Endoglin over-expression mediates gastric cancer peritoneal dissemination by inducing mesothelial cell senescence

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Running title

Endoglin mediates peritoneal dissemination

Key words:

Stomach cancer; peritoneal dissemination; mesothelial cell; senescence; Endoglin; TGF- β receptor

Abstract

Peritoneal dissemination (PD), which is highly frequent in gastric cancer (GC) patients is the main cause of death in advanced GC. Senescence of human peritoneal mesothelial cells (HPMC) may contribute to gastric cancer peritoneal dissemination (GCPD). In this study of 126 patients, we investigated the association between Endoglin expression in GC peritoneum and the clinicopathological features. The prognosis of patients was evaluated according to Endoglin and ID1 expression. *In vitro*, gastric cancer cell- HPMC co-culture was established. Endoglin and ID1 expression was evaluated by Western blot. Cell cycle and HPMC senescence were analyzed after harvesting HPMC from the co-culture. Gastric cancer cells (GCCs) adhesion and invasion to HPMC were also assayed. Our results showed that positive staining of Endoglin (38%) was associated with a higher TNM stage and higher incidence of GCPD (both $p < 0.05$). Kaplan-Meier analysis showed that the patients who were Endoglin-positive had a shorter survival time compared with Endoglin-negative patients ($p = 0.02$). Using the HPMC and GCCs adherence and invasion assay, we demonstrated that TGF- β 1-induced HPMC senescence was attenuated by silencing the Endoglin expression, which also prevented GCC attachment and invasion. Our study indicated a positive correlation between Endoglin over-expression and GCPD. Up-regulated Endoglin expression induced HPMC senescence via TGF- β 1 pathway. The findings suggest that Endoglin induced HPMC senescence may contribute to peritoneal dissemination of GCCs.

1. Introduction

Gastric cancer is a common malignant tumor of the gastrointestinal system and is associated with poor prognosis [1-3]. Gastric cancer peritoneal dissemination (GCPD) frequently occurs in late-stage gastric cancer and is a serious and life-threatening challenge [4, 5]. GCPD is a metastatic cascade of a series of events, with the most critical step involving disruption of the human peritoneal mesothelial cell (HPMC) barrier [6-8]. Intact HPMC monolayer forms a peritoneal barrier to prevent free gastric cancer cells (GCCs) from infiltrating into the sub-mesothelial connective tissue [9]. HPMC injuries, such as senescence, apoptosis and fibrosis were attributed to damaged HPMC barrier [7-8, 10-11]. These HPMCs are also referred to as tumor-associated human peritoneal mesothelial cells (TA-HPMC). Most of the HPMC injuries are caused by peritoneal-free GCCs exfoliating from the primary tumor. Our previous studies clarified the molecular pathway of HPMC apoptosis and fibrotic transition [8, 10-11]. However, the precise mechanism of GCC-induced HPMC senescence is still largely unknown.

Senescence results in permanent cell cycle arrest and disturbed HPMC. TGF- β 1 mediates a broad array of biological activities such as epithelial cell growth, differentiation, migration, senescence, extracellular matrix production, and angiogenesis [12, 13]. These functions are mediated via binding to a TGF- β -specific membrane receptor complex that contains 3 types of receptors: types I (T β RI), types II (T β RII) and an auxiliary receptor known as Endoglin [14]. Endoglin (CD105) is a 180-kDa homodimeric glycoprotein composed of two 95-kDa disulfide-linked subunits, including the long (L)-Endoglin and short (S)-Endoglin [15]. Endoglin is up-regulated in numerous tumors and correlated with proliferation, invasion as well as metastasis [16, 17]. Endoglin is also highly expressed in endothelial cells and is correlated with endothelial cell senescence by interacting with the T β RI and II [18, 19]. We previously demonstrated that TGF- β 1 was up-regulated in peritoneal dissemination of GCCs [8-10]. TGF- β 1 plays a vital role in mesothelial cell senescence. However, whether Endoglin expression mediated this process is still unknown.

