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RESEARCH ARTICLE

Gastric-cancer-derived mesenchymal stem cells: a promising target for resveratrol in the suppression of gastric cancer metastasis

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Abstract

The tumor microenvironment (TM) is an essential factor of tumor progression. Mesenchymal stem cells (MSCs) are important components of the TM and play critical roles in cancer metastasis. Resveratrol (RES) is a potential antitumor drug that has attracted extensive attention. However, it remains unclear whether RES can exert its antitumor activity by targeting MSCs located in the TM. In this study, we demonstrated that the conditioned medium of gastric-cancer-derived MSCs (GC–MSCs) promoted gastric cancer (GC) metastasis and facilitated the progression of epithelialmesenchymal transition (EMT) of GC cells. However, after pretreatment with RES, the prometastatic effect of GC–MSCs on GC cells was reversed. Furthermore, RES reduced GC–MSC (IL-6, IL-8, MCP-1, VEGF) gene expression and protein secretion, and counteracted the activation of the GC–MSC-induced Wnt/β-catenin signaling of GC cells, with less β-catenin nuclear transport and declined expression of β-catenin, CD44, and CyclinD3 in GC cells. Re-expression of β-catenin impaired the inhibitory effect of RES on GC cells. In conclusion, RES restricted the mobility increase of GC cells and reversed the progress of EMT induced by GC–MSCs by inactivating the Wnt/β-catenin signaling. GC–MSCs are promising target for RES in the inhibition of GC metastasis.

Keywords Resveratrol · Gastric-cancer-derived mesenchymal stem cells · Gastric cancer · Metastasis · Wnt/β-catenin

Abbreviations

GC	Gastric cancer
GC–MSCs	Gastric-cancer-derived mesenchymal stem cells
RES	Resveratrol
GC–MSC–CM	Conditioned medium of GC–MSCs
RES0-CM	Conditioned medium of GC–MSCs pretreated with 0.4 ‰ DMSO for 24 h
RES20-CM	Conditioned medium of GC–MSCs pretreated with 20 μM RES for 24 h

Introduction

Gastric cancer (GC) is one of the principal causes of cancer mortality worldwide. Reports state that nearly 783,000 GC-associated deaths occurred globally in 2018 [1], and approximately 52.4% of the cases were recorded in China [2]. Although the global mortality of GC has steadily declined over several decades, the conventional treatment such as chemotherapy, surgery, and radiotherapy remain unsatisfactory [3]. Therefore, the search for emerging molecular targets to eliminate GC is urgent.

Recently, increasing compact evidence indicates that the tumor microenvironment (TM) is a promising target for cancer treatment [4, 5]. The TM is an ecological niche that harbors endothelial cells [6], macrophages [7], granulocytes [8], and mesenchymal stem cells (MSCs) [4]. As an important component of the TM, MSCs can favor tumor growth and metastasis. We have previously successfully isolated GC-derived MSCs (GC–MSCs) [9, 10] and demonstrated that GC–MSCs promote tumor growth by secreting PDGF-DD [11]. Another study proved that GC–MSCs prompted the progression of GC through their abundant secretion of IL-8. However, neutralizing antibodies blocked the IL-8 and

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counteracted the tumor-promoting effect of the GC–MSCs [12]. These findings collectively suggest that the GC–MSCs is a promising target to control the progression of GC.

Resveratrol (3,5,4′-hydroxystilbene, RES) is a preventive agent against cancer with a variety of biological functions, such as inducing cancer cell apoptosis [13], blocking cell cycle progression [14], and inhibiting cell proliferation [15]; however, no research has addressed the effect of RES on the GC–MSCs.

In this present study, we found that RES suppressed GC metastasis and reversed the progression of epithelial–mesenchymal transition (EMT) by targeting GC–MSCs, and Wnt/β-catenin signaling played a crucial role in the entire process. By regulating GC–MSCs in the TM, RES exerted a considerable negative effect on the motility of the GC cells. The results demonstrated that GC–MSCs is a candidate target for RES in suppressing gastric cancer metastasis.

Materials and methods

Cell culture

GC–MSCs were isolated from the gastric cancer tissues obtained from the patients with gastric adenocarcinoma in The Affiliated People's Hospital of Jiangsu University, and identified as described previously [9]. A primary culture of GC–MSCs was cultivated in low-glucose Dulbecco's modified Eagle's medium (L-DMEM; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Gibco), which is special for stem cell culture. GC–MSCs at passages 3–5 were used for the subsequent experiments. The expression of specific surface antigens [CD44 (BD Pharmingen), CD105 (Miltenyi), CD34 (BD Pharmingen), CD45 (BD Pharmingen)] of GC–MSCs was detected by flow cytometry, and multi-directional differentiation potential was assessed through osteogenic and adipogenic differentiation assays according to the manufacturer's instructions (Cyagen). Human GC cell lines (HGC-27, AGS) were purchased from the China Academia Sinica Cell Repository (Shanghai, China).

Preparation of conditioned medium and enzyme-linked immunosorbent assay

RES (Sigma) was dissolved in dimethylsulfoxide (DMSO) to prepare a 50 mM stock solution and diluted with L-DMEM subsequently. After treatment with 0.4 ‰ DMSO or 20 μM RES for 24 h, the conditioned medium of GC–MSCs was discarded, and the cells were cultured for 24 h in a new culture medium. A normally conditioned medium was used as a control. The conditioned media of GC–MSCs pretreated with 0.4 ‰ DMSO and 20 μM RES were referred to as RES0-CM and RES20-CM, respectively. The conditioned media were collected and centrifuged at 1000×*g* for 10 min and subsequently filtered through a 0.22-μm filter (Millipore) and stored at − 80 ℃until used. A human IL-6 enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems, and the ELISA kits of MCP-1, IL-8 and VEGF were purchased from ExCell Bio (China). The secretion level of these cytokines in the conditioned media of GC–MSC was assessed according to the manufacturer's instructions.

