THBS4/Integrin $\alpha 2$ axis mediates BM-MSCs promote angiogenesis of gastric cancer associated with chronic *Helicobacter pylori* infection

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Research

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Abstract

Background

BM-MSCs contribute to *Helicobacter pylori* (*H. pylori*)-induced gastric cancer, but its mechanism is still unclear. The aim of our study is to investigate the specific role and mechanism of BM-MSCs in *H. pylori*-induced gastric cancer.

Main methods

Mice were received total bone marrow transplantations and then infected with *H. pylori*. BM-MSCs were extracted and transplanted to gastric serosal layer of mice infected with *H. pylori* chronically. Hematoxylin and eosin staining, immunohistochemistry staining and immunofluorescence were performed to detect the tumor growth and angiogenesis in mice stomach tissues. Chicken chorioallantoic membrane assay, xenograft tumor model, and Human umbilical vein endothelial cells tube formation assay were used for *in vivo* and *in vitro* angiogenesis study. THBS4 was screened out from RNA-SEQ Analysis with gastric tissues of BM-MSCs transplantation into *H. pylori* infected mice.

Results

BM-MSCs can migrate to the site of chronic mucosal injury and promote tumor angiogenesis associated with chronic *H. pylori* infection. Migration of BM-MSCs to the site of chronic mucosal injury induced the up-regulation of THBS4, which was evident also in human gastric cancer, and correlated with more blood vessel formation and worse outcome. THBS4/Integrin $\alpha 2$ axis exhibited promoting angiogenesis by facilitating PI3K/AKT pathway in endothelial cells.

Conclusions

Our results discover that BM-MSCs have a new pro-angiogenic effect in chronic *H. pylori* infection microenvironment, primarily through THBS4/Integrin $\alpha 2$ axis, which activated PI3K/AKT pathway in endothelial cells and eventually induced formation of new tumor vessels.

Introduction

Gastric cancer is the fifth most common cancer and the third most common cause of cancer death globally(1). Many factors are implicated in the carcinogenesis and progression of gastric cancer, including prevalence of *Helicobacter pylori* (*H. pylori*) infection, the main cause of gastric cancer(2). Chronic *H. pylori* infection of the gastric mucosa leads to stepwise progression from atrophic gastritis and intestinal metaplasia, and eventually adenocarcinoma (3) However, the mechanism by which *H. pylori* infection mediates gastric cancer progression is incompletely defined.

Recent studies on gastric cancer demonstrated that cancer stem cells (CSCs) exist in gastric cancer. These cells could originate from bone marrow-derived cells (BMDCs) (4–7). BM-derived mesenchymal

stem cells(BM-MSCs) are among the BMDCs that have been shown to be recruited to gastric cancers and to promote tumor growth(8, 9). Houghton et al demonstrated chronic infection of C57BL/6 mice with *Helicobacter*, induces an environment conducive to BMDCs recruitment, with the BM-MSCs the most likely candidate(7). Varon demonstrates that infection by *H. pylori* leads to BM stem cell and particularly BM-MSCs recruitment and homing in the gastric mucosa and that BM-MSCs reconstituted gastric glands can evolve toward metaplasia and dysplasia(10). Quante et al demonstrated that a significant percentage of carcinoma-associated fibroblasts (CAFs) in *Helicobacter felis* infected inflammation-associated gastric cancer originates from BM-MSCs, which promote cancer growth and progression(11). Studies using bone marrow transplantation in mice have shown that MSCs display a remarkable feature called tumor-specific tropism, in which they actively migrate to tumor sites(12). However, the specific mechanism of BM-MSCs involved in the occurrence of *H. pylori*-related gastric cancer remained uninvestigated.

Several studies have pointed to BM-MSCs promote tumor growth by enhancing angiogenesis(13–15). Nevertheless, whether BM-MSCs participate in the occurrence of gastric cancer associated with chronic *H. pylori* infection by promoting tumor angiogenesis is unknown. Since the in vivo distinct functional role of facilitating blood vessel formation of BM-MSCs in spontaneous primary gastric tumors associated with chronic *H. pylori* infection remain unexplored, the aim of this research was to investigate the role of BM-MSCs and its specific mechanism in angiogenesis associated with chronic *H. pylori* infection.

In this study, we demonstrate that BM-MSCs can migrate to the site of chronic mucosal injury with chronic *H. pylori* infection and promote tumor angiogenesis in vitro and in vivo. BM-MSCs in mouse models of chronic infection of BALB/c mice with *H. pylori* induced the up-regulation of Thrombospondin 4(THBS4), which was previously shown to affect the functions of microvascular endothelial cells and promote angiogenesis(16, 17). BM-MSCs derived THBS4 exhibits promoting angiogenesis by facilitating PI3K/AKT pathway in endothelial cells. Thus, our findings that BM-MSCs and its derived THBS4 promote angiogenesis may provide a therapeutic target to inhibit angiogenesis in *H. pylori* infection related gastric cancer.