In the present study, we demonstrate that peritoneal Endoglin over-expression is correlated with higher tumor node metastasis (TNM) staging, a higher tendency for GCPD and poor prognosis. **P16 and Id1 are important markers of senescence [20,21], We found a negative**

correlation between Endoglin and ID1 suggests that GCPD is induced by peritoneal mesothelial cell senescence. *In vitro*, co-culture models showed that GCCs up-regulated Endoglin expression but down-regulated P16 expression and induced HPMC senescence via TGF- β 1 pathway.

2. Materials and Methods

2.1. Tissue sample

Peritoneal specimens were obtained from 126 patients undergoing a radical surgery of gastric cancer including resection of the peritoneum at the Department of Surgical Oncology, First Affiliated Hospital of China Medical University during the period 2003-2008. All patients provided informed consent, to use their clinical and pathological data.

2.2. Immunohistochemistry

The sections were then boiled for 10 min in 0.01M citrate buffer and endogenous peroxidase was blocked by incubating with 0.3% H₂O₂ in methanol for 30 min. The sections were incubated overnight at 4 °C with a mouse monoclonal anti-Endoglin antibody (1:400, Abcam) or rabbit monoclonal anti-Id1 antibody (1:200, Abcam). The sections were exposed to a biotin-labeled secondary antibody (1:200, Santa Cruz Biotechnology) for 1h. Staining was scored according to the following scale: 0, no staining; +, minimal staining; ++, moderate to strong staining in at least 20% of cells; +++, strong staining in at least 50% of cells. Cases with 0 or + staining were classified as negative, and cases with ++ or +++ staining were classified as positive.

2.3. Confocal imaging of tissue immunofluorescence

Specimens were fixed in 4% paraformaldehyde and embedded in paraffin. 4- μ m-thick histological sections were deparaffinized with xylene and rehydrated through a graded series of alcohol. The sections were then blocked by incubation with 0.3% H₂O₂ in methanol for 30 min. Sections were incubated with rabbit monoclonal anti-Id1 antibody (1:200, Abcam) and mouse monoclonal anti-Endoglin antibody (1:400, Abcam), overnight at 4 °C followed by secondary antibodies including Alexa fluor-594 conjugated goat anti-mouse and Alexa fluor-488 conjugated goat anti-rabbit antibodies (Invitrogen, both diluted 1:200). Nuclear counterstaining was conducted with 100 ng/ml of 4, 6-diamidino-2-phenylindole (DAPI,

Sigma-Aldrich).

2.4. Small interfering-RNA (siRNA)

HPMCs were grown to 70% confluence in culture dishes and transient transfection was performed with sequence-specific small interfering-RNA (siRNA) against Endoglin using Lipofectamine 2000 according to the manufacturer's instructions. Cells were harvested for further experiments. HPMC with Endoglin knockdown was denoted as Endoglin^Δ.HPMC

2.5. Primary GCC culture and establishment of the TA-HPMC Model

Primary GCC culture was described previously [22]. The HPMC cell-line HMR-SV5, human normal gastric glandular epithelial cell-line GES-1 was obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). GCCs were cultured in F12 media containing 10% FBS; HMR-SV5, and GES-1 cell- lines were both cultured in RPMI-1640 media containing 10% FBS. All cell cultures were incubated continuously under 5% CO₂ at 37°C. HMR-SV5 cells (at a density of 5×10^5 cells/well) were seeded into a six-well plate. After 80% confluence, a co-culture insert was added (Corning), and approximately 2×10^5 GES-1 or GCCs were plated into the upper chamber, respectively. The whole co-culture system was incubated for 48h. HMR-SV5 cells in the lower chamber were collected after co-culture for morphology analysis, Western blot assay, SA-β-Gal staining and confocal imaging. HPMCs co-cultured with GES-1 cells were referred to as the N-HPMC; HPMCs co-cultured with GCCs were referred to as the TA-HPMC; Endoglin^Δ HPMC co-cultured with GES-1 cells was referred to as the N- Endoglin^Δ; and Endoglin^Δ HPMC co-cultured with GCCs was referred to as the TA- Endoglin^Δ.