Cell counting Kit-8 assay

After treatment with RES for 24 h or 48 h, the activity of the GC–MSCs was assessed with a Cell Counting Kit-8 assay (CCK8, MCE) according to the manufacturer's instructions.

Quantitative reverse-transcription polymerase chain reaction

Total RNAs were extracted from the GC–MSCs with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Next, 1 μg of RNA was converted to cDNA according to the manufacturer's protocol (Vazyme, Nanjing, China), and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed to evaluate gene expression. The primer sequences are listed in Table 1.

Transwell migration assay and Matrigel Transwell invasion assay

After treatment with RES0-CM, RES20-CM, and CP21 (Selleck, 3 uM) for 24 h, the HGC-27 and AGS cells were suspended in serum-free RPMI 1640 medium and placed into the top chamber of transwell dishes (Corning Inc., Corning, NY, USA). RPMI 1640 medium containing 10% FBS was added into the lower chamber. After incubation for 24 h, the cells on the lower surface of the top chamber were fixed in paraformaldehyde, stained with crystal violet and counted under a microscope. For the Matrigel Transwell invasion assay, the top chambers were coated with matrigel before the HGC-27 and AGS cells were plated, and the experiment was performed similarly to that of the Transwell migration assay.

Western blot

HGC-27 and AGS cells were processed in a lysis buffer with proteinase inhibitors (Pierce) and analyzed through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (15%). The proteins were transferred to a polyvinylidene fluoride membrane; the nonspecific sites were blocked, and then incubated with primary and secondary antibodies, respectively (1:3000; Invitrogen). The primary antibodies

Table 1 Primer Sequences of Target Genes

Genes	Primer sequence (5′-3′)	Amplicon size (bp)	Annealing temperature (℃)
IL-6-For	AGCTCTGGCTTGTTCCTCAC	252	60
IL-6-Rev	TACATCCTCGACGGCATCTC		
VEGF-For	ATCTGCATGGTGATGTTGGA	280	58
VEGF-Rev	CCTTGCTGCTCTACCTCCAC		
IL-8-For	TTCTGTGTTGGCGCAGTGT	144	62
IL-8-Rev	GCTCTGTGTGAAGGTGCAGTTT		
MCP-1-For	GAACCGAGAGGCTGAGACTA	151	62
MCP-1-Rew	GCCTCTGCACTGAGATCTTC		
β-actin-For	CATACTCCTGCTTGCTGATC	265	60
β-actin-Rev	CACGAAACTACCTTCAACTCC		
Wnt1-For	GATCGTCAACCGAGGCTGTC	115	64
Wnt1-Rev	CGTGCAGGATTCGATGGAAC		
Wnt2-For	AGCTGGCAGGAAGGCTGTAA	91	63
Wnt2-Rev	CAGCCAGCATGTCCTGAGAG		
Wnt3-For	GGCGCCTCTTCTAATGGA	188	60
Wnt3-Rev	AGAAGCGCAGTTGCTTGG		
Wnt3a-For	GGCATGATCTCCACGTAGTT	167	63
Wnt3a-Rev	TACTCCTCTGCAGCCTGAAG		
Wnt4-For	GCGAGCAACTGGCTGTACCT	119	64
Wnt4-Rev	AGGTTCCGCTTGCACATCTG		
Wnt5a-For	CTCGCCATGAAGAAGTCCA	157	59
Wnt5a-Rev	TACCTAGCGACCACCAAGAA		
Wnt6-For	GACGCATCCTGCAACAGGAC	106	65
Wnt6-Rev	AGCAGCTCGCCCATAGAACA		
Wnt7b-For	CGAAGCGGAACTGGTACTGG	177	64
Wnt7b-Rev	TGAAGCTCGGAGCACTGTCA		
Wnt10b-For	GGCGCCAGGTGGTAACTGAA	178	66
Wnt10b-Rev	GCTCCAGAATTGCGGTTGTG		
Wnt11-For	ACAAGACAGGCAGTGCAACA	135	61
Wnt11-Rev	ACGTAGCAGCACCAGTGGTA		

For Forward primer, *Rev* Reverse primer

included anti-E-cadherin (1:800; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-β-catenin (1:800; CST), anti-N-cadherin (1:1000; CST), anti-α-SMA (1:1000; CST), anti-vimentin (1:500; Bioworld), anti-t-GSK3β (1:500; Bioworld), anti-CD44 (1:1000; Bioworld), anti-CyclinD3 (1:500; Bioworld), and anti-GAPDH (1:2000; Bioworld). The target proteins were visualized with chemiluminescence.

Immunofluorescence

After treatment with RES0-CM, RES20-CM, and CP21 (3 μM) for 24 h, HGC-27 and AGS were immobilized with 4% paraformaldehyde and punched with 0.1% Triton X-100, and then incubated with primary antibody (1:100, anti-CD44; 1:100, anti-β-catenin; 1:100, anti-E-cadherin; anti-vimentin, 1:100), secondary antibody (1:250, Alexa Fluor 555, Invitrogen), and Hochest 33,342. Immunofluorescence was observed with through a DeltaVision™ Elite microscope (GE, USA).

Luciferase reporter activity assay

The TOP-Flash or FOP-Flash luciferase reporter plasmid was co-transfected into HGC-27 or AGS cells combined with the Renilla luciferase gene governed by the β-actin promoter. At 8 h post transfection, the prepared conditioned media of GC–MSCs were added and incubated with the GC cells for 24 h. The cells were collected to quantify Wnt reporter activity using TOP/FOP according to the manufacturer's manual (Promega).

Tumor metastasis in vivo

The male BALB/c nu/nu mice (aged 4–6 weeks) were purchased from the Laboratory Animal Center of Shanghai (Shanghai, China) and were randomly divided into four groups ($n = 6$). HGC-27 cells were preincubated with different media (Control, RES0-CM, RES20-CM, RES20-CM combined with CP21) for 24 h, and then intraperitoneally injected (2.5×10^6 cells in 300 µl PBS) into the mice. Mice were killed and the metastatic tumor nodes were recorded at 35 days after injection. The excisional tumor tissues were immobilized with 4% paraformaldehyde and analyzed by routine hematein eosin staining.