Materials And Methods

Helicobacter strain and infection experiments

H. pylori strain SS1 was cultured in *H. pylori* fluid nutrient medium (HB8647, Hopebio, QingDao, China) under microaerobic conditions (N₂: 85%; O₂: 5%; CO₂: 10%) at 37°C. *H. pylori* strain SS1 was collected in phosphate buffered saline PBS (Gibco, ThermoFisher, USA) and male BALB/ c mice (4-6 weeks of age) were fasted for 6H and inoculated by oral gavage of the bacterial suspension ($1 \sim 2 \times 10^9$ CFU/mL, 100μl per mice) every other day for 3 days. Non-infected control groups were given PBS alone.

Total bone marrow transplantations (BMT)

Balb/c mice were lethally irradiated using an x-ray machine. 24 h post-irradiation, mice were injected i.v. with unfractionated bone marrow cells harvested aseptically from age-matched Isldtomato- female mice.

Following transplantation, mice received antibiotics for 4 wk in water (Enrofloxacin; 0.2 mg/ml). After three months of chronic *H. pylori* infection, engraftment of Isltdtomato marrow-derived cells was tracked with Isltdtomato staining.

BM-MSCs extraction and transplantation

After 3 months of chronic *H. pylori* infection, GFP fluorescent-labeled BM-MSCs transplantation model was established. BM-MSCs were obtained by femoral lavage of 4-week-old male GFP transgenic BALB/c mice. Cells were cultured and identified by flow cytometry for cell surface antigens including CD45, CD73, CD105, Stem cell antigen-1 (Sca1), CD11b (antibodies all from BD-Pharmingen, PaloAlto, CA) (Fig.S1). 2×10^6 collected BM-MSCs from GFP mice was resuspended and washed in PBS then injected into the serosal layer of mice gastric antrum using a 50 μ l Hamilton syringe. After 3 months, the level of engraftment and migration was assessed by confocal microscope observation of GFP fluorescence in gastric tissues.

HUVEC extraction and identification

Human umbilical vein endothelial cells (HUVEC) were obtained from human umbilical vein using Type I collagenase Hank's solution. HUVEC were cultured in Endothelial Cell Medium (ECM) (cat. No.1001, ScienceCell, San Diego, California, USA) containing 25 ml of fetal bovine serum (FBS, Cat. No. 0025), 5 ml of endothelial cell growth supplement (ECGS, Cat. No. 1052) and 5 ml of antibiotic solution (P/S, Cat. No. 0503). Cells were identified by immunofluorescence staining of anti-von Willebrand factor (VWF) (Bs-0586R, Bioss) and anti-CD31(ab9498, Abcam) for cell surface antigens (Fig.S2).

Cell culture, animals and patient samples

The GC cell lines SGC7901 used in our study were purchased from American Type Culture Collection (ATCC). BALB/c mice and BALB/c nude mice were purchased from the Beijing Huafukang Biological Co., Ltd and Beijing Viton Lihua Biological Co., Ltd., and housed under standard laboratory conditions in Experimental Animal Center of Tongji Medical College, Huazhong University of Science & Technology. The human sample comes from the Endoscopy Center of Union Hospital. All samples were obtained with the patients' informed consent, and the samples were processed histologically.

SGC7901 xenograft tumor model

Four-week-old male BALB/c nude mice housed under standard laboratory conditions. Human gastric cancer SGC7901 were harvested and resuspended in PBS. 2×10^6 Cells were injected subcutaneously into the right forelimb armpit of BALB/c nude mice. The tumor size was measured every two days. After 21 days, mice were sacrificed and the tumor growth was evaluated by weight.

Chick embryo chorioallantoic membrane (CAM) assay

The CAM assay was performed to evaluate in vivo angiogenic activity as previously described(18). Briefly, 100 fertilized eggs were incubated at 38 °C and 36.5-38.5% relative humidity in a forced draught incubator. A small window was punctured on each egg of 8-day-old fertilized eggs. 30µl of cell suspension was dropped onto a gelatin sponge and then added onto the CAM. The eggs were sealed with transparent tape and incubated at 37.8 °C and 60% humidity for 4 days. On day 12, the eggs were opened and the chorioallantoic membrane vasculatures were imaged using a stereo microscope equipped with a digital camera. Angiogenesis was quantified by counting the blood vessel density (percentage of blood vessel area over the whole area under a microscopic field) using the image analysis program IPP 6.0 (Image-Pro Plus, version 6.0, Media Cybernetics).

Tube formation assay

HUVECs were resuspended to a final concentration of 2×10^5 cells/ml. µ-Slide Angiogenesis (ibidi, Martin Reid, Germany) were pre-coated with 10µl Matrigel (Corning, BD) and then incubated at 37 °C and 5% CO₂ for 30 min. After Matrigel was solidified, resuspended HUVECs (approximately 1×10^4 cells) were seeded into the µ-Slide Angiogenesis. After a 7-h incubation, the tube network was photographed using a phase contrast microscope and confocal microscope. The NIH Image J with the Angiogenesis plugin software were used on the tube network to detect total nodes/tubes /mesh in combination.