2.6. Western blot

Cells were incubated for 20 min on ice in 50 μL RIPA buffer and then centrifuged for 20 min at $12,000 \times g$ at 4 °C. Next, 50 μg protein from each sample was resolved on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated with mouse monoclonal anti-Endoglin (1:2000; Abcam), rabbit monoclonal anti-Id1 (1:5000; Abcam), or rabbit monoclonal anti-p16 (1:10000; Abcam) primary antibodies in TBST solution overnight at 4 °C. Following overnight incubation, membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000; Santa Cruz) in TBST for 2 h at room

temperature.

2.7. Senescence-associated β -galactosidase (SA- β -Gal) staining

Senescence-associated β -galactosidase (SA- β -Gal) staining was performed using an SA- β -Gal Staining Kit (Genmed Scientifics Inc) according to the manufacturer's protocol.

2.8. MTT assay

HPMCs (approximately 1×10^4 cells/well) were seeded into 96-well culture plates and cultured in DMEM with 10% FBS for 12h to 48h. Cells were then incubated with 20 μ L MTT (10 mg/ml) for 4 h at 37 °C, and 200 μ L DMSO was added for 20 min at room temperature to solubilize the formazan product. The optical density (OD) was determined using a spectrophotometer (Bio-800, Bio-Rad) at a wavelength of 570 nm. Approximately 1×10^4 cells/well of untreated HPMCs, were treated with serum-free DMEM medium as a control. Cell proliferation was calculated using the following equation:

$$\text{Cell proliferation rate (\%)} = \text{OD (Experimental group - Control group)} / \text{OD control group} \times 100\%$$

2.9. Confocal imaging of cell immunofluorescence

Cells were fixed in 4% paraformaldehyde in 20 mM HEPES (pH 7.4) for 20 min and then incubated with rabbit monoclonal anti-Id1 antibody (1:500; Abcam) and mouse monoclonal anti-Endoglin antibody (1:200; Abcam) for 1 h at room temperature before washing three times. They were incubated with goat anti-mouse conjugated Alexflour 594 and goat anti-rabbit conjugated Alexa fluor 488 secondary antibody (1:200, Invitrogen) for 30 min at room temperature in the dark.

2.10. Cell cycle analysis

Harvested cells were washed twice with phosphate-buffered saline (PBS) and fixed in ice-cold 70% ethanol at 4 °C overnight. The cell pellet was then suspended in 0.2 mL DNA extraction buffer (192 mL 0.2 M Na₂HPO₄ and 8 mL 0.1 M citric acid, pH 7.8) and incubated in a 37°C shaker for 30 min. Cells were centrifuged at 1500 rpm for 10 min. The supernatant was carefully discarded. The pellet was stained with a propidium iodide (PI) solution (0.08 mg PI, 0.1% triton X-100 and 0.1 mg/mL RNaseA in PBS) and incubated in the dark for 30 min at 37 °C. In total, 10,000 cells (adherent and non-adherent) were harvested. Cellular DNA contents were measured by exciting PI at 488 nm and measuring the emission at 580 nm

using a flow cytometer (Aria II, BD Biosciences).

2.11. GCC-HPMC adhesion assay

The adhesion of tumor cells to HPMCs was determined as previously described [23]. GCC cells were pretreated with or without 50 µg/mL RGD and stained with 15 µM calcein AM for 30 min at 37°C. Subsequently, GCC cells (1×10^4 /well) were added to the 24-well plates with confluent TA-HPMCs or N-HPMCs, and incubated for the indicated time at 37°C. To remove non-adherent GCCs, the plates were washed three times with 200 µL PBS. The fluorescent intensity in each well was recorded by spectrofluorometry at 485 nm and 535 nm for excitation and emission, respectively. As a positive control, HPMC monolayers were seeded with labeled GCC cells and maintained for 30 min without any PBS wash steps. Untreated HPMC monolayers were used as negative controls. Adhesion capability was calculated as follows:

$$\text{Adhesion capability} = (\text{fluorescence intensity of experimental group} - \text{negative control} / \text{fluorescence intensity of positive control} - \text{negative control}) \times 100\%$$