Immunohistochemistry

The protein levels of β-catenin in GC tissues were detected by immunohistochemistry. Briefly, the sections of GC tissues were incubated with anti-β-catenin antibody, secondary antibody, 3,3′-diaminobenzidine (DAB) and hematoxylin according to the manufacturer's instructions (Boster, Wuhan, China).

Statistical analysis

All data are represented as the mean ± standard deviation. The statistically significant differences between groups were assessed by One-way or Two-way ANOVA, followed by Tukey's test, as appropriate. $P < 0.05$ was considered statistically significant.

Results

The characteristics of GC–MSCs

GC–MSCs were isolated from the gastric cancer tissues through adherent culture. After the initial 1–2 weeks of primary culture, GC–MSCs adhered to the surface of the culture dish and displayed a small population of cells with spindle shape (Fig. 1a). Differentiation of GC–MSCs was evaluated after 16 days of induction in the conditioned media. GC–MSCs presented the ability of differentiating into either osteocytes (Fig. 1b) or adipocytes (Fig. 1c), displayed by positive staining of Alizarin Red S and Oil Red O. In addition, GC–MSCs were positive for CD44 and CD105 but negative for CD45 and CD34 (Fig. 1d).

RES impaired the promigratory and proinvasive effects of GC–MSCs on GC cells

In this study, we found that the conditioned medium of GC–MSCs (GC–MSC–CM) increased the migration (Fig. 2a) and invasion (Fig. 2b) ability of GC cells, suggesting that GC–MSCs could be a candidate of anti-tumor target. To prove this point, we assessed the effect of RES on GC–MSCs activity by exposing cells treated with increasing concentrations of RES for 24 h or 48 h. After treatment with RES for 48 h, GC–MSC activity showed a significant reduction (20%–30%) (Fig. 2c). Paracrine action is a crucial process in MSC promotion of cancer progression; therefore, we investigated the effect of RES on the cytokine synthesis of GC–MSCs. We previously proved that IL-6, IL-8, MCP-1, and VEGF were the most abundant of the 10 soluble cytokines presented in the GC–MSC–CM [8]. Therefore, these four cytokines were

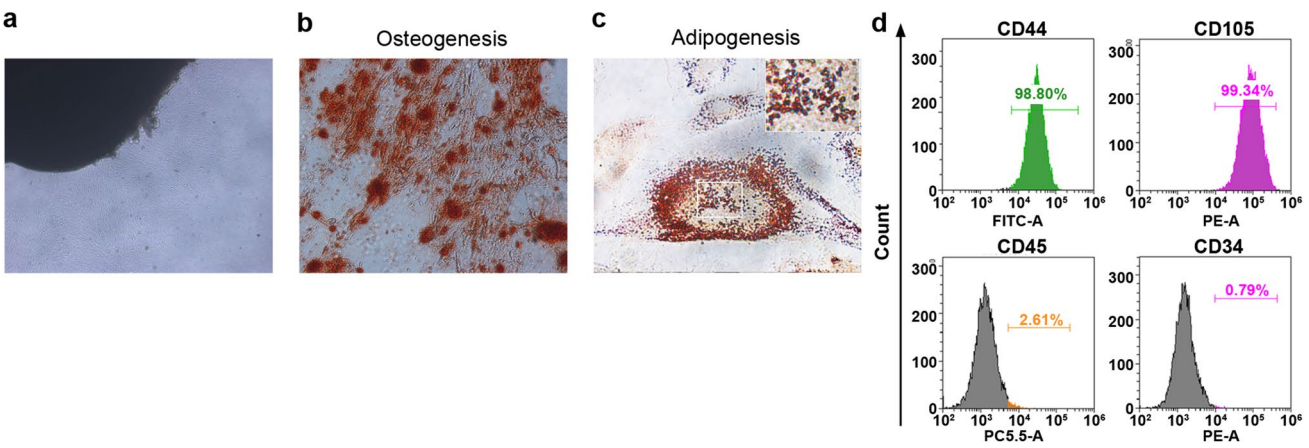

Fig. 1 The characteristics of GC–MSCs. **a** The morphology of GC–MSCs after 9 days of primary culture (40×). **b** Osteogenic differentiation of GC–MSCs (100×). **c** Adipogenic differentiation of GC–MSCs (400×). **d** The surface antigens of GC–MSCs. All the data are from three independent experiments

