Helicobacter pylori-derived Heat shock protein 60 enhances angiogenesis via a CXCR2-mediated signaling pathway

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ABSTRACT

Helicobacter pylori is a potent carcinogen associated with gastric cancer malignancy. Recently, H. pylori Heat shock protein 60 (HpHSP60) has been reported to promote cancer development by inducing chronic inflammation and promoting tumor cell migration. This study demonstrates a role for HpHSP60 in angiogenesis, a necessary precursor to tumor growth. We showed that HpHSP60 enhanced cell migration and tube formation, but not cell proliferation, in human umbilical vein endothelial cells (HUVECs). HpHSP60 also indirectly promoted HUVEC proliferation when HUVECs were co-cultured with supernatants collected from HpHSP60-treated AGS or THP-1 cells. The angiogenic array showed that HpHSP60 dramatically induced THP-1 cells and HUVECs to produce the chemotactic factors IL-8 and GRO. Inhibition of CXCR2, the receptor for IL-8 and GRO, or downstream PLCβ2/Ca2+-mediated signaling, significantly abolished HpHSP60-induced tube formation. In contrast, suppression of MAP K or PI3 K signaling did not affect HpHSP60-mediated tubulogenesis. These data suggest that HpHSP60 enhances angiogenesis via CXCR2/PLCβ2/Ca2+ signal transduction in endothelial cells.

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1. Introduction

Helicobacter pylori is a gram-negative microaerophilic bacterium that colonizes the human stomach [1] and is associated with numerous pathologies, including gastritis, peptic ulcer, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma [2]. Importantly, epidemiological studies reveal that H. pylori infection is found in over half of the world population [3]. Persistent infection by H. pylori can trigger chronic inflammation and ultimately cancer malignancy [4,5]. H. pylori Heat shock protein 60 (HpHSP60) is an adhesion molecule that interacts with host gastric epithelial cells and mucin [6] and acts as a potent immunogen to induce host inflammation [7]. HpHSP60 stimulates human monocyte cells, macrophages, and gastric epithelial cells to

produce pro-inflammatory cytokines, including IL-1β, IL-6, and IL-8 [7–11]. We have previously shown that HpHSP60 plays a role in gastric carcinogenesis. HpHSP60 induces monocyte inflammation, gastric epithelial cell migration, and endothelial cell proliferation. Gastric cancer patients are found to have low anti-HpHSP60 antibody titers. This clinical finding correlates with our in vitro results, because HpHSP60 may down-regulate host immunity and accelerate tumor malignancy [11].

Angiogenesis, the process of new blood vessel formation, is critical for tumor growth and metastasis. Numerous studies demonstrate a positive correlation between chronic inflammation, angiogenesis, and cancer development. Thus, the dysregulation of inflammation and angiogenesis are likely prerequisites to aggressive tumor progression [12]. Patients with H. pylori-positive gastritis have significantly higher vessel density in the gastric mucosa compared to those with H. pylori-negative gastritis [13]. It suggested that inflammation promotes angiogenesis during H. pylori infection [14]. By interacting with TLR2/TLR9, H. pylori significantly increase cyclooxygenase-2 (COX-2) expression, and up-regulation of COX-2 is critical for the inflammatory changes and tissue

damage associated with chronic *H. pylori* infection [15]. Additionally, *H. pylori* may promote the expression of angiogenesis-promoting factors such as IL-8, IL-6, TNF-α, and growth related oncogene (GRO) in endothelial cells [16]. As previously mentioned, HpHSP60 induces expression of pro-inflammatory genes related to angiogenesis, and recent evidence suggests that HpHSP60 directly enhances blood vessel formation [11].

