAMs by co-culturing THP-1 monocytes with GC cells to clarify the effect of GC cell-derived exosomal miR-15b-5p on the formation of TAMs, and the role and specific mechanism of TAMs in the malignant phenotype of GC cells and angiopoiesis of vascular endothelial cells (VECs). This article provides some references for the clinical research of TAMs and the treatment of GC.

MATERIALS AND METHODS

Cell culture

THP-1 monocytes and AGS human gastric carcinoma cells were bought from the Cell Center of the Chinese Academy of Sciences (Shanghai, China). Then, they were cultured in RPMI1640 (Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, MA, USA) and 1% penicillin/streptomycin (Invitrogen, CA, USA) in an incubator at 37° C with 5% CO_2. During the logarithmic growth phase, cells were treated with 0.25% trypsin (Thermo Fisher HyClone, Utah, USA) and sub-cultured.

Cell transfection

After trypsinization and passage, the AGS or THP1 cells at the logarithmic growth stage were seeded in 6-well plates at 5×10^6 cells/well and transfected after stable cell growth. The miR-15b-5p mimics, miR-15b-5p inhibitors, si-WNT5A and corresponding negative control group (miR-NC-in or si-NC) were obtained from GenePharma (Shanghai, China). AGS or THP1 cells were transfected using Lipofectamine® 3000 (Invitrogen; ThermoFisherScientific, Inc.) according to the supplier's specifications. The cells were incubated at 37° C with 5% CO_2. After transfection for 24 hours, the total RNA was isolated for quantitative real-time PCR (qRT-PCR) to detect the expression changes of miR-15b-5p in the transfected cells.

The co-culture of THP-1 monocytes with AGS cells

First, THP-1 cells were treated with 100 ng/mL phorbol myristate acetate (PMA) (Sigma-Aldrich, USA) for 24 hours to produce THP-1 macrophages (M0 macrophages). Then, they were induced with IL-4 (50 ng/ml, R&D Systems) or IL-10 (20 ng/ml, R&D Systems) for 48 hours to obtain M2 or M1 macrophages. M0 macrophages and AGS human gastric carcinoma cells were cultured in the 6-well Transwell co-culture system (0.4 μM pore size, Corning, USA) to simulate the formation of TAMs. TAMs were obtained from the co-cultured macrophages 48 hours later.

Exosome isolation by the ultracentrifugation method

The AGS cell-derived exosomes were isolated by differential centrifugation at 4° C. Briefly, cells were centrifuged at 300 rpm for 10 min at 4° C, and the

supernatant was obtained. Afterward, the supernatant was centrifuged for 10 min at 2000 rpm and harvested. Subsequently, the harvested supernatant was centrifuged at 10000 rpm for 30 min at high speed and collected. Next, the collected supernatant was centrifuged at 140000 rpm for 90 min. After that, the supernatant was removed, and the precipitation obtained was exosomes. Finally, the precipitation was washed and suspended with PBS buffer and recentrifuged at 140000 rpm for 90 min. The precipitation was resuspended with 100 μL PBS buffer and frozen at -80° C for further use.

Cell counting kit-8 (CCK-8) assay

Three independent experiments were performed for the cell proliferation assay. AGS cells (5×10^3) were treated in 96-well plates for the indicated times. Then, 10 μL CCK-8 solution (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) was added to 100 μL culture media. Cells were incubated at 37° C for 1-4 hours, and then the plates were read at 450 nm by a microplate reader (Model 550, Bio-Rad Laboratories, Richmond, CA, USA). The following formula was used to calculate cell viability (%) = OD value of TAMs group/OD value of control group 100×.

Flow cytometry

After treatment with different factors, AGS cells were trypsinized and collected through centrifugation (1500 rpm,3 min). The obtained cells were treated by observing the following Cell Apoptosis Detection Kit (Shanghai Zeye Biotechnology Co., Ltd, China, Article No.: ZY140626) instructions. Cells were washed with PBS twice, and 400 μL pre-cooled PBS was added. Then, 10 μL AnnexinV-FITC and 5 μL PI were added, respectively. After the cells were incubated at 4° C in the dark for 30 min, the cell apoptosis was measured by flow cytometry immediately. The apoptosis rate was calculated by computer software. Apoptosis rate = apoptotic cell number/(apoptotic cell number + normal cell number) ×100%. The calculation was made according to the kit instructions.

Transwell assay

Transwell assay was implemented to test cell migration and metastasis. AGS human gastric carcinoma cells were trypsinized with 0.25% trypsin, centrifuged, resuspended, and seeded in each well of the 24-well plates. 8 μM pore size matrigel chambers (Corning, Beijing, China) were adopted in the invasion experiment while they were not used in the migration experiment. 5×10^4 transfected cells were placed in the upper chamber, and the matrigel was then coated. In contrast, 10% FBS medium was placed in the lower chamber, and 400 μL RPMI-1640 was supplemented. After incubation at 37° C for 24 hours, the unmigrated cells were wiped off from the upper chamber. Transwell membranes were fixed with 4% paraformaldehyde for 10 min and stained with 0.5% crystal violet. After being rinsed with tap water, the cells were calculated under an inverted microscope. All tests were done three times.

Tube formation assay

Matrigel (BD Biosciences) was melted at 4° C overnight. On the second day, 50 μL melted matrigel solution was added to the pre-cooled 96-well plates and maintained at 37° C for 40 min for solidification. Then, VECs were added on the gel surface in each well and further cultured for 10 hours in an incubator with 5% CO_2 at 37° C. The angiogenic activity of TAMs was studied using VEC capillary-like structures on a basement membrane matrix. VECs were then seeded onto the Matrigel bed (1.5×10^4 cells/well) and cultured for 6 hours. The tube formation *in vitro* was observed under an inverted microscope.

Reverse transcription-quantitative real-time PCR (qRT-PCR)

In initial studies of candidate primers, singleplex qRT-PCR was performed using the AgPath mastermix kit as described by the manufacturer (Thermo Fisher Scientific, Waltham, MA) in the presence of LCGreen DNA-binding dye as described by the manufacturer (BioFire, Salt Lake City, UT). Some such qRT-PCR products were evaluated by 1.5% agarose gel electrophoresis. Briefly, 15 μL of eluate was combined with 35 μL of mastermix as described in the SensiFAST LO-ROX one-step qRT-PCR kit product manual (Bioline, Taunton, MA). Cycling conditions were reverse transcription 10 min at 48° C, 95° C for 2 min followed by 45 cycles of 95° C for 5 s followed by 50° C for 35 s. For pfs25/18S rRNA multiplex RT-PCR, total nucleic acids were DNase-treated (TURBO DNA-free Kit, Ambion/Life). All of the fluorescence data were converted into relative quantification. U6 was the internal reference of miR-15b-5p, and β-actin was that of the rest of the detected molecules. All qRT-PCR was repeated three times. The primers were designed and synthesized by Guangzhou Ribo Biotechnology Co., Ltd. Primer sequences are shown in Table 1.

Western blot (WB)

THP-1 monocytes and VECs were collected and washed with cold PBS three times. Then, 100~200 μL RIPA lysate (Beyotime Biotechnology, Shanghai, China) was added for ultrasonic water-splitting. The protein concentration was verified by the Bradford

Table 1. Primer sequences of each gene.

Genes	primer sequence (5`→3`)
miR-15b-5p	product code:MmiR3463
MMP1	forward:GCTTGACCCTCAGAGACCTT
	reverse:GATGAAGCAGCCCAGATGTG
MMP2	forward:GTGCCCTCTTGAGACAGTCT
	reverse:AATCCCACCAACCCTCAGAG
MMP9	forward:AAAGGTGAGAAGAGAGGGCC
	reverse:GAGTTCCCGGAGTGAGTTGA
IL-4	forward:TTTCAGTGTTGTGAGCGTGG
	reverse:CAACAAGGAACACCACGGAG
IL-10	forward:TTTCTGGGCCATGGTTCTCT
	reverse:ATAACTGCACCCACTTCCCA
VEGF	forward:TTCCACACGTAGCCTTCCTT
	reverse:AGGGCGTTACGTGTATGGAA
GAPDH	forward:TGATCTTCATGGTCGACGGT
	reverse:CCACGAGACCACCACCTACAACT

method. An equivalent amount of protein in each group was subjected to 10% SDS-PAGE electrophoresis, and the proteins on the gel were transferred to PVDF membranes (Millipore, Bedford, MA, USA). Afterward, the membranes were blocked at 4° C for 1 hour and incubated with the primary antibodies (1:1000) of Anti-CD63 antibody (ab217345), Anti-CD9 antibody (ab92726), Anti-Tsg101 antibody (ab125011), Anti-iNOS antibody (ab15323), Anti-CD68 antibody (ab125212), Anti-CD163 antibody (ab182422), Anti-CD20 antibody (ab78237), Anti-WIF1 antibody (ab89935), Anti-WNT5A antibody (ab229200), Anti-VEGFA antibody (ab52917), Anti-FGF 23 antibody (ab56326), Anti-eNOS antibody (ab76198), Anti-PI3K antibody (ab191606), Anti-PI3k (phosphoY607) antibody (ab182651), Anti-pan-AKT antibody (ab18785), Anti-AKT (phospho T308) antibody (ab38449) and Anti-GAPDH antibody (ab128915) at 4° C overnight. After the membranes were washed twice with TBST, they were incubated with the fluorescein-labeled Goat Anti-Rabbit IgG (ab205718,1:2500) for 1 hour at room temperature. The above antibodies were obtained from Abcam (MA, USA). After the membranes were rinsed three times, they were exposed with the ECL chromogenic agent (Millipore, Bedford, MA, USA) and imaged with a membrane scanner.

Dual-luciferase reporter assay

The purpose fragments of wild-type and mutant WIF1 were constructed and integrated into pGL3 vectors (Promega, Madison, WI, USA) to induce pGL3-WIF1-wild type (WIF1-WT) and PGL3-WIF1-mutant (WIF1-MUT) reporter vectors. THP-1 monocytes were co-transfected with WiF1-WT or WIF1-MUT and miR-15b-5p or the negative control. Forty-eight hours after the transfection, the luciferase activity was measured following the manufacturer's instructions. All experiments were done three times.