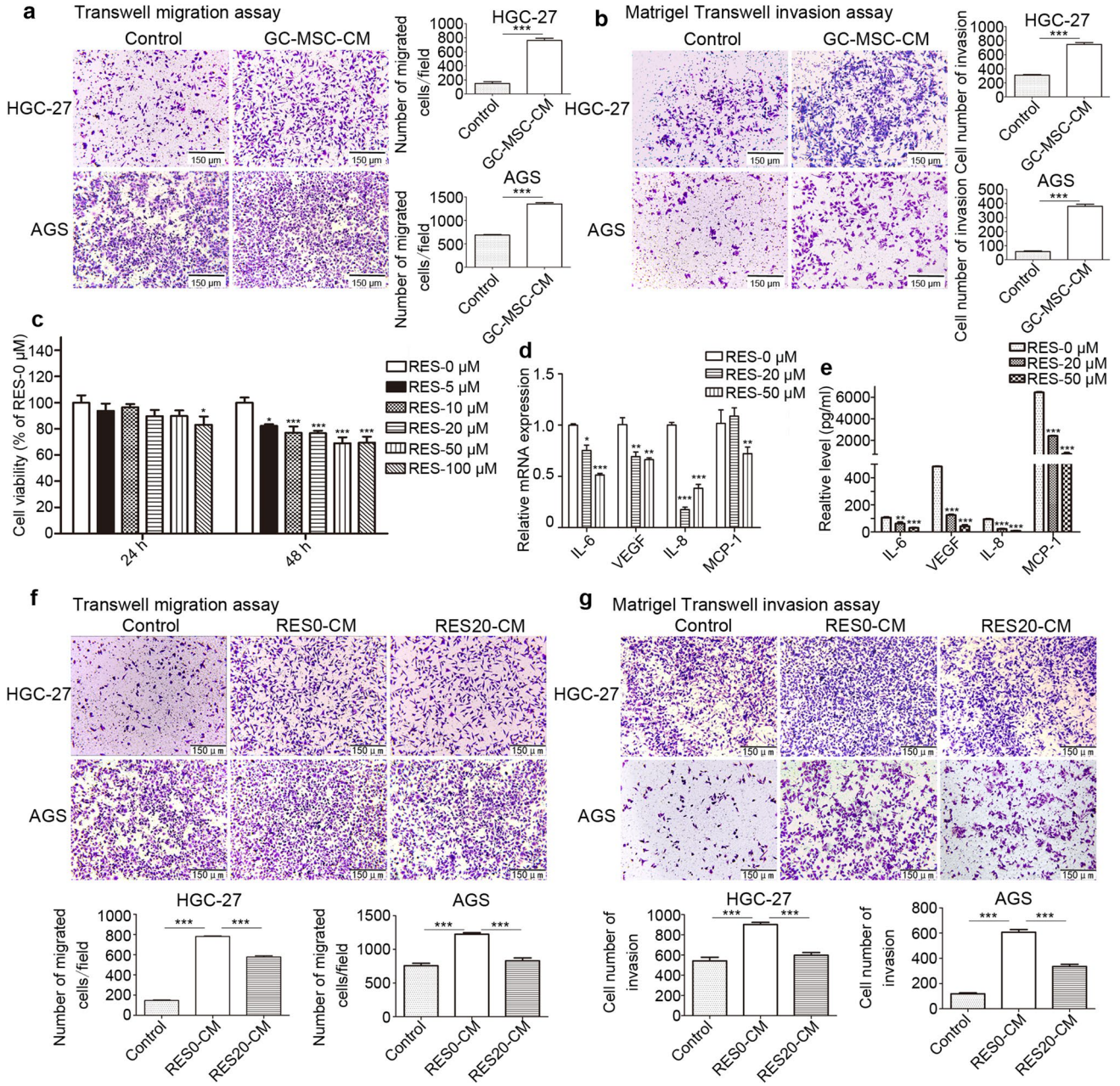

Fig. 2 RES impaired the promigratory and proinvasive effect of GC–MSCs on GC cells. HGC-27 and AGS were treated with GC–MSC–CM for 24 h (**a**, **b**). **a** The migration ability was measured with Transwell migration assay. **b** The invasion ability was detected by Matrigel Transwell invasion assay. **c** After treatment with different concentrations of RES for 24 h or 48 h, the activity of GC–MSCs was measured with a CCK8. GC–MSCs were treated with 20 μM or 50 μM RES for 24 h (**d**, **e**). **d** The IL-6, IL-8, VEGF, and MCP-1 mRNA expression of GC–MSCs was evaluated through qRT-PCR. **e** The IL-6, IL-8, VEGF, and MCP-1 secretion of GC–MSCs was measured using ELISA. HGC-27 and AGS were treated with different media (Control, RES0-CM, RES20-CM) for 24 h (**f**, **g**). **f** The migration ability was detected by Transwell migration assay. **g** The invasion ability was evaluated through Matrigel Transwell invasion assay. All the data are from three independent experiments; *P<0.05; **P<0.01; ***P<0.001; Control: normally conditioned medium; GC–MSC–CM: conditioned medium of GC–MSCs; RES0-CM: conditioned medium of GC–MSCs pretreated with 0.4 ‰ DMSO; RES20-CM: conditioned medium of GC–MSCs pretreated with 20 μM RES

explored in the current study. We found that RES inhibited the gene expression (Fig. 2d) and protein secretion (Fig. 2e) of these GC–MSC factors after treatment with RES for 24 h. For their important role in MSC-mediated tumor progression, RES sharply reduced these four cytokines' secretion, and thus may alleviate their undesirable effects within the GC microenvironment. As shown in Fig. 2f, RES significantly inhibited the promigratory behavior of GC–MSCs, and the migrated cell

numbers of the two GC cell lines dramatically decreased after treatment with RES20-CM compared with those treated with RES0-CM. The Matrigel Transwell invasion assay was carried out to further evaluate cell mobility. Consistently, significantly fewer migrated HGC-27 and AGS cells were noticed when the cells were treated with RES20-CM compared with treatment with RES0-CM (Fig. 2g).

RES counteracted the EMT of GC cells induced by GC–MSCs

Because EMT has the ability to enhance cancer cell migration and invasion [16], we investigated whether RES could regulate the EMT process of GC cells. Using western blot analysis, we discovered that higher expression of N-cadherin, vimentin, and α-SMA, and lower expression of E-cadherin were induced by RES0-CM in GC cells, relative to the control. However, RES20-CM neutralized this process (Fig. 3a). In addition, immunofluorescence proved that RES0-CM reduced E-cadherin expression (Fig. 3b) and increased vimentin expression (Fig. 3c) in the HGC-27 and AGS membranes, but this effect was impaired when the GC–MSCs were pretreated with RES (Fig. 3b, c). RES may possibly counteract the promigratory and proinvasive effects of GC–MSCs on GC cells by regulating EMT.