Chemokines comprise a family of approximately 50 low molecular weight chemotactic cytokines that were initially described as being important for leukocyte recruitment to sites of infection and inflammation [17]. Interestingly, a subset of these cytokines has been shown to promote blood vessel growth and repair. In human tissues, the glutamic acid-leucine-arginine (ELR+) CXC chemokines, such as GRO and IL-8, are known to promote angiogenesis via G-protein coupled receptor binding to CXCR1 and CXCR2 to promote systemic endothelial cell proliferation and migration [18,19]. Both *H. pylori* and HpHSP60 stimulate the secretion of angiogenic CXC chemokines, including IL-8, growth related oncogene-alpha (GRO-α, and epithelial neutrophil activating protein-78 (ENA-78) [11,20].

We previously showed that HpHSP60 promotes tumor-associated angiogenesis; however, the underlying mechanism behind this observation remains unclear. Here, we elucidate the signal transduction pathways responsible for HpHSP60-mediated angiogenesis. Our findings are important for the development of tumor malignancy prevention treatments.

2. Materials and methods

2.1. Cell culture

AGS gastric epithelial cells (BCRC, Hsinchu, Taiwan) were cultured in RPMI 1640 medium (Invitrogen, MD, USA) supplemented with 2 g/L sodium bicarbonate (BIO BASIC Inc., Canada), 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, MD, USA), and 50 μg/ml penicillin/streptomycin (Biological Industries, Beithaemek, Israel). Human Umbilical Vein Endothelial Cells (HUVECs) (BCRC, Hsinchu, Taiwan) were cultured in M199 medium (Invitrogen, MD, USA) supplemented with 10% heat-inactivated FBS, 50 μg/ml penicillin/streptomycin, 30 μg/mL endothelial cell growth supplement (Sigma–Aldrich, Steinheim, Germany), and 25 U/mL heparin (Sigma–Aldrich, Steinheim, Germany). Human acute monocytic leukemia (THP-1) cells (BCRC, Hsinchu, Taiwan) were cultured in RPMI 1640 medium supplemented with 0.05 mM 2-mercantoethanol (Amresco Inc., OH, USA), 2 g/L sodium bicarbonate, 50 μg/ml penicillin/streptomycin, and 10% heat-inactivated FBS.

2.2. Protein preparation of HpHSP60

HpHSP60 was prepared as described previously [7]. Briefly, a DNA fragment containing the Hsp60 gene was amplified from *H. pylori* genomic DNA and cloned into the T7 promoter-driven pET 30a (+) expression vector (Novagen, Darmstadt, Germany). The gene was expressed in *Escherichia coli* (BL21 strain), and the protein was purified using HisTrap affinity (Ni–NTA) chromatography (General Electric, NY, USA) followed by a Sephadex G-25 column (General Electric, NY, USA) to remove any salts associated with the proteins.

2.3. MTT assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium bromide (MTT) assay (Sigma–Aldrich, Steinheim, Germany) [11]. AGS and THP-1 cells were seeded in 24-well plates (1×10^5 cells/well) with 1 ml growth medium. Cells were incubated at 37 °C for 2 h, after which 10 μg of HpHSP60

was added. After 24 h, supernatants were harvested. HUVECs were seeded in 96-well plates (2×10^4 cells/well) and incubated in previously-collected HpHSP60-treated AGS or THP-1 supernatant for 48 h at 37 °C. Next, 0.5 mg/ml MTT solution was added to each well, and the cells were cultured for 4 h at 37 °C. Conversion of MTT into purple formazan by metabolically active cells indicated the extent of cell viability. Formazan crystals were dissolved with DMSO, and the optical density was measured at 570 nm using a microplate reader.

2.4. Cell migration assays

HUVECs were incubated in serum-free medium for 24 h prior to seeding in Transwell cell culture chambers (Corning, NY, USA). The cell suspensions with or without HpHSP60 were added to the upper side of the Transwell chambers (3×10^4 cells) for 8 h at 37 °C and 5% CO_2. After incubation, the wells were washed, the cells were stained with 50 μg/mL propidium iodide (PI) (Sigma–Aldrich, Steinheim, Germany), and the membranes were examined using a fluorescent microscope. The migration rate was calculated as the number of cells that moved to the lower side of the Transwell insert as a fraction of the total number of cells seeded.