Xenograft tumor experiment

Ten BALB/c nude mice (female, 4-6 weeks old, 16-20g) were obtained from the Laboratory Animal Research Center of Shandong Province (Shandong, China). All animal experiments were implemented in accordance with the "Guidelines for the Care and Use of Experimental Animals" issued by Linyi people's hospital. Each mouse was subcutaneously injected with 5×10^6 AGS human gastric carcinoma cells on the right side. The liposomal clodronate was intravenously injected to clear macrophages [18]. The xenografted nude mice were randomized into two groups (5 mice in each group). MiR-15b-5p-mimics or miR-NC (RiboBio, Guangzhou, China) were injected directly into the xenograft tumor in the mice via the tail vein at the dose of 10^6 cells/50 μL for eight times over three days. After the injection, the mice were anesthetized on day 5, 10, 15, 20 and 25, respectively, and the tumors were removed and weighed. The longest diameter (a) of the tumor and the shortest diameter (b) perpendicular to it were determined with a vernier caliper, and the tumor volume was calculated according to the formula V (mm^3) =0.5× a × b^2. All experiments were approved by the Animal Experiment Ethics Review Committee of the Institute of Medicine of Linyi people's hospital. All experiments were carried out strictly abide by the National Institutes of Health

Laboratory Animal Care and Use Guidelines (NIH Publication No. 8023).

Data analysis

Data were analyzed with the SPSS20.0 statistical software (SPSS Inc., Chicago, IL, USA) and presented as mean ± standard deviation (x±s). The multi-factor comparison was made by One-way ANOVA, and *t* test was adopted for pairwise comparison. $P<0.05$ indicated statistical significance.

RESULTS

Gastric cancer cells promoted the formation of tumor-associated macrophages and enhanced the miR-15b-5p expression in tumor-associated macrophages

THP-1 monocytes were co-cultured with AGS cells to test the effect of GC cells on TAM formation. First, the expression of TAM molecular markers (MMP1, MMP2, MMP9, IL-4, IL-10, and VEGF) was examined by qRT-PCR, and it was found that the above molecular markers were up-regulated compared with that of the control group ($P<0.05$, Figure 1A). WB demonstrated that the expression of molecular markers (iNOS, CD68, CD163, and CD20) of TAMs was up-regulated in the co-culture system compared with that in the control group ($P<0.05$, Figure 1B). Moreover, the miR-15b-5p level was monitored by qRT-PCR, and the results confirmed that GC cells significantly promoted the miR-15b-5p expression in TAMs compared with that in the control group ($P<0.05$, Figure 1C). Also, WB results testified that compared with the control group, WIF1 was down-regulated, and WNT5A was up-regulated ($P<0.05$, Figure 1D). These findings stated that the co-culture of GC cells and THP-1 monocytes induced the formation of TAMs and facilitated the miR-15b-5p expression in TAMs.

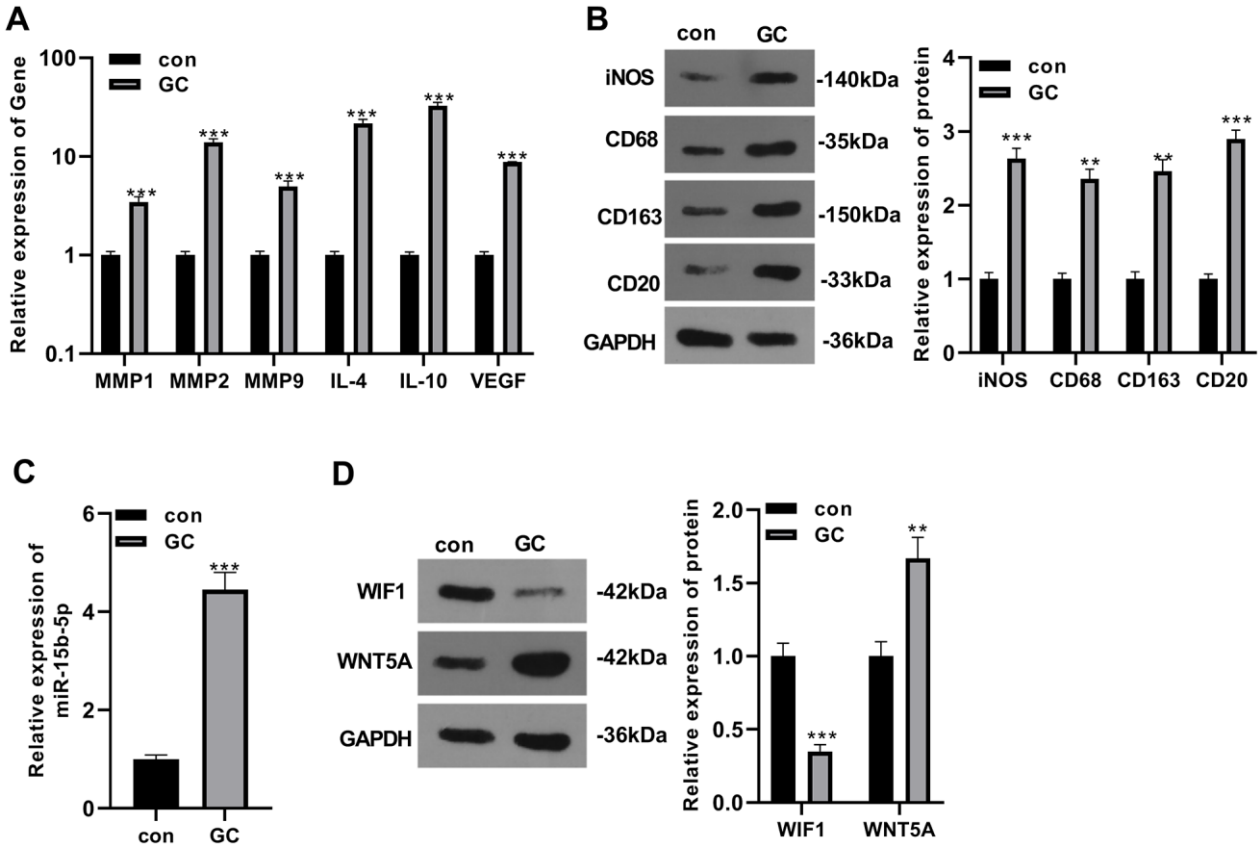

Figure 1. Gastric cancer cells promoted the formation of tumor-associated macrophages and enhanced the miR-15b-5p expression in tumor-associated macrophages. THP-1 monocytes were co-cultured with GC cells for 24 hours to obtain TAMs. (**A**) QRT-PCR was adopted to calculate the expression of molecular markers (MMP1, MMP2, MMP9, IL-4, IL-10, and VEGF) of TAMs. (**B**) WB was implemented to verify the expression of molecular markers (iNOS, CD68, CD163 and CD20) of TAMs. (**C**) The miR-15b-5p level was monitored by qRT-PCR. (**D**) The WIF1/WNT5A expression was examined by WB. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (vs. con group), N=3.

Tumor-associated macrophages strengthened the malignant phenotypes of gastric cancer cells and angiogenesis of vascular endothelial cells

Cell processing is shown in Figure 2A. First, AGS human gastric carcinoma cell proliferation and apoptosis were detected by CCK-8 and flow cytometry, respectively, to examine the effect of TAMs on AGS human gastric carcinoma cells. The results showed that compared with the control group, GC cell proliferation in the TAMs group was increased while the apoptosis was reduced (P<0.05, Figure 2B, 2C). Meanwhile, the