RES inhibited the activation of the GC–MSC-induced Wnt/β-catenin signaling of GC cells

Aberrant activation of Wnt/β-catenin pathway in TM may facilitate EMT and tumor progression [17, 18]. In this research, we found that RES suppressed various Wnt gene expression, including Wnt1, Wnt2, Wnt3a, and Wnt5a in GC–MSCs (Fig. 4a), and RES pretreatment blocked the activation of GC–MSC-induced Wnt/β-catenin pathway of GC cells (Fig. 4b, c), as measured by luciferase reporter assay. In addition, we indicated that RES0-CM promoted the expression of CD44 and CyclinD3, which are the downstream proteins of Wnt/β-catenin pathway, but RES20-CM reduced these proteins' expressions (Fig. 4d). Immunofluorescence was carried out to further clarify that RES0-CM induced β-catenin nuclear transport of GC cells and that RES20-CM reversed this process (Fig. 4e). Additionally, RES restrained the GC–MSC-induced CD44 expression in the membranes of HGC-27 and AGS cells (Fig. 4f). Taken all these together, it is demonstrated here that, RES inactivates Wnt/β-catenin signaling of GC cells by regulating GC–MSCs.

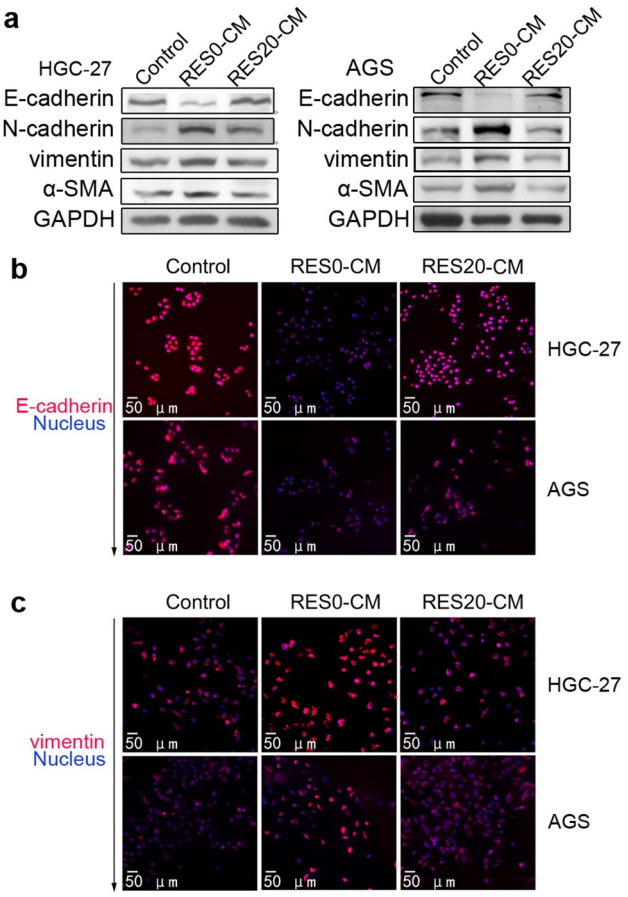

Fig. 3 RES counteracted the EMT of GC cells induced by GC–MSCs. HGC-27 and AGS were treated with different media (Control, RES0-CM, RES20-CM) for 24 h. **a** The expression of N-cadherin, vimentin, α-SMA and E-cadherin were detected by western blot. **b** The expression of E-cadherin was appraised through immunofluorescence (Blue, nucleus; red, E-cadherin; 200×). **c** The expression of vimentin was evaluated through immunofluorescence (Blue, nucleus; red, vimentin; 200×). All the data are from three independent experiments; Control: normally conditioned medium; RES0-CM: conditioned medium of GC–MSCs pretreated with 0.4‰ DMSO; RES20-CM: conditioned medium of GC–MSCs pretreated with 20 μM RES

RES impaired the promigratory and proinvasive effects of GC–MSCs on GC cells by regulating Wnt/β-catenin signaling

Although RES blocked the Wnt/β-catenin signaling of GC cells induced by GC–MSCs, it was not certain whether the blockage of this signal is related to the alteration of motility and EMT of GC cells. After treatment with CP21, which is a selective GSK3 inhibitor that can suppress GSK3β expression and activate canonical Wnt signaling (Fig. 5a), GC cells restored the expression of β-catenin in the nucleus and cytoplasm (Fig. 5b), along with the re-expression of CD44, CyclinD3, N-cadherin, and vimentin (Fig. 5c). Additionally, CP21 treatment impaired RES-20CM-induced expression