2.5. Human angiogenesis antibody array

HUVEC, AGS, or THP-1 cells were seeded in 24-well plates (1×10^5 cells/well) with 1 ml growth medium and incubated at 37 °C for 2 h. Next, 10 μg of HpHSP60 was added to each well and cultured with the cells for 24 h. After incubation, the supernatants were harvested and assayed using the human angiogenesis antibody array (RayBiotech, GA, USA) according to the manufacturer instructions. Briefly, membranes were treated with blocking buffer for 30 min, incubated with 1 ml of supernatant from HpHSP60-treated or untreated cells and cultured at room temperature for 2 h. After incubation, the membranes were treated for 1 h with biotin-conjugated anti-cytokine antibodies, reacted with horseradish peroxidase–streptoavidin, and then developed using ECL detection. To determine the relative concentrations of angiogenic factors in the media, the densities of individual spots were measured by the ImagePro software (Media Cybernetucs, Silver spring, MD, USA) for image capturing and analysis. The results were expressed as relative densities compared with positive controls included in each membrane.

2.6. In vitro capillary-like tube formation assays

To examine whether HpHSP60 enhances angiogenesis via CXCR2-dependent signaling, HUVECs were seeded on matrigel-coated 96-well plates (1.5×10^4 cells) and incubated in 100 μl of serum free-medium 199 with or without 50 nM SB225002 (Calbiochem, Darmstadt, Germany), 3 μM BAPTA (Invitrogen, MD, USA), 5 μM PD98059 (Calbiochem, Darmstadt, Germany), or 10 nM wortmannin (Calbiochem, Darmstadt, Germany) for 1 h. Next, cells were treated with 100 μl of medium with or without 20 μg/ml HpHSP60 for 6 h. Tube formation was then assessed by quantification of tube branch points with a phase contrast microscope at 100$\times$ magnification. Branch points were defined as area where a single point gave rise to two divergent outgrowths. The results were expressed as percentage compared with controls (HUVECs cultured in normal media) whose tube numbers were set as 100%.

2.7. Statistical analysis

Results are expressed as the mean ± SEM, and statistical significance was determined using the two-tailed Student's *t* test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. HpHSP60 promotes HUVEC migration and proliferation

We previously showed that HpHSP60 promotes gastric tumorigenesis together with increased blood vessel tube formation. Because endothelial cell proliferation and migration are important steps during angiogenesis, we wondered if HpHSP60 promotes endothelial cell migration and proliferation. Co-culturing HpHSP60 with HUVECs found that cell migration was significantly enhanced in the presence of HpHSP60 (Fig. 1, $p < 0.01$) compared to untreated cells. HUVECs incubated with HpHSP60 did not display increased cell proliferation (Fig. 2A); however, a significant increase in proliferation rate was observed when HUVECs were cultured in the supernatants harvested from HpHSP60-treated THP-1 or AGS cells (Fig. 2B, $p < 0.05$; Fig. 2C, $p < 0.01$). Together, these data suggest that HpHSP60 directly stimulates HUVEC migration and indirectly promotes HUVEC proliferation by inducing the secretion of proliferation-promoting factors in AGS or THP-1 cells.

3.2. HpHSP60 stimulates the differential expression of angiogenic factors in HUVEC, THP-1, and AGS cells

Thus far, we showed that HpHSP60 enhanced endothelial cell migration and cell proliferation; however, the downstream molecules involved in HpHSP60-induced angiogenesis are still unknown. We performed an angiogenesis-related protein array to determine the factors involved in HpHSP60 signaling in HUVEC, THP-1, and AGS cells. IL-8 and GRO were both up-regulated in HpHSP60-treated HUVEC and THP-1 cells, while IL-6 was increased only in HUVECs. Additionally, HpHSP60 was found to stimulate epidermal growth factor expression in AGS cells, albeit at very low levels (Fig. 3 and Table 1).