2.12. GCC-HPMC invasion assay

TA-HPMCs or N-HPMCs were plated in the upper chamber of 24-well transwell inserts (8µm pore, Corning), which were pre-coated with 100µl matrigel. Once HPMCs reached 95% confluence, GCCs (1×10^5) were seeded into the upper chamber and the lower chamber was filled with 0.5 mL of DMEM with 10% FBS as a chemoattractant. After incubation for the indicated time, non-invading cells (upper chamber) were gently removed using a cotton-tipped swab. The invading cells (lower chamber) were fixed using methanol and stained with trypan blue. The invasive ability was determined by counting the number of penetrating cells in 10 random fields in each well, microscopically at 200× magnification.

2.13. Statistical analysis

All statistical analyses were performed using SPSS 16.0 software. Overall survival rates were determined using the Kaplan-Meier estimator. The log-rank statistics were used to identify differences between the survival curves of different patient groups. In univariate analysis, continuous data obtained from two groups were analyzed by 2-tailed Student's T-test, and categorical data were analyzed by a 2-tailed χ^2 test. All data were expressed as mean $\pm$ SD. Statistical significance was set at an alpha value of $p < 0.05$.

3. Results

3.1. Endoglin expression in peritoneum is correlated with poor prognosis and incidence of GCPD in gastric cancer patients

We determined Endoglin and Id1 expression in the peritoneal tissues of patients with gastric cancer. Endoglin and Id1 expression was both negative (Figure. 1A, C) and positive in gastric cancer (Figure. 1B, D). We also investigated the correlation between Endoglin expression and clinicopathological features in 126 patients. A positive Endoglin expression was significantly associated with tumor size, TNM staging as well as GCPD incidence (Table 1). Positive Endoglin expression was more frequently detected in larger tumors (> 5cm) and stage III and IV cancer (both $p < 0.05$). The protein expression of Endoglin was significantly correlated with the incidence of GCPD ($p < 0.05$). Chi-square analysis revealed a negative correlation between these two senescence-related proteins. Tissue confocal imaging was conducted in peritoneum tissue with Endoglin expression score +++ or 0. As shown in figure 1E, a strongly positive Endoglin expression (+++) in the peritoneum resulted in a minimum Id1 expression. By contrast, negative Endoglin (0) and strongly positive Id1 expression co-existed in another group of patients. Kaplan-Meier analysis of the 126 patients revealed that a positive Endoglin expression was associated with a shorter survival when compared with Endoglin-negative expression (Figure. 1F, $p = 0.02$). Similarly, absence of Id1 expression was also an independent factor related to poor prognosis in patients with gastric cancer (Figure. 1G, $p = 0.045$).

3.2. GCCs up-regulated Endoglin expression and induced HPMC senescence via TGF- β 1 pathway

Endoglin is a crucial receptor in TGF- β pathway, which responds to the TGF- β 1 ligand [16]. We first performed primary cultures of GCCs in 5 patients who suffered from GCPD and their TGF- β 1 expression was analyzed by Western blot. As shown in Figure 2A, GC3 cells exhibited the highest level of TGF- β 1 expression. Therefore, GC3 was selected as the inducer of HPMC injury model. The protein expression in senescence and TGF- β pathway was analyzed by Western blot, and compared between TA-HPMC and N-HPMC at different time intervals. After 24h co-culture, TGF- β pathway-related proteins Endoglin and phosphorylated Smad2/3 (pSmad2/3) were significantly up-regulated in TA-HPMC. In contrast, no variation