Angiogenesis fluorescence imaging in vivo

The AngioSense 750EX Fluorescent Imaging Agent (Perkin Elmer) was injected into the mouse model of gastric cancer cell xenograft by tail vein (2 nmol/100µl per mouse, respectively). After 4H, the probe was accumulated in tumors and enabled imaging of vascularity, perfusion, and vascular permeability at a wavelength of 750nm. The angiogenesis fluorescence imaging in vivo was detected by Bruker In-Vivo Xtreme (Germany) and quantified by measuring the tumor uptake of the probe AngioSense750 EX.

RNA-SEQ

Total RNA was obtained using Tripure Reagent (Roche Applied Science, Indianapolis, IN, USA), following manufacturer's instructions. Image analysis, per-cycle base-calling and quality score assignment was made using Illumina Real Time Analysis software (Illumina, San Diego, CA). Firstly, the magnetic beads with Poly(T) probes were hybridized with total RNA to purify mRNA. Then the mRNA with Poly(A) was eluted from the magnetic beads and broken into fragments with a magnesium ion solution. The mRNA fragments were reversely transcribed to cDNA and synthesized to double-stranded cDNA. The amplified cDNA was repaired and purified. Directional RNA-SEQ libraries resulting from these analyses were sequenced in paired-end format in two different rounds (Illumina HiSeq2000).

RNA extraction and gene expression analysis

Total RNA was isolated from triplicate wells in each condition with RNAiso Plus reagent (Code No.: 9109, TAKARA, Japan) and reversely transcribed into cDNA using a Reverse Transcription system (Code No.:

RR036A, TAKARA, Japan) according to the manufacturer's instructions. The quality and quantity of RNA were measured with a Nanodrop (Thermo Scientific). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the TB Green *Premix Ex Taq* (Tli RNaseH Plus) (Code No.: RR420A, TAKARA, Japan). The fold changes in gene expression were obtained after normalized to GAPDH and the $2^{-(\Delta\Delta Ct)}$ was used to calculate the relative abundance of mRNA. Primers used in this study are shown in Supplemental Table1.

SDS–PAGE and Western blotting analysis

Proteins were extracted in RIPA buffer containing phosphatase and protease inhibitor mixes (Beyotime Biotechnology, China) at 4°C for 10-20 min. The quantity of proteins was measured using BCA protein concentration determination kit (Beyotime Biotechnology, China). After removing DNA and impurities, proteins were separated by SDS–PAGE (WB2102, Biotides, BeiJing, China) and transferred to nitrocellulose membranes. The membrane was blocked using 10% skimmed milk before detecting with specific antibodies and HRP-conjugated secondary antibodies in combination with enhanced chemiluminescence. The Signal was acquired with Millipore immobilon western chemilum HRP substrate and grey value was quantified with Image J. The primary antibodies used were sheep anti-mouse THBS4(AF7860-SP, R&D Systems), rabbit anti-AKT (#4691T, CST), and rabbit anti-Phospho-AKT(p-AKT) (Ser473) (#4060T, CST).

Histology, immunohistochemistry (IHC) and Immunofluorescence (IF) staining

The tissues were embedded and sectioned. After dewaxed and hydrated, the sections were incubated overnight with antibodies against human THBS4(YT4646, immunoWay), human PECAM-1(ab9498, Abcam), mouse CD31(AF3628-SP, R&D Systems), mouse NG2(sc-5389, Santa Cruz), mouse THBS4(AF7860-SP R&D Systems), mouse Ki67(ab1667, Abcam), and identified with Alexa 488, Alexa 594 or cy3 secondary antibodies or HRP-conjugated secondary antibodies the next day. The sections were detected by phase contrast microscope and confocal microscope and raw image data quantity were processed with Image-Pro Plus.

Cell migration assay

The twenty-four-well plate Transwell was performed to determine the ability of HUVEC migration. 1000 serum-starved HUVEC were plated on the upper chambers of 3.0-μm pore size Transwell inserts and culture ECM medium containing 20% FBS was introduced to the lower chambers. After incubating the cells for 24h at 37° incubator, unmigrated cells on the top membrane was removed with cotton swabs. The remaining cells were fixed in 4% paraformaldehyde for 15min and stained with 1% crystal violet for 10 min at room temperature and then washed in PBS for 3 times. The image of migratory cells was acquired with inverted light microscope and the cells were counted to quantify cell migration.

Lentivirus construction and transfection

Lentivirus for THBS4 short hairpin RNA (shRNA) and negative control (NC) sequences were designed and constructed by Obio Technology Co. Ltd., Shanghai, China. The shRNA Lentivirus were transfected into BM-MSCs using HitransG A (Genechem, Shanghai, China) according to the manufacturer's protocol. Selection of stably transfected cells was performed with puromycin (8 µg/ml). The targeted THBS4 sequences are as follows: GGATGAGTGCAAATACCAT.