3.3. HpHSP60 enhances angiogenesis via a CXCR2-dependent signaling pathway

Angiogenic array results indicated that HpHSP60 triggers high expression of IL-8 and GRO in HpHSP60-treated HUVEC and THP-1 cells. IL-8 and GRO are ELR$^+$ CXC chemokines and share the same CXCR2 receptor. Therefore, inhibitors of CXCR2 signaling were used to determine if HpHSP60 signals through CXCR2 to promote angiogenesis (Fig. 4A). Our data showed that the increased tube formation induced by HpHSP60 was rescued by the CXCR2 inhibitor SB225002
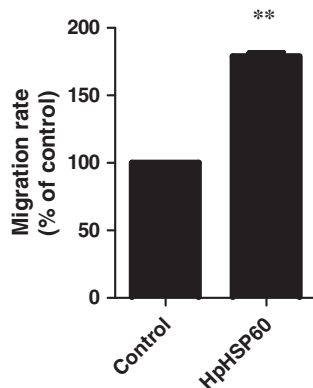

Fig. 1. HpHSP60 promotes HUVEC migration. HUVEC migration assays were performed in basal medium with or without (control) 10 μg/ml HpHSP60 for 8 h. Cells were stained with PI, and the number of migrated cells was scored in three random microscopic fields (100× magnification). Migration rate was expressed as percent migration following HpHSP60 treatment, where untreated cell migration equals 100%. The data are expressed as the mean ± SEM, **$p < 0.01$. The experiments were performed in triplicate.
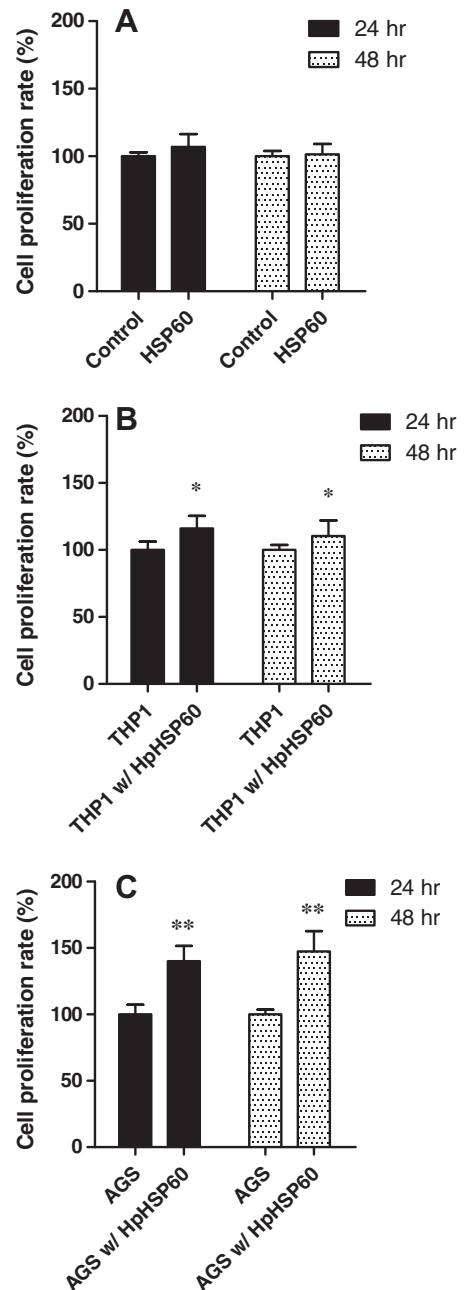

Fig. 2. The effects of HpHSP60 on HUVECs proliferation. (A) HUVECs were treated with or without (control) 10 μg/ml of HpHSP60 for 24 or 48 h. (B,C) HUVECs were cultured for 24 or 48 h in supernatants from THP-1 or AGS cells that were cultured with or without 10 μg/ml HpHSP60 for 24 h. Proliferation rates are expressed as percent proliferation, where untreated cell proliferation is equal to 100%. The data are expressed as the mean ± SEM, *$p < 0.05$ and **$p < 0.01$. All experiments were performed in triplicate.

and the calcium signaling inhibitor BAPTA but not by the MEK inhibitor PD98059 or the PI-3 inhibitor wortmannin (Fig. 4B). These data suggest that HpHSP60-mediated angiogenesis is dependent on CXCR2 signaling. Furthermore, the calcium chelator BAPTA suppressed angiogenesis, revealing that phospholipase C (PLC) β2 / Ca2+ pathway acts downstream of CXCR2 to promote angiogenesis.