in expression was found in N-HPMC. The cell-cycle regulatory protein P16 was up-regulated in TA-HPMC, and senescence inhibitory protein Id1 was remarkably down-regulated, which indicated the initiation of HPMC injury (Figure 2B). Next, immunofluorescence confocal imaging was conducted in N-HPMCs and TA-HPMCs. After 48h co-culture, Endoglin was significantly up-regulated in TA-HPMCs compared with N-HPMCs. The expression of senescence inhibition protein ID1 reached a minimum in TA-HPMCs (Figure 2C). As shown in figure 2D, TA-HPMCs showed distinct senescence and stained bright blue, indicative of positive β -Gal staining (red arrow). HPMCs proliferation was detected by MTT assay. The proliferation of TA-HPMCs was reduced following 12h co-culture and was completely inhibited in 48h. N-HPMCs displayed normal levels of cell proliferation. Proliferation rates of TA-HPMCs were as follows: 12h, $11.8 \pm 0.9\%$; 24h, $21.8 \pm 3.6\%$; and 48h, $29.7 \pm 5\%$ (all $p < 0.05$ compared with N-HPMCs at each time point, Fig. 2E). Cell cycle was also analyzed to confirm our MTT results. TA-HPMC exhibited apparent G1/S and G2/M arrest compared with N-HPMC, indicating authentic senescence transition in HPMCs (Fig 2F).

In the GCC-HPMC adhesion assays, the attachment of GC3 to the TA-HPMC monolayers was significantly greater than that of N-HPMC monolayers at multiple time points ($44.6 \pm 5.66\%$ for 12h; $62.2 \pm 11.4\%$ for 24h; $69.5 \pm 9.7\%$ for 48h; all $P < 0.05$ compared with N-HPMCs, Figure 2G). We also established a transwell model to compare the ability of GC3 to cross the different HPMC monolayer barriers. As shown in Figure 2H-I, TA-HPMC monolayer barrier was more likely to be breached as more GC3 resided in the lower chamber of transwell insert. The invasive ability of GC3 was significantly increased compared with N-HPMC model (4.14 ± 0.8 fold in 24h; 6.23 ± 1.1 fold in 48h both $p < 0.05$).

3.3. Knockdown of Endoglin expression attenuates TGF- β 1-induced HPMC senescence and prevents GCC attachment and invasion

To confirm the role of TGF β 1/Endoglin/Smad pathway in GC3-induced HPMC senescence, we knocked down Endoglin expression using Endoglin siRNA. As shown in Figure 3A, Endoglin expression was complete silenced. Untreated HPMC and Endoglin^Δ HPMC were cultured with GC3 or negative control cells, and pSmad2/3 and Id1 expression were assayed using Western blot. Smad2/3 protein phosphorylation was largely inhibited following Endoglin knockdown. Id1 expression was uniform in Endoglin^Δ HPMC co-cultured with

GES-1 or GC3, which indicated that HPMC senescence transition was completely inhibited following Endoglin knockdown (Figure 3B). The SA- β -Gal assay indicated positive β -Gal staining of TA-HPMC, with flat and enlarged cells. However, the typical senescence transition was absent in TA-Endoglin^Δ (Figure 3C). Consistent with the Western blot result, no significant Id1 down-regulation was found in N-Endoglin^Δ or TA-Endoglin^Δ compared with that of TA-HPMC (Figure 3D). To further investigate the role of Endoglin silencing on GCCs-induced senescence transition, we repeated the GCC-HPMC adhesion and invasion assays. As shown in Figure 3E, TA-HPMC monolayers exhibited a significant higher attachment rate than N-HPMC after GC3 were added at multiple time points (all $P < 0.05$ compared with N-HPMCs). The enhanced adhesion in TA-HPMC was largely reduced in both N-Endoglin^Δ and TA-Endoglin^Δ (both $P < 0.05$ at each time point compared with TA-HPMC). Similarly, the enhancement of invasion ability of GC3 break through TA-HPMC monolayers was also reduced when the expression of Endoglin was silenced in TA-Endoglin^Δ (both $P > 0.05$ at each time point compared with TA-HPMC, Figure 3F).

4. Discussion

Senescence of HPMCs plays a critical role in peritoneal dissemination associated with mesothelial cell damage. However, the molecular mechanisms of GCC-induced HPMC senescence are still poorly understood. Here, we report that GCC up-regulated Endoglin expression, which induced HPMC senescence via TGF- β 1 pathway for the first time. We provide evidence *in vivo* and *in vitro* that Endoglin expression contributes to GCC-associated expression of TGF- β 1. Our data support that Endoglin is induced during HPMC senescence and may contribute to peritoneal dissemination of GCCs.