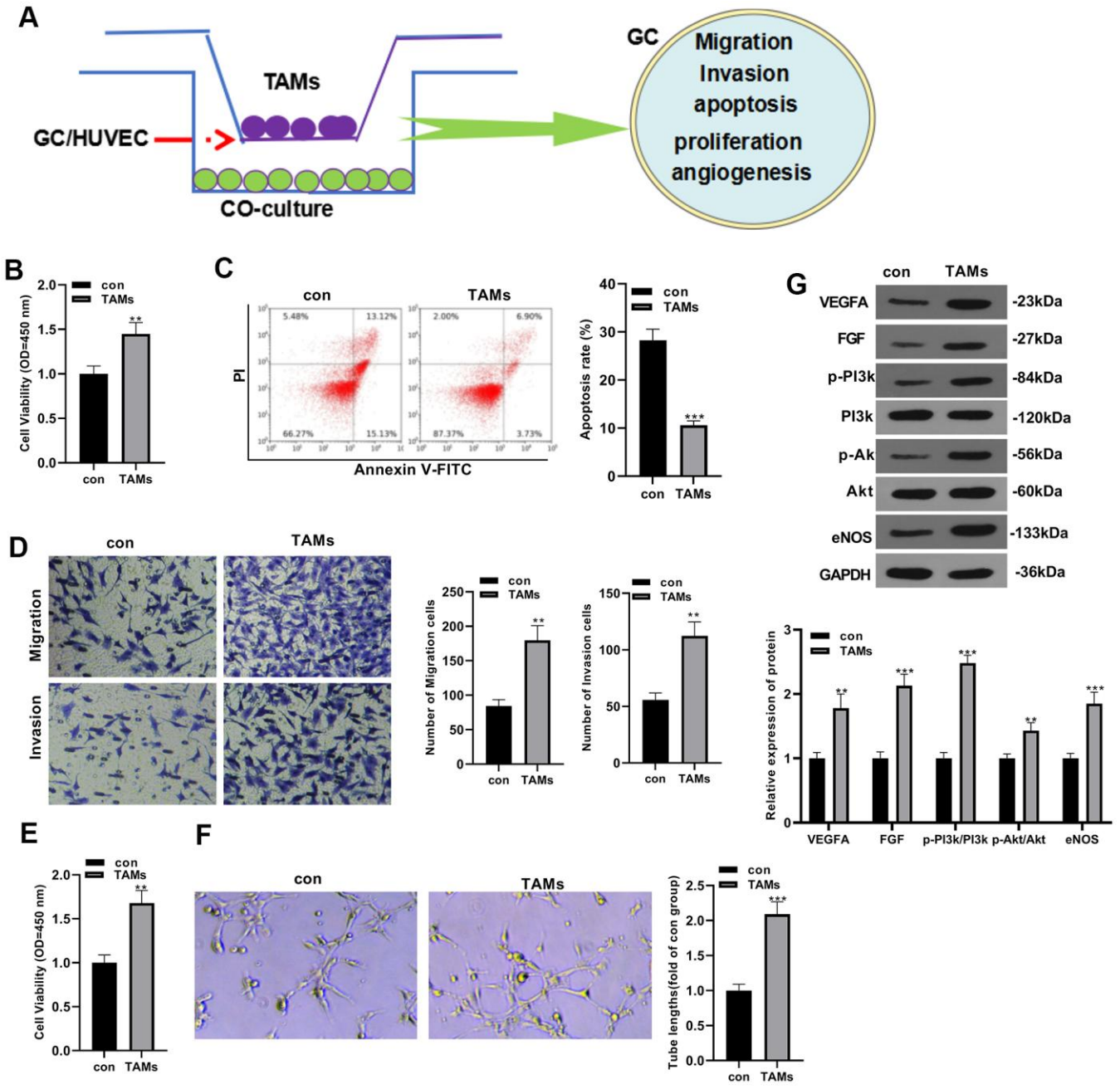

Figure 2. Tumor-associated macrophages strengthened the malignant phenotypes of gastric cancer cells and angiogenesis of vascular endothelial cells. (**A**) The cell processing method. (**B**, **C**) AGS human gastric carcinoma cells proliferation and apoptosis were detected by CCK-8 and flow cytometry, respectively. (**D**) Transwell assay was employed to determine the influence of TAMs on GC cell migration and invasion. (**E**) CCK-8 method was conducted to determine the impact of TAMs on VEC proliferation. (**F**) Tube formation assay was employed to test VEC angiogenesis. (**G**) The protein expression VEGFA, FGF, and PI3k/Akt/eNOS were compared by WB. **P<0.01, ***P<0.001 (vs. Con group), N=3.

Transwell assay confirmed that GC cell migration and invasion in the TAMs group were facilitated (P<0.05, Figure 2D). Angiogenesis is closely related to TAMs, which is also the key to tumor development [19]. Therefore, we tested VEC proliferation by the CCK-8 method and found that cell proliferation was enhanced in the TAMs group (P<0.05, Figure 2E). Also, the tube formation experiment testified that TAMs promoted angiogenesis of VECs (P<0.05, Figure 2F). Besides, studies have demonstrated that TAMs can promote angiogenesis through various pro-angiogenic factors [20]. Therefore, we further monitored the protein levels of vascular endothelial growth factor A (VEGFA), fibroblast growth factor (FGF) and PI3k/Akt/eNOS by WB, and discovered that TAMs elevated the expression of the above factors (P<0.05, Figure 2G). These findings suggested that TAMs aggravated the malignant biological behaviors of GC cells and enhanced the angiogenesis of VECs.

Gastric cancer cell-derived exosomes overexpressing miR-15b-5p accelerated the "M2" polarization of tumor-associated macrophages

First, we transfected miR-15b-5p mimics into AGS cells co-cultured with THP-1 monocytes. Then we isolated the exosomes from AGS cells (Supplementary Figure 1A–1C). Next, the exosome were treated with THP1 cells. As a result, miR-15b-5p was up-regulated in the exosomes of the GC+ miR-15b-5p group compared to that of the GC+miR-NC group (P<0.05, Figure 3A). Furthermore, we checked the expression of molecular markers (MMP1, MMP2, MMP9, IL-4, IL-10, and VEGF) of

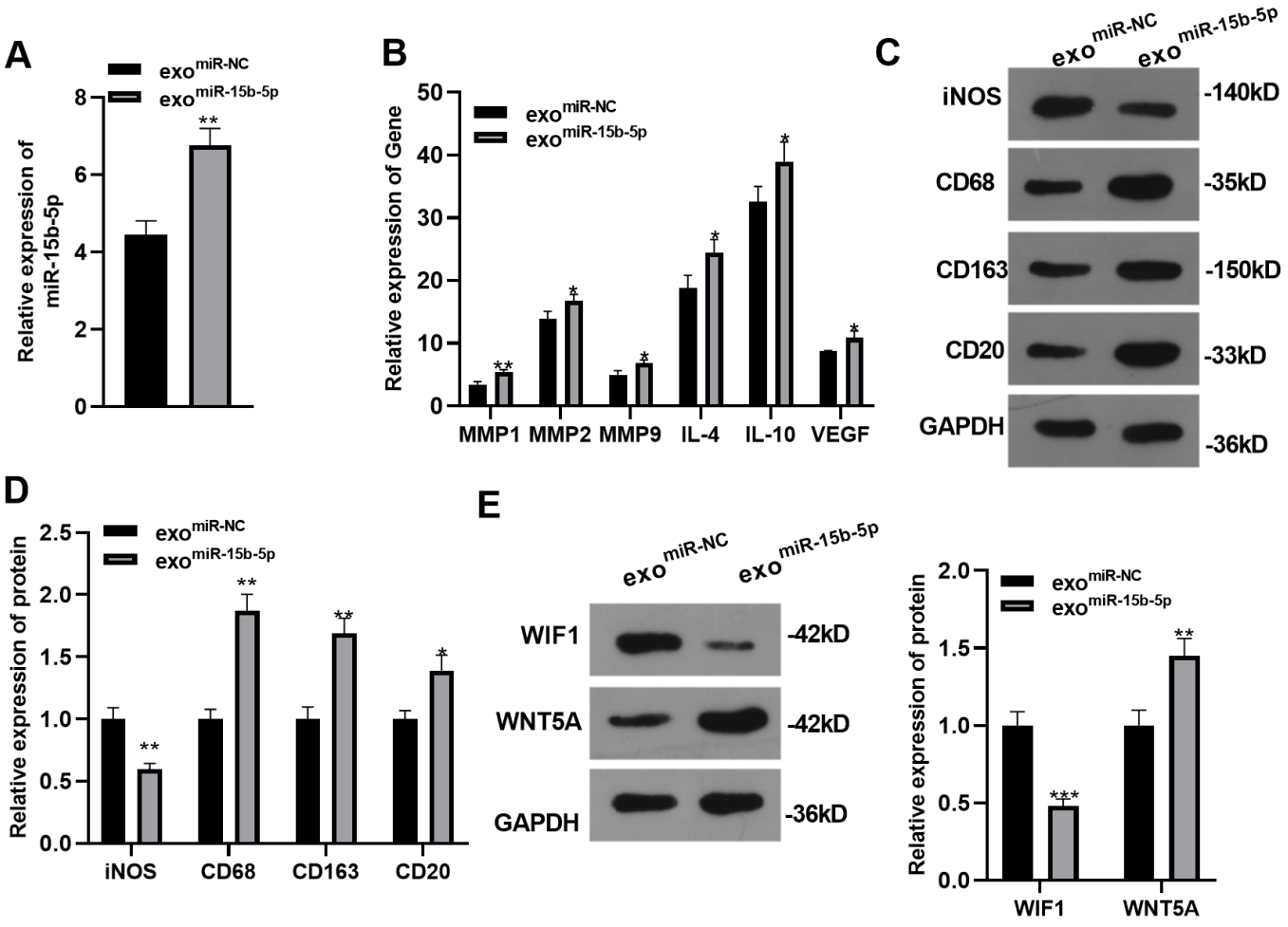

Figure 3. Gastric cancer cell-derived exosomes overexpressing miR-15b-5p accelerated the "M2" polarization of tumor-associated macrophages. miR-15b-5p mimics were transfected into GC cells co-cultured with THP-1 monocytes. (**A**) The miR-15b-5p level was detected by qRT-PCR. (**B**) QRT-PCR was carried out to verify the levels of molecular markers (MMP1, MMP2, MMP9, IL-4, IL-10, and VEGF) of TAMs. (**C**–**E**) WB was conducted to examine the expression of molecular markers (iNOS, CD68, CD163, and CD20) of TAMs and the WIF1/WNT5A pathway. *P<0.05, **P<0.01, ***P<0.001 (vs.GC+ miR-NC group), N=3.

TAMs through qRT-PCR. The results also showed that the above molecular markers were up-regulated in the GC+miR-15b-5p group (P<0.05, Figure 3B). Next, the expression of polarization markers (iNOS, CD68, CD163, and CD20) of TAMs and the WIF1/WNT5A pathway was measured by WB. Interestingly, compared with the GC+miR-NC group, the "M2" polarization markers including CD68, CD163, and CD20 were all promoted, while iNOS, an "M1" polarization marker, was inhibited. Meanwhile, WNT5A was up-regulated after the transfection with miR-15b-5p mimics, while WIF1 was down-regulated (P<0.05, Figure 3C–3E). Moreover, we found that miR-15b-5p was upregulated in GC tissues (Supplementary Figure 1D). Herin, GC cell-derived exosomal miR-15b-5p promoted the development of TAMs.