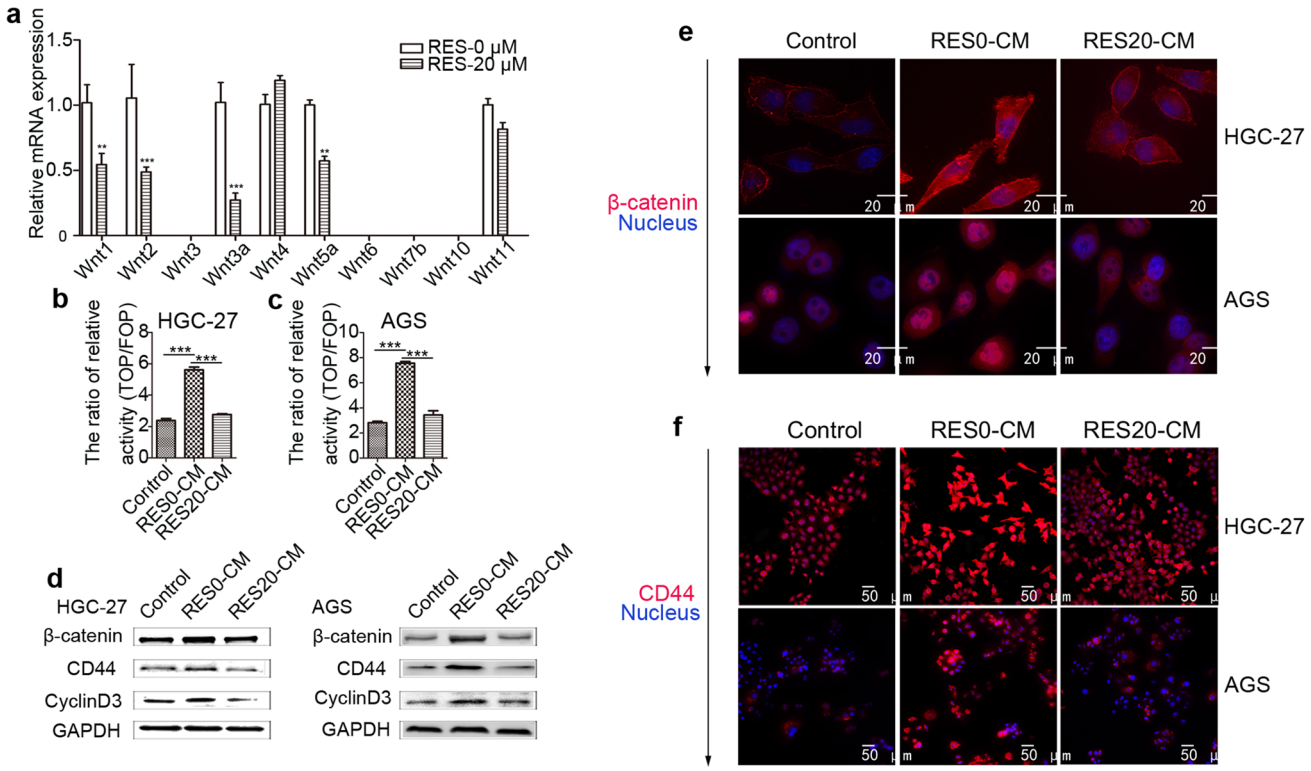

Fig. 4 RES inhibited the activation of the GC–MSC-induced Wnt/β-catenin signaling of GC cells. **a** GC–MSCs were treated with 20 μM RES for 24 h, mRNA expression of Wnt molecules were evaluated through qRT-PCR. HGC-27 (**b**) or AGS (**c**) cells transfected with the TOP- or FOP-Flash luciferase reporter were treated with different media (Control, RES0-CM, RES20-CM) for 24 h. **b** The ratio between TOP- and FOP-Flash luciferase activity of HGC-27 (***$p < 0.001$). **c** The ratio between TOP- and FOP-Flash luciferase activity of AGS (***$p < 0.001$). HGC-27 and AGS were treated with different media (Control, RES0-CM, RES20-CM) for 24 h (**d–f**). **d** The expression of β-catenin, CD44, and CyclinD3 was detected by western blot. **e** The expression of β-catenin was appraised through immunofluorescence (Blue, nucleus; red, β-catenin; 600×). **f** The expression of CD44 was evaluated through immunofluorescence (Blue, nucleus; red, CD44; 200×). All the data are from three independent experiments; Control: normally conditioned medium; RES0-CM: conditioned medium of GC–MSCs pretreated with 0.4 ‰ DMSO; RES20-CM: conditioned medium of GC–MSCs pretreated with 20 μM RES

of E-cadherin in GC cells (Fig. 5c) and almost completely abrogated the inhibitory effect of RES-20CM on GC cell motility (Figs. 5d, e). This means that, the inactivation of Wnt/β-catenin signaling by RES prevents GC cells' migration, invasion, and as well reverses the progress of EMT induced by GC–MSCs.

RES suppresses the prometastatic effect of GC–MSCs on GC cells in vivo

To evaluate the influence of RES on prometastatic property of GC–MSC in vivo, HGC cells pretreated with different media (Control, RES0-CM, RES20-CM, RES20-CM combined with CP21) were utilized to establish peritoneal metastasis models in nude mice. As shown in Fig. 6, the RES0-CM significantly promoted GC metastasis (Fig. 6a–e) and β-catenin expression (Fig. 6f), but the number of metastatic tumor nodes in RES20-CM group was obviously less than that in the Res0-CM group (Fig. 6a–e), which was reversed in RES20-CM combining with CP21 group (Fig. 6a–e). In addition, RES20-CM group displayed lower expression of β-catenin than that in RES0-CM (Fig. 6f). Altogether, these results suggest that GC–MSCs is a promising target for Res in the suppression of GC metastasis and Wnt/β-catenin signaling plays an important role in this process.

Discussion

The TM provides a safe sanctuary to protect tumor cells from radiotherapy and chemotherapy [19]. GC–MSCs in the TM seems to be promising in the fight against GC. GC–MSCs regulate a variety of biological characteristics and functions of neutrophils in the TM through IL-6–STAT3–ERK1/2 signaling. The communication between GC–MSCs and neutrophils accelerated GC development [8]. Our results provided here also demonstrated that GC–MSC promoted the