Statistical analysis

All experiments were repeated at least three times, and the results were expressed as means ± SD. All statistical analyses were performed using GraphPad Prism software. Student's t-test or the one-way analysis of variance (ANOVA) were used to analyze the data, and chi-square test was used to analyze differences in other variables, as appropriate. P value of ≤ 0.05 was considered statistically significant for all datasets. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

1. BM-MSCs exacerbated tumorigenicity and angiogenesis of H. pylori-induced GC

To verify the role of BM-MSCs by depleting or inhibiting the intrinsic BM-MSCs, we conducted a total BM transplantations models (BMT) and then infected the mice with *H. pylori* for 3 months. In BMT mice, engraftment of Isldtomato marrow-derived cells were detectable in mice mucosal layer with Isldtomato staining at 3 months of *H. pylori* infection (Fig. 1A). HE staining revealed that intraepithelial neoplasia but no tumors was detected in BMT mice (Fig. 1B). Then we conducted BM-MSCs transplantation to Iserosal layer of mice gastric model in order to allow more BM-MSCs cells to reach the gastric mucosal layer, which has a magnifying effect to better study the role of BM-MSCs in inducing gastric cancer. Mice were infected with *H. pylori* for 3 months. Gastric serosal layer was transplanted with BM-MSCs marked by green fluorescent GFP. After 3 months after transplantation. Confocal microscope confirmed that BM-MSCs can be transplanted into the stomach for a long time under chronic *H. pylori*-infection, and migrate to the site of chronic mucosal injury (Fig. 1C). HE staining revealed that in mice with *H. pylori*-infection chronically, transplantation and engraftment of BM-MSCs leads to more dysplasia and gastric carcinoma (Fig. 1D-E). Engraftment of BM-MSCs increased vasculogenic markers, such as CD31, NG2 in stomach tissues in mice with *H. pylori*-infection. (Fig. 1F-H). These findings point to a role for BM-MSCs engraftment in the tumor microenvironment of chronic *H. pylori* infection inflammation-induced gastric cancer and contributes to tumor tumorigenicity and angiogenesis.

2. BM-MSCs promotes GC angiogenesis in vivo and the tube formation and migration of HUVEC following H. pylori stimulation

To explore the specific role of BM-MSCs in the angiogenesis of chronic *H. pylori*-related gastric cancer, we pre-intervened BM-MSCs by *H. pylori* culture supernatant (MOI = 50).

We performed a gastric cancer assay on the CAM, which has emerged as a standard for in vivo angiogenesis assay(18). BM-MSCs and gastric cancer cells were mixed and injected into CAM to detect its role in tumor angiogenesis. Compared with the control, BM-MSCs significantly increased vascular density of cancer xenografts on the CAM (Fig. 3A-B). In addition, we subcutaneously transplanted SGC cells and mixed with BM-MSCs into BALB/c nude mice. As shown in Fig. 3C-E, The SGC cells mixed with BM-MSCs group obtains the subcutaneous tumors with larger volume and weight, compared with that of SGC cells group. Ki-67 IHC staining supports BM-MSCs obtained a stimulatory role in gastric tumor growth. CD31 IHC staining revealed that formation of microvessel in tumors from SGC cells mixed with BM-MSCs group was more developed (Fig. 3F-H). The tumor vascular permeability fluorescence imaging in vivo demonstrated that BM-MSCs significantly promoted the degree of tumor neovascularization in the SGC-induced tumor xenografts (Fig. 3I-J).

We established an in vitro co-culture system, in which HUVEC was indirectly cocultured with SGC cells and separated from SGC cells by a semipermeable membrane. CM from BM-MSCs stimulated by *H. pylori* was add to co-culture system. Transwell assays revealed that treatment with CM from BM-MSCs stimulated by *H. pylori* promoted the migration of HUVEC (Fig. 2K-L). Meanwhile, HUVEC tube formation was evaluated, and CM from BM-MSCs stimulated by *H. pylori* was proved to promote HUVEC to form a spider web-like microvascular network structure (Fig. 2M-N).

3. BM-MSCs engraftment led to THBS4 overexpression during H. pylori infection

To explore the specific mechanism of BM-MSCs promoting chronic *H. pylori* infection-related gastric cancer angiogenesis, we performed RNA-SEQ Analysis to screen for differential genes. The results showed that BM-MSCs transplantation caused the up-regulation of many differential gene expressions (Fig. 3A). Genes related to angiogenesis were obtained through enrichment analysis (Gene Ontology) (Fig. 3B). In order to further verify the differential gene, the gastric tissues were verified at the mRNA level. The results of qRT-PCR experiments demonstrated that compared with the chronic *H. pylori*-infection group, the expression of Kng1, THBS4, Prodh2, Serpina1d, Myl3 and Angptl3 genes increased significantly by BM-MSCs transplantation (Fig. 3C). Since THBS4 is the most expressed gene, western blot further indicated THBS4 protein overexpression after BM-MSCs transplantation (Fig. 3D-E). Subsequently, the same result was detected by IHC and IF of THBS4 in stomach tissue (Fig. 3F-H). At the same time, the effect of *H. pylori* on THBS4 protein expression of BM-MSCs was further tested, and it was found that *H. pylori* pretreatment increased the expression of THBS4 protein of BM-MSCs (Fig. 3I-J).