Tumor-associated macrophages overexpressing miR-15b-5p deteriorated the malignant phenotype of gastric cancer cells and angiogenesis of vascular endothelial cells

Cell processing is shown in Figure 4A. To explore the influence of TAMs overexpressing miR-15b-5p on the

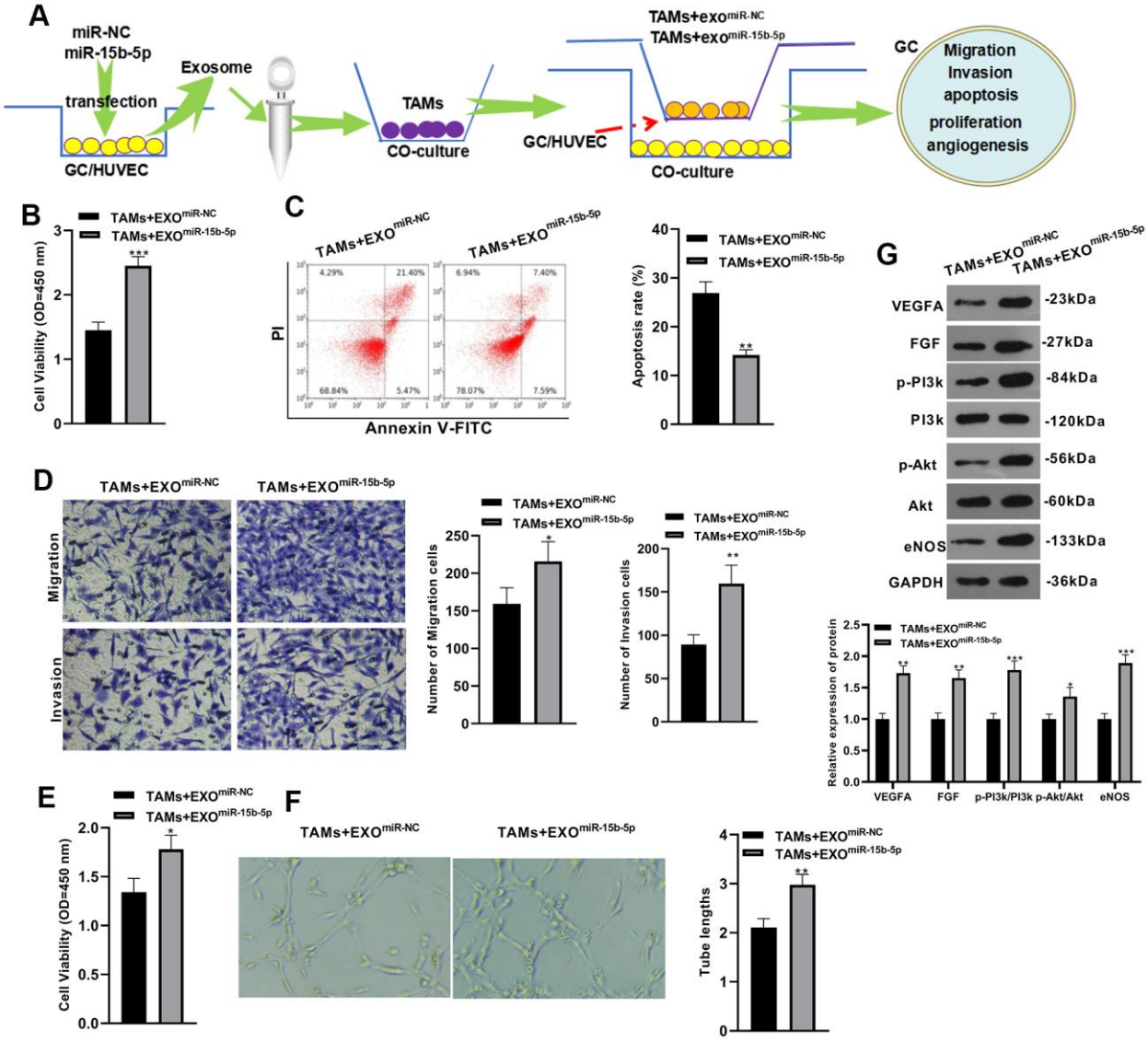

Figure 4. Tumor-associated macrophages overexpressing miR-15b-5p deteriorated the malignant phenotype of gastric cancer cells and angiogenesis of vascular endothelial cells. (**A**) The cell processing method. (**B**, **C**) AGS human gastric carcinoma cells proliferation and apoptosis were monitored by CCK-8 and flow cytometry, respectively. (**D**) Transwell assay was applied to verify GC cell migration and invasion. (**E**) VEC proliferation was measured by the CCK-8 method. (**F**) Tube formation assay was implemented to examine VEC angiogenesis. (**G**) Protein profiles of VEGFA, FGF and PI3k/Akt/eNOS were compared by WB.*P<0.05, **P<0.01, ***P<0.001 (vs.TAMs+EXO^{miR-NC} group), N=3.

malignant biological behavior of GC cells, we verified AGS human gastric carcinoma cells proliferation and apoptosis in TAMs transfected with the mimics of GC cell-derived exosomal miR-15b-5p using the CCK-8 method and flow cytometry, respectively. It turned out that compared with the TAMs+EXO^{miR-NC} group, the cell proliferation increased and apoptosis was reduced after the miR-15b-5p mimic transfection (P< 0.05, Figure 4B, 4C). Meanwhile, the Transwell assay illustrated that GC cell migration and invasion in the TAMs+EXO$^{miR-15b-5p}$ group were raised (P<0.05, Figure 4D). Similarly, the CCK-8 method showed that VEC proliferation in the TAMs+ EXO$^{miR-15b-5p}$ group was elevated (P<0.05, Figure 4E). Also, the tube formation experiment confirmed that the cell angiogenesis was further enhanced after the miR-15b-5p mimic transfection in TAMs (P<0.05, Figure 4F). Furthermore, the expression of VEGFA, FGF, and PI3k/Akt/eNOS was compared by WB. It was found that these factors were up-regulated in the TAMs+ EXO$^{miR-15b-5p}$ group compared with that in the TAMs+EXO^{miR-NC} group (P<0.05, Figure 4G). It was illustrated that the miR-15b-5p mimic transfection in TAMs promoted GC cell proliferation and metastasis and VEC angiogenesis.

Gastric cancer cell-derived exosomes knocking down miR-15b-5p weakened the "M2" polarization

First, we transfected the miR-15b-5p knockdown plasmids into GC cells co-cultured with THP-1 monocytes and examined the miR-15b-5p expression by qRT-PCRT to clarify the effect of GC cell-derived exosomes knocking down miR-15b-5p on TAMs. It turned out that miR-15b-5p was down-regulated after knocking down miR-15b-5p compared with the GC+miR-NC-in group (P<0.05, Figure 5A). Moreover, the expression of molecular markers (MMP1, MMP2, MMP9, IL-4, IL-10, and VEGF) of TAMs was detected by qRT-PCR. The results illustrated that the above molecular markers of TAMs in the GC+miR-15b-5p-in

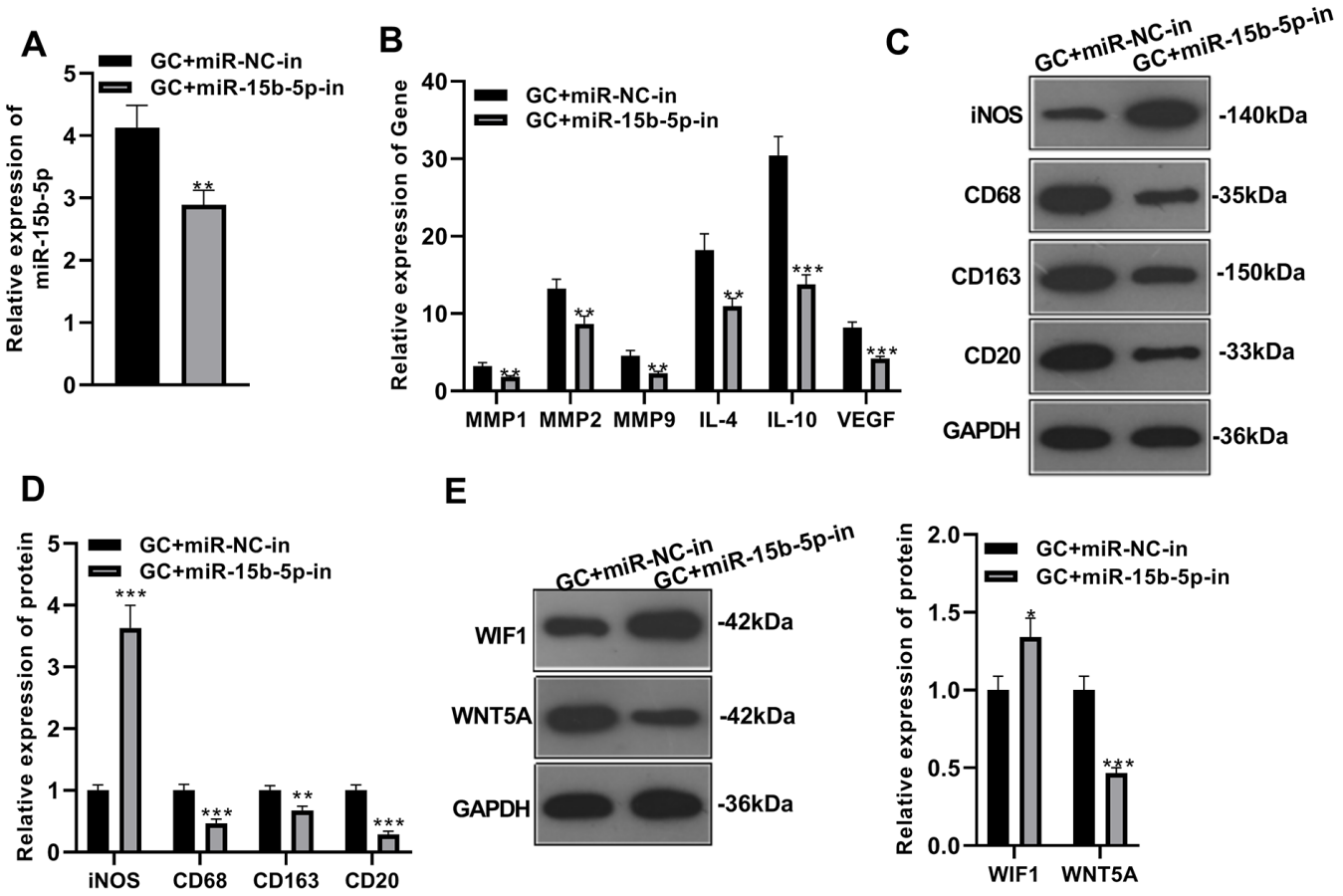

Figure 5. Gastric cancer cell-derived exosomes knocking down miR-15b-5p weakened the "M2" polarization. A miR-15b-5p knockdown model was constructed in GC cells co-cultured with THP-1 monocytes. (**A**) The miR-15b-5p expression was monitored by qRT-PCR. (**B**) QRT-PCR was carried out to examine the profiles of molecular markers (MMP1, MMP2, MMP9, IL-4, IL-10, and VEGF) of TAMs. (**C**–**E**) WB was applied to test the expression of molecular markers (iNOS, CD68, CD163, and CD20) of TAMs and WIF1/WNT5A. * P < 0.05, * *P < 0.01, * * * P < 0.001, (vs GC + miR-NC-in group), N = 3.