Although intact monolayer HPMC can prevent peritoneal GCCs invasion, it may be injured by factors released by GCCs during metastasis. We have previously demonstrated that GCCs secrete soluble factors such as TGF- β 1 [24-26] into the abdominal cavity, which cause HPMC damage. HPMC damage leads to exposure of sub-mesothelial connective tissue and collagen fibers, promoting tumor dissemination [27].

It has been accepted that Endoglin modulates adhesion and migration of both cancer and endothelial cells [28, 29]. Meanwhile, migration and adhesion are critical steps of GCPD. Thus, we hypothesis that Endoglin may involve in GCPD. In our study, we reported that

Endoglin expression occurring in gastric cancer peritoneum, was associated with higher TNM staging and higher incidence of GCPD ($p < 0.05$). Further, Endoglin-positive patients had significantly lower cumulative postoperative 5-year survival rates when compared with Endoglin-negative patients ($p = 0.02$).

Regulation of cell migration by Endoglin may involve TGF- β -dependent mechanisms. In the HPMC injury model *in vitro*, after 24h co-culture, TGF- β pathway-related proteins Endoglin and phosphorylated Smad2/3 (pSmad2/3) were significantly up-regulated in TA-HPMC. In our study, Id1-negative expression was also an independent factor related to poor prognosis in patients diagnosed with gastric cancer. HPMC injury is initiated by up-regulation of cell-cycle regulatory protein P16 and significant down-regulation of senescence inhibitory protein Id1. Interestingly, contrary to our results, Craft [30] reported that Endoglin suppresses invasion and metastasis of cancer cells by activating T β RI ALK2. The inconsistency may be related to our focus on the expression of Endoglin in HPMCs and not in cancer cells. TGF-beta 1 can produce opposite effects depending on the receptors to which it binds, this may also explain "inconsistent" effect observed by Craft, in whose study, endoglin suppressed invasion and metastases, by activating the receptor T β RI ALK2. The invasive ability of GC3 was significantly increased in the *in vitro* transwell model compared with the N-HPMC model. To confirm whether GCCs induced HPMC senescence via TGF β 1/Endoglin/Smad pathway, we knocked down the Endoglin expression, and repeated the GCC-HPMC adhesion and invasion assays. Our results confirm that Endoglin silencing reduced the invasive ability of GC3 cells to cross TA-HPMC monolayers and indicated that Endoglin was the key receptor mediating TGF β 1-induced HPMC senescence.

In conclusion, our results demonstrate that GCCs escaping from primary foci secrete TGF- β 1 that induces senescence in HPMC and promotes GCPD through Endoglin receptor. Our study highlights the importance of HPMC senescence in GCPD and establishes Endoglin as a potential therapeutic target in GCPD.

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Figure and Figure Legends

Fig.1 Expression of Endoglin in peritoneum is correlated with poor prognosis and incidence of GCPD in gastric cancer patient.

A Showing the negative expression of Endoglin analyzed by IHC in the patients' peritoneum tissues. (magnification, $\times 200$)

B Showing the positive expression of Endoglin analyzed by IHC in the patients' peritoneum tissues (magnification, $\times 200$)

C Showing the negative expression of Id1 analyzed by IHC in the patients' peritoneum tissues (magnification, $\times 200$)

D Showing the positive expression of Id1 analyzed by IHC in the patients' peritoneum tissues. (magnification, $\times 200$)

E Immunofluorescence analysis of Endoglin and Id1 expression in the peritoneum. In this image, DAPI stained blue, Id1 stained green, and Endoglin stained red (magnification, $\times 400$).

F Showing the survival duration of 126 involved patients that was compared by the Kaplan–Meier method between Endoglin-positive patients and Endoglin-negative patients ($p=0.02$).

G Showing the survival duration between Id1-positive patients and Id1-negative patients ($p=0.045$).

Fig.2 GCCs up-regulated Endoglin expression and induce HPMC senescence via TGF- β 1 pathway.

A Western blot analysis of the expression of TGF- β 1 in GCCs. GAPDH served as a protein loading control.