4. Overexpression of THBS4 is correlated with the vessel density in human GC tissues and predicts poor prognosis of GC patients

THBS4 is a member of the extracellular calcium-binding protein family and is involved in cell adhesion and migration(19). Bioinformatic analysis of THBS4 expression profiles through The Cancer Genome Atlas (TCGA) indicated that the THBS4 were significantly increased in majority of tumor types (Fig. 4A). Notably, THBS4 were expressed at significantly higher levels in gastric carcinoma compared with non-cancerous stomach tissues (Fig. 4B). Furthermore, TCGA database analysis showed that THBS4

expression was negatively correlated with survival of gastric cancer (Fig. 4C-D). We then detected THBS4 protein expression in *H. pylori* (+) GC tissues and corresponding *H. pylori* (+) nontumor tissues by IHC. THBS4 was located in the tumor stroma in gastric adenocarcinoma. In adjacent non-tumor tissues, no positive expression was detected (Fig. 4E-F). THBS4 mRNA expression has been demonstrated to be associated with GC progression(20). The IHC staining of PECAM1 demonstrated that the blood vessel density in *H. pylori* (+) tumor tissues was significantly higher than that in *H. pylori* (+) non-tumor tissues (Fig. 4G-H). Further analysis revealed that the expression of interstitial THBS4 was positively correlated with the blood vessel density in tumor tissues (Fig. 4I). Similarly, bioinformatic analyses of the correlation between THBS4 and PECAM1 through GEPIA (Fig. 4J). Confirmed these findings, which indicated that THBS4 may play a vital role in the angiogenesis of GC.

5. THBS4 mediate BM-MSCs induced angiogenesis in GC

In order to investigate the biological behaviors of THBS4 in BM-MSCs promoting angiogenesis, we successfully constructed the Lentivirus vector (sh-THBS4 and sh-NC) (Fig. 5A). Real Time PCR and Western Blot were performed to detect whether expression level of THBS4 changed in BM-MSCs (Fig. 5B-E). In co-culture system as previously described, transwell assays and tube formation revealed that downregulation of THBS4 reduced the migratory ability and tube formation induced by CM from *H. pylori*-pretreated BM-MSCs (Fig. 5F-I). Results from xenograft tumor model revealed that injection of *H. pylori*-pretreated BM-MSCs could dramatically increase the growth of GC subcutaneous tumors, these effects were also notably inhibited when THBS4 was silenced (Fig. 6A-D). The results of IHC staining against CD31 and Ki67 showed that the silencing THBS4 in BM-MSCs related to microvessel density and gastric cancer proliferation reduction in subcutaneous tumors. (Fig. 6E-G). The tumor vascular permeability fluorescence imaging in vivo demonstrated that THBS4-knockdown *H. pylori*-pretreated BM-MSCs demonstrated weakened neovascularization density compared with the *H. pylori*-pretreated NC-knockdown MSCs (Fig. 6H-I). Moreover, *H. pylori*-pretreated BM-MSCs strongly accelerated gastric cancer angiogenesis in CAM assay, while these effects were dramatically inhibited when THBS4 was silenced (Fig. 6J-K). We then performed THBS4-knockdown MSCs and NC-knockdown MSCs transplantation into mice gastric serosa layer with chronic *H. pylori* infection. IF of CD31 and NG2 revealed that NC-knockdown BM-MSCs were more efficient in inducing angiogenesis (Fig. 6L-M).

6. Integrin $\alpha 2$ mediates the effects of paracrine THBS4 signaling

Integrin $\alpha 2$ was identified as a receptor of THBS4 involved in regulation of endothelial cell migration(17, 21). To test whether Integrin $\alpha 2$ contribute to promoting angiogenesis in response to rTHBS4, we cultured HUVEC for 48 hours in the presence of 10ng/ml rTHBS4. Western Blot and IF showed that integrin $\alpha 2$ in HUVEC was significantly increased after the treatment of rTHBS4 (Fig. 7A-C). Furthermore, molecular mechanisms by which integrin $\alpha 2$ receptor participates in paracrine THBS4 signaling was investigated using an $\alpha 2$ -specific antibody. The $\alpha 2$ -specific antibody was able to alleviate rTHBS4 induced migration and tube formation capacities of HUVEC (Fig. 7D-G). Additionally, the $\alpha 2$ -specific antibody also can abrogate rTHBS4 induced proliferation in HUVEC (Fig. 7H).