group were down-regulated (P<0.05, Figure 5B). Then, the expression of TAM polarization markers (iNOS, CD68, CD163, and CD20) was tested by WB. As a result, compared with the GC+miR-NC-in group, CD68, CD163, and CD20 in TAMs were down-regulated and iNOS was up-regulated after the miR-15b-5p knockdown (P<0.05, Figure 3C, 3D). Meanwhile, WB revealed that WIF1 was up-regulated, while WNT5A was down-regulated in the GC+ miR-15b-5p-in group (P<0.05, Figure 5E). These results suggested that knocking down GC cell-derived exosomal miR-15b-5p slowed down the development of TAMs.

miR-15b-5p inhibition in tumor-associated macrophages attenuated the malignant phenotypes of gastric cancer cells and angiogenesis of vascular endothelial cells

Cell processing is shown in Figure 6A. To study the effect of TAMs knocking down miR-15b-5p on the

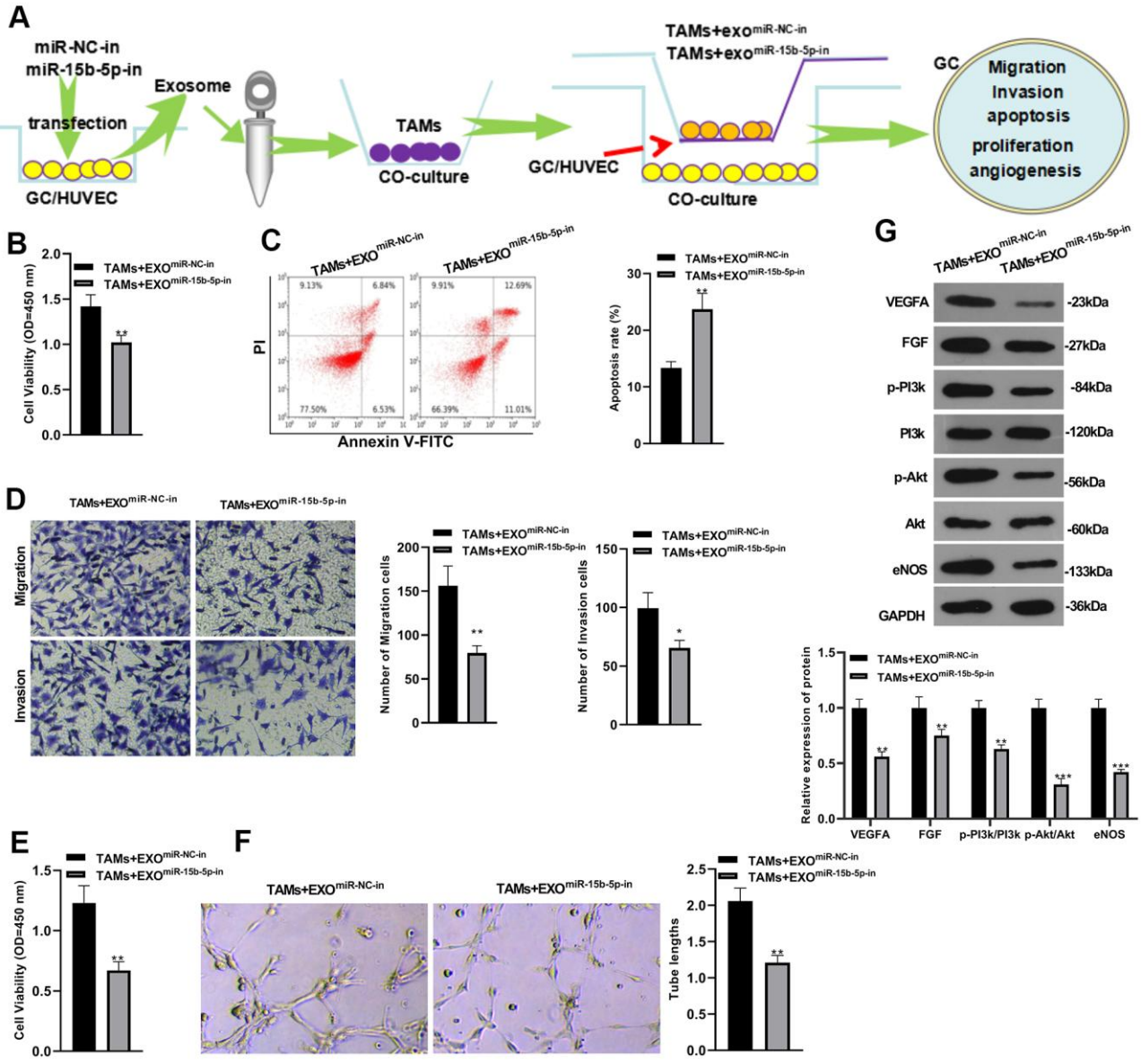

Figure 6. miR-15b-5p inhibition in tumor-associated macrophages attenuated the malignant phenotypes of gastric cancer cells and angiogenesis of vascular endothelial cells. (**A**) The cell processing method. (**B**, **C**) AGS human gastric carcinoma cells proliferation and apoptosis were testified by CCK-8 and flow cytometry, respectively. (**D**) Transwell assay was applied to detect GC cell migration and invasion. (**E**) VEC proliferation was measured by the CCK-8 method. (**F**) Tube formation assay was employed to verify VEC angiogenesis. (**G**) Protein profiles of VEGFA, FGF and PI3k/Akt/eNOS in VEC were compared by WB. **P<0.01, ***P<0.001 (vs.TAMs+ EXO$^{miR\text{-}NC\text{-}in}$ group), N=3.

malignant biological behaviors of GC cells, we conducted the CCK-8 method and flow cytometry in TAMs supplemented with the inhibitors of GC cell-derived exosomal miR-15b-5p to verify AGS human gastric carcinoma cell proliferation and apoptosis. The results demonstrated that compared with the TAMs+EXO$^{miR\text{-}NC\text{-}in}$ group, cell proliferation was reduced and apoptosis was increased after knocking down miR-15b-5p (P<0.05, Figure 6B, 6C). Meanwhile, the Transwell assay illustrated that GC cell migration and invasion in the TAMs+ EXO$^{miR\text{-}15b\text{-}5p\text{-}in}$ group were also significantly dampened (P<0.05, Figure 6D). Similarly, the CCK-8 method showed that VEC proliferation in the TAMs+EXO$^{miR\text{-}15b\text{-}5p\text{-}in}$ group was abated (P<0.05, Figure 6E). The results of the tube formation experiment confirmed that the cell angiogenesis was attenuated in the TAMs knocking down miR-15b-5p (P<0.05, Figure 6F). Furthermore, the expression of VEGFA, FGF and PI3k/Akt/eNOS was tested by WB. It was found these factors in the TAMs+EXO$^{miR\text{-}15b\text{-}5p\text{-}in}$ group were knocked down compared with that in the TAMs+EXO$^{miR\text{-}NC\text{-}in}$ group (P<0.05, Figure 6G). These results manifested that TAMs knocking down miR-15b-5p repressed GC cell proliferation and metastasis and weakened VEC angiogenesis.

Inhibiting miR-15b-5p or WNT5A in the tumor-associated macrophages reversed "M2" phenotype alteration

We first transfected miR-15b-5p inhibitor or si-WNT5A in TAMs, which were treated with the exosomes from AGC cells with overexpressed miR-15b-5p. Compared with the miR-NC-in+exo$^{miR\text{-}15b\text{-}5p}$ group, the expression of miR-15b-5p was significantly down-regulated after transfection with miR-15b-5p inhibitor, while the expression of miR-15b-5p was not significantly different after inhibition of WNT5A (P<0.05, Figure 7A). We further detected the expression of molecular markers of TAMS (MMP1, MMP2, MMP9, IL-4, IL-10, VEGF) by qRT-PCR, and the results also showed that, the expression of these molecular markers of TAMS was significantly down-regulated after