B Western blot analysis of the expression of TGF- β pathway related proteins (Endoglin and phosphorylated Smad2/3) and proteins related to senescence (p16 and Id1) in HPMCs after 24h co-culture between TA-HPMC and N-HPMC . GAPDH served as a protein loading control.

C Immunofluorescence analysis of senescence-related protein expression in the TA-HPMC and N-HPMC groups. (Id1 shows green staining; Endoglin shows red staining, DAPI shows blue staining, magnification, $\times 600$).

D SA- β -Gal staining of HPMCs in the TA-HPMC and N-HPMC (red arrow indicates senescent HPMCs).

E HPMC proliferation was analyzed by MTT assay. Results are expressed as fold of change (mean $\pm$ SD, *P < 0.05 compared with N-HPMC; **P < 0.05 compared with TA-HPMC at indicated time interval).

F Cell cycle assayed shows G1/S and G2/M arrest of HPMCs in the co-culture system.

G Rate of GC3 adhered to confluent HPMCs at multiple time points (44.6 $\pm$ 5.66% for 12h; 62.2 $\pm$ 11.4% for 24h; 69.5 $\pm$ 9.7% for 48h; all P < 0.05 compared to N-HPMCs).

H Showing a transwell model to compare the ability of GC3 to transverse the different HPMC monolayers barrier.

I Number of GC3 in the back side of lower chamber of transwell insert between TA-HPMC and N-HPMC groups (4.14 $\pm$ 0.8 fold-increased in 24h; 6.23 $\pm$ 1.1 fold-increased in 48h both P<0.05).

Fig 3. Knockdown Endoglin expression attenuate TGF- β 1 induced HPMC senescence and prevent GCC attachment.

A Western blot showing Endoglin expression was complete silenced in Endoglin $^{\Delta}$ HPMC group.

B Western blot showing the pSmad2/3 and Id1 expression after HPMC and Endoglin $^{\Delta}$ HPMC were cultured with GC3 or GES-1.

C SA- β -Gal staining of HPMCs in the N-HPMC, TA-HPMC, N- Endoglin $^{\Delta}$ and TA-Endoglin $^{\Delta}$ groups(red arrow indicates senescent HPMCs).

D Immunofluorescence analysis of senescence-related protein expression in the TA-HPMC, N-HPMC, N-Endoglin $^{\Delta}$ and TA-Endoglin $^{\Delta}$ groups (Id1 shows green staining; Endoglin shows red staining, DAPI shows blue staining, magnification, $\times$ 600).

E Rate of GC3 adhered to confluent HPMCs in the TA-HPMC, N-HPMC, N-Endoglin $^{\Delta}$ and TA-Endoglin $^{\Delta}$ groups at multiple time points(*P>0.05 at each time point compared with N-HPMC; ** P>0.05 compared with TA-HPMC in 12h).

F Number of GC3 in the back side of lower chamber of transwell insert in the TA-HPMC, N-HPMC, N-Endoglin $^{\Delta}$ and TA-Endoglin $^{\Delta}$ groups (*P>0.05 at each time point compared with N-HPMC; ** P>0.05 compared with TA-HPMC in 12h).

Figure 1

Figure 2

Figure 3

Table 1

	Endoglin expression		P value
	Negative(n=77)	Positive(n=49)	
Tumor size (cm)			
≤ 5	41	18	P<0.05*
> 5	36	31	
TNM stage			
I	23	0	P<0.05*
II	31	9	
III	16	26	
IV	7	14	
Histological grade			
Differentiated	42	21	P=0.273
Undifferentiated	35	28	
Lauren grade			
Intestinal	29	23	P=0.355
Diffuse	48	26	
Lymph node metastasis			
Negative	31	21	P=0.853
Positive	46	28	
Hematogenesis metastasis			
Negative	68	45	P=0.500
Positive	9	4	
Peritoneal metastasis			
negative	59	20	P<0.05*
positive	18	29	
ID1 expression			
negative	20	31	P<0.05*
positive	57	18	

*P<0.05

Table 1 Association between Endoglin expression and clinic-pathologic characteristics of patients with gastric cancer (n=126)