7. Targeting PI3K/AKT pathway abrogate THBS4/integrin $\alpha 2$ axis induced promotion of angiogenesis

The THBS4/integrin $\alpha 2$ axis is known to activate a number of downstream signaling molecules including PI3K/AKT, ERK, P38(22–25). Then we evaluated these downstream signaling molecules of the THBS4/integrin $\alpha 2$ axis in HUVEC. Results demonstrated that p-AKT level was positively correlated with rTHBS4 treatment (Fig. 8A-B). HUVEC were treated with rTHBS4 for 15–60min to assess the levels of p-Akt to further verify the results with activation of Akt. The results indicated that the p-AKT activation is in a time-dependent manner of rTHBS4(Fig. 8C-D). Next, we investigate the role and requirement of Akt signaling in rTHBS4 facilitating the migration and tube formation of HUVEC. rTHBS4 treatment enhanced Akt phosphorylation, while this effect was dramatically restrained by pretreatment with integrin $\alpha 2$ -specific antibody or the PI3K inhibitor LY294002(Fig. 8E-F). In addition, the promotion effects on migration and tube formation of HUVEC mediated by rTHBS4/integrin $\alpha 2$ axis were partly counteracted by PI3K inhibition. (Fig. 8G-J). It turned out that THBS4/integrin $\alpha 2$ axis accelerates angiogenesis via activating PI3K/AKT signaling.

Discussion

Our results show for the first time that BM-MSCs participate in angiogenesis in gastric cancer associated with chronic *H. pylori* infection of the gastric mucosa in mice. Our results demonstrated that *H. pylori* induced BM-MSCs recruitment and producing THBS4, which induced PI3K/AKT signaling activating in vascular endothelial cells, contribute to *H. pylori*-induced GC angiogenesis. The results revealed the specific role of BM-MSCs promoting angiogenesis in the occurrence of *H. pylori* related gastric cancer.

Chronic gastric inflammation, which develops as a consequence of *H. pylori*, leads over time to repetitive injury repair resulting in hyperproliferation, an increased rate of mitotic error, and progression to adenocarcinoma. Recent studies have suggested that in response to chronic *H. pylori* infection, BM-derived cells especially BM-MSCs can home to and repopulate the gastric mucosa and contribute over time to metaplasia, dysplasia, and cancer(7, 11). Nevertheless, the specific role and mechanism of BM-MSCs in the occurrence of gastric cancer are unknown. Several studies have pointed to BM-MSCs as a dominant stromal component within the tumor microenvironment and contribute in many ways to tumor progression; these include extracellular matrix remodeling, metastasis, and angiogenesis(13, 14, 26). In the present study, in BM transplantation mice, engraftment of Isldtomato marrow-derived cells were detectable in mice gastric at 3 months of *H. pylori* infection. We then showed that transplanted BM-MSCs could have the ability to migrate to the site of chronic mucosal injury under chronic *H. pylori* infection. Furthermore, engraftment of BM-MSCs leads to dysplasia and gastric carcinoma in mice with chronic *H. pylori* infection and vasculogenic markers detected in mucosa and submucosa gastric tissues. Thus, the precise effects of BM-MSCs on tumor growth and angiogenesis and the mechanism underlying these effects should be elucidated.

We further found that *H. pylori*-pretreated BM-MSCs significantly promote vessel formation of gastric cancer within the CAM and xenograft tumor model. The oncogenicity of GC cells and number of new

blood vessels was enhanced by co-implantation with *H. pylori*-pretreated BM-MSCs; Thus, BM-MSCs may affect angiogenesis of gastric cancer tumor microenvironment. Therefore, more studies are necessary to examine whether BM-MSC harbor this property in endothelial cells. In addition, in vitro experiments, we observed that BM-MSCs promote the angiogenic potential of HUVEC in vitro. It appears that the ability of BM-MSCs promoting angiogenesis increased after *H. pylori* treatment.

It has been shown that paracrine factors secreted by BM-MSCs may be involved in tumor angiogenesis (4, 27, 28). Better understanding the molecular mechanisms of BM-MSCs promoting angiogenesis is important. Analysis of RNA-SEQ and GO enrichment revealed THBS4 may involve with BM-MSCs promoting angiogenesis of gastric cancer associated with chronic *H. pylori* infection. We also found that THBS4 expression of BM-MSCs increased with *H. pylori* intervention. THBS4 was shown to belong to a group of secreted matricellular ECM proteins and was involved in wound healing and tissue remodeling(29, 30). Tissue remodeling, inflammation, and angiogenesis often occur simultaneously in the tumor microenvironment and are regulated by the same stimuli. THBS4 was reported to be expressed in some types of solid cancers(31, 32). The emerging role of THBS4 in tumor–stroma interactions, particularly in gastric cancer(31) suggest that it may play an important role in the tumor microenvironment(33). THBS4 was reported to contribute to the proliferation and metastasis of GC in vitro(34). Muppala S recently reported that THBS4 affects the functions of microvascular endothelial cells and promotes angiogenesis (16, 17) with EMT6 mouse breast cancer cells. So what role does THBS4 expressed by BM-MSCs play in *H. pylori*-related gastric cancer angiogenesis? Our data indicated that THBS4 derived from BM-MSCs plays a facilitative role in regulating tumor angiogenesis of *H. pylori*-related GC in vitro and in vivo. These findings suggest that BM-MSCs may stimulate GC angiogenesis by THBS4.