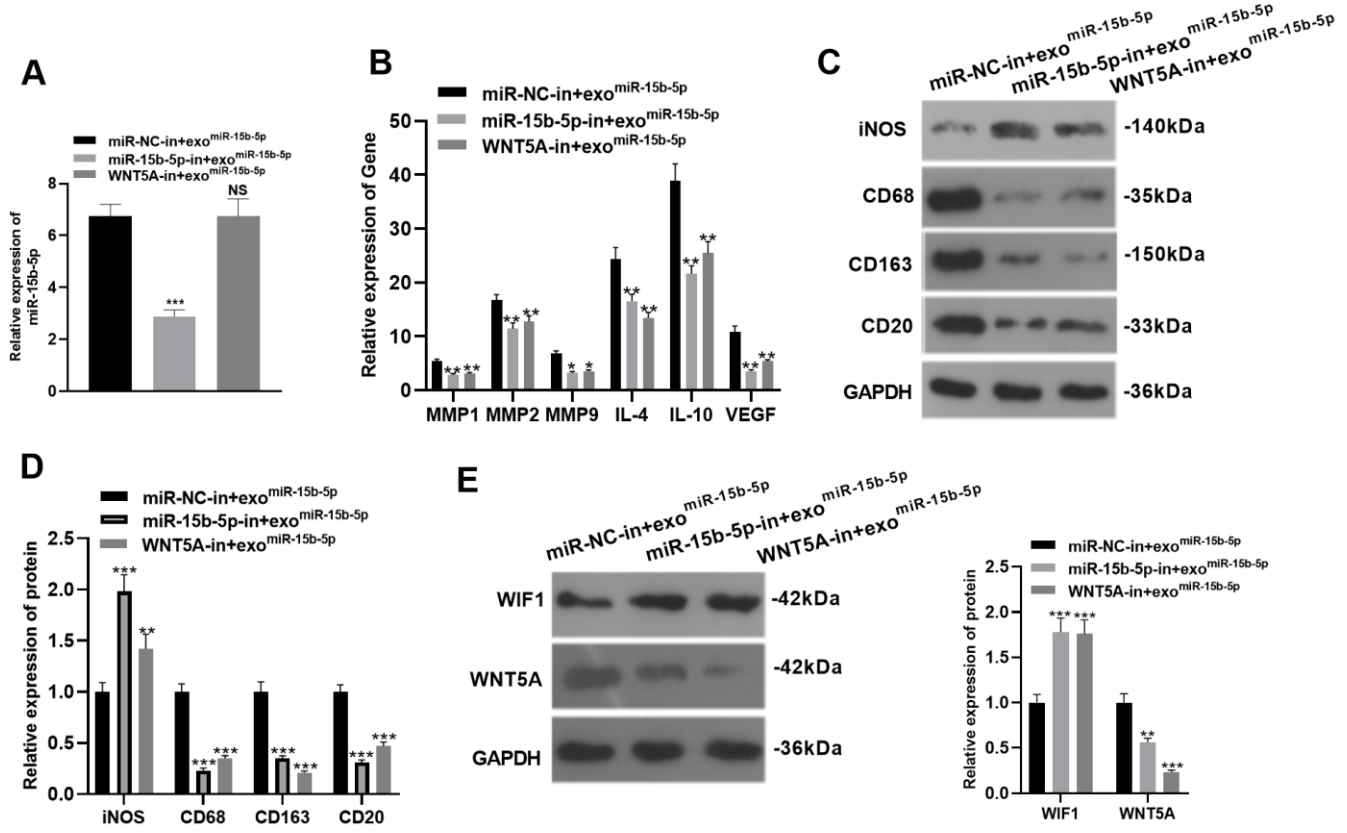

Figure 7. Inhibiting miR-15b-5p or WNT5A in the tumor-associated macrophages reversed "M2" phenotype alteration. miR-15b-5p inhibitor or WNT5A inhibitor was transfected into TAMs, which were then treated with exosomes of GC cells with overexpressing miR-15b-5p. (**A**) The miR-15b-5p expression in TAMs was monitored by qRT-PCR. (**B**) qRT-PCR was carried out to examine the profiles of molecular markers (MMP1, MMP2, MMP9, IL-4, IL-10, and VEGF) of TAMs. (**C**–**E**) WB was applied to test the expression of molecular markers (iNOS, CD68, CD163, and CD20) and WIF1/WNT5A of TAMs. * P < 0.05, * *P < 0.01, * * * P < 0.001, (vs miR-NC-in+exo$^{miR\text{-}15b\text{-}5p}$ group), N = 3.

transfection with miR-15b-5p inhibitor or inhibition of WNT5A (compared with the miR-NC-in+exo$^{miR-15b-5p}$ group, P<0.05, Figure 7B). Meanwhile, the protein expression of iNOS was significantly up-regulated, while the protein expressions of CD68, CD163 and CD20 were significantly down-regulated (P<0.05, Figure 7C, 7D). Western blot results also showed that compared with the miR-NC-in+exo$^{miR-15b-5p}$ group, the expression of WIF1 in the miR-15b-5p-in+exo$^{miR-15b-5p}$ group was significantly up-regulated, while the expression of WNT5A was down-regulated (P<0.05, Figure 7E). This suggests that inhibition of miR-15b-5p or WNT5A in TAMs weakens the transformation of TAMS to the "M2" phenotype.

The inhibition of miR-15b-5p or WNT5A in tumor-associated macrophages weakens their tumor-promoting effects on AGS cells

The cell treatment methods were shown in Figure 8A. CCK-8 method and flow cytometry were used to detect

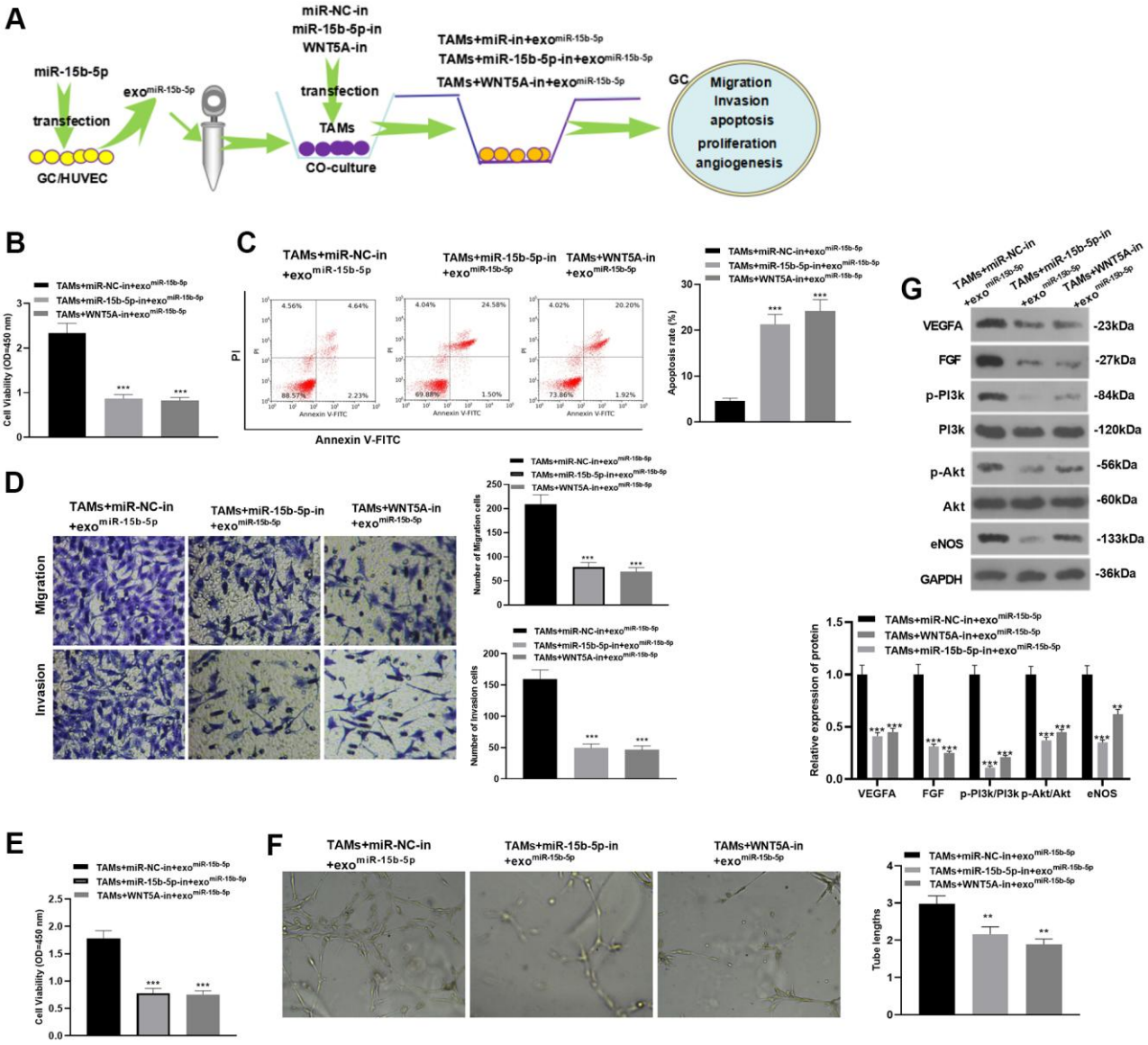

Figure 8. The inhibition of miR-15b-5p or WNT5A in tumor-associated macrophages weakens their tumor-promoting effect in AGS cells. (**A**) The cell processing method. MiR-15b-5p inhibitor or WNT5A inhibitor was transfected into TAMs, which were then treated with exosomes of GC cells with overexpressing miR-15b-5p. Then the TAMs were coculture with AGS cells or VECs. (**B**, **C**) AGS human gastric carcinoma cells proliferation and apoptosis were testified by CCK-8 assay and flow cytometry, respectively. (**D**) Transwell assay was applied to detect GC cell migration and invasion. (**E**) VEC proliferation was measured by the CCK-8 method. (**F**) Tube formation assay was employed to verify VEC angiogenesis. (**G**) Protein profiles of VEGFA, FGF and PI3k/Akt/eNOS in VECs were compared by WB. **P* < 0.05, ***P*<0.01, ****P*<0.001 (vs.TAMs+miR-NC-in+ exo$^{miR-15b-5p}$ group), N=3.

the proliferation and apoptosis ability of AGS cells, respectively. The results showed that compared with the TAMs+miR-NC-in+exo$^{miR-15b-5p}$ group, cell proliferation, migration and invasion were significantly reduced and apoptosis was significantly increased in the TAMs+miR-15b-5p-in+exo$^{miR-15b-5p}$ group or TAMs+WNT5A-in+exo$^{miR-15b-5p}$ group ($P<0.05$, Figure 8B–8D). Similarly, in the vascular endothelial cells, the results of the CCK-8 method also showed that the proliferation of VECs in the TAMs+miR-15b-5p-in+exo$^{miR-15b-5p}$ group and the TAMs+WNT5A-in+exo$^{miR-15b-5p}$ group was significantly reduced ($P<0.05$, Figure 8E). The tubule formation assay results also showed that the angiogenesis was significantly reduced when inhibiting miR-15b-5p or WNT5A in TAMs ($P<0.05$, Figure 8F). The protein expressions of VEGFA, FGF and PI3K /Akt/eNOS were further detected by Western blot assay. Compared with TAMs+miR-NC-in+exo$^{miR-15b-5p}$ group, the protein expressions of TAMs+miR-15b-5p-in+exo$^{miR-15b-5p}$ group and the TAMs+WNT5A-in+exo$^{miR-15b-5p}$ group were significantly down-regulated ($P<0.05$, Figure 8G). These results indicate that repressing miR-15b-5p or WNT5A in TAMs weakens the proliferation and metastasis of GC cells and the angiogenesis of vascular endothelial cells, which were induced by TAMs.