4. Discussion

In this study, we provide the first evidence that HpHSP60 activates a CXCR2/PLCβ2/Ca2+ signaling pathway to promote angio-

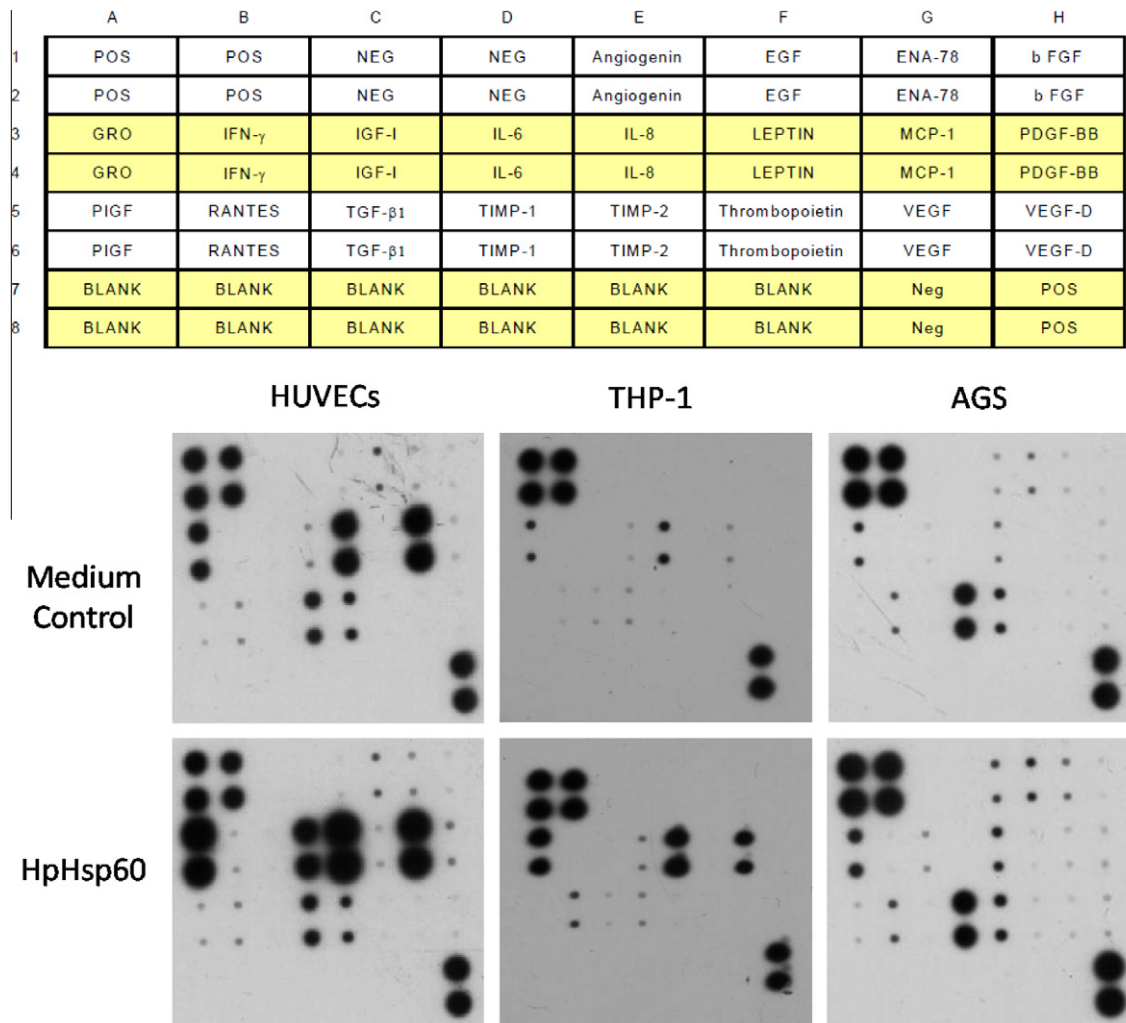|	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b FGF
2	POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b FGF
3	GRO	IFN-γ	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
4	GRO	IFN-γ	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
5	PIGF	RANTES	TGF-β1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
6	PIGF	RANTES	TGF-β1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
7	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Neg	POS
8	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Neg	POS