THBS4 is known as a secreted extracellular matrix (ECM) protein, which exerts its effect in multiple ways: by binding to its extracellular ligands and receptors (16, 17, 35), by initiating intracellular signaling(25) (30, 36) and transiting through secretory pathways(37). Moreover, we found that integrin $\alpha 2$ in HUVEC was significantly increased after rTHBS4 treatment. Integrin $\alpha 2$ was implicated in endothelial cell migration and tube formation on THBS4 using an $\alpha 2$ -specific antibody. THBS4/integrin $\alpha 2$ axis is known to activate a number of downstream signaling molecules, such as PI3K/Akt(24), ERK(25), p38(30, 36). Thus, we further explored whether the downstream intracellular signaling participates in the THBS4/integrin $\alpha 2$ axis-induced migration and tube formation in HUVEC. We observed that AKT activation is essential for the BM-MSCs derived THBS4 induced migration and tube formation of HUVEC. Sustained endothelial Akt activation caused increased blood vessel size and chronic vascular permeability, which induced formation of structurally and functionally abnormal blood vessels that recapitulate the aberrations of tumor vessels (38, 39). Collectively, these results led us to propose a model whereby BM-MSCs-derived THBS4 interacts with its receptor integrin $\alpha 2$ to activate the PI3K-Akt pathway, which mediates the migration and tube formation of endothelial cells.

In this study, the association of BM-MSCs derived THBS4 with angiogenesis of gastric cancer associated with *H. pylori* infection was observed in vivo, in xenograft tumor models, and in CAM, as well as in

cultured HUVEC. THBS4/integrin $\alpha 2$ axis promote migration and tube formation of endothelial cells by facilitating PI3K/AKT pathway, which induced formation of new tumor vessels in *H. pylori* infection microenvironment. Insight into the role of BM-MSCs derived THBS4 in angiogenesis may provide a therapeutic target to inhibit angiogenesis in *H. pylori* infection related gastric cancer.

Conclusions

Our results provide the basis for the view that BM-MSCs derived THBS4 may play an important role in angiogenesis of gastric cancer associated with chronic *H. pylori* infection, suggesting that THBS4/integrin $\alpha 2$ axis may be a new prognostic and therapeutic biomarker for GC associated with chronic *H. pylori* infection.

Abbreviations

BM-MSCs: bone marrow (BM)-derived mesenchymal stem cells; THBS4: Thrombospondin 4; GC: Gastric cancer; *H. pylori*: *Helicobacter pylori*; HUVEC: Human umbilical vein endothelial cells; CAM: Chick embryo chorioallantoic membrane; qRT-PCR: Quantitative real-time polymerase chain reaction; GO: Gene Ontology.

Declarations

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Ethical considerations

All procedures followed were in accordance with the ethical standards of the Ethical Committee of Tongji Medical College, Huazhong University of Science & Technology and with the Helsinki Declaration of 1964 and later versions. All human samples were obtained with the patients' written informed consent. All institutional and national guidelines for the care and use of laboratory animals were followed.

Author contribution

RL conceived the study and performed the project design. LNH, WJW and HYS performed most of the experiments. CJ, HLY and YRZ helped in some of the animal experiments. LNH analyzed the data and wrote the paper. RL, WJW and HYS revised the paper. WQ provided technical assistance in in vitro experiments, and evaluated the development of methodology.

LNH, WJW and HYS contributed equally to this work.

Consent for publication

Not applicable.

Disclosure

The authors declare that they have no conflicts of interests.

Availability of data and materials

Please contact the corresponding author for all data requests.

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Figures

Figure 1

Figure 1

BM-MSCs promotes tumorigenicity and angiogenesis of *H. pylori*-induced GC (A) In BMT mice, engraftment of Is1tdtomato marrow-derived cells in mice gastric tissues was tracked with Is1tdtomato staining. (B) Representative gastric mucosa histopathology of GIN in BMT mice. (C) IF of GFP in *H. pylori*-infected stomachs with GFP-labeled BM-MSCs transplantation. (D) Representative gastric mucosa histopathology in *H. pylori*-infected stomachs with (HP+MSC) or without (HP) BM-MSCs transplantation.

(E) Quantification of dysplasia and gastric cancer in D. (F) IHC of CD31 and NG2 in HP or HP+MSC group. (G) Quantification of F. Multiple fields (at least six) from three different mice were analyzed. (H) IF of CD31 and NG2 in HP or HP+MSC group. Cell nuclei, DAPI; CD31, Cy3; NG2, Cy3. HP: H. pylori. MSC: Bone marrow-derived mesenchymal stem cells. IF: Immunofluorescence. Results show mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$.