Overexpressing miR-15b-5p facilitated gastric cancer cell proliferation and tumor-associated macrophages M2 polarization *in vivo*

We conducted *in vivo* experiments to verify the influence of overexpressing miR-15b-5p on GC cell proliferation. It was found that overexpressing miR-15b-5p promoted tumor volume and weight (vs. the control group) ($P<0.05$, Figure 9A–9C). Then, the expression of molecular markers (MMP1,2,9, IL-4,

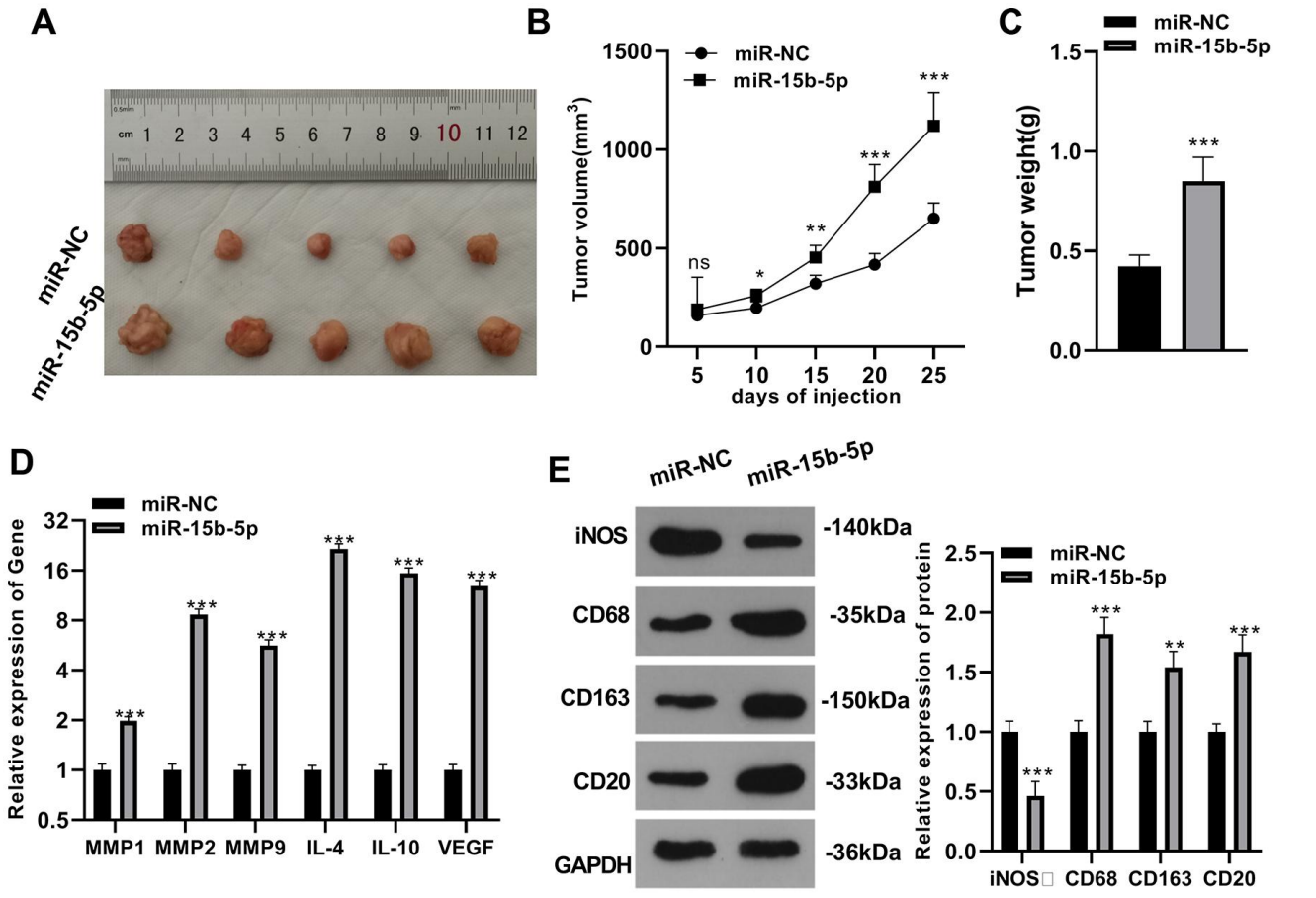

Figure 9. Overexpressing miR-15b-5p facilitated gastric cancer cell proliferation and tumor-associated macrophages invasion *in vivo*. 5×10^6 AGS human gastric carcinoma cells were injected subcutaneously in each mouse at the right side, and miR-15b-5p-mimics or miR-NC were directly injected into the xenograft tumor at a dose of 10^6 cells/50μL, with eight times over three days. After injection, the mice were anesthetized on day 5, 10, 15, 20 and 25, respectively, to collect the tumors. (**A–C**) The tumor volume and weight. (**D**) QRT-PCR was implemented to testify the profiles of molecular markers (MMP1,2,9, IL-4, IL-10, and VEGF) of TAMs. (**E**) The expression of TAM polarization markers (iNOS, CD68, CD163, and CD20) of TAMs was monitored by WB. Ns$P > 0.05$, *$P < 0.05$, **$P < 0.01$, * * * $P < 0.001$, (vs miR - NC group), N = 5.

IL-10, and VEGF) of TAMs was verified by qRT-PCR. Interestingly, overexpressing miR-15b-5p distinctly up-regulated these molecular markers (P<0.05, Figure 9D). Also, WB proved that the protein expression of "M2" polarization markers (CD68, CD163, and CD20) of TAMs was up-regulated and iNOS was down-regulated after overexpressing miR-15b-5p (P<0.05, Figure 9E). These findings confirmed that overexpressing miR-15b-5p enhanced GC cell proliferation and TAM invasion *in vivo*.

miR-15b-5p targeted WIF1 and up-regulated WNT5A

Through the Starbase database (http://starbase.sysu.edu.cn/), we discovered that the binding sites between miR-15b-5p and WIF1 were shown as that in Figure 10A. Then, the dual-luciferase reporter assay was adopted to clarify the targeted association between the two. As a result, miR-15b-5p mimics attenuated the luciferase activity of cells transfected with the WIF1-WT vector but had little effect on that of WIF1-MUT (P <0.05, Figure 10B). Additionally, qRT-PCR was implemented to verify the mRNA expression of WIF1 and WNT5A after transfecting miR-15b-5p mimics into THP-1 monocytes. It was found that compared with the control group, WIF1 was down-regulated, while WNT5A was significantly up-regulated after overexpressing miR-15b-5p (P<0.05, Figure 10C, 10D). These findings indicated that miR-15b-5p inhibited WIF1 and up-regulated WNT5A.

DISCUSSION

TAMs are mainly derived from monocytes in peripheral blood. Constrained by chemokines (CCL2, CCL5, and CXCL12) and cytokines (VEGF, FGF, TGFa, and M-CSF), these monocytes aggregate in tumor tissues and differentiate into TAMs [21]. As reported, TAMs are closely related to the low survival rate of tumor patients. By promoting tumor angiogenesis and lymphoangiogenesis, TAMs regulate the transduction of tumor growth promotion signals and produce various activated molecules that enhance the proliferation, invasion and migration of tumor cells, thus shortening the survival time of patients [22–24]. Therefore, TAMs may be an important factor leading to the poor prognosis of tumor patients. In the current study, THP-1

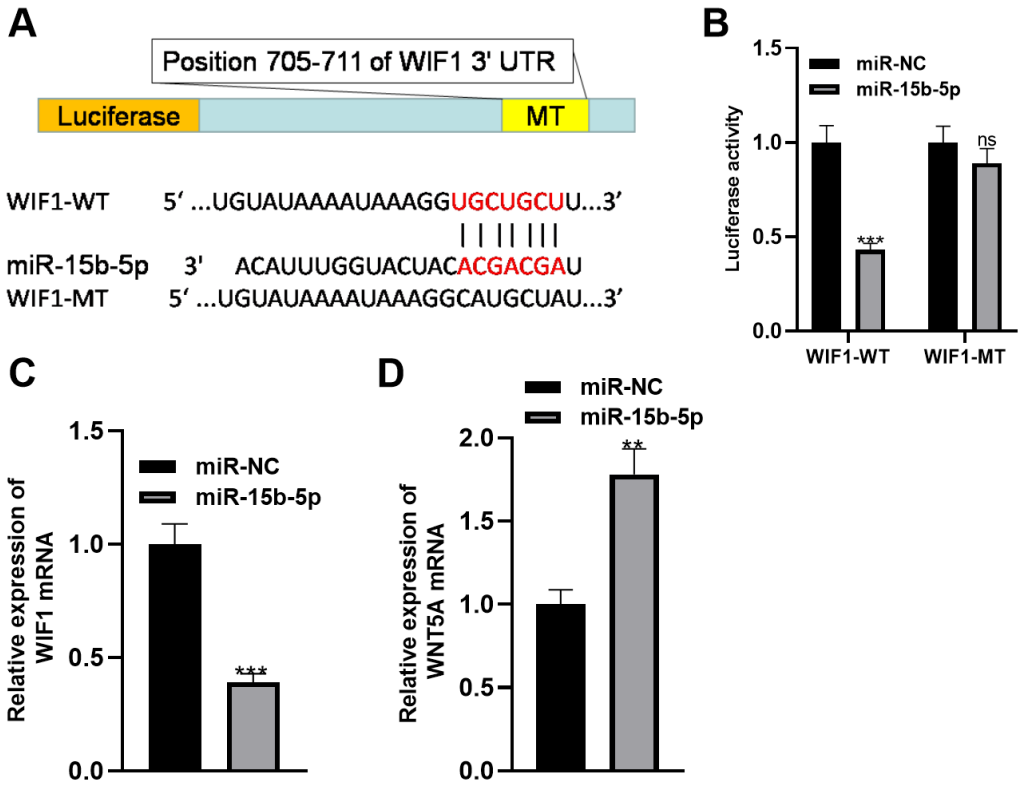

Figure 10. MiR-15b-5p targeted WIF1 and up-regulated WNT5A. (**A**) The combining sites between miR-15b-5p and WIF1 were predicted by the Starbase database. (**B**) Dual-luciferase reporter assay was conducted to verify the binding association between miR-15b-5p and WIF1. (**C**, **D**) QRT-PCR was utilized to determine the mRNA expression of WIF1 and WNT5A after transfecting miR-15b-5p mimics into THP-1 monocytes. NsP>0.05, **P<0.01, ** *P<0.001 (vs miR-NC group), N=3.

monocytes were co-cultured with GC cells to obtain TAMs, and it was found that the co-culture of the two induced the formation of TAMs. In addition, we found that TAMs significantly facilitated the development of GC cells and expedited the angiogenesis of VEC. At the same time, GC cell-derived exosomal miR-15b-5p enhanced the immunodiffusion of TAMs via the WIF1/WNT5A axis, which provided more references for the molecular mechanism of the interaction between TAMs and GC cells.