Fig. 3. Protein array analysis of HpHSP60-treated HUVEC, THP-1, and AGS cells. Supernatants collected from HpHSP60-treated HUVEC, THP-1, and AGS cells were subjected to angiogenic arrays to assess expression profiles of angiogenesis-promoting factors.

Table 1
Angiogenic factors released from HpHSP60-treated HUVEC, THP-1, and AGS cells. Expression intensities of angiogenic cytokines stimulated by HpHSP60 in HUVEC, THP-1, and AGS cells were recorded by densitometer analysis and normalized relative to the intensities of untreated cell cytokine levels. Fold change for specific cytokines are indicated. "−" indicates undetectable changes in expression.

Symbol	Name	Biological function	Fold change		
			HUVECs	THP-1	AGS
IL-8	Interleukin-8	Pro-inflammatory cytokine	**4.453**	**7.185**	0.387
GRO	Growth related oncogene	Neutrophil chemoattractants	**5.318**	**5.811**	0.566
IL-6	Interleukin-6	Pro-inflammatory cytokine	**3.23**	0.251	−
MCP-1	Monocyte chemoattractant protein-1	Monocyte chemoattractants	0.625	**3.924**	0.017
RANTES	Regulated on activation normal T cell expressed	Chemoattractants of T cells, eosinophils and basophils	0.002	0.477	0.055
EGF	Epidermal growth factor	Mitogen for endothelial cells	−0.026	−	0.248
TIMP1	Tissue inhibitor of metalloproteinase-1	Prevent extracellular matrix degradation (ECM)	−0.065	0.122	1.131

genesis, and that chemokines play an important role in HpHSP60-induced angiogenesis. CXCL8/IL-8 and GRO, which are expressed by HpHSP60-treated HUVEC and THP-1 cells, are important ELR+ CXC chemokines (Table 1). Both of these chemokines bind to CXCR2 to trigger endothelial cell chemotaxis and angiogenesis [21]. CXCR2 signaling can promote tumor malignancy by promoting tumor angiogenesis, and CXCR2 antagonists are found to inhibit tumor growth [22]. Both MEK1-ERK1/2 and PI3K-AKT signaling pathways are known to act downstream of CXCR2 [23,24]. In addition, PLCβ2/Ca2+ signaling is also activated by IL-8 binding to CXCR2 [25] and able to promote endothelial progenitor cell homing [26]. Our results indicate that HpHSP60 only activates the CXCR2/PLCβ2/Ca2+ signal transduction arm to enhance angiogenesis (Fig. 4).