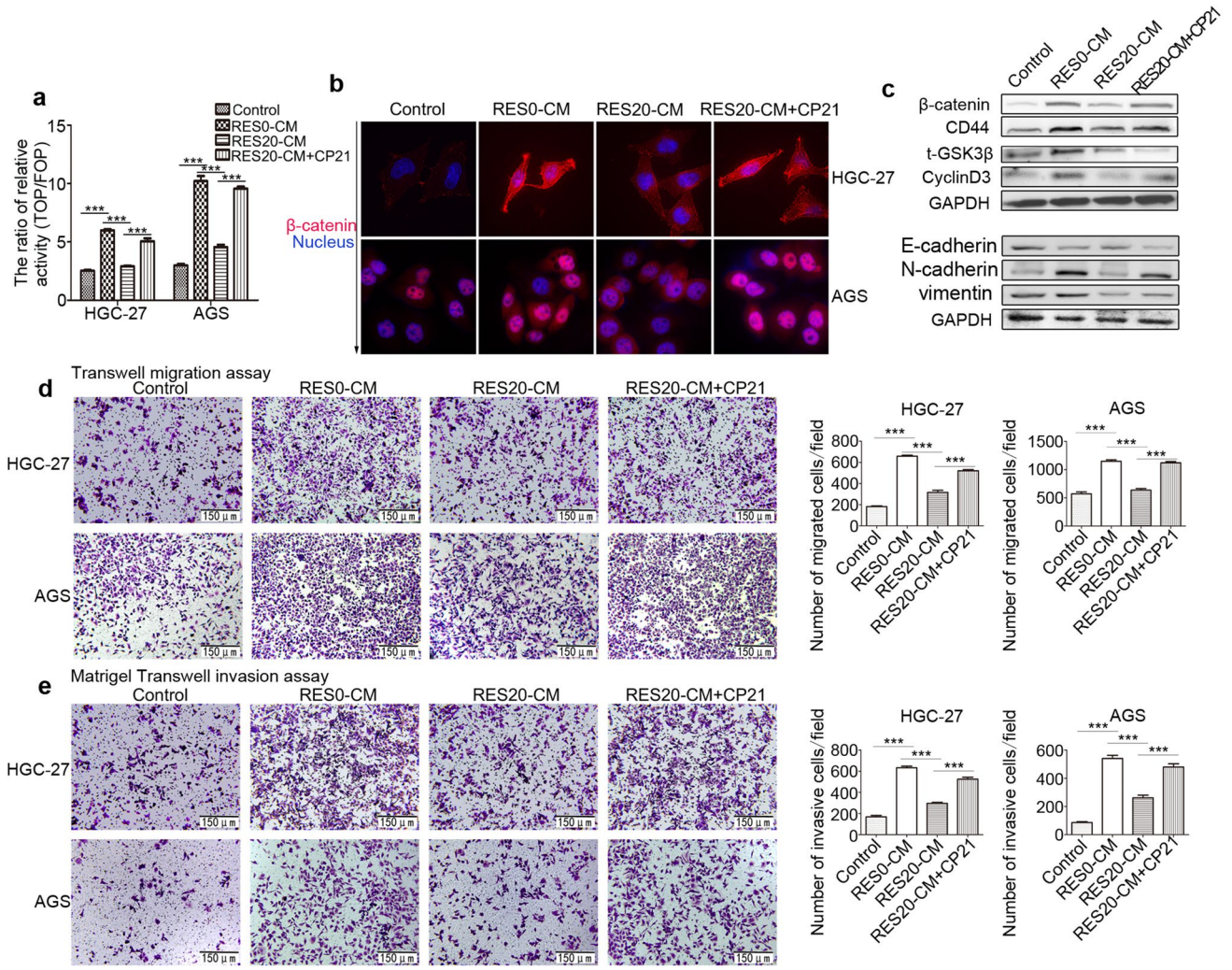

Fig. 5 RES impaired the promigratory and proinvasive effects of GC–MSCs on GC cells by regulating Wnt/β-catenin signaling. **a** HGC-27 or AGS cells transfected with the TOP- or FOP-Flash luciferase reporter were treated with different media (Control, RES0-CM, RES20-CM, RES20-CM combined with CP21) for 24 h, the ratio between TOP- and FOP-Flash luciferase activity was measured. GC cells were incubated with different media (Control, RES0-CM, RES20-CM, RES20-CM combined with CP21) for 24 h (**b–f**). **b** The expression of β-catenin of GC cells in the nucleus and cytoplasm was determined using immunofluorescence. **c** The expression of β-catenin, CD44, CyclinD3, GSK-3β, E-cadherin, N-cadherin, and vimentin of HGC-27 were detected through western blot. **d** The migration ability of GC cells was tested by Transwell migration assay. **e** The invasion ability of GC cells was evaluated through Matrigel Transwell invasion assay. All the data are from three independent experiments; $^{***}P < 0.001$; Control: normally conditioned medium; RES0-CM: conditioned medium of GC–MSCs pretreated with 0.4 ‰ DMSO; RES20-CM: conditioned medium of GC–MSCs pretreated with 20 μM RES

GC metastasis. IL-8-neutralizing antibody could counteract the tumor-promoting effect of GC–MSCs [12].

RES, as a promising natural agent, has prominent functions to treat a variety of tumors, including those of GC [15], breast cancer [20], and intestinal adenoma [21]. However, the specific effect of RES on the GC–MSCs is unclear.

We found that the supernatant from RES-preconditioning GC–MSCs had no effect on the proliferation and apoptosis of GC cells (dates not shown), but exhibited a weaker ability to promote GC cell migration and invasion. EMT plays a critical role in the process of tumor metastasis. Therefore, we explored the expression of EMT -associated proteins in GC cells. As shown in Fig. 3, RES20-CM increased E-cadherin expression and reduced the expression of N-cadherin, α-SMA, and vimentin. This means that RES could inhibit the EMT processes of GC cells mediated by GC–MSCs.

Wnt/β-catenin signaling is closely associated with the EMT process and tumor metastasis [21]. As a key component of the canonical Wnt/β-catenin pathway, β-catenin connects E-cadherin with α-catenin in the cytoplasm and, thus, causes E-cadherin to become anchored in the cell membrane to help promote cell adhesion and prevent cell