TAMs are key drivers for tumor development and are closely associated with invasion, migration and poor prognosis of diversified cancers [25–27]. Similarly, TAMs have been testified to promote GC cell invasion and metastasis via TGFβ2/NF-κB/ Kindlin-2 [28]. Furthermore, Guo J et al. discovered that TAMs motivate GC cell EMT, invasion and migration by inducing the FOXQ1 expression [29]. More importantly, Wang F et al. reported in 2018 that GC cell-derived exosomes can effectively induce the formation of M2-polarized TAMs, which in turn strengthen the malignant phenotype of GC by promoting tumor angiogenesis and metastasis and regulating T cell functions [30]. These reports suggest that TAMs and GC cells can complement and reinforce each other. Similarly, this article proved that TAMs promoted GC cell proliferation, migration and invasion and dampened apoptosis. Also, the tube formation assay test showed that TAMs promoted VEC angiogenesis. Meanwhile, GC cells co-cultured with human monocytes significantly induced the production of TAMs, which was basically consistent with previous studies in colorectal cancer [31].

It has been reported that exosomes are rich in non-coding RNA, and miRNAs are a class of endogenous non-coding RNA found in eukaryotes with regulatory functions. Studies have demonstrated that tumor-derived exosomal miRNAs are widely involved in TAM "M2" polarization. For example, Ying X et al. reported that exosomal miR-222-3p secreted by epithelial ovarian cancer (EOC) induces M2 polarization of TAMs through the SOCS3/STAT3 pathway [32]. Besides, the exosomal miR-222-3p of colorectal cancer cells induces "M2" polarization of macrophages and enhances the metastasis of colorectal cancer cells by increasing the levels of CD163 and STAT3 [33]. Moreover, previous studies have manifested that TAMs can be divided into classic M1-polarized macrophages and selectively M2-polarized macrophages. M2 polarization can express a large number of scavenger receptors, which is related to the high expression of IL-4, IL-10, VEG, and MMP [34]. It was also found that MMP is a proteolytic enzyme that degrades the extracellular matrix and basement membrane and is involved in tissue reconstruction, tissue infiltration and tumor neovascularization. MMP1, MMP2 and MMP9 are synthesized by M2-polarized macrophages, so MMPs related to CD68, CD163 and CD20 are often used to identify M2-polarized macrophages in TAMs [35]. MiR-15b-5p is also a critical member of the miRNA family. Therefore, we isolated miR-15b-5p from GC-derived exosomes to explore whether miR-15b-5p influenced the "M2" polarization of TAMs. In this research, an overexpression and knockdown model of GC cell-derived exosomal miR-15b-5p was constructed, and qRT-PCR and WB were adopted to test the expression of TAM molecular markers (MMP1, 2, 9, IL-4, IL-10, and VEGF), iNOS, CD68, CD163, and CD20. The results demonstrated that overexpressing miR-15b-5p facilitated the "M2" polarization of TAMs. However, after inhibiting the content of miR-15b-5p in GC cell-derived exosomes, the impact of GC cell-derived exosomes on the "M2" polarization of TAMs was reduced, which was also demonstrated *in vivo*.

Based on the above studies, we further probed the impacts of TAMs on the malignant phenotype of GC cells after regulating the expression of miR-15b-5Pp. Therefore, we conducted the CCK-8 assay, Transwell assay and flow cytometry and testified that TAMs overexpressing miR-15b-5p promoted GC cell proliferation, migration, and invasion and abated apoptosis, while TAMs knocking down miR-15b-5p exerted an opposite effect. In addition, Lin L et al. reported that the infiltration of TAMs is closely associated with extensive angiogenesis [36]. Hence, we further tested VEC angiogenesis and found that TAMs overexpressing miR-15b-5p heightened VEC angiogenesis, while TAMs knocking down miR-15b-5p attenuated the effect. Based on this, we posited that GC cell-derived miR-15b-5p promoted tumor progression by facilitating angiogenesis in the GC microenvironment. Besides, Liu Q et al. held that WNT5A leads to the TAM M2 polarization by modulating the CaKMII-ERK1/2-STAT3-mediated IL-10 secretion, thus accelerating the growth and metastasis of colorectal cancer [37]. The results of the mechanism study in this study also indicated that miR-15b-5p dampened the WIF1 level by targeting the 3 'UTR of WIF1 and up-regulated WNT5A, thereby exerting the above-mentioned effects on GC cells and TAMs. These findings further confirmed the great contribution of GC cell-derived exosome miR-15b-5p to invasion and immunodiffusion of TAMs and the malignant phenotype of GC cells through the WIF1/WNT5A pathway.

Overall, this article proved that miR-15b-5p in GC cell-derived exosomes promoted the invasion of

TAMs by inhibiting the WIF1 expression and up-regulating WNT5A, thus promoting GC evolvement. This paper provides clues for molecular targeting of GC cells, TAMs and their interactions as well as antivascular therapy. However, more downstream molecular targets and treatment strategies need to be further explored.

Ethics statement

Our study was approved by the Ethics Review Board of Linyi People's Hospital.

Data availability statement

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Chengkun Qin. Performed the experiments: Qinhui Sun; Hongjun Liu; Hongxia Zhang Statistical analysis: Xiaobo Guo. Wrote the paper: Chengkun Qin All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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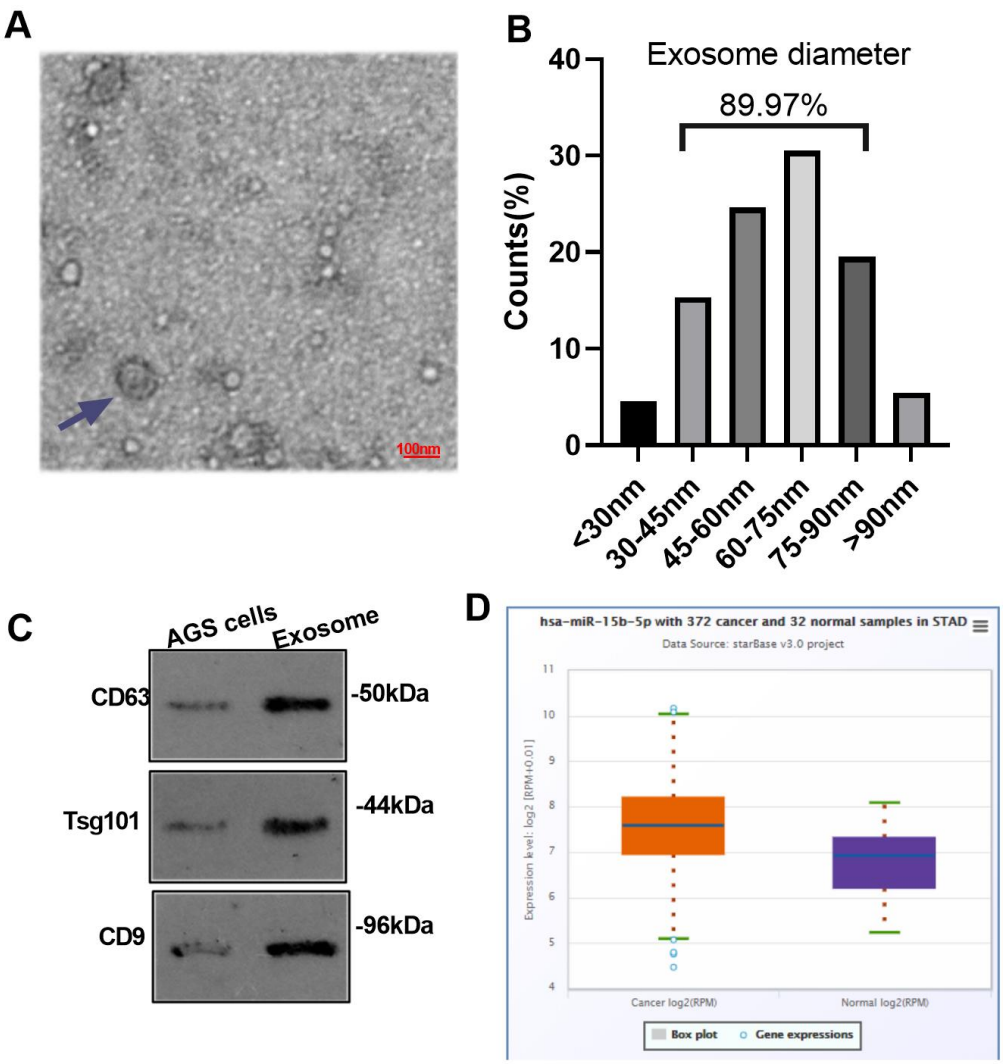
SUPPLEMENTARY MATERIALS

Supplementary Figure



Supplementary Figure 1. (**A**) The images of the isolated extracellular vesicles scanning electron microscope. (**B**) The diameter distribution of the isolated extracellular vesicles. (**C**) Western blot was used for detecting CD63, CD9 and Tsg101 in GC cells or the isolated extracellular vesicles. (**D**) Starbase (http://starbase.sysu.edu.cn/) was used for analyzing miR-15b-5p expression in Stomach Adenocarcinoma.