During tumor progression, mammalian HSP family members are known to play essential roles in tumor growth both by promoting autonomous cell proliferation and by inhibiting death pathways [27]. Several reports also suggest that microbial HSPs affect carcinogenesis. *Chlamydia trachomatis*, an obligate intracellular bacterium, is associated with the development of cervical and
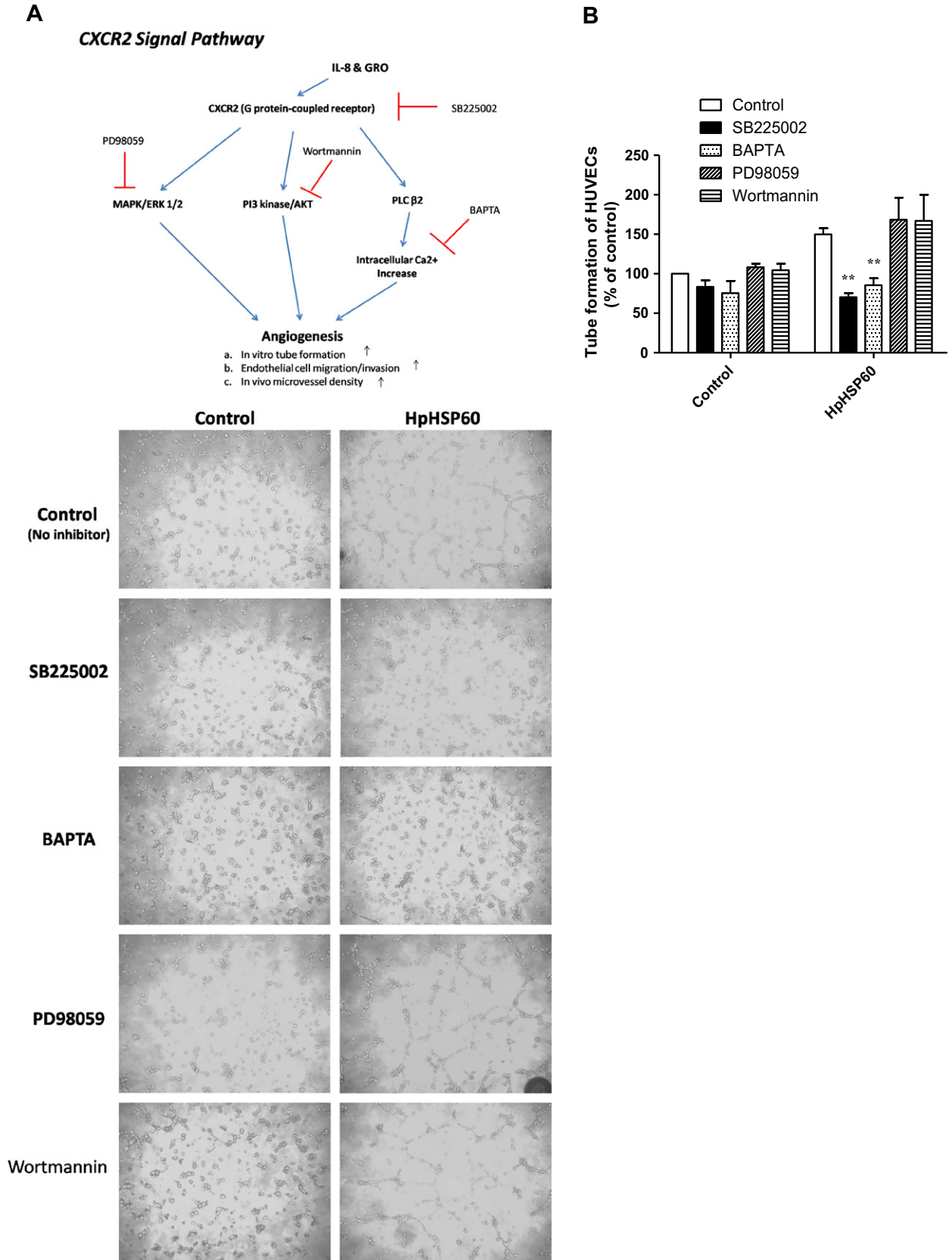
A

B

Fig. 4. Signaling through CXCR2 is required for HpHSP60-mediated angiogenesis. (A) Potential signaling pathways for HpHSP60-induced angiogenesis. IL-8 and GRO could engage with CXCR2 and turn on downstream molecules including MAP kinase [23,38], PI3 kinase [23,39], and PLCβ2 [26] to promote angiogenesis. Inhibitors to these specific signal pathways are used to investigating underlying molecular mechanisms. (B) HUVECs were treated with or without (control) 10 μg/ml HpHSP60 for 6 h after pre-treatment with SB225002 (50 nM), BAPTA (3 μM), PD98059 (5 μM), or wortmannin (10 nM) for 1 h. Tube branch points were then scored and are represented as % tube branch points where un-stimulated cells were equal to 100%. The data are expressed as the mean ± SEM, $^*p < 0.01$ compared to HpHSP60 treatment alone. All experiments were performed in triplicate.