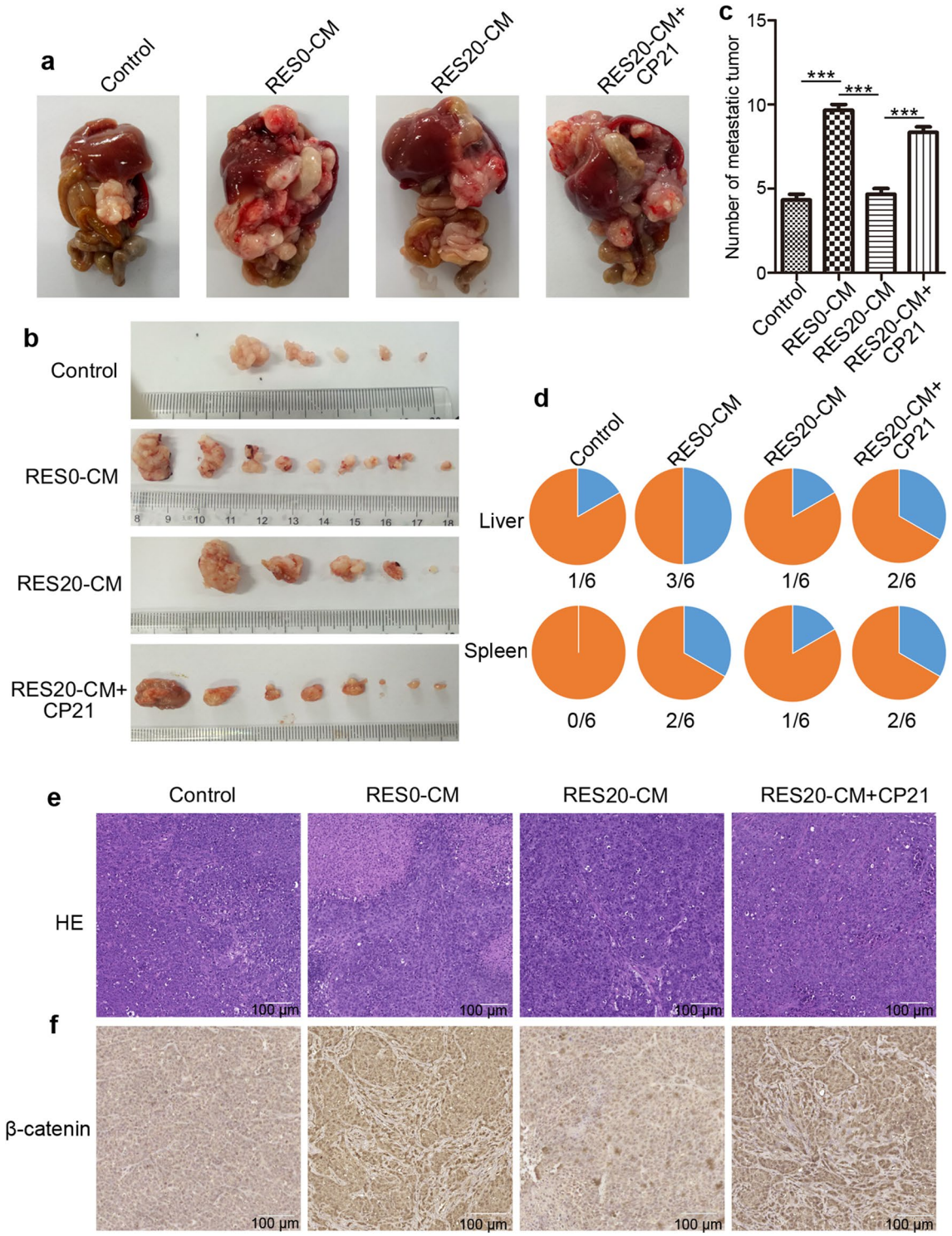

Fig. 6 RES suppresses the prometastatic effect of GC–MSCs on GC cells in vivo. HGC-27 cells were incubated with different media (Control, RES0-CM, RES20-CM, RES20-CM combined with CP21) for 24 h, and then intraperitoneally injected into the BALB/c nu/nu mice (**a–f**). **a** Macroscopic appearance of the tumor in peritoneal cavity. **b** Disseminated tumor isolated from the abdominal cavity. **c** The number of disseminated tumor isolated from the abdominal cavity. **d** The proportion of tumor invading the liver or spleen ($n=6$). **e** Histological images of the tumor tissue. **f** The expression of β-catenin in the tumor tissue and the data is from three independent experiments. ***$P<0.001$; Control: normally conditioned medium; RES0-CM: conditioned medium of GC–MSCs pretreated with 0.4 ‰DMSO; RES20-CM: conditioned medium of GC–MSCs pretreated with 20 μM RES

metastasis. We found that RES20-CM blocked the nuclear transport and expression of β-catenin of GC cells (Figs. 4, 5). In addition, CD44 and CyclinD3, as downstream proteins of Wnt/β-catenin signaling, also decreased in expression (Figs. 4, 5). Restoring the expression of β-catenin with CP21 almost completely abrogated the suppressive effect of RES20-CM on the progress of EMT and GC metastasis (Figs. 5, 6).

Furthermore, we demonstrated the association between molecule(s) within GC–MSC–CM and RES treatment in the suppression of GC metastasis. We found that RES inhibited the gene expressions (Fig. 2d) and protein secretions (Fig. 2e) of IL-6, IL-8, VEGF and MCP-1 of GC–MSCs, which may partially reverse the tumor-promoting effect of GC–MSCs through immune regulation or anti-angiogenesis. Additionally, we proved that RES20-CM has no significant effect on the expression of IL-6 receptor (dates not shown). Therefore, RES is unlikely to suppress GC metastasis by reducing the secretion of IL6. Moreover, we showed that GC–MSCs express various Wnt genes, including Wnt1, Wnt2, Wnt3a, Wnt4, Wnt5a, and Wnt11, which may activate the Wnt/β-catenin pathway to accelerate GC metastasis. Among those Wnt genes, the mRNA expression of Wnt5a is the highest in GC–MSCs. Wnt5a can promote GC invasion, and the patients with low expression of Wnt5a in gastric cancer tissues have a better prognosis than that with high expression [22]. In our study, we demonstrated that RES significantly suppressed Wnt5a mRNA (Fig. 4a) and protein expression (dates not shown) of GC–MSC, which may inactivate the Wnt/β-catenin pathway of GC and reduce GC metastasis.

In conclusion, this study demonstrated that GC–MSCs accelerate GC metastasis and promote EMT progression by activating Wnt/β-catenin signaling. Excitingly, RES could block these processes and restrain the development of GC by regulating the GC–MSCs. Therefore, GC–MSCs is a promising target for RES to suppress GC metastasis.

Author contributions LY, RZ and HQ designed and performed the research, and conducted data analysis and manuscript writing; YH, and ZL performed the apoptosis analysis and Transwell migration assay; WL and MW performed the western blot; and ZS, RJ, and WX provided technical guidance. All authors have read and approved the final manuscript for publication.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the medical ethics committee of Jiangsu University and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study were approved by the medical ethics committee of Jiangsu University (2012258).

Informed consent Informed consent was obtained from all individual participants included in the study.

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