Figure 2

Figure 2

BM-MSCs promotes GC angiogenesis in vivo and the tube formation and migration of HUVEC following H. pylori stimulation (A) Representative images of the blood vessels of SGC co-injected with or without BM-MSCs or H. pylori -pretreated BM-MSCs on CAM for 4 days. (B) Quantification of vascular density growth rate on CAM. (C-D) SGC cells mixed with or without BM-MSCs or H. pylori-pretreated BM-MSCs were injected into BALB/c nude mice and tumor volumes were monitored every two days. (E) Average tumor weights from nude mice. (F) Representative images of H&E staining, Ki-67 and CD31 (PECAM1) IHC of tumors. (G-H) Quantification of Ki-67 and CD31. (I) Angiogenesis fluorescent agent AngioSense750 EX in tumors. (J) Quantification of I. (K) The migration ability of HUVEC was evaluated using a Transwell assay. (L) Quantification of K. (M) In vitro tube formation to investigate the effect of CM from BM-MSCs cells. (N) Quantification of M.

Figure 3

Figure 3

BM-MSCs engraftment led to THBS4 overexpression during *H. pylori* infection (A) Volcano Plot presentation of differentially expressed genes of gastric tissues in HP versus HP+MSC mice. (B) Enrichment analysis of differential genes. (C) RT-PCR verified the expression of differential genes associated with angiogenesis, THBS4 is the most expressed gene. (D-E) Western Blot showed BM-MSCs transplantation led to THBS4 overexpression. (F-G) IHC (F) and IF (G) of THBS4 in HP+MSC or HP mice.

Cell nuclei, DAPI.THBS4, FITC. Magnification: × 200 and × 400. (H) Quantification of F. (I-J) Western Blot of THBS4 revealed that H. pylori pretreatment increased the expression of THBS4 protein of BM-MSCs.

Figure 4

Figure 4

Overexpression of THBS4 is correlated with the vessel density in human GC tissues and predicts poor prognosis (A) The expression of THBS4 in pan-cancer. (B) Volcano Plot revealed upregulation of THBS4 mRNA expression in GC compared with normal tissue. (C-D) The overall survival and disease-free survival

analyses of THBS4 were plotted for patients with GC in TCGA. (E) Representative IHC staining with THBS4 antibody in GC and adjacent nontumor tissues. (F) Quantification of E. (G) Representative IHC staining with PECAM1 antibody in GC and adjacent nontumor tissues. (H) Quantification of G. (I) Correlation analysis of THBS4 and vessel density showed that THBS4 was positively correlated with vessel density in GC. (J) Validation of the positive correlation between THBS4 and PECAM1 in GEPIA.

Figure 5

Figure 5

THBS4 mediates BM-MSCs promotes migration and tube formation of HUVEC in vitro (A) Transfection of recombinant lentiviral vectors into BM-MSCs. (B-E) qRT-PCR and Western blot analysis of THBS4 expression in BM-MSCs of both experiments, transfected as indicated. (F, H) Transwell assay and quantification of migrated cells showed THBS4 knockdown in BM-MSCs inhibited its ability to promote HUVEC migration. (G, I) Knockdown of THBS4 in BM-MSCs alleviated its ability to promote HUVEC tube formation.

Figure 6

THBS4 mediates BM-MSCs promotes GC angiogenesis in vivo (A-C) 4-wk-old nude male mice were injected with either SGC cells only or SGC and BM-MSCs transfected with recombinant lentiviral vectors. 3 wk following injected, mice were euthanized and tumors were analyzed. (D) Tumor weight of injected tumors at end-point. (D) Growth curve of tumors describes in A. (E) H&E staining, Ki-67 and CD31 immunostaining of tumors describes in A. (F-G) Quantification of staining presented in E performed with image plus. (H-I) Following AngioSense750 EX injection, the tumor vascular permeability fluorescence imaging in vivo was performed to detect the neovascularization density of tumors in A. (J-K) Representative images and Quantification of the CAM assay. (L-M) IF of CD31, and NG2 in NC-knockdown BM-MSCs transplanted mice and THBS4-knockdown BM-MSCs transplanted mice.

Figure 7

Figure 7

Integrin $\alpha 2$ mediates the efficacy of paracrine of THBS4 signaling on migration and tube formation of HUVEC. (A-C) Western blot and IF showed that Integrin $\alpha 2$ in HUVEC was significantly increased by rTHBS4. (D) Representative images of the migration assay after rTHBS4, rTHBS4 + anti- $\alpha 2$ or anti- $\alpha 2$ treatments in HUVEC. (E) Representative images of the tube formation assay after rTHBS4, rTHBS4 + anti- $\alpha 2$ or anti- $\alpha 2$ treatments in HUVEC. (F) Quantification of D. (G) Quantification of E.

Figure 8

Figure 8

Activation of AKT by THBS4/integrin α 2 axis in HUVEC (A-B) rTHBS4 induced phosphorylation of AKT in HUVEC. (C-D) p-AKT activation is in a time-dependent manner of rTHBS4. (E-F) Western blot analysis showed that rTHBS4 increased Akt phosphorylation in HUVEC, while this effect was alleviated by blocking integrin α 2 or inhibiting Akt. (G) Transwell migration assay of investigate the effect of PI3K inhibition and blocking integrin α 2 on HUVEC migration after treated with rTHBS4. (H) In vitro tube

formation to investigate the effect of PI3K inhibition and blocking integrin $\alpha 2$ on HUVEC tube formation.
(I) Quantification of G. (J) Quantification of H.

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