ovarian cancers and, *C. trachomatis* HSP60 inhibits apoptosis during persistent infection [28]. Large quantities of *C. trachomatis* HSP60s were produced and transported to the cytosol of the host cells [29], which formed a complex with cleaved caspase-3 and Bax and Bak to inhibit apoptosis [30]. Aside from its anti-apoptosis role, HSP60 also promotes tumorigenesis by inducing inflammatory cytokine/ chemokine secretion. *Chlamydial pneumoniae* HSP60 promotes diffuse pneumonia by inducing IL-6 production, which promotes infiltration of neutrophils into lung tissue and results in increased production of bronchoalveolar lavage fluid. HSP65 of *Mycobacteria leprae* induced release of TNF-α, IL-6, and IL-8 from human monocytic cells [31]. Moreover, *H. pylori* HSP60 has been reported to induce the expression of pro-inflammatory cytokines, including IL-6 and IL-8, from human monocytic cells and/or gastric epithelium cells [10,11,32]. Inflammatory responses are associated with cell migration and angiogenesis [12], and the data presented herein further confirmed these findings, because HpHSP60s induces IL-8 and GRO expression in HUVEC and THP-1 cells and activates CXCR2-mediated signal transductions to enhance endothelial cell migration, proliferation, and tube formation (Fig. 4).

IL-8 is secreted by both normal and tumor cells that are exposed to pro-inflammatory cytokines such as IL-1 and TNF-α [12]. *H. pylori* infection was found to induce chronic inflammation with persistent IL-1, TNF-α, and IL-8 production, and HpHSP60 induces the expression of these pro-inflammatory cytokines [7]. It seems that IL-8 itself is sufficient to promote angiogenesis, while IL-1 and TNF-α act to promote IL-8 production in host cells to synergize the neovascularizing effects. IL-8 was the first angiogenic chemokine to be characterized, and it is the prototype ELR+ CXC chemokine. IL-8 mediates endothelial cell proliferation *in vitro* and angiogenic activity *in vivo* by up-regulating MMP-2 and MMP-9 to promote the degradation of extracellular matrix, a prerequisite to endothelial cell migration [33,34]. IL-8 also regulates angiogenesis in an autocrine manner. Wuyts et al. demonstrated that in addition to non-endothelial cell expression of IL-8, endothelial cells themselves also produce CXCL8/IL-8 and CXCL6/GCP-2 to further promote angiogenesis [35].

Both HpHSP60-treated AGS and THP-1 cells secrete soluble factors that stimulate HUVEC proliferation (Fig. 2). While THP-1 cells produce IL-8 and GRO, AGS cells produce trace amount of the endothelial mitogen epidermal growth factor (EGF) (Table 1). EGF is primarily expressed in luminal and glandular epithelium and in stromal cells [36]. Interestingly, EGF/EGF receptor engagement promotes angiogenesis both directly, by inducing HUVEC migration/ invasion and proliferation, and indirectly, by promoting VEGF expression in tumor cells [37]. AGS cells are derived from gastric epithelium, while THP-1 cells originate from monocytes, and it is possible that the cell lines elicit different responses to EGF stimulation because they are different cell types. Our results indicate that HUVEC proliferation is triggered by HpHSP60-induced chemokine and/or EGF production in the surrounding non-endothelial cells.

Taken together, extracellular microbe-derived HpHSP60 was proven as a potential promoter of carcinogenesis. The clarification of angiogenic mechanism of HpHSP60 would provide one of the promising solutions to postpone tumor formation. Our serial investigations of HpHSP60s also offer the declaration that microbial HSPs are more than foreign immune activators, they also intervene physiological functions of host cells and lead them to abnormal cell transformation.

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